JOURNAL OF VIROLOGY, July 1981, p. 290-294
0022-538X/81/070290-05$02.00/0

Vol. 39, No. 1

NOTES

Transfection of KB Cells by Liposomes Containing Adenovirus Type 2 DNA

STEPHEN E. STRAUS,[1]* TAZEWELL WILSON,[2] AND HESCHEL J. RASKAS[3]

Medical Virology Section, Laboratory of Clinical Investigation, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20205[1]; Department of Pathology and Department of Microbiology and Immunology, Washington University School of Medicine, St. Louis, Missouri 63110[3]; and Department of Developmental Biology and Cancer, Albert Einstein College of Medicine, Bronx, New York 10461[2]

Received 16 January 1981/Accepted 8 April 1981

Adenovirus type 2 DNA was entrapped in liposomes which were then used to transfect KB cells with an efficiency of ~4,000 plaques per μg of encapsulated DNA.

Synthetic lipid vesicles (liposomes) have been utilized for entrapping a wide variety of drugs and macromolecules, including nucleic acids (12, 13). It has been demonstrated that poliovirus RNA can be encapsulated in lipid vesicles and the entrapped RNA can be transferred to cells, resulting in efficient transfections (20, 21). Ostro et al. (11) and Dimitriadis (3) reported that encapsulated rabbit globin mRNA can be introduced into cells and translated into globin-sized proteins.

Several groups (4, 5, 8-10) have recently reported the entrapment of high-molecular-weight DNA in large unilamellar vesicles (LUVs). The encapsulated DNA was shown to be largely nuclease resistant (4, 5, 8-10) and, upon recovery from the liposomes, it was biologically active (4). Fraley et al. (5) reported successful transformation of bacterial cells with liposome-entrapped plasmid DNA.

This report presents evidence that the large, linear adenovirus type 2 (Ad2) DNA (molecular weight, 23×10^6) can be encapsulated in phosphatidylserine vesicles [LUV(PS)] and recovered in an intact form. Moreover, addition of Ad2 DNA-containing liposomes to cultures of KB cells results in successful transfections.

In initial experiments, the entrapment of DNA in liposomes was studied. [^{32}P]Ad2 DNA (2) in phosphate-buffered saline was encapsulated in vesicles prepared from purified bovine brain phosphatidylserine (PS, Avante Biochemicals, Birmingham, Ala.) by the method of Papahadjopoulos et al. (13, 14). Briefly, PS in chloroform was evaporated under N_2, resuspended as multilamellar vesicles in phosphate-buffered saline, converted into small unilamellar vesicles by sonication, and precipitated as cochleate cylindrical forms (Ca^{2+}-PS complexes) during dialysis against phosphate-buffered saline with 1.5 mM $CaCl_2$. DNA was added to dilutions of the Ca^{2+}-PS complexes and entrapped during the change to LUVs, which was induced by chelation of the calcium with EDTA. The liposomes were repeatedly washed by blending in a Vortex mixer in 5 ml of phosphate-buffered saline and centrifuging at 48,000 × *g* for 20 min. At least three washings were required to remove the majority of the contaminating unencapsulated DNA. An average of only 18% of the [^{32}P]DNA counts associated with washed liposomes were converted to an acid-soluble form by treatment of the DNA-liposomes with 25 μg of DNase II per ml (Worthington Diagnostics, Freehold, N.J.) at 37°C for 60 min, indicating that most of the molecules were within the vesicle lumen. On the other hand, 93% of counts were solubilized by DNase II when [^{32}P]DNA was added to preformed liposomes. Earlier experiments utilizing DNase I gave more variable results, with solubilization of 20 to 77% of the vesicle-associated counts. It is probable that the magnesium (5 mM) recommended for enzymatic reactions with DNase I alters the structure of LUVs in a manner analogous to that which occurs with addition of calcium. Although that concentration of magnesium does not cause precipitation of LUVs(PS) (T. Wilson, unpublished data), it does cause vesicle fusion and rearrangements (15), which might expose entrapped DNA to the nuclease.

The percentage of input DNA encapsulated was determined to be a function of lipid concentration (Fig. 1). Maximal entrapment of DNA was achieved when lipid concentrations (as Ca^{2+}-PS complexes) of about 10 to 15 μmol/ml were used. In all subsequent preparations, a lipid concentration of 10 μmol/ml was used.

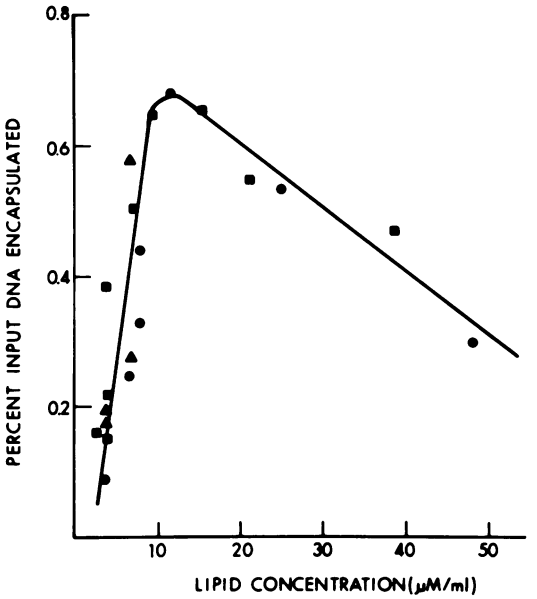

FIG. 1. *Percentage of input DNA encapsulated as a function of lipid concentration. Lipid concentrations in LUVs were estimated from the initial known amount of PS used in each preparation of vesicles. Various amounts of fresh Ca^{2+}-PS complexes were added to vials containing 2 μg of [^{32}P]Ad2 DNA. The lipid concentration ranged from 3 to 48 μmol/ml in a final volume of 0.25 ml. After encapsulation and washing, the vesicle-associated radioactivity was determined. The various symbols indicate determinations from separate experiments.*

The amount of DNA which could be successfully entrapped was also dependent upon the concentration of DNA present during formation of vesicles (Fig. 2). Using different concentrations of [^{32}P]Ad2 DNA in parallel vesicle preparations, it was determined that encapsulation increased linearly with DNA concentration up to about 350 μg/ml. No further increase in DNA uptake was observed when concentrations greater than 350 μg/ml were used. This may reflect saturation of the internal capacities of the vesicles, particularly when one considers the highly anionic nature of the DNA molecules and the PS head groups. It is also probable that the appreciable viscosity of concentrated solutions of unfragmented DNA slows the mixing and proper interaction of the aqueous solution and the lipid.

The entrapped DNA could be quantitatively recovered from washed liposomes by extraction with phenol, chloroform, and isoamyl alcohol (19). Labeled DNA recovered by this method was found to be intact. The sedimentation profiles of the DNA in sucrose gradients revealed that the majority of molecules were full-sized and lacked single-strand nicking (31S in neutral sucrose, 34S in alkaline sucrose; data not shown).

The results reviewed thus far are in general agreement with those reported previously in studies of smaller DNAs (4, 5, 8–10). The somewhat lower efficiency of entrapment observed here is probably a reflection of differences in the buffer and liposome systems employed and the

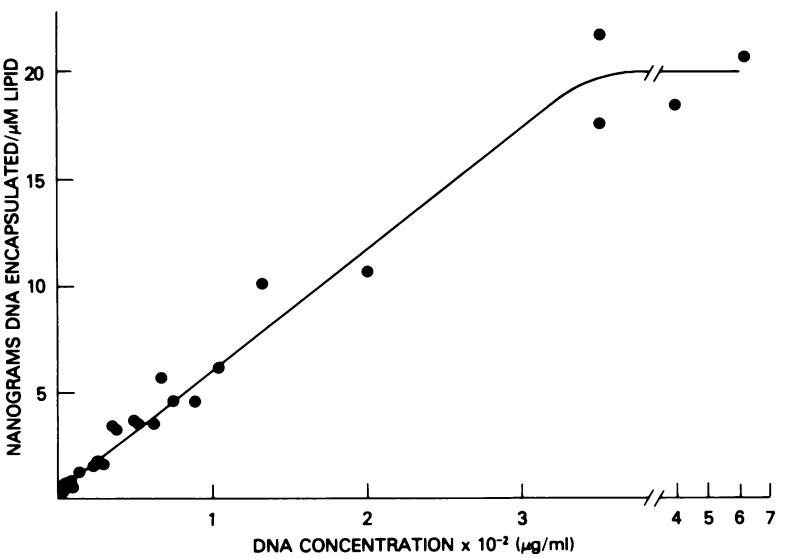

FIG. 2. *Amount of DNA (nanograms) encapsulated as a function of initial DNA concentration. Mixtures of unlabeled (17) and [^{32}P]Ad2 DNA were added to vials containing a concentration of 10 μmol of lipid (Ca^{2+}-PS complexes) per ml to give DNA concentrations ranging between 1 and 610 μg/ml in a final volume of 0.25 ml. After encapsulation and washing, the vesicle-associated radioactivity was determined.*

large size of the Ad2 DNA molecules (10).

Before testing the biological activity of encapsulated DNA, we ascertained that liposomes can interact with and transfer their contents to tissue culture cells. Liposomes were formed in the presence of 0.4 mg/ml of fluorescein isothiocyanate (FITC)-conjugated rabbit immunoglobulin G (16) and washed extensively. When viewed by fluorescence microscopy, these liposomes appeared as a swarm of countless miniature lights. After interaction of the vesicles with KB cells, large masses of fluorescent material were observed adhering to cell surfaces. Faint cytoplasmic fluorescence was noted, especially when large numbers of liposomes were applied to cells, suggesting that there was some release of liposome content into the cells (Fig. 3A).

A direct immunofluorescence assay was used to permit a qualitative analysis of the biological activity of injected material. In a control experiment, cells directly infected with Ad2 virions displayed bright nuclear fluorescence after staining with FITC-anti-Ad2 immunoglobulin G (data not shown) (18). Monolayer cultures of KB cells were treated with Ad2 DNA-liposomes and then incubated at 37°C for 27 h. The cell sheets were then washed, fixed, and stained with FITC-anti-Ad2 immunoglobulin G and examined by fluorescence microscopy. In cultures treated with DNA-liposomes, only about 1 in 5,000 cells (0.02%) displayed bright nuclear fluorescence (Fig. 3B). By 72 h postinfection about 1/500 (0.2%) of cells were positive. Uninfected cells and cells treated with empty liposomes, with or without free viral DNA, were all negative (data not shown).

For a quantitative and more accurate appraisal of the infectivity of DNA-liposomes, an infectious center assay was employed (7). The data in Table 1 demonstrate that this assay appropriately quantitated the infectivity of a previously titrated virus stock. Mock-infected cell sheets, or those treated with naked DNA, or DNA in the presence of empty liposomes manifested few or no plaques. The DNA-liposomes demonstrated an infectivity of about 4,000 plaques per μg of encapsulated DNA (Table 1). Plaques formed by transfection with DNA-lipo-

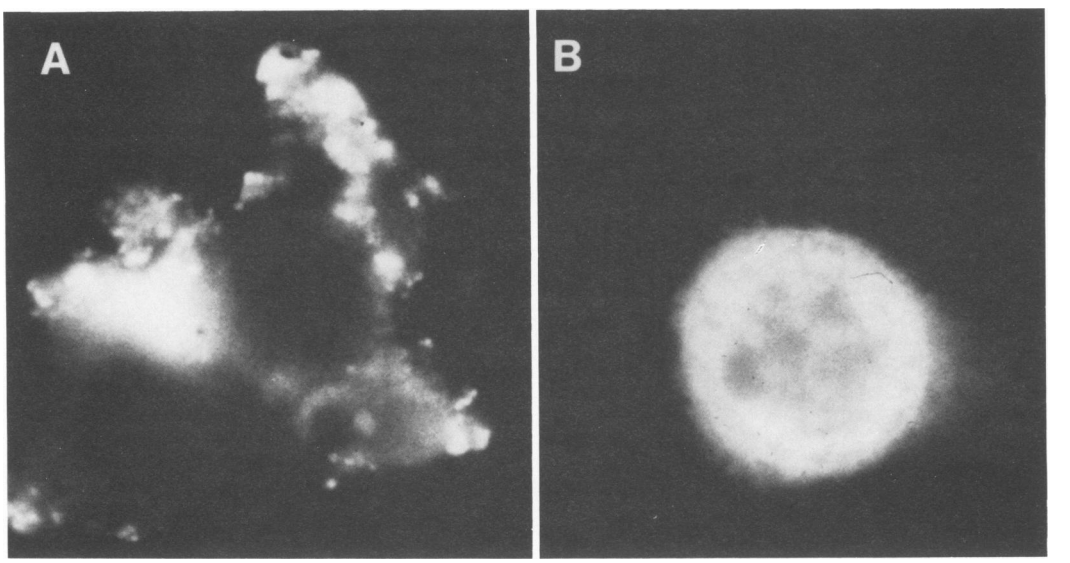

FIG. 3. *Fluorescence photomicrographs of cells after interaction with liposomes. Freshly confluent KB cell monolayers grown in Joklik's minimal essential medium with supplements (10% fetal calf serum, glutamine, penicillin, and streptomycin) were washed twice in medium containing 1.5 mM $CaCl_2$. Dilutions of liposomes were prepared in media. $CaCl_2$ was added to give a final concentration of 1.5 mM, and the liposomes (0.5 ml) were immediately plated onto 35-mm dishes of KB cells. The dishes were rocked several times every 10 min during a 2.5-h incubation at 37°C and then overlaid with 3 ml of medium with supplements. (A) KB cells in monolayers treated with a large excess of liposomes (approximately 10^4 to 10^5 liposomes/cell) containing FITC-anti-Ad2 immunoglobulin G display bright surface and faint cytoplasmic fluorescence. (B) KB cells in monolayers treated with liposomes containing Ad2 DNA were incubated for 27 h and stained with FITC-anti-Ad2 immunoglobulin G. This panel demonstrates nuclear fluorescence typical of that seen with Ad2-infected cells. Surrounding immunofluorescent-negative cells are not readily apparent in this reproduction. Original magnification, ×400.*

TABLE 1. *Infectivity of Ad2 virions, DNA, and liposomes for KB cells*

Sample	Dilution[a]	Treatment	DNA per dish (ng)	Plaques[b]	Plaques per μg of DNA
Virions[c]	10^{-9}			151.0 ± 36 (6)	
	10^{-10}			28.0 ± 11 (6)	
Mock-infected				0 (12)	
DNA			10,000	2.7 ± 1.4 (6)	0.27
Empty liposomes[d] plus DNA				0 (3)	
			1,500	1.0 ± 1 (3)	0.67
DNA-liposomes[d]	1		28.0	99.0 ± 11 (6)	3,540
	10^{-1}		2.8	11.0 ± 2 (6)	3,930
	10^{-2}		0.28	1.2 ± 0.7 (6)	4,280
	10^{-3}		0.028	0.2 ± 0.4 (6)	7,140
	1	DNase II[e]	28.0	23.0 ± 10 (3)	820

[a] Portion of original sample added to cells.

[b] Mean ± standard deviation. Number of determinations is indicated within parentheses.

[c] Purified Ad2 stock with previously determined infectivity of 2×10^{11} PFU/ml.

[d] KB cells were harvested from 75-cm^2 flasks for interaction of vesicles with cells in suspension. The cells were washed twice in medium and suspended at a final concentration of 5×10^6 cells/ml. A 0.25-ml amount of the liposome dilutions in medium was added to an equal volume of cells, which were then blended in a Vortex mixer. $CaCl_2$ was added to a final concentration of 1.5 mM. The cells were blended in a Vortex mixer, rocked continuously at 37°C for 2.5 h, diluted with 2 ml of medium with supplements, and plated onto washed subconfluent monolayers of KB cells. After a 12- to 18-h incubation at 37°C, the media were replaced with 3 ml of 0.9% agar-plaquing media. The monolayers were stained on day 10 and the plaques were counted on day 11 or 12.

[e] DNase II treatments, 25 μg/ml, for 30 min at 37°C.

somes had morphologies similar to those produced by viral infection.

DNA-liposomes were also plaqued directly on monolayers of KB cells but the efficiency of transfection was only about 820 plaques per μg of encapsulated DNA. The continuous agitation of liposomes in a very concentrated suspension of cells, as employed in the infectious center assay, may favor successful transfection.

For the infectious center assay, about 75% of the infectivity of DNA-liposomes was ablated by treatment with DNase II (Table 1). That level of reduction in infectivity exceeded the percentage of liposome-associated DNA that was digested by the nuclease in the earlier experiments (18%). This suggests that the DNase may be adsorbing to the liposomes and interfering with their interaction with the cells or digesting some of the DNA during release into the cells. Alternatively, a fraction of the infectivity of DNA-liposomes may result from DNA molecules which are not entirely entrapped within the vesicle.

The efficiency of liposome-mediated transfection with linear Ad2 DNA is higher than that achieved with routine calcium-phosphate systems (which average 10^1 to 10^2 plaques per μg of DNA [6]) or even that reported for dimethyl sulfoxide- or sucrose-augmented transfection methods (which average 10^2 to 10^3 plaques per μg of DNA [1]). The technique is about 10-fold more efficient than transfection by polyethylene glycol-induced fusion of cells with erythrocyte ghosts containing viral DNA (16). It remains slightly less efficient, however, than augmented transfection procedures with 293 cells or the Ad2 DNA-protein complex (which averages about 10^4 plaques per μg of DNA [1]) or both. Transfections mediated by liposomes containing the Ad2 DNA-protein complex were inexplicably highly variable and very inefficient.

One of the obvious limitations of these methods is the inefficiency of encapsulation of DNA. Although DNA which fails to be entrapped can be recovered easily for further experiments, a large initial quantity of DNA is required. In addition to improving the efficiency of encapsulation, new ways to boost infectivity must be sought. Recent studies by Fraley et al. (5a) indicate that glycerol treatment of cell-vesicle mixtures significantly enhances the efficiency of transfection by simian virus 40 DNA in liposomes. These and other refinements of liposome-mediated "microinjection" may make this technology more generally useful. In the interim, it does have interesting applications in providing a way to microinject large numbers of cells with a variety of macromolecules, particularly those

which may be sensitive to enzymes or other inimical components of the extracellular milieu and those which are not readily transported across the cell membrane.

Much of this work was supported by grant CA 16007 from the National Cancer Institute; grant VC-94D from the American Cancer Society; fellowship DRG-112-FT from the Damon Runyon-Walter Winchell Cancer Fund; grant 5 TO1 AI00459 from the National Institute of Allergy and Infectious Diseases; and grants from the Brown & Williamson Tobacco Corp., Larus and Brother Co., Ligget & Myer, Inc., Lorillard, a division of Loews Theatres, Inc., Philip Morris, Inc., R. J. Reynolds Tobacco Co., United States Tobacco Co., and Tobacco Associates, Inc. T.W. is a postdoctoral fellow of the American Cancer Society. Cell culture media were prepared in a cancer center facility funded by the National Cancer Institute.

The excellent technical support of Harold Simns, Holly Smith, and John Owens is gratefully acknowledged.

LITERATURE CITED

1. **Chinnadurai, G., S. Chinnadurai, and M. Green.** 1978. Enhanced infectivity of adenovirus type 2 DNA and a DNA-protein complex. J. Virol. **26**:195–199.
2. **Craig, E. A., and H. J. Raskas.** 1974. Two classes of cytoplasmic viral RNA synthesized early in productive infection with adenovirus 2. J. Virol. **14**:751–757.
3. **Dimitriadis, G. J.** 1978. Translation of rabbit globin mRNA introduced by liposomes into mouse lymphocytes. Nature (London) **274**:923–924.
4. **Dimitriadis, G. J.** 1978. Entrapment of plasmid DNA in liposomes. Nucleic Acids Res. **6**:2697–2705.
5. **Fraley, R. T., C. S. Fornari, and S. Kaplan.** 1979. Entrapment of a bacterial plasmid in phospholipid vesicles: potential for gene transfer. Proc. Natl. Acad. Sci. U.S.A. **76**:3348–3352.

5a. **Fraley, R., S. Subramani, P. Berg, and D. Papahadjopoulos.** 1980. Introduction of liposome-encapsulated SV40 DNA into cells. J. Biol. Chem. **255**:10431–10435.

6. **Graham, F. L., and A. J. van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52**:456–467.
7. **Green, M., M. Piña, and R. C. Kimes.** 1967. Biochemical studies on adenovirus multiplication. XII. Plaquing efficiencies of purified human adenoviruses. Virology **31**: 562–565.
8. **Hoffman, R. M., L. B. Margolis, and L. D. Bergelson.** 1978. Binding and entrapment of high molecular weight DNA by lecithin liposomes. FEBS Lett. **93**:365–368.
9. **Lurquin, P. F.** 1979. Entrapment of plasmid DNA by liposomes and their interaction with plant protoplasts. Nucleic Acids Res. **6**:3773–3784.
10. **Maninno, R. J., E. S. Allebach, and W. A. Strohl.** 1979. Encapsulation of high molecular weight DNA in large unilamellar phospholipid vesicles. Dependence on the size of the DNA. FEBS Lett. **101**:229–232.
11. **Ostro, M. J., D. Giacomoni, D. Lavelle, W. Paxton, and S. Dray.** 1978. Evidence for translation of rabbit globin mRNA after liposome-mediated insertion into a human cell line. Nature (London) **274**:921–923.
12. **Pagano, R. E., and J. N. Weinstein.** 1978. Interactions of liposomes with mammalian cells. Annu. Rev. Biophys. Bioeng. **7**:435–468.
13. **Papahadjopoulos, D., and W. J. Vail.** 1978. Incorporation of macromolecules within large unilamellar vesicles (LUV). Ann. N.Y. Acad. Sci. **308**:1–462.
14. **Papahadjopoulos, D., W. J. Vail, K. Jacobson, and G. Poste.** 1975. Cochleate lipid cylinders: formation by fusion of unilamellar lipid vesicles. Biochim. Biophys. Acta **394**:483–491.
15. **Portis, A., C. Newton, W. Pangborn, and D. Papahadjopoulos.** 1979. Studies on the mechanism of membrane fusion: evidence for an intermembrane Ca^{+2}-phospholipid complex, synergism with Mg^{+2}, and inhibition by spectrin. Biochemistry **18**:780–790.
16. **Straus, S. E., and H. J. Raskas.** 1980. Transfection of KB cells by polyethylene glycol-induced fusion with erythrocyte ghosts containing adenovirus type 2 DNA. J. Gen. Virol. **48**:241–245.
17. **Straus, S. E., A. Sergeant, M. A. Tigges, and H. J. Raskas.** 1979. Parental adenovirus type 2 genomes recovered early or late in infection possess terminal proteins. J. Virol. **29**:828–832.
18. **Thiel, J. F., and K. O. Smith.** 1967. Fluorescent focus assay of viruses on cell monolayers in plastic petri plates. Proc. Soc. Exp. Biol. Med. **125**:892–895.
19. **Tibbetts, C., K. Johansson, and L. Philipson.** 1973. Hydroxyapatite chromatography and formamide denaturation of adenovirus DNA. J. Virol. **12**:218–225.
20. **Wilson, T., D. Papahadjopoulos, and R. Taber.** 1977. Biological properties of poliovirus encapsulated in lipid vesicles: antibody resistance and infectivity in virus-resistant cells. Proc. Natl. Acad. Sci. U.S.A. **74**:3471–3475.
21. **Wilson, T., D. Papahadjopoulos, and R. Taber.** 1979. The introduction of poliovirus RNA into cells via lipid vesicles (liposomes). Cell **17**:77–84.