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Gene transfer from targeted liposomes to specific lymphoid cells by electroporation

(transfection/DNA targeting/monoclonal antibodies)

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ABSTRACT **Large unilamellar liposomes, coated with protein A and encapsulating the gene that confers resistance to mycophenolic acid, were used as a model system to demonstrate gene transfer into specific lymphoid cells. Protein A, which selectively recognizes mouse IgG2a antibodies, was coupled to liposomes to target them specifically to defined cell types coated with IgG2a antibody. Protein A-coated liposomes bound human B lymphoblastoid cells preincubated with a mouse IgG2a anti-HLA monoclonal antibody but failed to adhere to cells challenged with an irrelevant (anti-H-2) antibody of the same isotype or to cells incubated in the absence of antibody. Transfection of target cells bound to protein A-coated liposomes was achieved by electroporation. This step was essential since only electroporated cells survived in a selective medium containing mycophenolic acid. Transfection efficiency with electroporation and targeted liposomes was as efficient as conventional procedures that used unencapsulated plasmids free in solution but, in the latter case, cell selectivity is not possible. This technique provides a methodology for introducing defined biological macromolecules into specific cell types.**

Transfection of eukaryotic cells by DNA is a useful technique to increase our understanding of gene expression and regulation and of the function of given molecules in particular cell types. Early transfection studies relied upon two procedures to introduce exogenous genetic material into cells, (*i*) natural transfection, accomplished by infecting cells with genetically manipulated but intact virus (1, 2) and (*ii*) artificial procedures involving temporary physical or chemical perturbations of plasma membranes to permit entry of DNA. The second technique that uses complexes of DNA with calcium phosphate (3), DEAE-dextran (4, 5), or polyornithine (4) is efficient in introducing DNA into phagocytic cells (6) (e.g., fibroblasts or L cells) but is much less useful with cell populations lacking this property (e.g., lymphocytes). Other methods of introducing macromolecules into mammalian cells overcome this limitation by using direct microinjection into the nucleus (7, 8), DNA-transfer aided by polyethylene glycol (PEG)/dimethyl sulfoxide (9), by trypsin/EDTA/glycerol (10), or by osmotic shock (11). In addition, vehicle-mediated transfer with artificial carriers such as liposomes (12–15), erythrocyte ghosts (16–18), bacterial protoplasts (10, 19, 20), or reconstituted Sendai virus envelopes (21) have also proven useful in a variety of systems. Attention has focused on techniques that physically disrupt the cell membrane by laser microbeam (22, 23), electroporation (24, 25), or tungsten microprojectiles (26). Each of these procedures is distinguished by its own spectrum of advantages and disadvantages, including efficiency, toxicity, technical difficulty, equipment needs, and specificity.

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In this study we show that lymphocytes can be specifically transfected *in vitro* by using an electric field (electroporation) and targeted liposomes containing DNA. As a model system we used large unilamellar liposomes in which plasmid DNA carrying the bacterial xanthine guanine phosphoribosyltransferase (XGPRT) gene was encapsulated. These liposomes were directed to target cells by monoclonal antibodies and specific transfection was achieved by electroporation. The technique is simple and specific and can be used to introduce genetic material and macromolecules into other cell types.

MATERIALS AND METHODS

Cells. The human Burkitt lymphoma cell line BJAB was cultured in Dulbecco's modified Eagle medium (DMEM, GIBCO) supplemented with 5% (vol/vol) heat-inactivated fetal calf serum, 2 mM glutamine, and gentamicin at 37°C in an atmosphere of 7% CO_2/93% air.

Monoclonal Antibodies. B1.23.2 and H100-5/28 are monoclonal IgG2a mouse antibodies. B1.23.2 is directed against nonpolymorphic determinants expressed on human major histocompatibility complex-encoded molecules HLA-B and -C (27). H100-5/28 has affinity for H-2K murine major histocompatibility complex-encoded molecules (28). Antibodies were purified from culture supernatants by chromatography on protein A-Sepharose 4B (Pharmacia) columns (29).

Liposome Preparations. Dimyristoyl phosphatidylcholine (Avanti Polar Lipids, Birmingham, AL), dimyristoyl phosphatidylserine (Avanti Polar Lipids), cholesterol (Sigma), and dipalmitoyl phosphatidylethanolamine (Sigma) modified with *N*-hydroxysuccinimidyl 3-(2-pyridyldithio)propionate (Pharmacia) (30, 31) were used at a molar ratio of dimyristoyl phosphatidylcholine/dimyristoylphosphatidylserine/cholesterol/dipalmitoyl phosphatidylethanolamine-dithiopyridine of 54:10:35:1. Large unilamellar liposomes were prepared as described (32). Briefly, 40 μmol of total lipids were reverse-phase evaporated (33) with 1 ml of DSPK plasmid at 1.7–2 mg/ml (6.3 kilobase) (34) (linearized by *Sma* I digestion and containing in some experiments an aliquot of ^{32}P-labeled plasmid) or 1 ml of purified carboxyfluorescein (20 mM) (35). The preparations were then filtered through a 0.4-μm polycarbonate membrane (36). Liposomes were mixed with *N*-hydroxysuccinimidyl 3-(2-pyridyldithio)propionate-modified protein A (Pharmacia) (10 mol of *N*-hydroxysuccinimidyl 3-(2-pyridyldithio)propionate per mol of protein A; 200 μg of protein A plus an aliquot of ^{125}I-labeled protein A)

Abbreviation: XGPRT, xanthine guanine phosphoribosyltransferase.

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as described (30, 31, 37) and incubated with 50 μg of DNase I (Sigma) and 10 mM $MgCl_2$ for 30 min at room temperature before chromatography on Sepharose 4B columns to remove uncoupled and unencapsulated molecules. Liposomes were sterilized by filtration through 0.44-μm Millipore filters and stored at 4°C. The percentage of encapsulated DNA and carboxyfluorescein varied between 5 and 8% corresponding to 1–3 plasmid molecules per liposome. We calculated that 2–5 protein A molecules were coupled to the surface of each liposome (protein A-liposome).

Binding of Targeted Liposomes to Cells. Cells (5×10^6 cells) were incubated with 20 μg of anti-HLA or anti-H-2K (control) monoclonal antibodies. After 1 hr at 4°C, cells were washed twice with medium and incubated with protein A-liposomes containing carboxyfluorescein. In some experiments, 50 μg of free protein A was added prior to incubation. After 1 hr at 4°C, cells were washed three times with isotonic phosphate-buffered saline (PBS) and lysed with 1% Triton X-100. Samples were centrifuged and liposome binding was monitored by using a Perkin–Elmer model 650 10S spectrofluorimeter (excitation wavelength, 488 nm; emission wavelength, 520 nm) to measure carboxyfluorescein released into the supernatants.

Transfection Protocol. Cells were washed with PBS (Ca^{2+}/Mg^{2+}-free) and incubated at 5×10^6 to 10^7 cells per 0.5 ml in PBS with 5 μg of circular plasmid or various concentrations of linearized plasmid for 10 min on ice. The suspensions were then electroporated (25) by using an LKB 2197 power supply (2500 V, 0.9 mA, ≈100 μsec). Five successive shocks were delivered to each preparation. The cells were incubated on ice for an additional 10 min and distributed in normal culture medium into 96-well Costar (Cambridge, MA) microtiter plates at 5×10^4 to 10^5 cells per well.

Transfection with targeted cells was accomplished by incubating cultures for 1 hr at 4°C in medium with 20 μg of antibodies (anti-HLA or control anti-H-2K), washing with PBS, and incubating for 1 hr in ice with protein A-bearing liposomes containing linearized DNA and/or carboxyfluorescein. The cells were then electroporated.

In each case, after 24 hr at 37°C, cells were cultured in selective medium [DMEM supplemented with 10% fetal calf serum, 2 mM glutamine, xanthine at 0.25 mg/ml (Sigma), adenine at 25 μg/ml (Sigma), and mycophenolic acid at 5 μg/ml (Sigma)]. Every 2 days the medium was replaced and after 15 days the number of growth-positive wells was counted.

Southern Blots. DNA for Southern blot analysis was prepared by a modification of standard methods of phenol extraction and ethanol precipitation (9). Purified DNA (10–20 μg) was precipitated with ethanol and resuspended in reaction buffer, and 5 units of *Xba* I restriction enzyme per μg of DNA was added. The samples were incubated for 20 hr at 37°C, loaded onto the agarose gels (0.8%) by using a horizontal apparatus (Bethesda Research Laboratories, model M4), and electrophoresed for 14 hr at 40 V. DNA was stained with ethidium bromide and observed under UV light. GeneScreen*Plus* was used for the DNA transfer. The nick-translated probe (XGPRT insert, 680 base pairs) with a specific activity of 2.9×10^8 cpm/μg was hybridized for 18 hr at 42°C in 40% (vol/vol) formamide.

RESULTS

Transfection by Electroporation. We tested the relative abilities of circular or linearized plasmids to transfect lymphocytes by electroporation. The DSPK plasmid used in this model system is a eukaryotic expression vector containing the XGPRT gene under the control of the simian virus 40 promoter (34). After stable transfection, surviving cells expressing XGPRT can be selectively grown in medium containing mycophenolic acid, which blocks *de novo* purine nucleotide synthesis and provides xanthine as the sole precursor for guanine nucleotide formation. Fig. 1 summarizes the data obtained after transfection of the human BJAB cell line by electroporation. Linearized plasmid was at least 10 times more efficient than the same plasmid in circular form (25). Cells not subjected to electroporation, or electroporated in the absence of plasmid, failed to proliferate. Cell viability after electroporation was 80% as measured by trypan blue exclusion.

Specific Transfection of Cells by Targeted Liposomes. Protein A-liposomes were directed to HLA molecules by a monoclonal anti-HLA antibody bound to the target cells (Fig. 2). At least 90% of the cells bound liposomes (data not shown). The number of liposomes bound depended on their bulk concentration (Fig. 2 *Inset*). At the highest liposome concentration tested, ≈1000 of the 400-nm liposomes bound per cell, corresponding to 1000–3000 plasmids per cell.

Liposomes failed to bind untreated cells or cells preincubated with control anti-H-2 antibody (Fig. 2). Liposome binding was inhibited when target cells were preincubated with free protein A (data not shown).

Target cells incubated with the anti-HLA antibody and protein A-bearing liposomes containing the XGPRT gene were transfected by electroporation (Fig. 3, bar a). Cells bearing control antibodies or no antibody generated few transfectants (Fig. 3, bars b and c). Similar negative results were obtained in the absence of electroporation and by using liposomes without encapsulated DNA (Fig. 3, bars d and e).

Comparison of Liposome-Encapsulated and Free Plasmid. Fig. 4 shows that transfection efficiency increased proportionally with free plasmid concentrations and reached a plateau at 5 μg of DNA. Optimal efficiency was estimated at 1 in 5×10^5 cells. Targeted liposomes yielded similar data indicating a comparable efficiency of transfection. However,

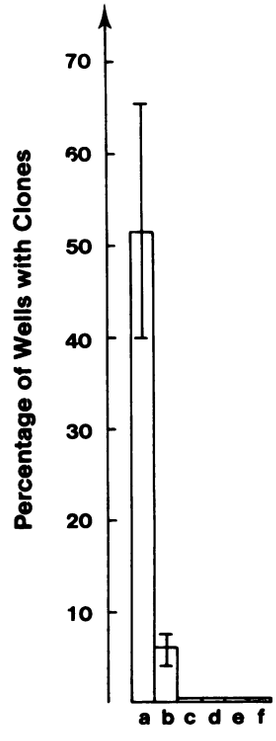

FIG. 1. Transfection of human lymphocytes by electroporation. BJAB cells were incubated with 5 μg of DSPK plasmid and electroporated. Bars: a, linearized plasmid with electroporation; b, circular plasmid with electroporation; c, cells alone with electroporation; d, linearized plasmid with no electroporation; e, circular plasmid with no electroporation; f, cells alone with no electroporation. Bars represent the mean value ± SD of three experiments.

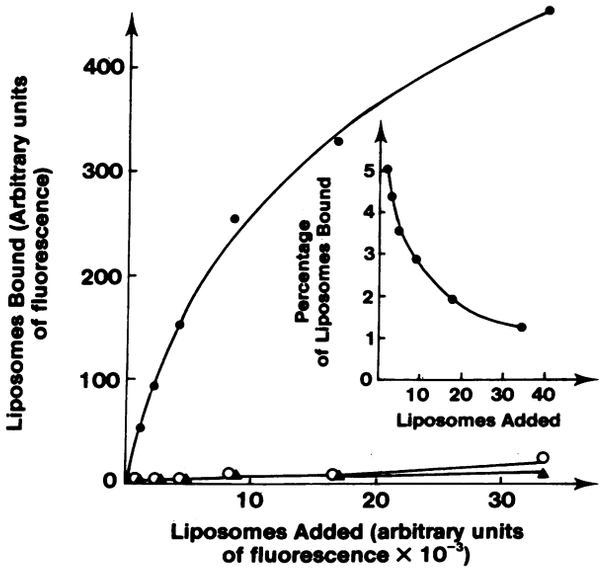

FIG. 2. Binding of targeted protein A-liposomes to cells. Cells were incubated without antibody (▲), with 20 μg of anti-HLA antibodies (●), or with anti-H-2 (a control irrelevant antibody) antibodies (○) before incubation with liposomes. (*Inset*) Percentage of total liposomes bound to the cells.

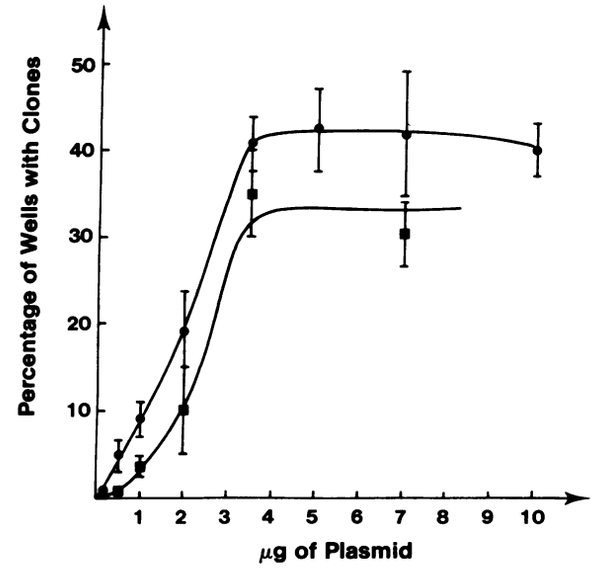

FIG. 4. Transfection efficiency for free plasmid and plasmids encapsulated in protein A-liposomes. Cells were electroporated with various concentrations of free plasmid (●) or with plasmid contained in targeted protein A-liposomes (■). Bars represent the mean value ± SD of three experiments.

it is important to note that in this experiment the efficiency of transfection is related to the total amount of DNA present in the sample in free or liposome-encapsulated form. Since only 1–5% of the total protein A-liposomes bind to cells, the efficiency of transfection with specific liposomes is much higher (20- to 100-fold).

Southern Blot. Hybridization with the nick-translated XGPRT gene revealed a 9.0-kDa DNA fragment, indicating the presence of this gene in cells transfected with free plasmid or with targeted liposomes containing the XGPRT gene (Fig. 5). DNA from untransfected cells failed to display cross-hybridizing bands. The three bands detectable in DNA from liposome-targeted cells may represent multiple insertions of the XGPRT gene or three clones in a single well. The latter explanation is possible because transfectants were not cloned after electroporation.

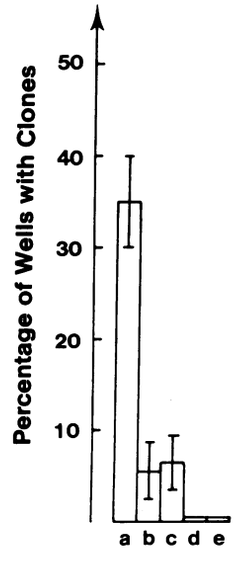

FIG. 3. Transfection of lymphocytes by DNA-containing targeted liposomes. Cells were incubated with antibodies and with targeted liposomes and were subjected to electroporation. The liposome concentration was equivalent to 5 μg of DNA. Bars represent transfectants obtained after electroporation of cells preincubated as follows: a, with anti-HLA relevant antibody; b, with anti-H-2 control antibody; c, without antibody plus protein A-liposomes; d, without electroporation of cells preincubated with anti-HLA antibody plus liposomes; e, cells incubated with anti-HLA antibody plus protein A-liposomes containing only carboxyfluorescein and subjected to electroporation. Bars represent the mean value ± SD of three experiments.

DISCUSSION

Electroporation is an efficient technique that can be used to transfect many cell types. However, no selectivity with

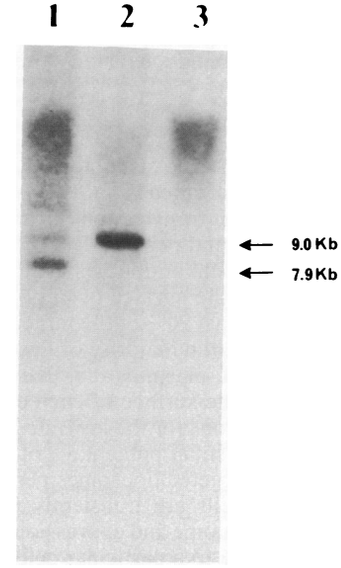

FIG. 5. Southern blot analysis of BJAB transfectants. Genomic DNA from BJAB transfected with DSPK plasmid encapsulated into anti-HLA antibody-targeted liposomes (lane 1), BJAB transfected with free DSPK plasmid (lane 2), and BJAB cell line (control, lane 3) was digested by *Xba* I, and 10 μg of DNA per well was applied to a 0.8% agarose gel. Nick-translated XGPRT gene probe (680 base pairs) was used for hybridization.

regard to cell type or stage of cell differentiation can be obtained by using standard electroporation techniques.

Liposomes have been used in gene transfer experiments because of their relative lack of toxicity and their utility in transfecting cells refractory to methods of classical gene transfer (12–15, 38–40). In *in vitro* experiments, binding of liposomes to cells was achieved by nonspecific (electrostatic) interactions (12–15, 41, 42). In this study we present evidence that selected cells can be transfected efficiently when electroporated in the presence of specifically bound liposomes.

Under certain conditions bound liposomes and their contents can be internalized by cellular endocytic pathways. The efficiency of this process depends on the target antigens recognized by protein A-liposomes, the cell type expressing these antigens, and liposome size (32, 37, 43). Liposomes of 400 nm were chosen because they encapsulate relatively large quantities of plasmid DNA. However, liposomes of this size are too large to enter nonphagocytic cells (e.g., lymphocytes) in endocytic vesicles (32, 44–46). Smaller liposomes recognized by the endocytic pathways may also have utility in gene transfer but since endocytosed liposomes end up predominantly in lysosomal compartments where they are destroyed (47), the potential of this technique in transfection studies remains to be established. Nevertheless, by using pH-sensitive lipids that induce fusion of liposomes with acidic endocytic vesicles, it could be possible to minimize degradation in lysosomes as has been shown with DNA-loaded targeted liposomes *in vitro* (48) and *in vivo* (49).

Since only a low percentage of the total number of protein A-liposomes present in transfection samples bound the target cells (between 1 and 5%), we estimate that 0.05–0.25 μg of plasmid DNA is sufficient for maximum transfection efficiency. This amount of plasmid DNA is too low for successful transfection when present as free plasmid DNA or when DNA-containing liposomes are incubated with target cells coated with control antibody (Figs. 3 and 4). This suggests that transfection depends on the creation of pores between the cell and specifically bound liposomes permitting transfer of locally high concentrations of plasmid DNA. Electroporation is known to form pores in both plasma membranes (24) and lipid vesicles (50). Alternatively, transfection can be the result of direct fusion of the cell and the bound liposome, since electric fields can also induce fusion of lipid membranes (50–54).

From our data we can conclude that under given electroporation conditions, the transfection efficiency will not improve when free plasmid DNA is increased to more than 5 μg. However, by using specifically targeted liposomes, we may be able to improve transfection efficiency if more targeted liposomes are bound per cell. This can be achieved by using specific monoclonal antibodies against more abundant epitopes present on the surface of targeted cells.

In our experiments at least 90% of the targeted cells bound liposomes. Assuming that the majority of liposomes contain at least one molecule of plasmid and that at least 1000 plasmids are brought to the surface of each cell by targeted liposomes, three explanations for the low rate of gene expression are possible. Only a small percentage of these cells are electroporated or more targeted liposomes per cell are necessary. Alternatively, all cells are transiently transfected but stable insertion into the genome and expression of the gene are limiting events, or specific to a particular cell subpopulation. Without ruling out the first two hypotheses, the third is possible, since it has been shown with DNA–calcium phosphate coprecipitation that cells transfectable by this method harbor DNA in their cytoplasm, but only some correctly express the gene (55, 56). This suggests that access to the nucleus and integration into the genome are probably the most significant barriers to gene transfer and expression (56).

In conclusion, the use of electroporation and targeted liposomes is an efficient technique to transfect specific cell populations and has the potential to be applied to a number of macromolecules of biological interest and a range of various cell types.

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