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Communication

Direct *in vivo* transfer of plasmid DNA into murine tumors: Effects of endotoxin presence and transgene localization✪

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The purpose of this study was to investigate the effect of endotoxin presence in plasmid DNA preparations on the efficiency of transfection achieved *in vivo* with B16(F10) and Renca tumors and to determine transgene localization. Our data show that endotoxin markedly decreases the efficiency of transfection. Furthermore, the transgene transferred *in vivo* can be found in both neoplastic and normal (most likely myofibroblast) cells lying in proximity of the administration site.

In vivo DNA transfer to cells, both normal and neoplastic, can be achieved by a variety of methods. Most often, attempts to transfer therapeutic DNA *in vivo* make use of viral and synthetic carriers or physical methods such as electroporation and gene gun (for the latest review see [1]). Numerous data indicate that plasmid DNA alone ("naked" DNA) may be transferred *in vivo* to various normal and neoplastic cells without the use of any carriers [2, 3]. Such an approach appears simple, relatively inexpensive and has been adapted to the administration of so-called DNA vaccines (see [4]). Unexpectedly, the efficiency of naked DNA transfection into neoplastic cells *in vivo* is higher than that obtained with cationic lipids [5]. Some data, also from our laboratory, indicate that this DNA transfer method may be successfully used in experimental gene therapy [5–7].

An essential problem in genetic manipulations involving plasmid DNA is the degree of

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Abbreviations: LPS, lipolysaccharide; PBS, phosphate-buffered saline, pH 7.2; RLU, relative luminescence unit; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

its purification from bacterial endotoxin (lipopolysaccharide, LPS) [8–10]. Endotoxin derived from Gram-negative bacteria is strongly toxic (LPS is an extremely potent stimulator of the mammalian immune system). Hence, in order to be used in gene therapy, plasmid DNA preparations isolated from *E. coli* should be of greatest purity available (the so-called pharmacological grade). The purpose of this study was to investigate the influence of endotoxin contamination in plasmid DNA preparations upon the efficiency of *in vivo* transfection with plasmid DNA alone, without any carriers. The presence of bacterial endotoxin in plasmid DNA preparations has been otherwise known to exert a clear effect on *in vitro* transfection using cationic lipids, decreasing its efficiency [11]. In this study we also attempted to investigate the localization of the inserted transgene in various types of cells that form tumors.

MATERIALS AND METHODS

Plasmids. In this study we used the following plasmids: pVR1255 [12] (obtained from Dr. R. Zaugg, Vical Inc., San Diego, U.S.A.) containing luciferase reporter gene under the control of CMV promoter and two plasmids containing the *lacZ* gene, i.e. pCMV*lacZ* (Clontech) with the *Eschericha coli* β-galactosidase gene under the control of CMV promoter and pCMVn*lacZ* showing nuclear localization obtained from Dr. L. Sadowska (Université de Génève, Faculté de Biologie Moléculaire, Génève, Switzerland).

Plasmid DNA isolation and purification from endotoxin. Plasmid DNA preparations were isolated from spheroplasts according to Wicks *et al.* [9]. Plasmid DNA was separated from endotoxin and other contaminants using Sephacryl S-1000-filled column (Sephacryl S-1000 Superfine, Pharmacia Biotech, column height = 100 cm, diameter = 1.6 cm). Other details were described by Horn *et al.* [10].

Determining endotoxin concentration in plasmid DNA preparations. Fifty μl of LAL solution (Limulus Amebocyte Lysate, BioWhittaker) was added to 50 μl aliquots of DNA solutions of varying concentrations (0.005 μg/μl, 0.001 μg/μl, 0.0005 μg/μl). Samples were incubated for 10 min at 37°C. Then, 100 μl of Chromogenic Substrate solution (BioWhittaker) was added and the sample incubated for 6 min at 37°C. The reaction was stopped by adding 100 μl of 10% SDS solution (BDH Biochemicals). Endotoxin concentration in the DNA preparations was checked by measuring absorbance at λ = 405 nm. The degree of DNA preparations' purification from endotoxin was 700–1000-fold. One μg of DNA contained from 0.03 to 0.04 LPS units.

Animals. **In vivo** ***transfection.*** Six- to eight-week-old BALB/c and C57BL6 mice from on-site Animal Facility were used throughout the experiments. Mice's left dorsal side was shaved and the animals were inoculated subcutaneously with either 5×10^5 Renca cells/100 μl PBS^- per mouse (PBS^-: standard PBS buffer, pH 7.2, without Ca^{2+} and Mg^{2+}) or with 2.5×10^6 B16(F10) cells/100 μl PBS^- per mouse. Ten μg of luciferase gene-containing pVR1255 plasmid DNA alone, or 10 μg of pVR1255 plasmid DNA complexed to varying amounts of cationic liposomes DC-Chol/ DOPE [13] were injected into tumors approximately 5–10 mm in diameter using 200 μl Ringer's solution with 0.05% lactose added. DC-Chol/DOPE cationic lipids (1:1, w/w) were prepared as described previously [14]. After 24 h mice were killed and tumor material collected in order to measure the activity of the reporter gene.

Measuring luciferase gene activity in tumor specimens. Tumors excised from animals treated with pVR1255 plasmid DNA were homogenized in 1.5 ml of lysis buffer (Promega) containing protease inhibitors (phenylmethylsulphonyl fluoride, benzamidine, EDTA, pH 8, each 1 mM). The homogenate was transferred to Eppendorf-type test

tubes, mixed vigorously and centrifuged (15 000 r.p.m. for 15 min, 4°C). Luciferase activity was measured in 20 μl of supernatant to which 100 μl luciferase substrate (Promega) was added. Measurements were performed using a LUMAT LB 9501/16 luminometer (Berthold). Protein was measured in 10 μl of supernatant using Dye Reagent Concentrate solution (Biorad). The results are expressed as relative luminescence units (RLU) per milligram of total cell protein.

β-Galactosidase activity detection. pCMVn*lacZ* plasmid DNA (50 μg) was transferred directly, without any carriers, to the growing neoplastic tumors. After 24 h, tumors were excised and fixed at 4°C in 4% paraformaldehyde in PBS^- containing two types of detergent: NP-40 (final concentration = 0.02%) and sodium deoxycholate (final concentration = 0.01%). After 4 h incubation tumors were immersed overnight in staining solution containing X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, Sigma) and the above detergents [6]. Stained tumors were freezed in isopentane and liquid nitrogen and then sliced using cryostat (–20°C) into slices 10 μm-thick that were subsequently placed on gelatin slides. The preparations were stained with hematoxylin-eosin, fixed, covered and dried. Stained tumor cells were examined under a microscope (total magnification 850×).

RESULTS AND DISCUSSION

The efficiency of *in vivo* transfection with plasmid DNA has a significant implication for the efficacy of prospective gene therapy: the higher the percentage of transfected cells the greater the production of the therapeutic protein. The impact of plasmid DNA preparations' contamination with bacterial endotoxin on the efficiency of *in vivo* transfection has not been reported so far. Experiments performed on rodents showing their remarkable resistance to LPS [9] have, as a rule, involved plasmid DNA preparations not purified from endotoxin. Nonetheless, a negative effect of the contamination of plasmid DNA with endotoxin was previously described in a report from *in vitro* studies performed on cell cultures transfected with cationic liposomes [11]. Studies on *in vivo* transfection of B16(F10) neoplastic tumors with either endotoxin-contaminated or purified DNA show (Fig. 1) that such contamination markedly decreases transfection efficiency with both naked DNA and DNA complexed to cationic lipids (in this case DC-Chol/DOPE). This finding does not appear to depend on neoplastic cell type (a similar effect was seen with Renca cells, data not shown). So far, we are unable to determine at which stage of DNA transfer (e.g. cellular uptake or trans-

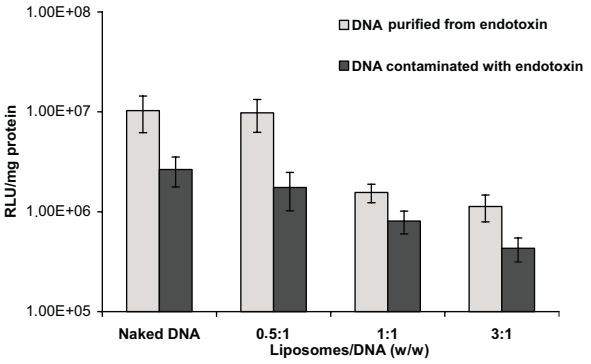

Figure 1. Enzymatic activity of luciferase in B16(F10) tumors transfected *in vivo* using lipoplexes and naked DNA.

Ten μg of pVR1255 plasmid DNA (containing luciferase gene) was injected using Ringer's solution supplemented with 0.05% lactose (200 μl total) into growing B16(F10) neoplastic tumors either as "naked DNA" without any carriers or using cationic liposomes DC-Chol/DOPE at various liposomes/DNA ratios (0.5:1, 1:1, 3:1, w/w). Plasmid DNA used in the experiments was either endotoxin-contaminated or endotoxin-free. Endotoxin-contaminated DNA contained 26.27 LPS units/μg DNA while purified DNA contained 0.03 LPS units/μg DNA. Luciferase activity was measured in B16(F10) tumor cell lysates 24 h post DNA transfer. Each data point represents average luciferase activity (±S.D.) in cell lysates obtained from 5 different tumors. Y-axis is logarithmic.

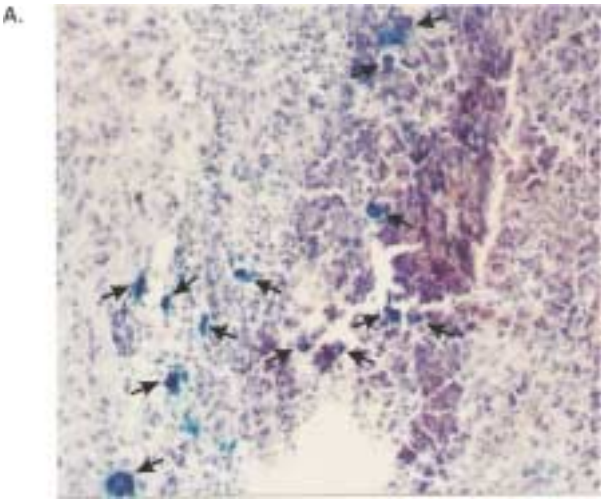

Figure 2A. Transfected neoplastic cells in B16(F10) tumor.

Ten μg pCMVn*lacZ* plasmid DNA containing β-galactosidase reporter gene was injected into growing B16(F10) tumor using Ringer's solution supplemented with 0.05% lactose (100 μl total). After 24 h the tumor was removed, rinsed in PBS^-, fixed for 4 h at 4°C in 4% formaldehyde with sodium deoxycholate (final concentration = 0.01%) and NP-40 (final concentration = 0.02%) added. Then, the tumor was rinsed three times in PBS^- and immersed in staining solution (X-gal) for 20 h (overnight) at room temperature. Following staining and rinsing (3×) with PBS^- the sample was frozen in isopentane and liquid nitrogen. The specimen was stored at −70°C and sliced at −20°C using a cryostat. Slices obtained were then hematoxylin-eosin stained, fixed in ethyl alcohol and xylene and covered with a cover slip. Transfected cells (blue) are indicated with arrows.

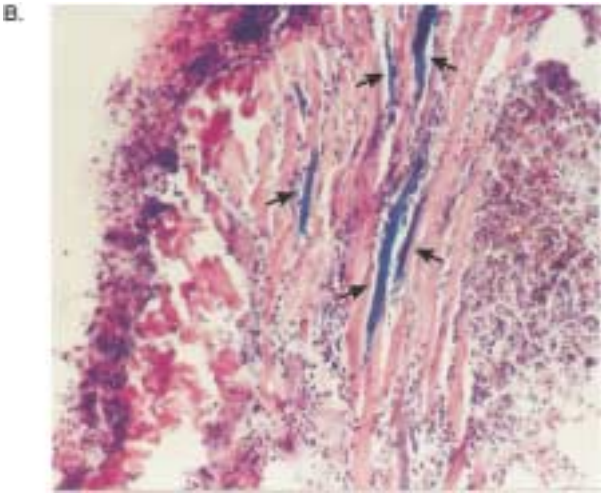

Figure 2B. Transfected normal cells in B16(F10) tumor.

Fifty μg pCMV*lacZ* plasmid DNA containing β-galactosidase reporter gene was injected 3 times (at 24 h intervals) into growing B16(F10) tumor using Ringer's solution supplemented with 0.05% lactose (100 μl total). For details see Fig. 2, part A.

port to the nucleus) is the endotoxin actually involved.

We cannot exclude the possibility that endotoxin has some influence on the luciferase gene expression rather than on the transfection efficiency itself. However, we believe that it is the transfection efficiency that is affected by endotoxin. Weber *et al.* [11] have shown that addition of endotoxin to previously purified plasmid DNA preparations exerts a negative effect on the efficiency of *in vitro* transfection.

Also, our own experiments *in vivo* have shown that when plasmid DNA was transferred into cells by electroporation, i.e. when transfer occurs *via* micropores formed and not due to endocytosis [15], no effect of endotoxin upon luciferase expression could be detected (Cichoń *et al.,* manuscript in preparation). Even if endotoxin gains entry to the cell together with plasmid DNA, its subsequent effect upon the reporter gene expression, following transfer to the nucleus, is negligible.

The second important issue brought up in this report concerns the localization of the transferred transgene. Some data indicate that transgene inserted into tumor as naked DNA is localized into neoplastic cells [3]. Our earlier studies of transgene transfer using cationic lipids also corroborate such localization [16]. An important observation made during this study was that transgene transferred into a tumor as naked plasmid DNA, without carriers, is found in the proximity of the injection site not only in cancerous cells but also in normal cells forming tumor mass (or infiltrating it) (Fig. 2A, B). The presence of transgene in normal cells does not depend, however, on the type of carrier used: the transgene was found in normal cells also when DNA–

cationic lipids complexes were used for *in vivo* transfection. Transgene presence in normal cells resembling muscle cells (smooth muscle-like cells or myofibroblasts) [17–20] is indeed intriguing (Fig. 2B). According to some authors, myofibroblasts may play a role in the development of tumors [6, 18]. Others claim that they play a major role in the inflammatory response [20]. Since the transgene is present in myofibroblasts it is possible that these cells contain more nucleic acids "receptors" than other cells forming the tumor. The presence of such receptors for oligonucleotides and nucleic acids has long been postulated for various types of normal cells; see Budker *et al.* [21].

The method of transferring plasmid DNA directly into cancer cells may be exploited in experimental tumor gene therapy [5–7]. In our opinion, better suited to such therapy are genes coding for immunomodulatory proteins (cytokines) or angiostatic proteins (inhibiting neoplastic angiogenesis) compared to genes coding for suicide or proapoptotic proteins. The latter two kill cells directly and thus their activity should rather be confined to neoplastic cells only. Actually, for transgenes, whose protein products take part in indirect killing of neoplastic cells, their localization is not critical; they may localize themselves in both normal and neoplastic cells. We used successfully the method of direct plasmid DNA transfer into murine tumors *in vivo* to treat such tumors with the IL-12 gene [5] or the endostatin gene [7].

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REFERENCES

1. Mountain, A. (2000) *Trends Biotechnol.* **18,** 119–128.
2. Wolff, J.A., Malone, R.W., Williams, P., Chong, W., Acsadi, G., Jani, A. & Felgner, P.L. (1990) *Science* **247,** 1465–1468.
3. Yang, J.-P. & Huang, L. (1996) *Gene Ther.* **3,** 542–548.
4. Moelling, K. (1998) *Gene Ther.* **5**, 573–574.
5. Budryk, M., Wilczyńska, U., Szary, J. & Szala, S. (2000) *Acta Biochim. Polon.* **47,** 385–391.
6. Coll, J.-L., Negoescu, A., Louis, N., Sachs, L., Tenaud, C., Girardot, V., Demeneix, B., Brambilla, E., Brambilla, Ch. & Favrot, M. (1998) *Hum. Gene Ther.* **9,** 2063–2074.
7. Szary, J. & Szala, S. (2001) *Int. J. Cancer* **91,** 835–839.
8. Cotten, M., Baker, A., Saltik, M., Wagner, E. & Buschle, M. (1994) *Gene Ther.* **1,** 239–246.
9. Wicks, I.P., Howell, M.L., Hancock, T., Kohsaka, H., Olee, T. & Carson, D.A. (1995) *Hum. Gene Ther.* **6,** 317–323.
10. Horn, N.A., Meek, J.A., Budahazi, G. & Marquet, M. (1995) *Hum. Gene Ther.* **6,** 565–573.
11. Weber, M., Möller, K., Welzeck, M. & Schorr, J. (1995) *BioTechniques* **19,** 930–940.
12. Hartikka, J., Sawdey, M., Cornefert-Jensen, F., Margalith, M., Barnhart, K., Nolasco, M., Vahlsing, H.L., Meek, J., Marquet, M., Hobart, P., Norman, J. & Manthorpe, M. (1996) *Hum. Gene Ther.* **7,** 1205–1217.
13. Gao, X. & Huang, L. (1991) *Biochem. Biophys. Res. Commun.* **179,** 280–285.
14. Szala, S., Missol, E., Sochanik, A. & Stróżyk, M. (1996) *Gene Ther.* **3,** 1026–1031.

15. Somiari, S., Glasspool-Malone, J., Drabick, J.J., Gilbert, R.A., Heller, R., Jaroszeski, M.J. & Malone, R.W. (2000) *Molec. Ther.* **2,** 178–187.

16. Missol, E., Sochanik, A. & Szala, S. (1995) *Cancer Lett.* **97,** 189–193.

17. Grégoire, M. & Lieubeau, B. (1995) *Cancer Metast. Rev.* **14,** 339–350.

18. Martin, M., Pujuguet, P. & Martin, F. (1996) *Path. Res. Pract.* **192,** 712–717.

19. Schürch, W., Seemayer, T.A. & Gabbiani, G. (1997) in *Histology for Pathologists,* 2nd edn. (Sternberg, S.S., ed.) pp. 129–165, Lippincott-Raven Publ., Philadelphia.

20. Powell, D.W., Mifflin, R.C., Valentich, J.D., Crowe, S.E., Saada, J.I. & West, A.B. (1999) *Amer. J. Physiol.* **277,** C1–C19.

21. Budker, V., Budker, T., Zhang, G., Subbotin, V., Loomis, A., Wolff, J.A. (2000) *J. Gene Med.* **2,** 76–88.