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QUARTERLY

Direct transfer of IL-12 gene into growing Renca tumors✪

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We investigated the feasibility of transferring naked plasmid DNA containing a therapeutic gene (IL-12) into mice harboring growing Renca tumors.

We found that naked DNA transferred into growing Renca and B16(F10) tumors gives higher expression level of reporter gene than complexes of DNA with DDAB/DOPE or DC-Chol/DOPE. Transfer of naked DNA carrying the IL-12 gene into growing Renca tumors causes a distinct therapeutic effect that depends on the time span between inoculation of mice with cancer cells and the beginning of the therapy. Therapy started on day 3 resulted in total cure (100%) of mice.

Experimental gene therapy of cancer involves transfer of therapeutic DNA by means of various carriers. These can be divided into two broad categories: viral (adenoviruses and retroviruses) (for review see [1]), and nonviral (mostly cationic lipids) ones: [2]. In addition naked DNA can be inserted into target cells by means of physical methods such as electroporation [3] or gene gun [4].

It has been shown, however, that plasmid DNA can also be transferred into cells of certain normal tissues (such as muscles [5], skin [6] and liver [7]) without recourse to any carriers or physical enhancement. It has recently been demonstrated that transfer of naked plasmid DNA is also possible in growing tumors [8].

We aimed to investigate whether plasmid DNA that contains a gene coding for IL-12, an immunomodulatory cytokine with known antitumor properties (for latest review see [9]), can be successfully transferred into grow-

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Abbreviations: CMV, cytomegalovirus; mIL-12, murine interleukin 12; PBS, phosphate buffered saline; RLU, relative luminescence units.

ing tumors thus increasing survival of treated mice. Cancer gene therapy trials involving IL-12 have until now relied on intradermal gene transfer [10], gene gun method [11, 12] or adenoviruses [13, 14]. The choice of IL-12 is not accidental as its proinflammatory and immunostimulatory modes of action are well-known. IL-12 induces proliferation of several subpopulations of lymphocytes including T_{H1} cells, natural killer (NK) and lymphokine-activated killer (LAK) cells. IL-12 induces production of TNF-α and IFN-γ [9]. IL-12 gene-based therapy appears to be a potentially safer alternative to systemic IL-12 protein-based therapy [15].

MATERIALS AND METHODS

Plasmids. We used in our therapeutic experiments the pBCMGSNeo/mIL-12 plasmid containing sequences encoding both subunits of murine IL-12 (p35 and p40, separated by an IRES sequence), under the control of cytomegalovirus promoter (construct obtained from Dr. H. Yamamoto) [16]. As a control we used the pBCMGSNeo plasmid (obtained from Dr. H. Karasuyama from the Basel Institute of Immunology). In some experiments we used the pVR 1255 plasmid which encodes a luciferase reporter gene under the control of CMV promoter as well as a kanamycin resistance gene (obtained from Dr. R.H. Zaugg, Vical, San Diego, CA) [17]. For preparative plasmid DNA isolation from spheroplasts we followed the protocol of Wicks and coworkers [18].

Cell lines and tissue culture. Renca cells (murine renal carcinoma line) and B16(F10) cells (murine melanoma line) were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (Gibco BRL). Cultures were kept at 37°C in 5% CO_2 incubator.

Cationic DNA carriers. As carriers of plasmid DNA we used cationic liposomes DDAB/DOPE [19] and DC-Chol/DOPE [20]. Liposomes were prepared at weight ratios of 0.6:1 (DDAB/DOPE) and 1:1 (DC-Chol/DOPE). Liposome preparations (sonicated emulsions at 1 μg total lipids/μl H_2O) were kept at 4°C and used within 2 weeks.

Animals. 6–8-week-old BALB/c and C57BL/6 mice were from our own animal facility. Mice had their left dorsal side shaved and were then inoculated subcutaneously with either Renca cells (5×10^5 per mouse) or B16(F10) cells (2.5×10^6 per mouse) suspended in 100 μl PBS (phosphate buffered saline). Administration of therapeutic DNA was begun either on day 3, day 7 or day 11 of experiments. Size of tumors was calculated from the following formula: Volume = $(\text{Width})^2 \times \text{Length} \times 0.52$. In some experiments, the luciferase gene-containing plasmid was administered. Transgene activity was determined in cell lysates from cancer tissue.

Luciferase activity determination. The activity of the reporter gene encoding luciferase (in the pVR 1255 plasmid) transferred into growing tumors *in situ*, was determined by measuring chemiluminescence in lysates of transfected cells obtained from isolated tumors (Promega kit). Protein content in lysates was determined spectrophotometrically (λ = 595 nm) using Dye Reagent Concentrate (BioRad) and bovine serum albumin standards. Chemiluminescence was measured using a LUMAT LB 9501/16 luminometer (Berthold).

Determination of IL-12 amount. Plasmid DNA encoding IL-12 (pBCMGSNeo/mIL-12) was injected into growing Renca tumors. The amount of IL-12 in lysates of transfected tumor cells was quantitated using ELISA (ELISA Kit for Quantification of Total Mouse Interleukin-12, Genzyme) [21]. The amount of protein in cancer cell lysates was determined spectrophotometrically, as described above. The results express the amount of IL-12 (pg per mg protein) generated in tumor cells following injection of pBCMGSNeo/mIL-12. Background IL-12, i.e. endogenous IL-12 as well as IL-12 induced in tumors following transfer of expression plasmid devoid of therapeutic IL-12 gene were both subtracted.

RESULTS

Transfer of naked plasmid DNA results in higher *in vivo* transfection level of neoplastic cells compared with lipoplexes

Ten μg of naked plasmid DNA (pVR 1255 containing the luciferase gene) as well as plasmid DNA complexed with cationic lipids DDAB/DOPE or DC-Chol/DOPE were transferred into growing Renca and B16(F10) tumors. Expression of the introduced luciferase gene in neoplastic cells transfected with naked plasmid DNA was one order of magnitude higher than in cells transfected by means of liposomal carriers (Table 1).

Table1. Expression level of luciferase transgene transferred into neoplastic cells of growing tumors either by means of cationic liposomes or directly naked DNA

Lipoplexes or naked DNA	Cells	
	Renca	B16(F10)
DNA-DDAB/DOPE	54 301 ± 13 792 RLU/mg protein	15 393 ± 1 539 RLU/mg protein
DNA-DC-Chol/DOPE	95 580 ± 27 379 RLU/mg protein	399 023 ± 89 693 RLU/mg protein
Plasmid DNA	211 580 ± 52 010 RLU/mg protein	2 461 355 ± 495 632 RLU/mg protein

Ten μg of DNA (pVR 1255 plasmid containing luciferase reporter gene) was transferred into growing Renca or B16(F10) tumors (5–10 mm of diameter) either complexed with cationic liposomes (DDAB/DOPE or DC-Chol/DOPE) or injected directly, without any carriers. DNA-liposome complexes contained 50 μg DDAB/DOPE liposomes or 20 μg DC–Chol/DOPE liposomes (in 200 μl of Ringer's solution with 0.05% lactose added). Enzymatic activity of luciferase was determined in lysates of neoplastic cells obtained from Renca tumors 24 h after DNA administration. Each value represents average luciferase activity in lysates from five tumors and expresses relative luminescence units (RLU) per mg of cell protein.

Transgene expression duration and optimum amounts of DNA transferred into growing tumors

Our studies show that the most suitable solutions to carry out DNA transfer are: isotonic PBS (without Ca^{2+} or Mg^{2+}), 15% mannitol containing heparin (2.5 unit/ml) in 0.9% NaCl, and Ringer's solution with 0.05% lactose. Transgene expression levels in neoplastic cells transformed using these solutions are similar (not shown).

Different amounts of pVR 1255 DNA carrying the luciferase reporter gene were transferred into growing tumors. The highest transgene activity was observed when about 50 μg of DNA was used (Fig. 1A).

We also determined the duration of plasmid DNA expression in Renca tumor cells. The enzymatic activity of luciferase in neoplastic cells was determined during consecutive days of the experiment. Transgene expression persists for about 22 days. The maximum activity was detected after four days following DNA transfer (Fig. 1B).

Amount of IL-12 in tumors

Amounts of IL-12 in transfected Renca tumor cells were determined in tumor lysates obtained following either single or fivefold administration of the transgene directly into a growing tumor. The amount of IL-12 following single DNA administration reached 800 pg/mg protein while fivefold administration resulted in about 2000 pg/mg protein (Fig. 2).

Effect of IL-12 therapeutic gene on survival of mice harboring Renca tumors

Syngenic BALB/c mice previously inoculated with Renca cells were then subjected to intratumoral injections of 50 μg pBCMG-

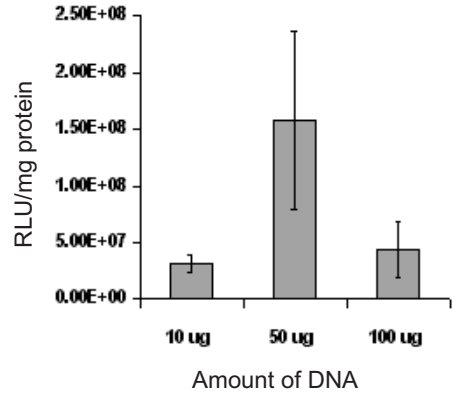

Figure 1A. Level of transgene expression in Renca tumor cells in relation to the amount of transferred DNA.

Varying amounts of pVR 1255 plasmid containing luciferase reporter gene were transferred (in 100 μl Ringer's with 0.05% lactose) into growing, 4–7 mm diameter Renca tumors (10, 50 or 100 μg DNA). Luciferase activity was determined in lysates of neoplastic cells obtained from tumors 24 h following DNA administration. Each data point represents average ±S.D. from luciferase activity determination in lysates from five tumors (RLU, relative luminescence unit).

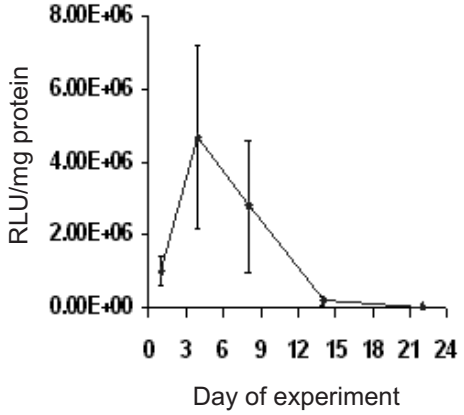

Figure 1B. Duration of transgene persistence in Renca tumors.

Fifty μg of pVR 1255 plasmid containing luciferase reporter gene was transferred, in 100 μl mannitol in 0.9% NaCl, containing heparin (2.5 units/ml), into growing 4–7 mm diameter Renca tumors. During following days luciferase activity was determined in lysates of neoplastic cells obtained from tumors. Each data point represents average ±S.D. from luciferase activity determination in lysates from five tumors.

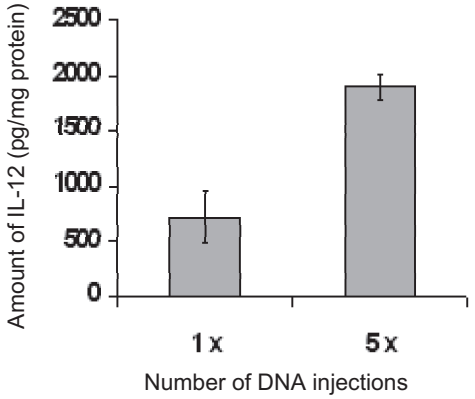

Figure 2. Amount of IL-12 in Renca tumors following intratumoral administration of IL-12 gene.

pBCMGSNeo/mIL-12 plasmid DNA (50 μg in 100 μl Ringer's with 0.05% lactose) was administered (once or five times) into growing 5–10 mm diameter Renca tumors. Amount of IL-12 was determined in Renca tumor lysates 24 h following last DNA administration. Each data point represents average ±S.D. from measurements of IL-12 amounts in lysates from three tumors. Differences between groups shown were statistically significant ($P < 0.0003$, Student' *t*-test).

SNeo/mIL-12 DNA which includes the IL-12 therapeutic gene. The therapy schemes were started either on day 3, day 7 or day 11 following inoculation of mice with cancer cells (Fig. 3). Control animals were treated with an "empty" pBCMGSNeo vector (without the IL-12 gene).

Therapy started on the third day gave a clear-cut therapeutic effect (100% cure rate). When the therapy was started on day 7 of the experiment its effectiveness decreased to 40% cure rate. If started on day 11, however, no therapeutic results were seen (i.e. the therapeutic effect was similar to that observed in mice receiving the "empty" vector without IL-12 gene).

The therapeutic effect, i.e. inhibition of tumor growth and lengthened survival of treated mice depends on time elapsed between inoculation of mice with cancer cells and beginning of treatment. In other words, smaller tumors respond to treatment better than larger ones.

DISCUSSION

Our experiments confirmed earlier reports about the feasibility of transferring naked

DNA into neoplastic cells *in vivo* [8]. The level of luciferase gene expression in Renca and B16(F10) cells is higher in the case of direct intratumoral injection of plasmid DNA, compared with the use of liposomal carriers to transfer the DNA (Table 1). The maximum amount of DNA transferable *via* liposomes in a similar setup does not exceed 10 μg [22]. The feasibility of direct transfer of plasmid DNA into the tumor makes it possible to significantly increase the dose of therapeutic DNA. In our study we found that the optimum amount of naked plasmid DNA was about 50 μg (Fig. 1A). The persistence of transgene expression in tumor cells depends on tumor type and ranges from about 1 week for B16(F10) cells (data not shown) to about 3 weeks for Renca cells (Fig. 1B). It is likely a consequence of different tumor growth dynamics: faster-dividing B16(F10) cells can lose their transgene faster.

On the basis of these results we carried out therapeutic experiments involving the transfer of the IL-12 gene. When this gene was administered directly into growing Renca tumors we observed an evident therapeutic effect. The sooner the treatment started the better its efficacy was (Fig. 3). Presumably, cancer cells in large tumors grow at a rate vastly exceeding that of their depletion and/or the amount of IL-12 produced is not sufficient to generate the therapeutic effect (see [23]). Only mice treated from the third day following inoculation with cancer cells could be cured with 100% efficiency, i.e. complete tumor regression could be seen (Fig. 3).

So far, there have been no reports concerning direct transfer of naked plasmid DNA containing the IL-12 gene into growing tumors. The only report describing direct transfer of therapeutic plasmid DNA into growing tumors concerns the *bax 2* and *p53* genes [24].

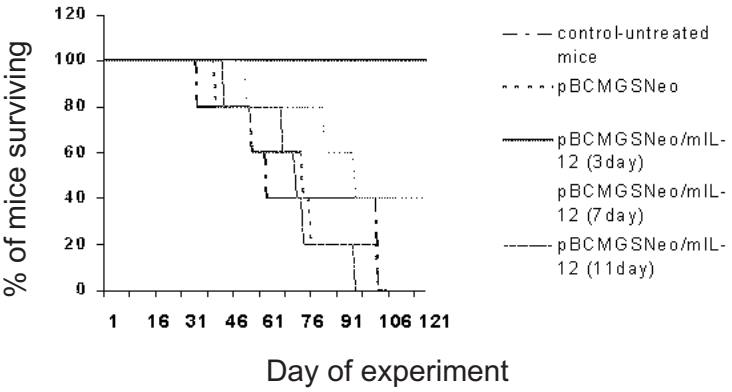

Figure 3. Survival time of mice harboring Renca tumors and treated with plasmid DNA carrying mIL-12 gene.

BALB/c mice were inoculated subcutaneously with Renca cells' suspension (5×10^5 cells/animal). Mice then received injections of either control plasmid pBCMGSNeo or therapeutic plasmid pBCMGSNeo/mIL-12, directly into the tumor. Therapy was started on day 3, day 7 or day 11 of experiment. DNA preparations (50 μg in 100 μl PBS/animal) were administered daily through day 25 of experiment. Each experimental group comprised 5 animals.

It seems that besides its simplicity, the therapy relying on direct transfer of plasmid DNA (carrying a therapeutic gene) into growing tumors *in situ* has some other advantages. The use of naked plasmid DNA allows the introduction of much larger doses of therapeutic DNA in comparison with lipoplex formulations. The amount of DNA complexed with liposomes is rather fixed and much lower ([22]). Furthermore, plasmid DNA is relatively straightforward to handle. Last but not least, it can be administered repeatedly.

According to Yang *et al.* [8] and Coll *et al.* [24] the fraction of tumor cells transfectable with naked DNA does not exceed 1%. Nonetheless, even with transfection efficiencies this low, a distinct therapeutic effect can be observed under certain conditions [24]. This coincides with increased IL-12 level following repeated direct intratumoral DNA injections (Fig. 2). In our experiments the therapeutic effect of IL-12 may be additionally amplified thanks to short immunostimulatory sequences (called ISS and containing the CpG

dinucleotide in a particular base context) within the ampR gene present in pBCMGSNeo [25, 26]. Unmethylated CpG sequences present in plasmid DNA may costimulate the activation of T_{H1} cells [27].

Experiments with naked DNA may be useful in preclinical investigations to test prospective gene therapy constructs (mainly those with immunomodulatory properties). In addition, its costs are lower than those incurred in implementing therapies based on viral, liposome or polypeptide carriers or therapies requiring sophisticated equipment (such as a gene gun).

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REFERENCES

1. Anderson, W.F. (1998) Human gene therapy. *Nature* **392,** 25–30.

2. Lasic, D.D. & Templeton, N.S. (1996) Liposomes in gene therapy. *Adv. Drug Deliv. Rev.* **20,** 221–266.

3. Muramatsu, T., Nakamura, A. & Park, H.M. (1998) *In vivo* electroporation: A powerful and convenient means of nonviral gene transfer to tissues of living animals. *Int. J. Molec. Med.* **1**, 55–62.

4. Yang, N.S. & Sun, W.H. (1995) Gene gun and other non-viral approaches for cancer gene therapy. *Nature Med.* **1,** 481–483.

5. Wolff, J.A., Malone, R.W., Williams, P., Chong, W., Acsadi, G., Jani, A. & Felgner, P.L. (1990) Direct gene transfer into mouse muscle *in vivo*. *Science* **247,** 1465–1468.

6. Raz, E., Carson, D.A., Parker, S.E., Parr, T.B., Abai, A.M., Aichinger, G., Gromkowski, S.H., Singh, M., Lew, D., Yankauckas, M.A., Baird, S.M. & Rhodes, G.H. (1994) Intradermal gene immunization: The possible role of DNA uptake in the induction of cellular immunity to viruses. *Proc. Natl. Acad. Sci. U.S.A.* **91,** 9519–9523.

7. Hickman, M.A., Malone, R.W., Lehmann-Bruinsma, K., Sih, T.R., Knoell, D., Szoka, F.C., Walzem, R., Carlson, D.M. & Powell, J.S. (1994) Gene expression following direct injection of DNA into liver. *Hum. Gene Ther.* **5,** 1477–1483.

8. Yang, J.P. & Huang, L. (1996) Direct gene transfer to mouse melanoma by intratumor injection of free DNA. *Gene Ther.* **3,** 542–548.

9. Trinchieri, G. (1998) Interleukin-12: A cytokine at the interface of inflammation and immunity. *Adv. Immun.* **70,** 83–243.

10. Tan, J., Newton, C.A., Djeu, J.Y., Gutsch, D.E., Chang, A.E., Yang, N.S., Klein, T.W. & Yu, H. (1996) Injection of complementary DNA encoding interleukin-12 inhibits tumor establishment at a distant site in a murine renal carcinoma model. *Cancer Res.* **56**, 3399–3403.

11. Rakhmilevich, A.L., Turner, J., Ford, M.J., McCabe, D., Sun, W.H., Sondel, P.M., Grota, K. & Yang, N.S. (1996) Gene gun-mediated skin transfection with interleukin 12 gene results in regression of established primary and metastatic murine tumors. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 6291–6296.

12. Rakhmilevich, A.L., Janssen, K., Turner, J., Culp, J. & Yang, N.S. (1997) Cytokine gene therapy of cancer using gene gun technology: Superior antitumor activity of interleukin-12. *Hum. Gene Ther.* **8**, 1303–1311.

13. Bramson, J.L., Hitt, M., Addison, C.L., Muller, W.J., Gauldie, J. & Graham, F.L. (1996) Direct intratumoral injection of an adenovirus expressing interleukin-12 induces regression and long-lasting immunity that is associated with highly localized expression of interleukin-12. *Hum. Gene Ther.* **7**, 1995–2002.

14. Caruso, M., Pham-Nguyen, K., Kwong, Y.L., Xu, B., Kosai, K.I., Finegold, M., Woo, S.L.C.

& Chen, S.H. (1996) Adenovirus-mediated interleukin-12 gene therapy for metastatic colon carcinoma. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 11302–11306.

15. Rakhmilevich, A.L., Timmins, J.G., Janssen, K., Pohlmann, E.L., Sheehy, M.J. & Yang, N.S. (1999) Gene gun-mediated IL-12 gene therapy induces antitumor effects in the absence of toxicity: A direct comparison with systemic IL-12 protein therapy. *J. Immunother.* **22,** 135–144.

16. Obana, S., Miyazawa, H., Hara, E., Tamura, T., Nariuchi, H., Takata, M., Fujimoto, S. & Yamamoto, H. (1995) Induction of anti-tumor immunity by mouse tumor cells transfected with mouse interleukin-12 gene. *Jpn. J. Med. Sci. Biol.* **48,** 221–236.

17. 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) An improved plasmid DNA expression vector for direct injection into skeletal muscle. *Hum. Gene Ther.* **7,** 1205–1217.

18. Wicks, I.P., Howell, M.L., Hancock, T., Kohsaka, H., Olee, T. & Carson, D.A. (1995) Bacterial lipopolysaccharide copurifies with plasmid DNA: Implications for animal models and human gene therapy. *Hum. Gene Ther.* **6,** 317–323.

19. Rose, J.K., Buonocore, L. & Whitt, M.A. (1991) A new cationic liposome reagent mediating nearly quantitative transfection of animal cells. *BioTechniques* **10,** 520–525.

20. Gao, X. & Huang, L. (1991) A novel cationic liposome reagent for efficient transfection of mammalian cells. *Biochem. Biophys. Res. Commun.* **179,** 280–285.

21. Bramson, J.L., Hitt, M., Addison, C.L., Muller, W.J., Gauldie, J. & Graham, F.L. (1996) Direct intratumoral injection of an adenovirus expressing interleukin-12 regression and long-lasting immunity that is associated with highly localized expression of interleukin-12. *Hum. Gene Ther.* **7,** 1995–2002.

22. Szala, S., Missol, E., Sochanik, A. & Stróżyk, M. (1996) The use of cationic liposomes DC-CHOL/DOPE and DDAB/DOPE for direct transfer of *Escherichia coli* cytosine deaminase gene into growing melanoma tumors. *Gene Ther.* **3,** 1026–1031.

23. Colombo, M.P., Vagliani, M., Spreafico, F., Parenza, M., Chiodoni, C., Melani, C. & Stoppacciaro, A. (1996) Amount of interleukin 12 available at the tumor site is critical for tumor regression. *Cancer Res.* **56**, 2531–2534.

24. Coll, J.L., Negoescu, A., Louis, N., Sachs, L., Tenaud, C., Girardot, V., Demeinex, B., Brambilla, E., Brambilla, C. & Favrot, M. (1998) Antitumor activity of *bax* and *p53* naked gene transfer in lung cancer: *In vitro* and *in vivo* analysis. *Hum. Gene Ther.* **9,** 2063–2074.

25. Sato, Y., Roman, M., Tighe, H., Lee, D., Corr, M., Nguyen, M.D., Silverman, G.J., Lotz, M., Carson, D.A. & Raz, E. (1996) Immunostimulatory DNA sequences necessary for effective intradermal gene immunization. *Science* **273**, 352–354.

26. Moelling, K. (1998) Naked DNA – the poor man's gene therapy? *Gene Ther.* **5**, 573–574.

27. Krieg, A.M. (1999) Direct immunologic activities of CpG DNA and implications for gene therapy. *J. Gene Med.* **1**, 56–63.