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(54) **COMPOSITION POUR LA TRANSFECTION DE CELLULES EUCARYOTES SUPERIEURES**

(54) **COMPOSITION FOR THE TRANSFECTION OF HIGHER EUCARYOTIC CELLS**

(57) L'invention concerne une composition pour la transfection de cellules eucaryotes supérieures. Un complexe comprenant un acide nucléique à exprimer dans la cellule et un lipide cationique présent dans une concentration sous-optimale pour la transfection, contient un ou plusieurs peptides acides à action membranaire, ainsi qu'éventuellement un(des) lipide(s) auxiliaire(s). Le rapport du nombre total des charges positives au nombre total des charges négatives de la composition est compris entre approximativement 0 et approximativement 3.

(57) The invention concerns a composition for the transfection of higher eucaryotic cells. A complex of a nucleic acid to be expressed in the cell and a cationic lipid present in a suboptimal concentration for transfection contains one or a plurality of membrane-active acid peptides and optionally helper lipid(s). The ratio of the total number of positive charges to the total number of negative charges in the composition is between approximately 0 and approximately 3.

(57) Abstract

The invention concerns a composition for the transfection of higher eucaryotic cells. A complex of a nucleic acid to be expressed in the cell and a cationic lipid present in a suboptimal concentration for transfection contains one or a plurality of membrane-active acid peptides and optionally helper lipid(s). The ratio of the total number of positive charges to the total number of negative charges in the composition is between approximately 0 and approximately 3.

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Composition for the transfection of higher eukaryotic cells

The invention relates to the transfection of higher eukaryotic cells, particularly mammalian cells.

There is a need for an efficient system for introducing nucleic acid into living cells particularly within the scope of gene therapy. In this, genes are locked into cells in order to synthesise therapeutically effective gene products *in vivo*.

The technologies which have progressed furthest and become most widespread hitherto for using nucleic acids in gene therapy make use of systems for the transfer of genes into the cell functioning on the basis of viruses, particularly retroviruses and adenoviruses (Miller, 1992; Mulligan, 1993; Berkner; 1988), and cationic lipids (Behr, 1994). Alternative strategies for gene transfer are based on mechanisms which the cell uses for transporting macromolecules. An example of this is the import of genes into the cell via receptor-mediated endocytosis (e.g. Wu and Wu, 1987, Wagner et al., 1990, EP-A1 0388 758).

Of the synthetic gene transfer vehicles which have been developed in the last decade, mono- and polycationic amphipathic lipids which are capable of complexing and condensing DNA have proved particularly promising (Behr, et al., 1989; Felgner, et al., 1987 and 1994; Leventis, et al., 1990; Gao, et al., 1991; Rose, et al., 1991; Hawley-Nelson, et al.,1993; Weibel, et al., 1995; Solodin, et al., 1995).

Efforts to improve the performance of gene transfer methods based on cationic lipids have hitherto been

concentrated on the direct modification of the cationic lipid, e.g. by acyl groups, spacer arms or hydrophilic sections (Remy, et al., 1994; Felgner, et al., 1994). In spite of improved results as a result of such measures there is still much that is unexplained regarding the mechanism of entry of lipid/DNA particles into the cell. More recent publications (Zabner, et al., 1995) would seem to indicate that the main method of transportation of these particles is via endocytosis.

Cationic lipids exhibit the best transfection results in vitro with a significantly positive excess of charge. Thus, for example, for the lipospermin DOGS (dioctadecylamidoglyclyspermin, commercially obtainable under the brand name "Transfectam") a three- to sixfold excess of positive charges in relation to the DNA has proved to be the optimum level for the efficiency of transfection (Barthel, et al., 1993). The positive charges promote the binding of the complex and its absorption into the cells. Additionally, Transfectam has a buffer action on the endosomes (the pKa value of the at least basic secondary amine of lipospermin, which is crucial to the buffer action, is about 5.4; Behr, 1994) and therefore on the one hand protects the DNA from enzymatic degradation, and on the other hand causes osmotic swelling and subsequent destabilisation of the buffered endosomes. A strongly positive charge on the lipid/DNA complexes thus has the disadvantage, when used in vivo, that the lipid/DNA particles have only a very short half life.

In order to improve the efficiency of the lipopolyamines DOGS and DPPES (palmitoylphosphatidylethanolamine) at a small positive excess of charge (ratio of positive to negative charges ≤ 2, preferably 0.5 to 1.5), it was proposed to add adjuvants which can associate with the

lipopolyamine/nucleic acid complex, e.g. the addition of so-called "helper lipids".

Kamata, et al., 1994, increased the efficiency of gene transfer carried out with lipofectin, a 1:1 mixture of the monocationic lipid DOTMA (N-[1-(2,3-dioleyl-oxy)propyl]-N,N,N-trimethylammoniumchloride) and the helper lipid DOPE (dioleylphosphatidylethanolamine), at a positive excess of charge of 1.25, by a factor of 3 to 5, by means of peptides.

Similarly, in WO 95/02698 it was proposed to use cationic lipids in conjunction with membrane-active peptides of coated viruses, influenza viruses *inter alia*, for the transfection of cells, using one of the peptides described by Kamata et.al., 1994, or a peptide derived from the glycoprotein of Vesicular Stomatitis Virus in combination with lipofectamine, a 3:1 mixture of the polycationic lipid 2,3-dioleyloxy-N-[2(spermincarboxamido)ethyl]-N,N-dimethyl-1-propanaminiumtrifluoroacetate (DOSPA) and DOPE and an improvement in the efficiency of transfection by a factor of up to 12 was achieved with a positive excess of charge of about 20, found to be the optimum level.

On the other hand, cationic lipids which were conjugated with viral peptides in order to give them the fusogenic or karyophilic properties of viruses produced disappointing results at the optimum lipid concentration (Remy et al., 1995).

The aim of the present invention was to provide a new gene transfer system based on cationic lipids.

In setting out to solve the problem, the question first arose as to whether it is the steps which follow the endocytosis, such as the transportation of DNA from the endosome into the cytoplasm and then on into the nucleus,

which constitute the stumbling block for successful gene transfer with cationic lipids.

It was found that the addition of membrane-active influenza peptides brings about only a slight increase in gene expression (1.5 to 5-fold) with Transfectam if the latter is used at the optimum concentrations, i.e with a high positive excess of charge. This leads one to conclude that, in this context, the stumbling block for the gene transfer is not the release from the endocytotic vesicles, which also accords with the results obtained by Kamata, et al., 1994 (see above).

The objective was achieved according to the invention by means of a composition for the transfection of higher eukaryotic cells, the composition containing a complex which contains a nucleic acid to be expressed in the cell and, in a suboptimum concentration for the transfection, one or more cationic lipids, optionally as well as helper lipid(s). The composition is characterised in that it contains one or more membrane-active acid peptides, the ratio of the total number of positive to the total number of negative charges in the composition being about 0 to about 3.

Preferably, the ratio of positive to negative charges in the composition is about 0 to about 2.

By "suboptimum concentration" is meant the quantity of cationic lipid at which the ratio of the positive charges of the cationic lipid to the negative charges of the nucleic acid is different from the ratio found to be the optimum ratio for the particular transfection, with the result that the efficiency of transfection achieved with cationic lipid, optionally with the addition of helper lipid, is less than that achieved under optimum conditions, the term "optimum" referring to the expression

of nucleic acid achieved by transfection (or, when using inhibiting RNA, to the extent of the biological effect intended) in the cell. The suboptimum concentration may be higher or lower than the optimal concentration.

The suboptimum concentration of cationic lipid, optionally mixed with helper lipid, preferably corresponds to a concentration at which the efficiency of transfection is lower than at the optimum concentration by a factor of at least about 2, preferably by a factor of about 5 to about 2,000.

The optimum concentration of cationic lipid for the particular transfection or the suboptimum concentration at which the efficiency of transfection reaches at most half the value it reaches at the optimum concentration, depends on the type of cell; it can be determined in each case by titration, by using the cationic lipid, optionally in admixture with helper lipid, in increasing (or decreasing) concentrations; appropriately, a reporter gene, e.g. a luciferase gene, is used to determine the efficiency of transfection.

Membrane-active peptides are defined by their ability to destabilise endosome membranes; they are also known as "endosomolytically active peptides", "endosome breaking peptides" or "fusogenic" peptides. These peptides have an amphipathic nature and are capable of forming α-helices; by virtue of their membrane-active properties they are suitable for use in gene transfer methods in which the release of the genetic material transported into the cell from the endosomes constitutes a limiting step, e.g. in the importing of nucleic acid into the cell by receptor-mediated endocytosis.

Suitable membrane-active peptides within the scope of the present invention are peptides of natural origin or synthetic peptides, e.g. the peptides published in WO 93/07283, by Plank et al. 1994, or by Zauner et al. 1995.

The question of whether peptides have suitable membrane-active properties and may therefore be considered for use within the scope of the present invention can be decided by means of assays which simulate the process which occurs in the cell during the breaking open of endosomes. Suitable assays are the liposome and erythrocyte permeability assays, which were described e.g. by Plank et al., 1994.

In a preferred embodiment the composition contains a peptide designated INF6 with the sequence GLF GAI AGFI ENGW EGMI DGWYG.

In another preferred embodiment the composition contains a peptide designated INF10 with the sequence GLF ELA EGLA ELGW EGLA EGWYGC.

In another preferred embodiment the composition contains a peptide designated INF5 with the sequence [GLF EAI EGFI ENGW EGnIDG]$_2$ K.

In another preferred embodiment the composition contains a peptide designated EGLA-I with the sequence GLFL GLA EGLA EGLA EGLA EGLA EGL EGLA GGSC.

In another preferred embodiment the composition contains a peptide designated INFA with the sequence GLF EAI EAFI ENAW EAMI DAWYG.

Other suitable peptides are synthetic peptides designated INF8 with the sequence [GLF EAI EGFI ENGF EGMI DGGG]$_2$ K; designated INF9 with the sequence GLF ELA EGLA ELGA EGLA

EGWYGC; designated EGLA-II with the sequence WEA GLA EGLA EGLA EGLA EGLA EGL EGLA GGSC; designated EGLA-III with the sequence GLF EGA EGLA EGA EGLA EGLA EGWY GAC and designated EGLA-IV with the sequence GLF EGA EGLA EGW EGLA EGLA EGWY GAC.

Other synthetic membrane-active peptides which are suitable within the scope of the present invention are those described by Plank et al., 1994, particularly peptides designated INF4, INF4DI and INF7.

In another embodiment of the invention the membrane-active peptide is modified with a lipid, e.g. with dipalmitoylphosphatidylethanolamyl (DPPE); the composition may also contain modified and unmodified peptide. If a lipid-modified peptide is used there is no need to add helper lipid.

Lipids which can be used for the modification of the membrane-active peptide are basically the same lipids which are also used as helper lipids; for practical reasons the lipid and the peptide are generally chosen particularly with regard to the method of coupling, on account of the presence of reactive groups. The coupling of the components is carried out according to methods known from the literature, e.g. as described by Martin et al., 1989, or Remy et al., 1995.

The quantity in which the optionally lipid-modified, membrane-active peptide is added to the transfection complex depends on the total positive charge on the lipid/DNA complex as well as on the sum of the negative charges of the peptide and its molecular weight. The relative amount in relation to the cationic lipid (equivalents, given in mol peptide/mol cationic peptide) or the absolute amount used, is calculated in each case using these parameters. (In the case of INF6, e.g. for

2 charge-equivalents of Transfectam, corresponding to 6 nmol, 5 μg INF6, corresponding to 2 nmol, were used). With regard to a ratio of positive to negative charges of about 0 to about 3, preferably about 0 to about 2, the quantity of peptide can first be varied in preliminary tests, optionally by comparison with other peptides, and in this way the optimum effective amount can be determined.

Within the scope of the present invention basically any mono- or polycationic lipid can be used, although polycationic lipids are generally preferred. Numerous cationic lipids which may be used as constituents of the composition according to the invention are known from the prior art. Examples of suitable cationic lipids may be found, e.g. in WO 95/02698, WO 91/16024, as well as in the publications of Remy et al., 1994; Solodin et al., 1995; Felgner et al., 1994; Ruysschaert et al., 1994; Weibel et al., 1995; Le Bole'h et al., 1995); the reader's attention is hereby drawn to the disclosures therein.

Particularly preferred cationic lipids are lipopolyamines, e.g. those described in EP-A1 394 111, particularly DOGS (dioctadecylamidoglyclyspermin), obtainable under the brand name "Transfectam".

The cationic lipid, optionally in admixture with helper lipid, is present in the transfection complexes in a suboptimum amount, as stated above, i.e. the ratio between the positive charges of the lipid and the negative charges of the nucleic acid is greater or smaller than the ratio used for the optimum gene transfer efficiency, whilst if helper lipid is present its effect on the efficiency of transfection is also taken into consideration for the definition of the suboptimum amount of cationic lipid: for example, a helper lipid can improve the effect of cationic lipid at a concentration which would have only suboptimum

efficiency with no helper lipid, to such an extent that the transfection achieved corresponds to that obtained at the optimum lipid concentration; the total concentration of the cationic lipid/helper lipid partners would thus be optimum over all in this case, in spite of a suboptimum concentration of cationic lipid on its own.

The "helper lipids" are neutral lipids (of natural origin or synthetic), which are zwitterionic or free from charges under physiological conditions, e.g. cholesterol, dioleylphosphatidylethanolamine (DOPE), oleoyl-palmitoylphosphatidylethanolamine (POPE), phosphatidylglycerol, diacylglycerol, etc. Other examples of suitable helper lipids include *inter alia* those described in WO 95/18863 and in WO 95/02698; reference is hereby made to the disclosures therein.

According to the invention the composition may contain one or more helper lipids.

The preferred helper lipids are the lipids DOPE, POPE, DOG (1.2-di-oleoyl-rac-glycerol), MOG (1-mono-oleoyl-rac-glycerol), EPC (eiphosphatidylcholine), EPE (eiphosphatidylethanolamine).

Preferably, the helper lipids are used in a concentration, based on the cationic lipid, of from 0.1 to 10 equivalents (mol/mol).

The nucleic acids to be transported into the cell may be DNAs or RNAs, with no restrictions on the nucleotide sequence. For gene therapy the DNA preferably consists primarily of genes which are locked into the cell in order to express therapeutically active gene products which are not expressed or not expressed in sufficiently high concentrations in the cell. For therapeutic purposes nucleic acids with an inhibitory effect, e.g. antisense-

RNA molecules or ribozymes or the DNA molecules coding for them, may be considered. Examples of nucleic acids which may be used within the scope of the present invention are given e.g. in WO 93/07283 and WO 95/18863.

According to another aspect the invention relates to a process for the transfection of higher eukaryotic cells, which is characterised in that the cells are brought into contact with the composition according to the invention.

The process can be used for transfections *in vitro*, *ex vivo* or *in vivo*, preferably for the transfection of mammalian cells. *In vitro* the process is used particularly on cell cultures (adherent or in suspension). Uses *ex vivo* are gene-therapy applications in which cells are taken from the body to be treated and transfected *ex corpore* with a therapeutically effective molecule of nucleic acid, to be reintroduced into the body afterwards, where the gene product is expressed and develops its therapeutic effect. An example of an *ex vivo* application is the preparation of tumour vaccines from autologous cells which are transfected with a cytokine gene.

For applications *in vivo* the composition according to the invention is administered to the body in the form of a pharmaceutical preparation, to which the present invention also relates, preferably intravenously or, when treating tumoral diseases, intratumorally.

For the pharmaceutical preparation, pharmaceutically acceptable additives as well as inert carriers, e.g. saline or phosphate-buffered saline or any carrier in which the compositions are soluble, may be added to the composition according to the invention. For the formulation of pharmaceutical preparations, reference is hereby made to Remington's Pharmaceutical Sciences, 1980.

The composition of the present invention can be modified in order to be used with other anionic molecules as a nucleic acid, e.g. for conveying anionic proteins into higher eukaryotic cells.

Within the scope of the present invention it was found, surprisingly, that the efficiency of transfection of the cationic lipid Transfectam (Behr, et al., 1989), can be improved significantly by means of membrane-active acid peptides when it is used at a low positive excess of charge (1.5 and 2 charge-equivalents), i.e. at a suboptimum concentration.

The improvement was up to 1,000-fold in the case of the peptide INF6 (2 µg/1.5 charge-equivalents Transfectam), which is approaching the level of gene expression obtained with optimum amounts of Transfectam (high positive excess of charge). The increase in gene expression with membrane-active peptides was observed in several types of cell. Of the tested membrane-active peptides only the acidic ones gave good results in conjunction with Transfectam. This finding accords with the hypothesis that, for an improved release of DNA from the endosomes the peptides have to be located in the same endocytotic vesicles as the lipid/DNA complexes, which is guaranteed when the peptides are ionically bound to the transfection particles. When 2 charge-equivalents of Transfectam were used the order of performance of the peptides was INF6>INF10> EGLA-I≥INF5>INFA>Melittin. These results obtained with Transfectam for the peptides investigated differ from the findings obtained when the peptides were used in a transfection system based on receptor-mediated endocytosis as a part of transfection complexes containing transferrin-polylysine: in this system INF5 was the best peptide and about 50 times more effective than INF6,

whereas in conjunction with Transfectam it is 10 times less effective than INF6. The peptide INF5 has a membrane-breaching effect only at acid pH levels, while INF6 is also membrane-active at neutral pH values. In the lipid-free gene transfer system based on polylysine-conjugated cell ligands the activity of the peptide was accompanied by toxic side-effects at neutral pH values which were not observed when the peptide was used in the lipid system. The synthetic peptide EGLA-I as well as INF10 proved to be more suitable as additives to lipid/DNA complexes than in polylysine-containing complexes.

It was also found that the effect of the tested peptides on the efficiency of transfection of cationic lipids at a suboptimum charge ratio was of the same order of magnitude as the effect of helper lipids (Remy, et al., 1995; Zhou, et al., 1994; Felgner, et al., 1994; Leventis, et al., 1990).

The transfection complexes, containing cationic lipid and membrane-active peptide, can be prepared by various methods. In the tests according to the present invention the compositions were prepared by three different methods, which differed in the order of adding or combining the components of the complex as well as in the time at which dilution was carried out. In the first variant the DNA was added after the peptide had been combined with Transfectam; in the second variant, first of all Transfectam was complexed with DNA and then the peptide was added. In the third variant the peptide was added after dilution of the Transfectam/DNA complex. An investigation was carried out to discover whether the method of preparing the transfection complex influenced the degree of increase in the efficiency of transfection. It was found that the method of preparation affected the various peptides differently: for INF6 the three methods of preparation were equal. In the case of INF5 the

transfection complexes produced according to the second and third methods yielded better results, whereas in the case of INF10 the first and third variant were superior to the second.

Irrespective of the method of production, the combining of the mutant influenza peptides, which have an overall negative charge, with cationic lipids changes the state of charge of the transfection particles, some of these peptide-containing complexes being close to electroneutrality, since the mutant influenza peptides have an overall negative charge, (see insert in Fig. 1).

In the experiments in the present invention, highly active DNA complexes which contained the helper lipid DOPE at low charge (2 charge-equivalents Transfectam) (Remy, et al., 1995), were also compared with the complexes according to the invention containing a membrane-active peptide with regard to their efficiency of transfection. Since the increase brought about by DOPE had been traced back to its fusogenic activity (Allen, et al., 1990; Litzinger and Huang, 1992), the FACS data obtained within the scope of the present invention (Fig. 3) lead one to conclude that the helper lipid has another important effect: the cell association of the transfection complex is significantly more marked when 1.5 equivalents DOPE are added to 2 charge-equivalents Transfectam than when the lipospermin is used on its own. It was apparent that the membrane-active peptide did not cause any increase in the optimally effective complex containing the helper lipid, whereas when Transfectam was used in excess (6 charge-equivalents), i.e. when a suboptimum amount was used, there was a significant increase in gene expression compared with the values obtained when using optimum amounts of Transfectam alone.

It was also found that the membrane-active peptide reduces the sensitivity of the transfection complex to serum, which is important in connection with its use *in vivo*.

Summary of Figures

Fig. 1: Efficiency of transfection of Transfectam/DNA/INF6 complexes

Fig. 2: Effect of serum on the efficiency of transfection

Fig. 3: A: Throughflow cytometric analysis
B: Gene expression

Fig. 4: Effect of various membrane-active peptides on the efficiency of transfection

Fig. 5: Influence of the method of production of transfection complexes containing membrane-active peptide on the efficiency of transfection

Fig. 6: Efficiency of complexes containing membrane-active peptide during the transfection of various cell lines

Fig. 7: Influence of bafilomycin A1 on the efficiency of transfection

Fig. 8: Influence of helper lipids on the efficiency of transfection

Fig. 9: Influence of the combination of Transfectam, helper lipid and membrane-active peptide on the efficiency of transfection

Fig. 10: Effect of a lipid-modified membrane-active peptide on the efficiency of transfection

In the Examples which follow, unless stated otherwise, the following materials and procedures were used:

a) reporter gene plasmid pCMV-Luc

The construction of the plasmid pCMV-Luc, which carries the luciferase gene under the control of the CMV-promoter/enhancer, is described in WO 93/07283 under the name pCMVL.

b) Various reagents

The lipopolyamine DOGS (dioctadecylamidoglyclyspermin) with the brand name "Transfectam" was obtained from Promega, the helper lipids DOPE (1.2-dioleoyl-sn-glycero-3-phosphoethanolamine), MOG (1-mono-oleoyl-rac-glycerol), DOG (1.2-di-oleoyl-rac-glycerol), EPE (eiphosphatidylethanolamine), EPC (eiphosphatidylcholine), as well as chloroquine, bafilomycin A1 and melittin (from bee venom) were obtained from Sigma.

c) Peptide synthesis

The names of the peptides, their origins and their sequences are given in the Table.

The peptides designated INF5 and INF6 were synthesised as described by Plank, et al., 1994.

The peptides INFA, INF10 and EGLA-I were synthesised using the Fmoc strategy (N-(9-fluorenyl)methoxycarbonyl) with HBTU activation [O-(1H-benzotriazol-1-yl)-N,N,N',N'-tetramethyluroniumhexafluorophosphate], (Fastmoc™ - 0.25 mmol standard) on an Applied Biosystems Peptide

synthesizer Model 433A (Foster City, California) with feedback monitoring. Three deprotection steps were carried out per cycle. When the feedback monitoring showed that the Fmoc deprotection was not quantitative, double coupling of the next amino acid and blocking of the terminal NH_2 groups (using acetic anhydride) were carried out in the following step. The following amino acid protecting groups were used: (Trt)Asn, (Trt)Cys, (t-Bu)Cys, (t-Bu)Asp, (t-Bu)Glu, (Boc)Lys. A mixture of 70 % NMP/30 % DMF was used as solvent.

For EGLA-I an HMP resin was used (Tentagel R PHB, 0.22 mmol/g, Rapp Polymere). The peptides were synthesised together apart from the group Gly-30, then half the quantity of resin was used to synthesise EGLA-I.

The peptides INF10 and INFA were synthesised on a Cys(Trt)-precharged aminomethylated polystyrene resin with a p-carboxytrityl chloride linker (0.52 mmol/g; PepChem, Tübingen, Germany) using DMF as solvent.

The peptide designated INF7dimer (a dimer of the peptide INF7 descried by Plank et al., 1994) was synthesised using the Fmoc strategy on an Applied Biosystems Peptide synthesizer Model 431. A resin precharged with cysteine is used (Tentagel S PHB-Cys). The centrally positioned Lys DiFmoc- was coupled as the first amino acid. Then double couplings were made up to Ile-18. Single couplings were carried out from Met-17 to Ile-10; at Phe-9 there was a single coupling, then an additional coupling with 1% Triton in the coupling mixture. Single couplings were made from Gly-8 onwards.

The deprotection of the peptides and the cleaving from the resin were carried out with a mixture of phenol, ethanedithiol, thioanisol, water and trifluoroacetic acid (0.75:0.25:0.5:0.5:10). The crude peptides were

precipitated by adding dropwise to ether and were then centrifuged. The peptides thus obtained were washed three times with ether and subsequently dried under an argon current, followed by a high vacuum. The crude peptides were dissolved in 1 M TEAB, pH 9 and 1 % β-mercaptoethanol.

The purity of the peptides was determined by analytical reversed-phase HPLC using a C-18 column (Vydac 218ATP54, 2.1 mm x 25 cm, 5 µm). A binary solvent system (solvent A: aqueous 0.1 % trifluoroacetic acid; solvent B: acetonitrile, containing 0.1 % trifluoroacetic acid) was used at a gradient of 0 - 100 % in 45 minutes at a flow rate of 1 ml/min. Analytical ion exchange chromatography (SuperQ-Toyopearl 650, TosoHaas, 5 mm x 50 mm column) was carried out using a salt gradient (20 mM HEPES, pH 7.3, 0 - 1.5 M NaCl in 60 min, flow rate 0.5 ml/min).

The peptide EGLA-I was subjected to gel filtration (Sephadex G10, 20 mM TEAA, pH 7.3). The purified peptide fraction was freeze-dried in a Speedvac (Savant). The analytical reversed phase chromatography showed a purity of about 95 %.

The solutions of the crude peptides INF10 and INFA were fractionated by gel filtration on Sephadex G10 (10 mm x 300 mm column) in 20 mM TEAA, pH 7.3. The peptides were lyophilised; the analytical reversed phase chromatography yielded a purity of about 98 or 95 %.

The peptide INF7dimer was purified over Sephadex G 10 (buffer HBS; column 10mm x 300mm). Then ion exchange chromatography was carried out (column: (10mm x 100mm); equipment: Toso Haas Super Q 650 S; throughflow rate: 0.5 ml/min; eluant: A: 20 mM Hepes 7.3, B: 3 M NaCl/20 mM Hepes 7.3; gradient: 0 - 40 min 0% B, 40 -

140 min 100% B, i.e. 1%/min). The peptide eluted at 70 - 80 min. The peptide purified using an ion exchanger was purified again using a Sephadex G 10 column (10mm x 300mm) (buffer HBS/50% glycerol).

The identity of the peptides was determined as described by Plank, et al., 1994. The peptides were frozen in liquid nitrogen.

The Liposome Leakage Assay was carried out as described by Plank et al., 1994.

d) Cell culture and media

The media as well as foetal calf serum (FCS) and equine serum were obtained from Gibco-BRL. The culture media were supplemented with 2 mM L-glutamine and antibiotics. The cells used were primary human melanoma cells designated H225, murine embryo liver cells of the cell line TIB-73 (ATCC BNL CL.2), human lung carcinoma cells of the cell line A549 (ATCC CCL 185) and cells of the murine melanoma cell line Cloudman S91, clone M3 (ATCC No. CCL 53.1). The H225-cells were grown in RPMI 1640/10 % FCS/1 mM sodium pyruvate, the A549-cells in DMEM/10 % FCS, the BNL CL.2-cells in high glucose-DMEM/10 % FCS and the M3-cells in Ham's F10-Medium/15 % equine serum/5 % FCS.

e) Transfection of the cells

i) Preparation of cationic lipid/DNA complexes

The complexes were prepared as described by Barthel, et al., 1993, by dissolving 3 μg of plasmid DNA and the appropriate amount of Transfectam in 75 μl of 150 mM NaCl. After 10 to 20 min the two solutions were combined. After another 10 min the mixture was diluted with serum-free medium to a total volume of 2 ml.

The term "charge equivalent" specifies the amount of cationic lipid used for a transfection; 1 charge equivalent corresponds to the amount required to neutralise all the negative charges of the phosphate groups of the plasmid. 3 μg DNA correspond to 9 nmol of negative charges; when calculating the charge ratio it was borne in mind that 1 mol of Transfectam has 3 mol of positive charges which originate from the three ammonium groups protonated at the physiological pH value. Starting from a molar weight of about 1250 this means that, when 3 μg of DNA are used, 3 μl of a 2mM Transfectam solution containing 6nmol (=7.58 μg) Transfectam are required for a doubly-positive excess of charge (2 charge-equivalents Transfectam/3 μg DNA).

The peptides were added in an amount of 0.5 or 1 mg/ml of solution in HBS (HEPES-buffered saline, containing 150 mM NaCl, 20 mM HEPES pH 7.3) to the Transfectam/DNA complexes. After a 10 to 20 minute maturation period the transfection volume was made up to 2 ml with culture medium, and 1 ml of the transfection mixture obtained was pipetted onto the cells.

The helper lipids DOPE, DOG, EPC, EPE or MOG or cholesterol were diluted in ethanol containing a trace of dichloromethane. The Transfectam/helper lipid/DNA complexes were formed by mixing the appropriate amounts of Transfectam/helper lipid (the amount of helper lipid used, based on the Transfectam, is given as a molar ratio) before diluting with the DNA solution.

ii) Transfection and luciferase determination

50,000 to 75,000 cells per well of a 24 well plate were plated out one day before the transfection, the transfection volume being 1 ml in all the experiments. The

transfection was carried out with 1 ml of the complex prepared in i). After 3 to 4 h the transfection medium was replaced with fresh medium containing 10 % FCS. Unless otherwise stated, each experiment was carried out at least twice; the quantities of peptide shown in the Figures in these cases indicate the total amount used for the double measurement.

24 h after transfection the cells were harvested and taken up in 150 - 200 µl of 250 mM Tris, pH 7.3, 0.5 % Triton X-100. The cell lysate was then transferred into 1.5 ml Eppendorf tubes and centrifuged for 5 min at 14,000 g in order to pellet the cell debris. The luciferase activity was determined as described in WO 93/7283, the luciferase light units being determined from one aliquot of the supernatant (20 µl) with 10 sec integration after the injection of freshly prepared luciferin solution. The luciferase background (150 - 250 light units) was subtracted from each value; the efficiency of transfection was expressed as the total light units which represent the average of double measurements. The protein content was quantitatively determined using the Bradford assay (Bio-Rad).

f) Throughflow cytometry

For the throughflow cytometry the plasmid DNA was incubated with the intercalating fluorescent dye YOYO-1, (described by Rye, et al., 1992; Hirons, et al., 1994, obtainable from Molecular Probes; about 1 molecule of dye per 300 bp), then the complexes were prepared as described above. The complexes were added to 300,000 H225 cells per well of a 6 well plate, then the plate was incubated for 4 h either at 4°C (cell surface association) or at 37°C (cell surface association and absorption into the cells). The cells were then washed twice with cold PBS and

harvested with 1 mM EDTA in PBS and analysed on an FACScan apparatus (Becton Dickinson).

Example 1

Efficiency of transfection of Transfectam/DNA/INF6 complexes

Complexes were prepared from 1, 1.5, 2 or 4 charge-equivalents of Transfectam per 3 µg of pCMV-Luc combined with increasing amounts of the membrane-active acid peptide INF6 originating from the influenza virus and then mixed with RPMI 1640 culture medium. The resulting complexes were placed on H225 cells (75,000 cells per well of a 24 well plate). After 4 h the transfection medium was replaced by fresh RPMI medium containing 10% FCS. The results of the experiments are shown in Fig. 1 (dotted line with rhombi: 1 equivalent (eq.) Transfectam /x µg INF6; solid line with circles: Transfectam 1.5 eq./x µg INF6; broken line with squares: Transfectam 2 eq./x µg INF6; solid line with triangles: Transfectam 4 eq./x µg INF6). It was found that, when 2 or 1.5 charge-equivalents lipid were used, there was a 100- to 1,000-fold increase in the efficiency of transfection, measured as luciferase activity. When the conditions used were already optimum (4 charge-equivalents of cationic lipid) there was only a slight increase (about 1.5- to 5-fold). Since the peptide INF6 has four negative charges at pH 7, it is able to associate with the cationic lipid/DNA particles by means of electrostatic interactions. When neutral particles were used (1 charge equivalent) no increase was observed, presumably because only a few peptides can bind to these complexes. Interactions with the cell membrane might even be prevented because positive residual charges are masked by the association with the peptide. The insert in Fig. 1 shows the efficiency of transfection of the

Transfectam/DNA/INF6 complexes, plotted against the theoretical charge ratio of the complexes. On the assumption that nearly the entire peptide binds to the transfecting particles, a high luciferase expression is obtained with electroneutral (or nearly neutral) lipid vectors.

Example 2

Effect of serum on the efficiency of transfection

Transfections of H225 cells were carried out using the quantities of Transfectam (in charge-equivalents) specified in Fig. 2 and the peptide INF6. The transfections were performed on the one hand without any serum (empty bars), and on the other hand in the presence of 10% (dotted bats) or 20% (grey bars) of non-heat-inactivated FCS (Fig. 2). It was found that the peptide-containing complexes had lower sensitivity to serum.

Example 3

Association of Transfectam/DNA complexes and expression rate

a) Throughflow cytometry analysis of transfection complexes

The analysis was carried out with H225 cells as described in the method section. As shown in Fig. 3A, the association of the Transfectam/DNA complexes with the cell surface changes critically with the charge ratio: with 2 charge-equivalents a heterogeneous cell population was found, whereas with 4 charge-equivalents a conventional Gaussian curve was found. The addition of membrane-active peptides to 2 (or 4) charge-equivalents of Transfectam did

not bring any major change to the curve profile either at 4°C or at 37°C.

b) Gene expression

In parallel to the tests carried out in a), the gene expression of 300,000 cells was measured after incubation at 4°C and 37°C with different transfection complexes (the cells were incubated at 4°C or at 37°C, washed twice with PBS after 4 h, mixed with fresh medium containing 10% FCS and incubated for 20 h at 37°C and at 5% CO_2). The presence of peptide (1.5 µg/well; in this experiment a single measurement was taken) brought about a 15-fold increase in expression with the mixture at 4°C and a 100-fold increase in expression with the mixture at 37°C. (Fig. 3B. Empty bars: gene expression after incubation at 4°C; filled-in bars: gene expression after incubation at 37°C).

Example 4

Effect of different peptides

As described in the method section, Transfectam/DNA complexes were prepared from 2 charge-equivalents of Transfectam per 3 µg DNA. For some experiments the peptides specified in Fig. 4 were added to the complexes in the quantities stated. The peptides INF6, INFA and Melittin, which have a good haemolytic activity, without having a marked specificity for low pH values, exhibited different characteristics: INFA and INF6 increased the efficiency of transfection of 2 charge-equivalents of Transfectam by a factor of 10 or 200, whereas Melittin increased the luciferase expression only slightly. Moreover, Melittin was highly toxic in an amount of 5 µg/double measurement. The influenza peptide mutants

INF5 and INF10, which were shown in the liposome leakage test to release calcein efficiently at pH 5.0 (Plank, et al., 1994) gave better results than INFA, but were less effective than INF6. In addition, a peptide designated EGLA-I was tested, which is not derived from the HA2 sequence of the influenza virus, in which one of the alanine groups in the GALA repeat (Parente, et al., 1988) was replaced by a glycine group. This peptide gave just as good results as INF5.

Example 5

Influence of the method of preparation of Transfectam/DNA/peptide complexes on the efficiency of transfection of H225-cells

Complexes were prepared in three different ways (Fig. 5):

a) Combining peptide and lipid before complexing with DNA (dotted bars)

b) Adding the peptide after forming the Transfectam/DNA complex before diluting with medium (150 µl volume; grey bars)

c) Adding the peptide after diluting the complexes with the culture medium (2 ml volume; black bars)

As shown by the results in Fig. 5, the influence of the method of preparation on the gene expression rate depends on the peptide sequence. Combining the peptide with Transfectam before complexing with the plasmid made the peptide behave in the opposite way: whereas the gene expression rate remained unchanged with INF6, with INF10 the performance of the complexes thus produced was five times better than that of the complexes obtained by the standard method (see method section). By contrast, with INF5 this method of complex production led to a sharp reduction in gene expression.

Example 6

Effect of the peptides on the efficiency of transfection of various cell lines

The fact that the effect of the peptides on gene transfer by means of lipids is not restricted to specific cell types but is a general phenomenon became apparent from experiments with cells of different cell lines shown in Fig. 6 (H225 cells: empty bars; BNL CL.2-cells: dotted bars; A549-cells: light-grey bars; M3-cells: dark-grey bars). Complexes were prepared from Transfectam, pCMV-Luc 5 μg INF6 and the efficiency of the complexes on the cells was tested. (As a comparison Transfectam was used in an excess of charge of 4 charge-equivalents, although it does not give the highest values on all the cells tested.) For all the cell lines, increases in the efficiency of transfection were found when the transfection complexes contained a membrane-active peptide.

Example 7

Investigating the influence of bafilomycin A1 on the efficiency of transfection of Transfectam/DNA complexes

The presence of the specific inhibitor of the vacuole proton pump bafilomycin A_1 (Bowman, et al., 1988; Yoshimori, et al., 1991) in a concentration of 200 nM in the transfection of H225 cells (2 charge-equivalents of Transfectam or 2 charge-equivalents of Transfectam plus INF10 or INF6 were used; after 4 h incubation the medium was replaced by fresh medium containing 10 % FCS) reduced the gene expression by a factor of 7, 5 and 1.5, respectively (Fig. 7. Empty bars: no bafilomycin A1 added.

Dotted bars: in the presence of 200 nM of bafilomycin A1). When 4 charge-equivalents of Transfectam were used Bafilomycin could not bring about a reduction in gene transfer.

Example 8

Investigation of the influence of helper lipids on the efficiency of Transfectam/DNA complexes

a) Increasing the effect of Transfectam by means of helper lipids

The helper lipids specified in Fig. 8 were used in a molar ratio of 2 charge-equivalents of Transfectam to 1 to 3 equivalents of helper lipid. This led to a 10- to 20-fold higher gene expression for DOPE, EPE or DOG (see also Fig. 3B). MOG increased the expression by a factor of about 4, whereas EPC and cholesterol could not increase the efficiency of transfection onto H225 cells.

A further increase, up to 7-fold, could be achieved if 1 mol/% DOG was mixed with the Transfectam/DOPE formulation; this can presumably be put down to the ability of DOG to induce fusion, *inter alia* (Siegel, et al., 1989).

Throughflow cytometry was carried out with transfection complexes containing the helper lipid DOPE (1.5 mol per 2 mol Transfectam; i.e. 9 nmol DOPE, corresponding to 6.7 µg), as described in Example 3 a). There was a distinct change in the FACScan profile (Fig. 3A): the cell population is more homogeneous and the cell association (as well as the uptake into the cells at 37°C, not shown in the Figure) is more intensive than without helper

lipid. When the luciferase activity was measured 24 h later in an experiment carried out at 4°C with 2 charge-equivalents Transfectam and 1.5 equivalents DOPE, it was apparent that the DOPE-containing complexes were 8 and 280 times better, respectively, than 4 and 2 charge-equivalents of the cationic lipid alone (Fig. 3B). When the experiment was carried out at 37°C, the differences in the luciferase expression were 2-fold and 640-fold, respectively.

b) Increasing the effect of Transfectam by means of helper lipids and membrane-active peptides

After the preparation of Transfectam/DNA/DOPE complexes increasing amounts of INF10 were added. After a maturation period of 10 - 20 min, serum-free culture medium was added to make up a total volume of 2 ml; 1 ml of the transfection mixture was applied to each well for the double measurement. The result of these tests is shown in Fig. 9 (empty bars: Transfectam, dotted bars: Transfectam 6 eq.Transfectam/DOPE 0.36 eq., filled-in bars: Transfectam 6 eq.Transfectam/DOPE 0.36 eq./x μg INF10). The presence of membrane-active peptide did not result in any further increase, in the case of complexes with a high efficiency (Transfectam 2 eq./INF10 3 μg, 5 μg or 7.5 μg/1.5 eq. DOPE) (not shown in Fig. 9). However, when the peptide was used together with an excess of Transfectam (6 charge-equivalents), a significant increase in gene expression could be obtained compared with the values obtained with optimum amounts of Transfectam/DOPE; the values with 6 eq.Transfectam/0.36 eq. DOPE/10 μg INF10 were 6x better than those with 2 eq.Transfectam/1.5 eq. DOPE/7.5 μg INF10.

Example 9

Effect of a lipid-modified membrane-active peptide on the efficiency of transfection

a) Preparation of modified peptide (DPPE-INF7dimer)

The peptide designated INF7dimer (cf. Table), a dimer of the peptide INF7 described by Plank, et al., 1994, was coupled to the lipid derivative DPPE-(dipalmitoylphosphatidylethanolamyl)bromoacetamide (hexadecanoic acid-3-((2-(2-(2-(2-(2-(2-bromoacethylamino)ethoxy)ethoxy)ethoxy)-acetylamino)ethoxy)hydroxyphosphoryloxy)-2-hexadecanoyloxypropylester) by adding 0.95 equivalents of peptide (80 nmol; about 450 μg) to the lipid diluted in 1.3ml of diethanolamine buffer (pH 9.5/ethanol; 9/1; v/v). After 3 h at ambient temperature an Ellman test was carried out for quantitative determination of the thiol groups. Then β-mercaptoethanol was added in order to destroy the residual bromoacetamide group. The lipid-modified peptide obtained was used without further purification.

b) Transfection of BNL CL.2 cells

1.5 eq. Transfectam were diluted in 75 μl 0.15 M NaCl, mixed thoroughly, then the quantities of DPPE-INF7dimer specified in Fig. 10 were added (the values given in the Fig. are mol%, based on the total amount of Transfectam). This was then mixed thoroughly, after 10 min 3 μg DNA in 75 μl NaCl were added, the mixture was mixed again and after another 10 min topped up with medium to a total transfection volume of 2 ml. Of the transfection composition obtained, 1 ml per well was added to the cells. The results given in Fig. 10 show that the greatest

increase was obtained with a content of 7.5 mol% of DPPE-INF7dimer.

Table

Sequences and origin of the membrane-active peptides

Peptide	Origin	Sequence
melittin	Bee venom	GIGAVLKVLTTGLPALISWIKRKRQQ
INF6	Influenza virus	GLF GAI AGFI ENGW EGMI DGWYG
INF5	Influenza virus, acid mutant	[GLF EAI EGFI ENGW EGnIDG]$_2$K
INF10	Influenza virus, EGLA hybrid	GLF ELA EGLA ELGW EGLA EGWYGC
INFA	Influenza virus, acid alanine mutant	GLF EAI EAFI ENAW EAMI DAWYG
EGLA-I	Synthetic	GLFL GLA EGLA EGLA EGLA EGLA EGL EGLA GGSC
INF7dimer	Influenza virus, acid mutant	[GLF EAI EGFI ENGW EGMI DGWYG]$_2$ KC
INF8	Influenza virus, acid phenylalanine mutant	[GLF EAI EGFI ENGF EGMI DGGG]$_2$K
INF9	Synthetic	GLF ELA EGLA ELGA EGLA EGWYGC
EGLA-II	Synthetic	WEA GLA EGLA EGLA EGLA EGLA EGL EGLA GGSC
EGLA-III	Synthetic	GLF EGA EGLA EGA EGLA EGLA EGWY GAC
EGLA-IV	Synthetic	GLF EGA EGLA EGW EGLA EGLA EGWY GAC

n: norleucine

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Patent Claims

1. Composition for the transfection of higher eukaryotic cells, wherein the composition contains a complex which contains a nucleic acid to be expressed in the cell as well as one or more cationic lipids, in a concentration which is suboptimal for the transfection, optionally as well as helper lipid(s), characterised in that the composition contains one or more membrane-active acid peptides, the ratio of the total number of positive to the total number of negative charges in the composition being about 0 to about 3.

2. Composition according to claim 1, characterised in that the ratio of the positive to the negative charges of the composition is about 0 to about 2.

3. Composition according to claim 1, characterised in that the suboptimal concentration of cationic lipid, optionally in admixture with helper lipid, corresponds to a concentration at which the efficiency of transfection is lower than at the optimum concentration by a factor of at least about 2.

4. Composition according to one of claims 1 to 3, characterised in that it contains a peptide designated INF6 with the sequence GLF GAI AGFI ENGW EGMI DGWYG.

5. Composition according to one of claims 1 to 3, characterised in that it contains a peptide designated INF10 with the sequence GLF ELA EGLA ELGW EGLA EGWYGC.

6. Composition according to one of claims 1 to 3, characterised in that it contains a peptide designated INF5 with the sequence [GLF EAI EGFI ENGW EGnIDG]$_2$ K.

7. Composition according to one of claims 1 to 3, characterised in that it contains a peptide designated EGLA-I with the sequence GLFL GLA EGLA EGLA EGLA EGLA EGL EGLA GGSC.

8. Composition according to one of claims 1 to 3, characterised in that it contains a peptide designated INFA with the sequence GLF EAI EAFI ENAW EAMI DAWYG.

9. Composition according to one of claims 1 to 3, characterised in that it contains a peptide designated INF8 with the sequence [GLF EAI EGFI ENGF EGMI DGGG]$_2$ K.

10. Composition according to one of claims 1 to 3, characterised in that it contains a peptide designated INF9 with the sequence GLF ELA EGLA ELGA EGLA EGWYGC.

11. Composition according to one of claims 1 to 3, characterised in that it contains a peptide designated EGLA-II with the sequence WEA GLA EGLA EGLA EGLA EGLA EGL EGLA GGSC.

12. Composition according to one of claims 1 to 3, characterised in that it contains a peptide designated EGLA-III with the sequence GLF EGA EGLA EGA EGLA EGLA EGWY GAC.

13. Composition according to one of claims 1 to 3, characterised in that it contains a peptide designated EGLA-IV with the sequence GLF EGA EGLA EGW EGLA EGLA EGWY GAC.

14. Composition according to one of claims 1 to 3, characterised in that it contains a peptide designated INF7dimer with the sequence [GLF EAI EGFI ENGW EGMI DGWYG]$_2$ KC.

15. Composition according to one of the preceding claims, characterised in that the membrane-active peptide is modified with a lipid.

16. Composition according to claim 15, characterised in that the peptide is modified with dipalmitoylphosphatidylethanolamyl (DPPE).

17. Composition according to one of the preceding claims, characterised in that the cationic lipid is a lipopolyamine.

18. Composition according to claim 17, characterised in that the lipopolyamine is dioctadecylamidoglyclyspermin (DOGS).

19. Composition according to one of the preceding claims, characterised in that it contains one or more helper lipids, selected from the group comprising phosphatidylethanolamine, phosphatidylglycerine and diacylglycerine.

20. Composition according to claim 19, characterised in that it contains dioleylphosphatidylethanolamine (DOPE) as helper lipid.

21. Composition according to claim 19, characterised in that it contains oleoyl-palmitoylphosphatidylethanolamine (POPE) as helper lipid.

22. Composition according to claim 19, characterised in that it contains 1-mono-oleoyl-rac-glycerol (MOG) as helper lipid.

23. Composition according to claim 19, characterised in that it contains 1.2-di-oleoyl-rac-glycerol (DOG) as helper lipid.

24. Composition according to claim 19, characterised in that it contains eiphosphatidylcholine (EPC) as helper lipid.

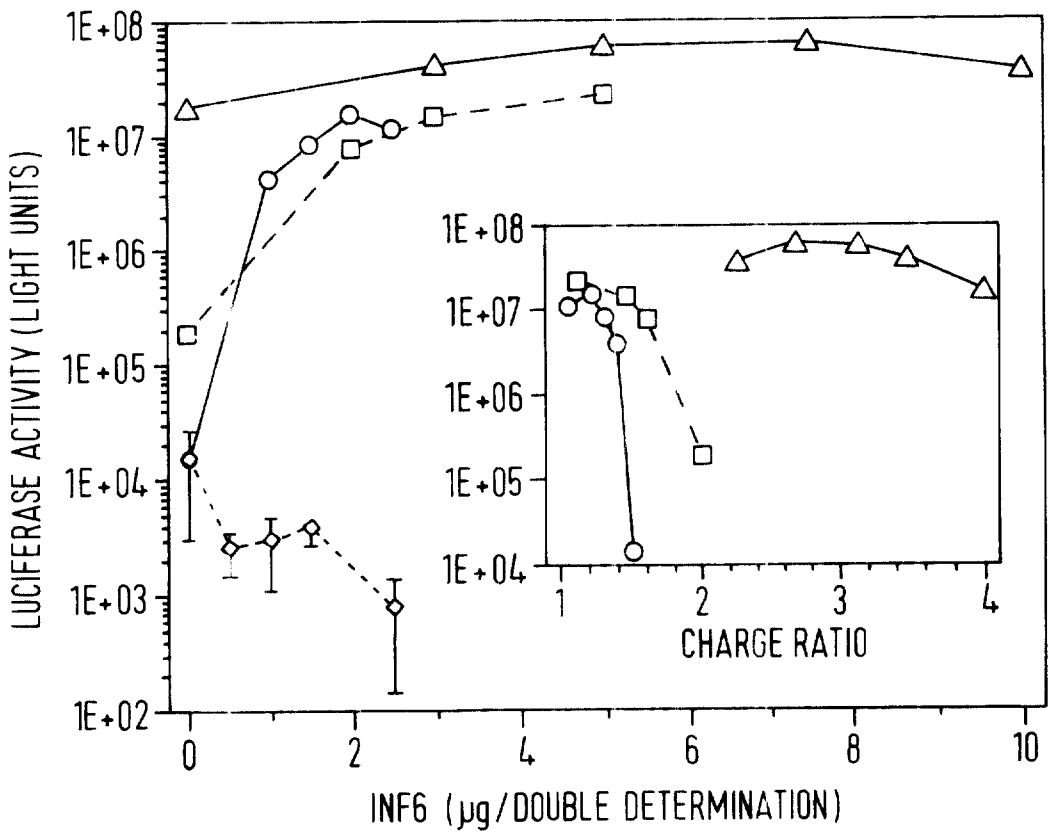
25. Composition according to claim 19, characterised in that it contains eiphosphatidylethanolamine (EPE) as helper lipid.

26. Composition according to claim 19, characterised in that it contains cholesterol as helper lipid.

27. Pharmaceutical preparation containing as active component a composition according to one of claims 1 to 25.

28. Process for transfecting higher eukaryotic cells, characterised in that the cells are brought into contact with a composition according to one of claims 1 to 26.

29. Use of the process according to claim 28 on mammalian cells *in vitro* or *ex vivo*.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1E+07
1E+06
1E+05
1E+04
1E+03
1E+02
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4
6
8
10
INF6 (µg/DOUBLE DETERMINATION)
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1E+06
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1E+04
1
2
3
4
CHARGE RATIO

FIG. 1

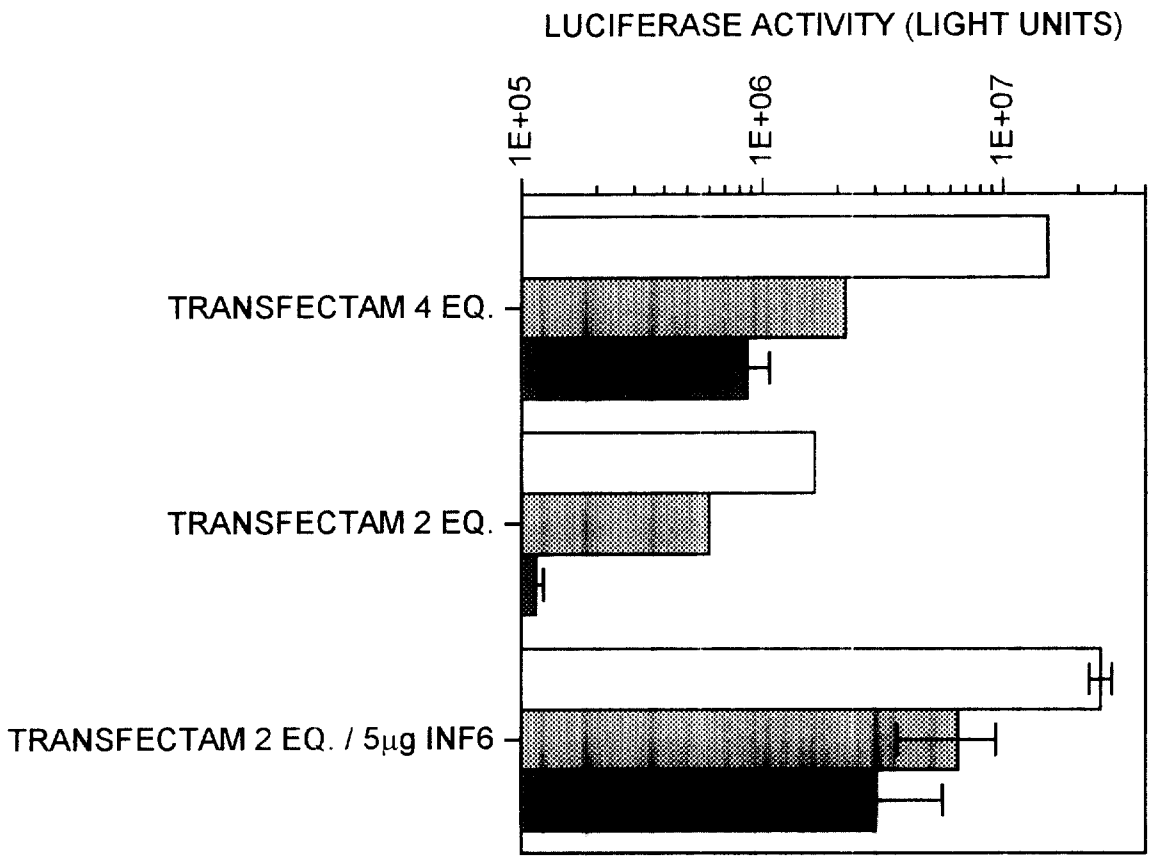
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FIG. 2

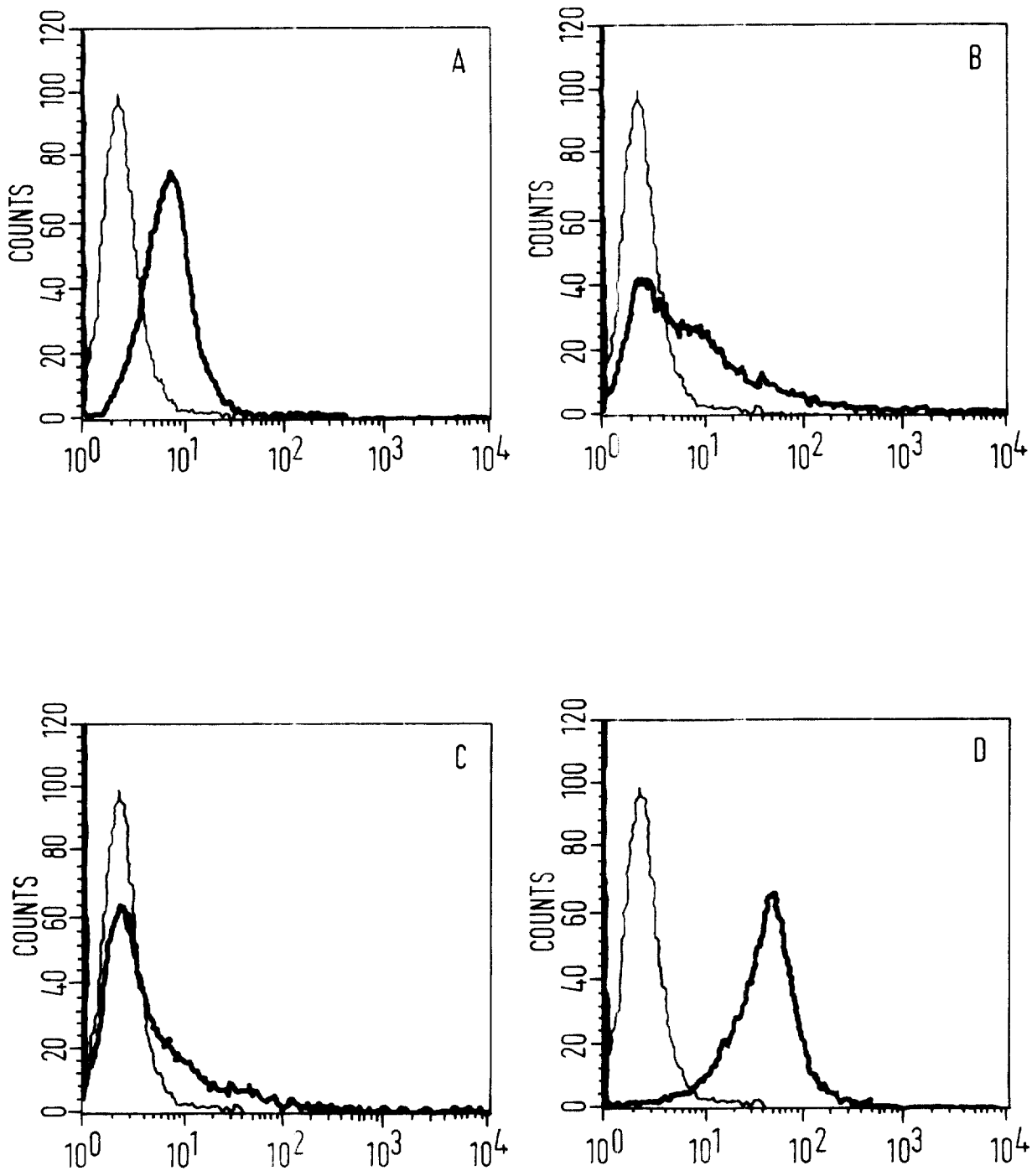
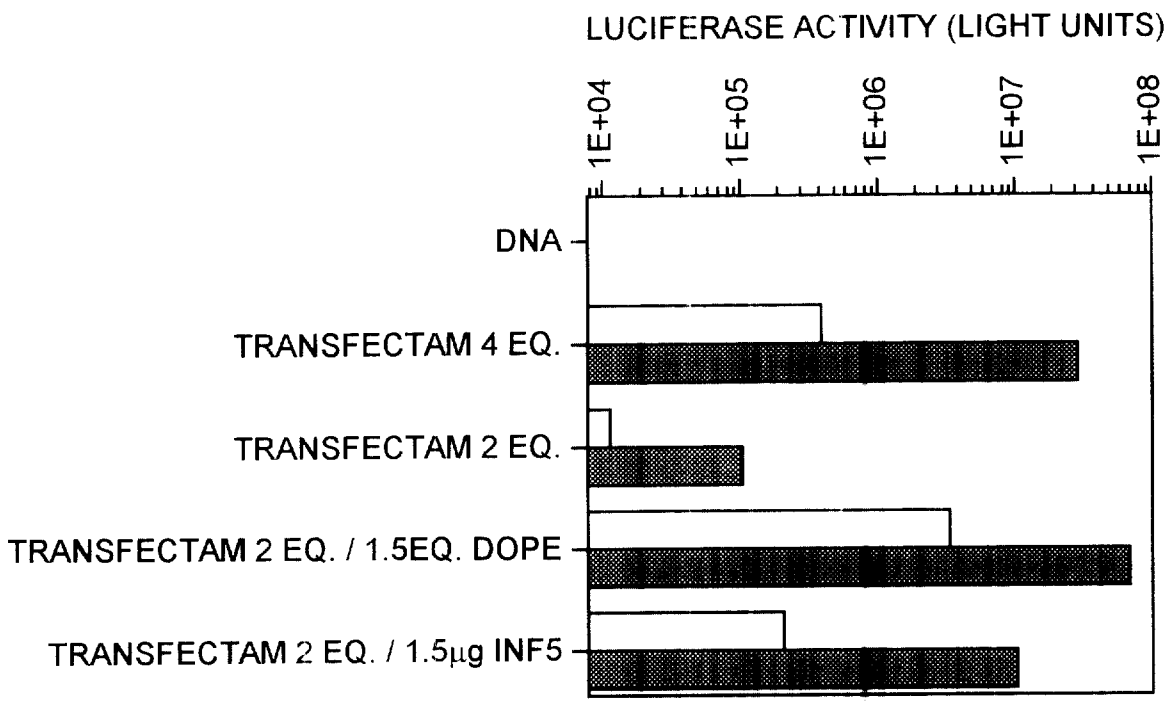


FIG. 3A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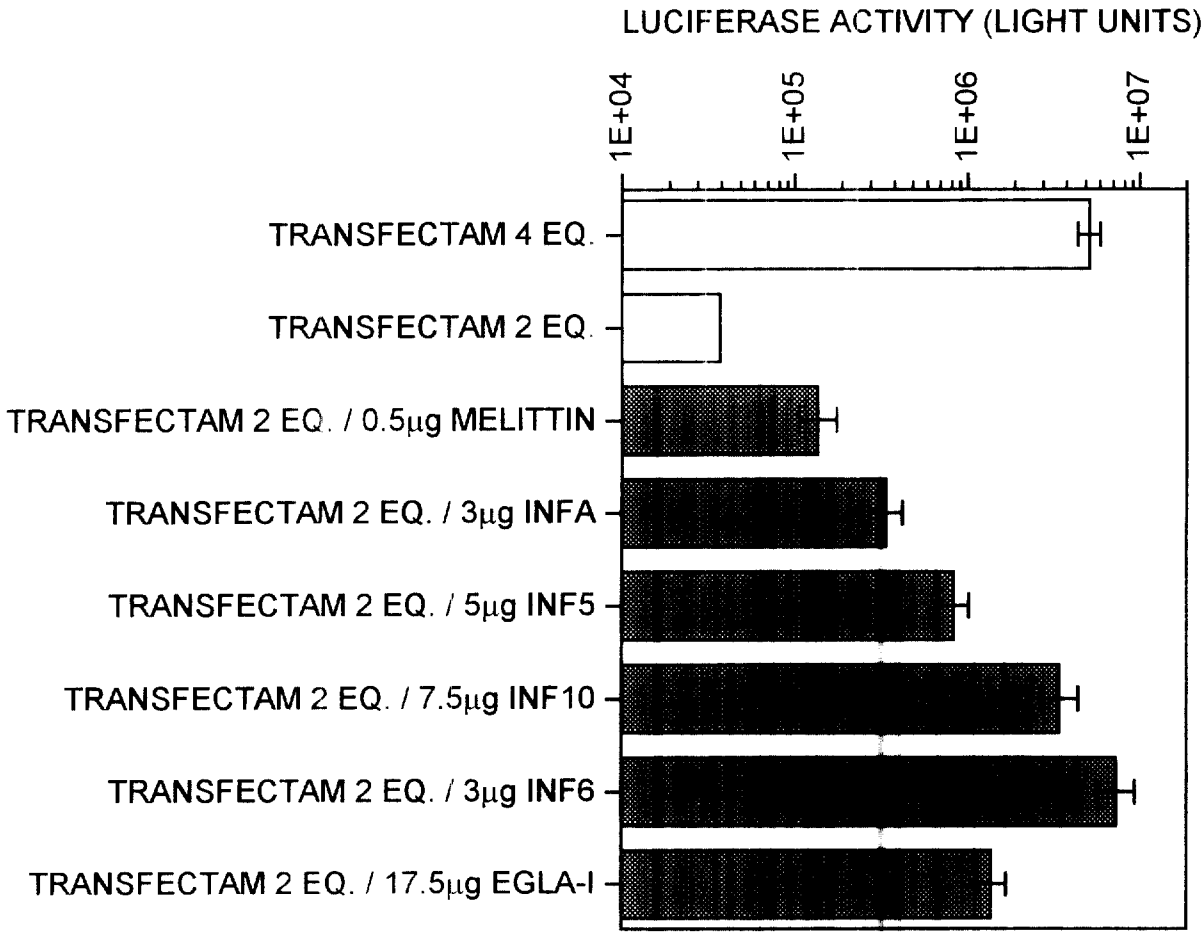
TRANSFECTAM 2 EQ. / 1.5EQ. DOPE
TRANSFECTAM 2 EQ. / 1.5µg INF5

FIG. 3B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1E+05
1E+06
1E+07
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TRANSFECTAM 2 EQ.
TRANSFECTAM 2 EQ. / 0.5µg MELITTIN
TRANSFECTAM 2 EQ. / 3µg INFA
TRANSFECTAM 2 EQ. / 5µg INF5
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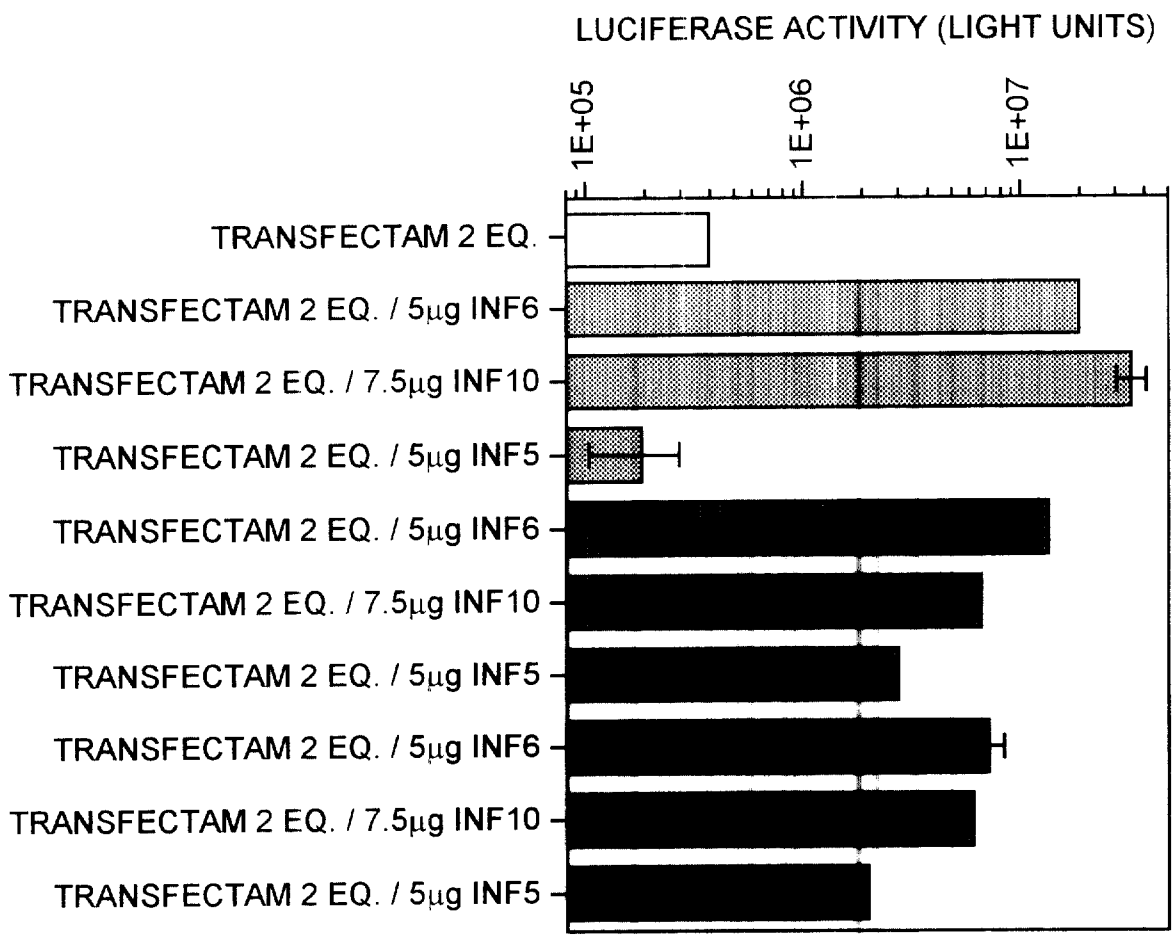
FIG. 4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TRANSFECTAM 2 EQ.
TRANSFECTAM 2 EQ. / 5µg INF6
TRANSFECTAM 2 EQ. / 7.5µg INF10
TRANSFECTAM 2 EQ. / 5µg INF5
TRANSFECTAM 2 EQ. / 5µg INF6
TRANSFECTAM 2 EQ. / 7.5µg INF10
TRANSFECTAM 2 EQ. / 5µg INF5
TRANSFECTAM 2 EQ. / 5µg INF6
TRANSFECTAM 2 EQ. / 7.5µg INF10
TRANSFECTAM 2 EQ. / 5µg INF5

FIG. 5

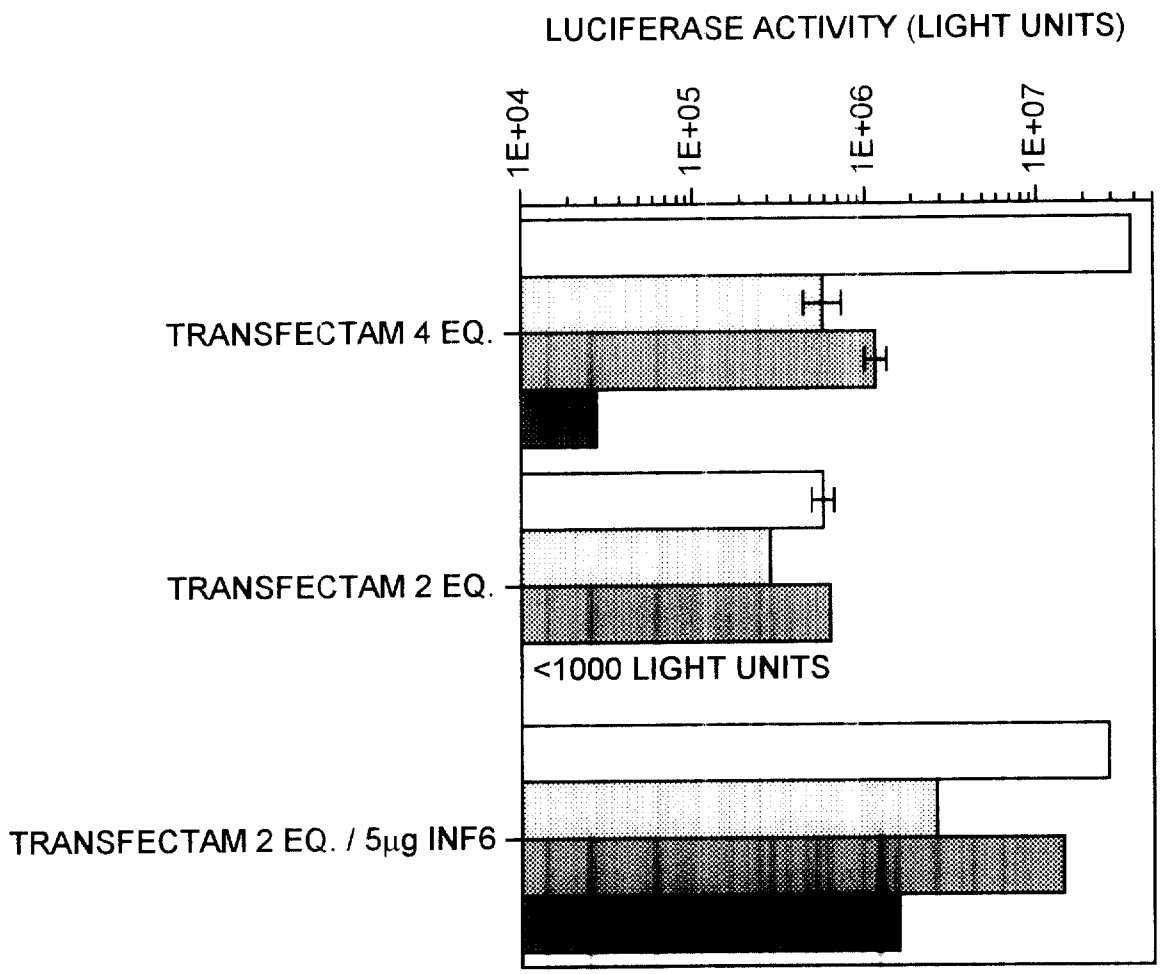
LUCIFERASE ACTIVITY (LIGHT UNITS)
1E+04
1E+05
1E+06
1E+07
TRANSFECTAM 4 EQ.
TRANSFECTAM 2 EQ.
<1000 LIGHT UNITS
TRANSFECTAM 2 EQ. / 5µg INF6

FIG. 6

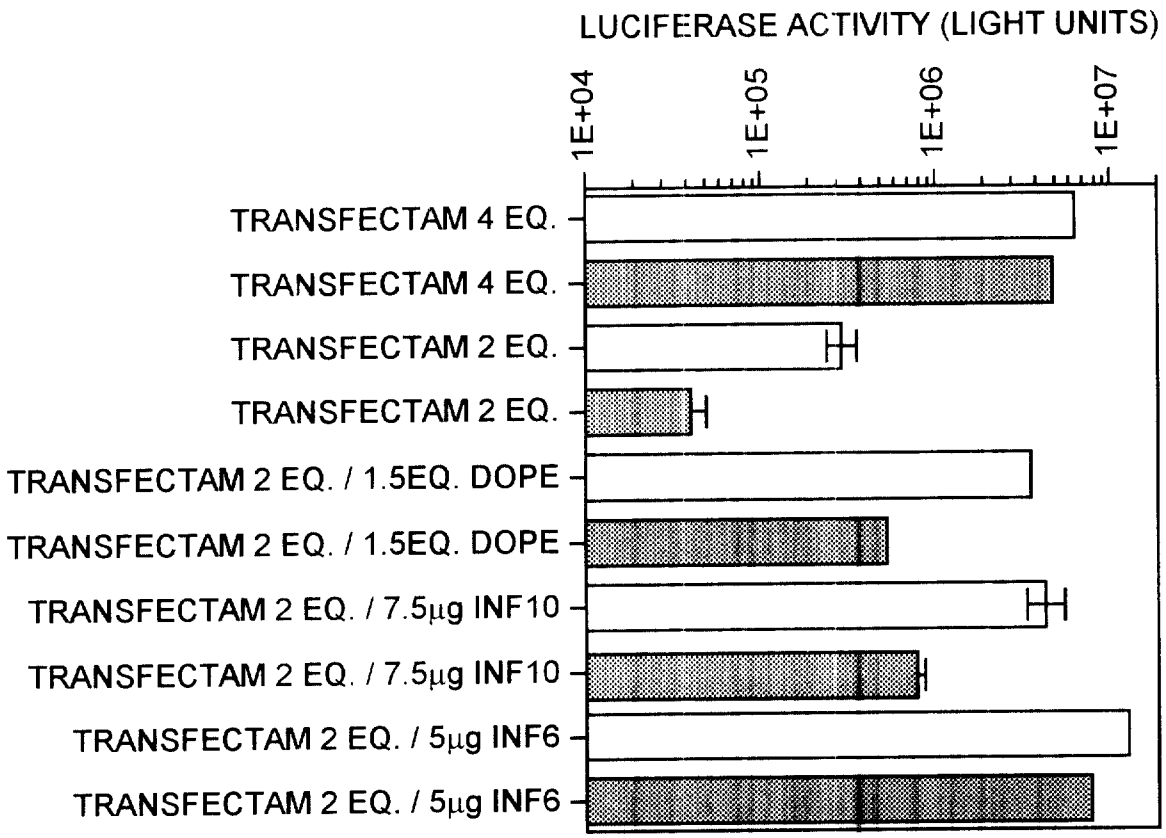
LUCIFERASE ACTIVITY (LIGHT UNITS)
1E+04
1E+05
1E+06
1E+07
TRANSFECTAM 4 EQ.
TRANSFECTAM 4 EQ.
TRANSFECTAM 2 EQ.
TRANSFECTAM 2 EQ.
TRANSFECTAM 2 EQ. / 1.5EQ. DOPE
TRANSFECTAM 2 EQ. / 1.5EQ. DOPE
TRANSFECTAM 2 EQ. / 7.5µg INF10
TRANSFECTAM 2 EQ. / 7.5µg INF10
TRANSFECTAM 2 EQ. / 5µg INF6
TRANSFECTAM 2 EQ. / 5µg INF6

FIG. 7

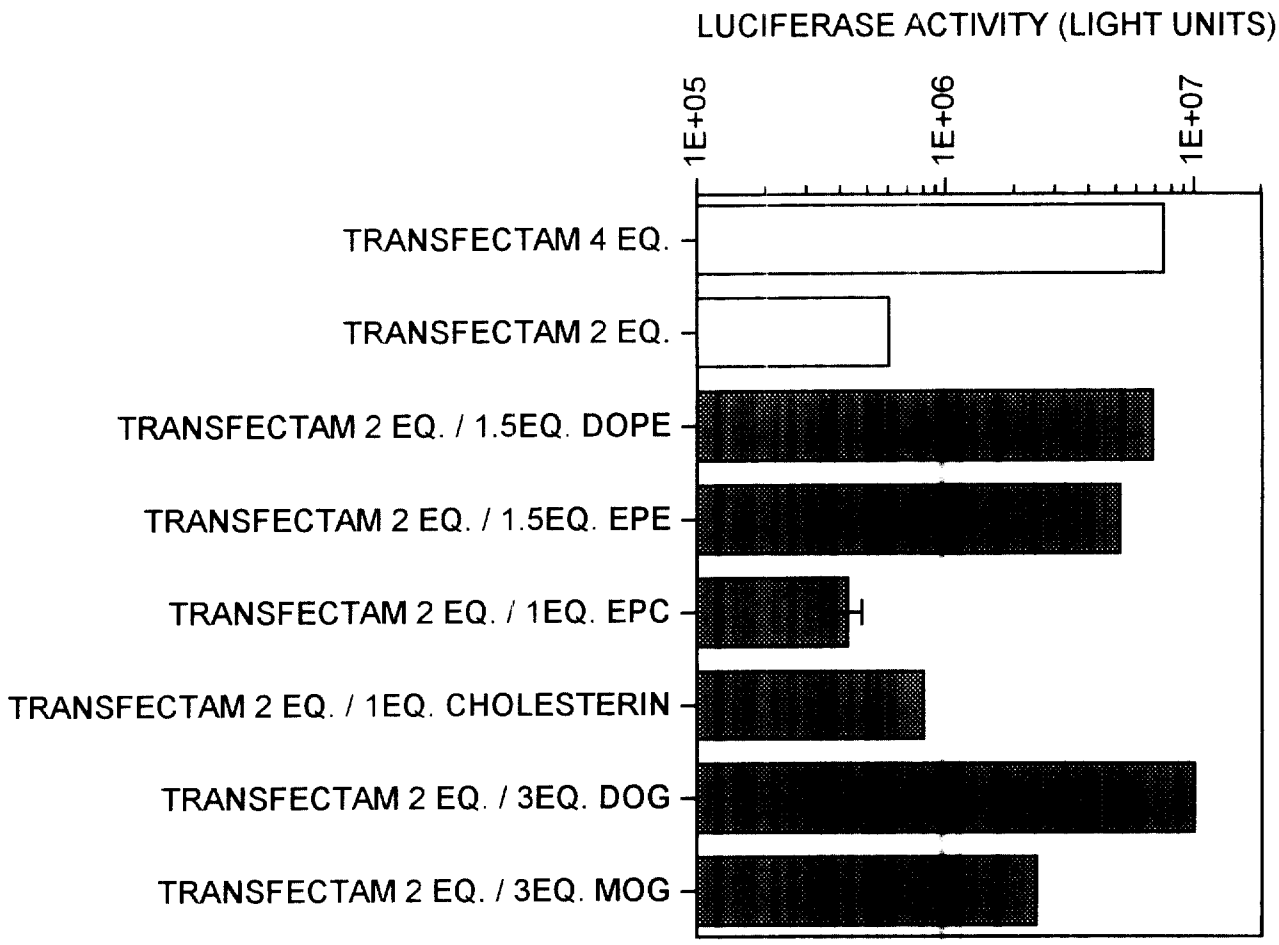
LUCIFERASE ACTIVITY (LIGHT UNITS)
1E+05
1E+06
1E+07
TRANSFECTAM 4 EQ.
TRANSFECTAM 2 EQ.
TRANSFECTAM 2 EQ. / 1.5EQ. DOPE
TRANSFECTAM 2 EQ. / 1.5EQ. EPE
TRANSFECTAM 2 EQ. / 1EQ. EPC
TRANSFECTAM 2 EQ. / 1EQ. CHOLESTERIN
TRANSFECTAM 2 EQ. / 3EQ. DOG
TRANSFECTAM 2 EQ. / 3EQ. MOG

FIG. 8

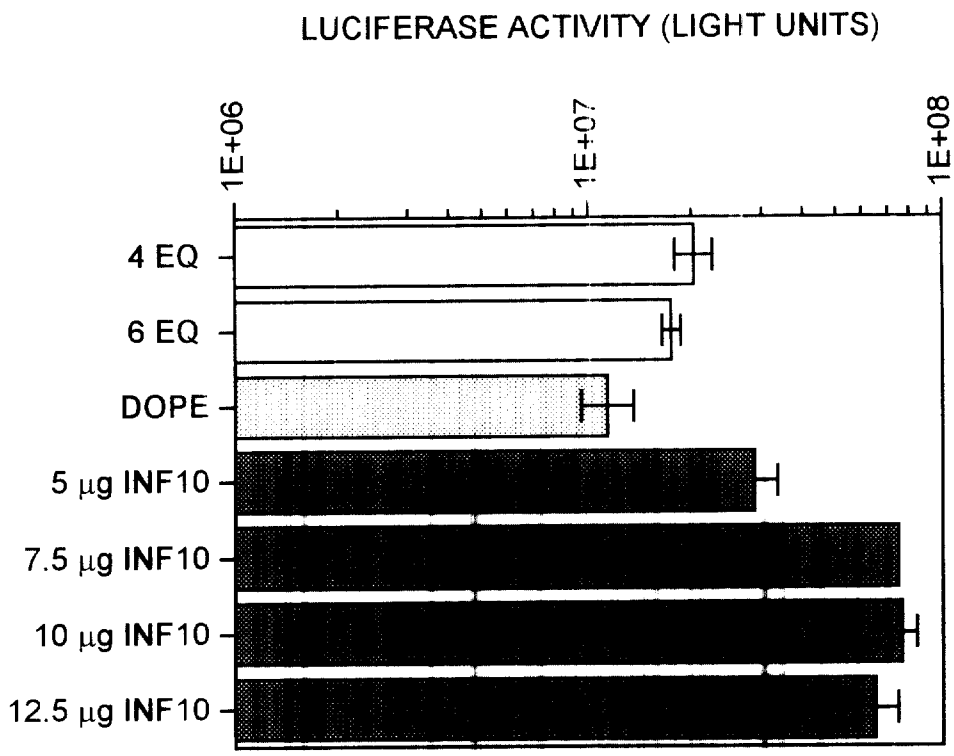
LUCIFERASE ACTIVITY (LIGHT UNITS)
1E+06
1E+07
1E+08
4 EQ
6 EQ
DOPE
5 μg INF10
7.5 μg INF10
10 μg INF10
12.5 μg INF10

FIG. 9

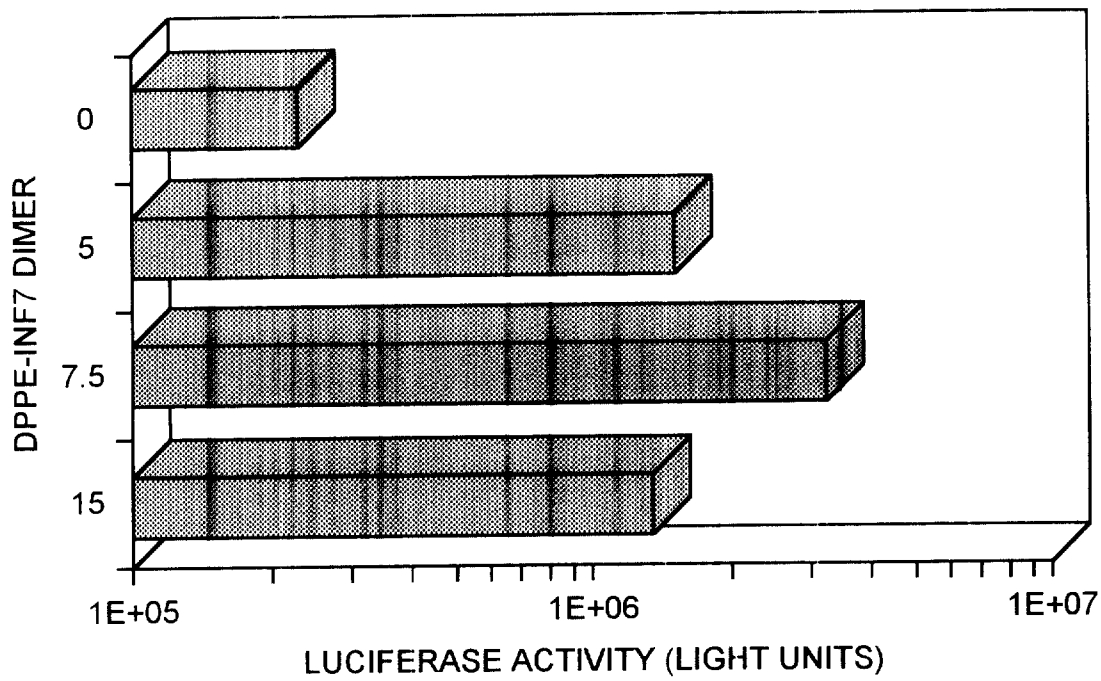
DPPE-INF7 DIMER
0
5
7.5
15
1E+05
1E+06
1E+07
LUCIFERASE ACTIVITY (LIGHT UNITS)

FIG. 10