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[54] **GENE TRANSFER IN INTACT MAMMALS**

[75] Inventors: **Winston A. Salser; Martin J. Cline,** both of Pacific Palisades; **Howard D. Stang,** Van Nuys, all of Calif.

[73] Assignee: **The Regents of the University of Calif.,** Berkeley, Calif.

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[58] **Field of Search** 424/94, 95, 101; 435/241, 172, 68

[56] **References Cited**

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Primary Examiner—Sam Rosen
Attorney, Agent, or Firm—Bertram I. Rowland

[57] **ABSTRACT**

Methods and compositions are provided for gene transfer to intact mammals with expression of the exogenous genetic material in the host. Mammalian host cells which are regenerative, normally highly proliferative or subject to induced proliferation, are transformed or modified in vitro with DNA capable of replication and expression in the host cell, wherein the DNA becomes incorporated into the cell. The modified cells are found to regenerate in the host with expression of the introduced DNA. Particularly, mammalian cells were modified with genes providing for overproduction of a particular enzyme. The modified cells were reintroduced in the host under conditions providing for selective advantage of the modified cells.

15 Claims, No Drawings

GENE TRANSFER IN INTACT MAMMALS

BACKGROUND OF THE INVENTION

1. Field of the Invention

The discovery that one could introduce exogenous genes into a bacterial host in vitro and observe expression of the exogenous genes in the bacterial host opened up vistas of new capabilities for the production of a wide range of compounds, particularly proteins, improved methods of treating waste, novel types of fertilizers, and new vaccines. While transformation of prokaryotes offer many new and yet envisaged opportunities, there is also great interest in being able to modify eukaryotes and particularly mammalian cells.

Many diseases are genetically related involving genetic deficiencies, which are usually either failure to produce a gene product or production of an abnormal product. Other situations involve treatment of a host with drugs which may have substantial toxicity to host cells. In these instances, it would be desirable to provide the host with the missing capability, the normal capability or a defense mechanism against the detrimental effects of the drug. The capability to modify a host's genetic structure to provide for either additional genetic capabilities or reparation of a defective capability on a temporary or permanent basis opens up wide avenues in the treatment of genetic deficiencies and disease.

2. Description of the Prior Art

Methods of introducing genetic material into a host cell include viral vectors Munyon et al. J. Virol, 7:813–820, 1971; cell-cell fusion, the fusion to cells of a limited number of chromosomes enveloped in nuclear membranes, Fournier et al. Proc. Natl. Acad. Sci. 74:319–323, 1977; and cellular endocytosis of microprecipitates of calcium-DNA complex, Bachetti and Graham, ibid. 74:1590–1594, 1977; Maitland and McDougall, Cell 11:233–241, 1977; Pellicer et al. ibid. 14:133–141, 1978 and Wigler et al. ibid. 14:725–731, 1978. Cell lines lacking thymidine kinase are readily transformed by appropriate DNA to a tk+ status when grown in the presence of a folic acid inhibitor and thymidine. Pellicer, supra and Wigler, supra.

SUMMARY OF THE INVENTION

Methods and compositions are provided for providing mammalian hosts with additional genetic capability, either a novel capability or enhancement of an existing one. Host cells capable of regeneration are removed and treated with genetic material under conditions whereby the genetic material is introduced into the host cells and becomes capable of replication and expression. The introduced genetic material includes at least one marker which allows for selective advantage for the host cells in which the introduced genetic material is capable of expression. The host cells are returned to the host under regenerative conditions, preferably of rapid proliferation of the cells, optionally with stressing of the host to provide a selective advantage for the genetically modified cells. It is found under these conditions, that the modified cells proliferate and express the genetic material which was introduced. Particularly, genetic material was employed which provided for expression of an enzyme. Either under the normal conditions of the host or subjecting the host to an enzyme antagonist, a selective proliferative advantage for the modified cells having overproduction of the enzyme resulted, in contrast to the normal cells incapable of such overproduction. By use of this approach, animals were obtaind in which the majority of the type of cells involved contained the added genetic material in a functionally active state.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

In accordance with the subject invention, a host is genetically modified by removing from the host or syngeneic source cells capable of regeneration when present in the host. The cells are then combined with DNA having genes capable of expression to provide a selective advantage for cells, under conditions where cells incorporate the DNA. The cells, which will include cells having the additional DNA, are then returned to the host. The genes providing the selective advantage can be combined with other genetic material which will be incorporated in conjunction with the gene supplying the selective advantage. The gene providing the selective advantage will be referred to as the selective marker.

Various methods may be employed for introduction of the genetic material, each of the methods having advantages and disadvantages. After introduction of the treated cells into the host, conditions are maintained in the host naturally, by administration of a physiologically active compound, or by dietary exclusion, to provide a selective advantage for the cells which have been genetically modified. In this way, genetic functions can be provided for a variety of purposes including treatment of genetic deficiencies, which includes providing a genetic capability which the host lacks or production of a normal product where the host produces an abnormal one; production of enzymes which can protect the host from cytotoxic agents; or for production of a wide variety of proteins e.g. hormones, globulins or the like.

In describing the invention, the host and host cells will be considered first, followed by the genetic material which may be employed for modifying the host cells and the manner in which the host cells are modified, and concluding with the regeneration of the modified cells and the purposes and effect of expression of the genetic material introduced into the modified cells.

Host and Host Cells

Various mammalian hosts may be treated in accordance with the subject invention, such as homo sapiens and domestic animals, particularly bovine, equine, ovine and porcine. The type of host cell which will be employed is one which is capable of regeneration, preferably rapid proliferation, either naturally or induced; can be isolated from the host or syngeneic source; can be modified by introduction of genetic material, which genetic material will then be capable of expression and replication; can be maintained in vitro, so as to be returned to the host in a viable state; are capable of being returned to the source in the host; and can provide the added genetic function in a form which is useful to the host.

Among potential cells which may be employed are bone marrow cells, particularly stem cells which provide hematopoietic functions. Other examples of tissues which have persistent stem cells included the intestinal mucosa and the germ line tissues. Use of these techniques to introduce genes into germ line cells may be of especial interest in breeding improved strains of domestic animals. Other cells which can be employed include cells of regenerative organs e.g. liver. Any body mem-

ber which is regenerative or can be induced to regenerate can be a source of cells.

Bone marrow cells chosen for modification should optimally be populations rich in stem cells. Furthermore, the cells chosen are preferably dividing, rather than stationary cells. To increase the fraction of these types of cells, the host may be treated by various techniques to increase the level of proliferating cells. For example, vinca alkaloids may be employed which inhibit mitosis, followed by rapid proliferation of the cells.

A wide variety of genetic material (DNA) may be employed to provide for the selective marker. The selective marker will allow for rapid proliferation of the modified cells in the host under normal conditions of the host or where rapid proliferation is subject to inhibition. The inhibition can be as a result of introduction of a drug which inhibits (a) proliferation because of interference with transcription of DNA or translation of RNA, that is, expression of one or more genes; (b) cell membrane formation; (c) cell wall formation, (d) enzyme activity; or (e) combination thereof.

A wide variety of drugs are known which are employed for the treatment of disease which inhibit cell replication, so as to favor the host against a parasitic invader such as bacteria, protozoa, or even a neoplastic variant of the host cell. The effectiveness of the drug may be inhibited in a cell by introducing into the cell genes which express an enzyme which reacts with the drug to deactivate it, genes which overproduce an enzyme involved in the metabolic pathway which the drug inhibits, so as to provide a selective advantage for the cells having higher concentrations of the enzyme(s), or genes which would provide for a metabolic pathway less affected by the drug, than the endogenous metabolic pathway.

Alternatively, the enzyme can provide for increased production of a metabolite essential to mitosis e.g. a metabolite on the biosynthetic pathway to DNA or RNA, for example, the formation of nucleosides. The modified cells having the selective marker which provides for enhanced enzyme production permits the modified cells to compete more effectively for a limited amount of metabolite precusor against the wild type cell.

The genetic material which is employed for recombination with the host cells may be either naturally occurring, synthetic, or combinations thereof. Depending upon the mode employed for introduction, the size of the genetic material introduced will vary. Furthermore, when two or more genes are to be introduced they may be carried on a single chain, a plurality of chains, or combinations thereof. Restrictions as to the size of a DNA fragment will be as a result of limitations due to the technical aspects of the vector: if a recombinant DNA is to be used, by the packaging requirements of a viral vector; the probability of transfer into the recipient cells by the method employed; the manner of preparation and isolation of the DNA fragments; or the like.

The selective markers employed can be chosen to deactivate an antimetabolite to mammalian cells, by reacting with the antimetabolite and modifying the antimetabolite to an ineffective product. Various enzymes and their genes are known and have been isolated for deactivating drugs. The most numerous examples are bacterial enzymes which deactivate antibiotics, such as those enzymes which confer resistance to aminoglycosides and polymyxines (streptomycin, kanamycin, neomycin, amikacin, gentamicin, tobramycin, etc.), and the like. Another drug which may find use is PALA. Where the drug does not provide a selective advantage, since the host metabolic pathways are not involved, a gene providing resistance to such a drug would not be useful. Illustrative of this situation are sulfonamides, which block a bacterial pathway, but not a mammalian metabolic pathway.

Alternatively, rather than providing a gene which expresses an enzyme, one could provide a gene which is not subject to interference by the drug. For example, one could employ DNA having a mutation at the site at which the drug binds or DNA which results in RNA or a protein, which substantially reduces the binding of the drug to the site at which the drug is active. Illustrative of drugs which are active by binding to specific sites are the macrolides, e.g. erythromycin and aminoglycosides, e.g. streptomycin.

The next group of drugs are chemotherapeutic agents. Protection of the host cells from the chemotherapeutic agents may be provided by introducing genes which overproduce the enzyme inhibited by the drug or deactivate the drug. Illustrative drugs include methotrexate, which inhibits dihydrofolate reductase, purine analogs, which interfere with the enzymes involved with inosinic acid, and pyrimidine analogs, such as fluorouracil, which inhibits thymidine monophosphate synthesis.

The selective marker may provide for enhanced production of one or more metabolites involved in proliferation, for example, production of nucleotides or nucleosides. An illustrative gene is the gene which codes for thymidine kinase, which is involved in the biosynthetic pathway to thymidylic acid. This selective advantage need not be associated with antimetabolite administration to the host.

In some genetic diseases the gene which corrects the genetic defect may itself confer a replicative advantage. For example, the insertion of genes for adenosine deaminase into cells of the marrow of certain patients with combined immunodeficiency disease may confer a selective advantage upon the replication of their stem cells leading to the production of a large population of immunocompetent cells which will ameliorate the effects of the disease.

Finally, one may employ genes which provide for production of a protein other than an enzyme, which allows for selective advantage of the modified cells. For example, this can be as a result of production of inducer which prevents repression of translation to provide semiconstitutive or constitutive production of an enzyme. In such cases a regulator gene may confer selective advantage even when no drug is employed.

In summation, the types of DNA which will be employed for selective markers include genes which react with drugs which interfere with regeneration so as to destroy activity of the drug; genes which provide sites which are not susceptible to drug action, so as to prevent the drug's action in the particular cell; genes which are repetitive for production of a desired protein e.g. an enzyme, which is inhibited by the drug; or genes which affect the regulatory function of the cell, so as to provide for overproduction of a particular enzyme by the natural processes of the cell, and which increase the normal replication of the cell genes to enable the cell to better compete for limited resources within the body.

If a drug is to be employed for providing the selective advantage the gene employed must be appropriately

related to the drug. The particular drugs employed must be considered as to level of toxicity and effect on the particular tissue which is being modified. Also to be considered is the purpose of the modification, which may limit the involved drug. In other cases the appropriate selective marker may be related to correction of the genetic deficiency involved with the disease or may alter the cells proliferation in any of various ways.

A number of ways have been developed for insertion of genetic materials into cells. Included among these techniques are viral vectors, Munyon et al., supra; cell-cell fusion involving the fusion to cells of a limited number of chromosomes enveloped in nuclear membranes, Fournier and Ruddle, supra; cellular endocytosis of microprecipitates of calcium-DNA complex, Bachetti and Graham, supra, Maitland and McDougall, supra, Pellicer et al., supra and Wigler et al., supra: minicell fusion; fusion with liposomes containing DNA; fusion with bacterial protoplasts containing plasmid DNA; and fusion with erythrocyte ghosts packaged with DNA. Each of the techniques has advantages and disadvantages, such as efficiency of information insertion, selectivity as to the particular nature or information of the DNA, permissible size of the DNA fragment, and the like.

When employing the microprecipitates of calcium-DNA complex, the DNA employed may provide for a single gene, a single set of genes, e.g. the beta-globin gene cluster, or a plurality of unrelated genes. As previously indicated, the size of the DNA fragments will vary, depending upon the particular manner used to introduce the genetic information. The mixtures of DNA which are not covalently linked may be introduced by congression, that is, different fragments of DNA will frequently concurrently enter a suspectible cell, so that those cells which have the selective marker are also likely to have the genetic capability of the additional genes.

The presence of a selective marker allows for selective pressure for preferential regeneration of the modified cell. Thus, in situations where gene deficiencies exist which would not provide for selective advantage of a modified cell, the selective marker affords this capability. With bone marrow cells, the cells could be modified by introducing genes which would provided for the correction of genetic deficiencies, by expression of products in which the host is deficient or provide for a wild type gene for correct expression of a protein. With bone marrow stem cells, genes could be provided with the correct sequence to correct hemoglobinopathes, such as sickle cell disease and thalassemia. Other defects could include defects in the production of plasma coagulation factors, e.g. fibrinogen, prothrombin and the various Factors, especially Factors VIII and IX. By introducing genes providing for structurally normal proteins fulfilling these functions, in conjunction with the ability to provide selective pressures for the modified cells, the modified cells may be maintained in the host of a high level for extended periods of time.

Depending upon the nature of the cells, the cells may be introduced into the host in various ways. With bone marrow or liver cells, the cells may be introduced intravenously. It may be desirable to treat the host to reduce the relevant cell population so that rapid cell replication will be favored. Various techniques can be employed to achieve this result, such as the use of mitotic inhibitors, e.g. vinca alkaloids, irradiation with X-rays, or other technique. It is desirable that prior to the introduction of the modified cells to the host, the host have a low level of the relevant cell type so that after introduction, there may be a rapid and expanding proliferation of the modified cells.

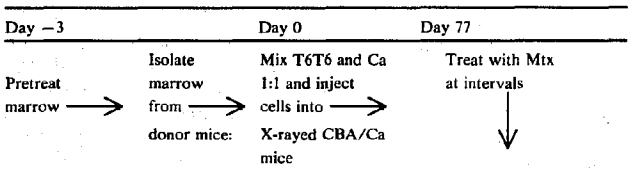
After introduction of the modified cells into the host, the host will be stressed with relevant drug(s) if these are to be employed to provide selective pressure for the modified cells. Appropriate levels of the drug may be maintained to insure proliferation of the desired cells. Depending upon the drug, the nature of the cells, and the concerns with repetitive introduction of modified host cells, the drug treatment may be of relatively short or long term duration. It is found that even after termination of the treatment with the drug providing the selective pressure, the cells continue to proliferate and may be maintained at a high level for extended periods of time.

The following examples are offered by way of illustration and not by way of limitation.

EXPERIMENTAL

The following is a flow chart of the progress of the experimentation:

FIG. 1



-continued

Day −3		Day 0	Day 77
donors with VLB	CBA/T6T6 ↑ Transform with Mtx^RDNA	CBA/Ca ↑ Mock transformation with "wild type" DNA	(1) Analyze karyotype (2) Transfer marrow to secondary irradiated CBA/Ca mice ↓ Mtx treatment ↓ Analyze karyotypes DHFR levels Hematologic status

Transformation of Mouse Bone Marrow In Vitro

Mouse fibroblast Swiss 3T6 cells highly resistant to Mtx and containing reiterated structural genes specifying DHFR were employed (See Kellems et al. J. Biol. Chem. 254, 309–318, 1979). They were maintained in 4×10^{-4} M methotrexate (Mtx) and designated 3T6 R1. DNA was isolated from 3T6 R1 and from non-resistant (wild type) mouse cell lines including 3T6 (fibroblastic) and L1210 (lymphocyctic leukemia) and in later experiments from salmon sperm (Sigma). The relative ratio of dihydrofolate reductase synthesis and number of gene copies in 3T6 R1 and 3T6 was approximately 30 to 1. DNA coprecipitated with calcium phosphate was used to transform wild type L1210 cells to methotrexate resistance by the method of Bachetti and Graman, supra, as modified by Wigler et al. supra.

Equal numbers of CBA/Ca and CBA/H-T6T6 mice were injected intraperitoneally with 3 or 4 mg/kg of the mitotic inhibitor vinblastine 3 days before marrow was removed for in vitro transformation. Mitotic inhibition by this treatment is followed by a burst of proliferation. Assays of colony-forming cells (CFU-S), when compared with total cell counts, showed that suspensions from animals thus treated were relatively depleted of mature cells and enriched approximately 3-fold in pluripotent spleen colony-forming cells (CFU-S). On the day of transformation (designated day 0, FIG. 1) single cell suspensions in McCoy's 5A medium with 15% fetal calf serum were obtained from femurs and tibias of sacrificed animals.

Cells from Ca and T6T6 animals were placed in separate pools. All T6T6 animals had the chracteristic marker chromosome abnormality. Cell suspensions of 5×10^6 in 10 ml complete medium were incubated with 1.0 ml Ca-precipitated DNA containing a total of 40 μg DNA as described by Wigler et al., supra, for 4 hours at 37° C. in 5% CO_2 in tissue culture flasks. For cells to be transformed to Mtx resistance, either 2 or 4 μg of DNA was from the 3T6R1 cell line. During this period differentiated phagocytic marrow cells firmly adhered to the flask.

T6T6 cells were incubated with DNA from 3T6 R1 Mtx-resistant cells, and CBA/Ca marrow cells were incubated with control DNA preparations from Mtx-sensitive cells. Thereafter, loosely adherent cells were collected and centrifuged at 150×g for 10 min and resuspended in DNA-free complete medium. After careful cell counts, Ca and T6T6 cells were combined in a ratio of 1:1 and between 5×10^6 and 5×10^7 of the combined cells were injected intravenously into recipient CBA/Ca mice in a volume of 0.3 to 0.4 ml in McCoy's medium with fetal calf serum. These recipients had received 850 rads irradiation from a cobalt source 24 hours previously to eradicate endogenous hematopoiesis. This dose or irradiation was selected because it had low lethality but virtually eradicated endogenous spleen colonly-forming cells (CFU-S). Thus an average of 2±1 endogenous CFU-S after 850 rads and 0.5±0.5 endogenous CFU-S after 900 rad whole body irradiation was observed in this mouse strain. Between 48 and 96 hours after injection, the recipient animals began treatment with the previously established Mtx protocol.

Hematopoietic Effects of Methotrexate Treatment in the Mouse

An appropriate schedule of Mtx treatment which would select for drug-resistant hematopoietic cells without lethality in control animals was established as follows. Groups of normal CBA or C3H mice weighing between 18 and 25 g were treated by a thrice weekly schedule of intraperitoneal injections of Mtx in doses varying between 0.5 and 8 mg/kg per injection. An escalating schedule of 0.5 mg/kg for 4 doses, 2 mg/kg for 4 doses and then 4 mg/kg thrice weekly was selected as not lethal but having profound suppressive effects on hematopoiesis. Tibial cellularity, peripheral white cell counts and hematocrits were all depressed in Mtx-treated animals and megaloblastic morphologic changes developed in the bone marrows. The hematocrit and tibial cellularity were found to be the easiest and most reliable hematologic parameter to follow and remained depressed in animals continuously treated with Mtx for at least 3 months. False elevations of hematocrit in Mtx-treated mice were occasionally observed in sick and dehydrated animals. No difference in sensitivity to Mtx was observed in the mouse strains CBA/Ca and CBA/H T6T6 as measured by standard hematologic parameters over 3 months of observation.

Selection of Drug Resistance Marrow Cells

The irradiated mice receiving mixtures of control Ca cells and T6T6 cells transformed with 3T6 R1 DNA were treated with Mtx for periods of 24 to 77 days. At intervals, animals were sacrificed or subjected to a limb amputation to obtain bone marrow samples. These were analyzed for karyotype distribution, cellularity, CFU-S content and injected into secondary irradiated CBA/Ca recipients. The results of two initial experiments are shown in Tables I and II.

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