elicited a very small current (Fig. 3A), which shows that the membrane resistance is high. Forty-five seconds after removing Ca^{2+} and adding Ba^{2+}, membrane resistance was very low, and there was a large instantaneous current, the typical effect of Ca^{2+} removal. The result is quite different if Ba^{2+} is added to the medium and allowed to block K^+ channels before Ca^{2+} removal (Fig. 3, C and D). In this case membrane resistance remained high, and current during the pulse was small. Interestingly, in 5 m*M* Ba^{2+} there was somewhat more inward current during the pulse and a very large tail current, which may be current through Ca^{2+} channels. It is known that Ca^{2+} channels in heart and skeletal muscle become abnormally permeable when the Ca^{2+} concentration is very low (*13, 14*), and the tails in Fig. 3D may result from a similar phenomenon. Regardless of the identity of the channels responsible for this current, they do not contribute to the instantaneous current because they close when the cell is polarized to −80 mV. Aside from the tails, we think it unlikely that Ca^{2+} channels play a part in the phenomena observed here. Ca^{2+} channels become unselective for Ca^{2+} in the low micromolar range, but the effect on K^+ channels described here is detectable when Ca^{2+} drops below 4 to 5 m*M*. Furthermore, unlike the abnormal channels described here, Ca^{2+} channels retain their gating properties after losing selectivity.

Our experiments show that K^+ channels in squid GFL neurons maintain their integrity with regard to gating and selectivity properties only in the presence of an adequate concentration of external Ca^{2+}. It is clear from previous work (*7*) that a major element in gating is the movement of charge intrinsic to the channel protein in response to a membrane voltage change. We suggest that a second major factor is the release of a Ca^{2+} ion, perhaps as the last step in opening, and the rebinding of Ca^{2+} as the channel closes. It is unknown whether calcium serves as a cofactor in gating for other channel types as well.

From previous results (*8*), we expected the K^+ channels to lose their ability to close, but their loss of selectivity was unexpected. We speculate that when a K^+ channel remains open for a long time, it slowly assumes an unusual conformation with abnormal selectivity. In the presence of Ca^{2+}, the channel is open so briefly that the probability of assuming this conformation is very small. On restoring Ca^{2+} in Fig. 1 the instantaneous current disappears quickly, but K^+ channel activity returns more slowly. This slow recovery suggests that extensive refolding is required to restore the normal properties of the channel.

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Implantation of Genetically Engineered Fibroblasts into Mice: Implications for Gene Therapy

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In a variety of human genetic diseases, replacement of the absent or defective protein provides significant therapeutic benefits. As a model for a somatic cell gene therapy system, cultured murine fibroblasts were transfected with a human growth hormone (hGH) fusion gene and cells from one of the resulting clonal lines were subsequently implanted into various locations in mice. Such implants synthesized and secreted hGH, which was detectable in the serum. The function of the implants depended on their location and size, and on the histocompatibility of the donor cells with their recipients. The expression of hGH could be modified by addition of regulatory effectors, and, with appropriate immunosuppression, the implants survived for more than 3 months. This approach to gene therapy, here termed "transkaryotic implantation," is potentially applicable to many genetic diseases in that (i) the transfected cell line can be extensively characterized prior to implantation, (ii) several anatomical sites are suitable for implantation, and (iii) regulated expression of the gene of therapeutic interest can be obtained.

BECAUSE IT IS KNOWN THAT AT LEAST some genes of therapeutic value can function normally in heterologous cells, there has been a major effort to develop a gene delivery system during the past few years (*1*). The model system widely used at present is retroviral-mediated genetic transformation of mouse bone marrow cells and subsequent transplantation of the marrow into lethally irradiated mice (*2–6*). The advantages of this approach are that the marrow cells are readily accessible, can be cultured outside the animal, and can be efficiently infected with recombinant retroviruses. However, this approach is limited in that retroviral vectors might activate endogenous retroviral genomes in the target cells by recombining with them (*4*); the batch-infected marrow cells are subject to a large number of random retroviral integration events, some of which might be harmful to the recipient of those cells; expression of the gene inserted into the retroviral vector has proved difficult, particularly in human bone marrow cells (*7*); and the marrow is probably not an acceptable site for production of many proteins of clinical relevance. Although some of these limitations may be eliminated in the future [see (*6*), for example], there are cogent reasons to search for alternative models for gene therapy.

An alternative approach to gene therapy would be to remove and transfect cells from a desired anatomical source in the affected individual, isolate a clone of transfected cells, test the clonal population for proper regulation of the transfected gene and for the absence of deleterious integration events, and finally, reintroduce the cells into the individual in a physiologically suitable location. As a first step toward evaluating this approach, we have introduced a human growth hormone (hGH) fusion gene into cultured mouse Ltk^- (thymidine kinase–deficient) fibroblasts and have subsequently implanted these cells into various locations in mice. We report here that the function of such implanted cells depends on the location and size of the implant, and on the histocompatibility of the donor cells with their

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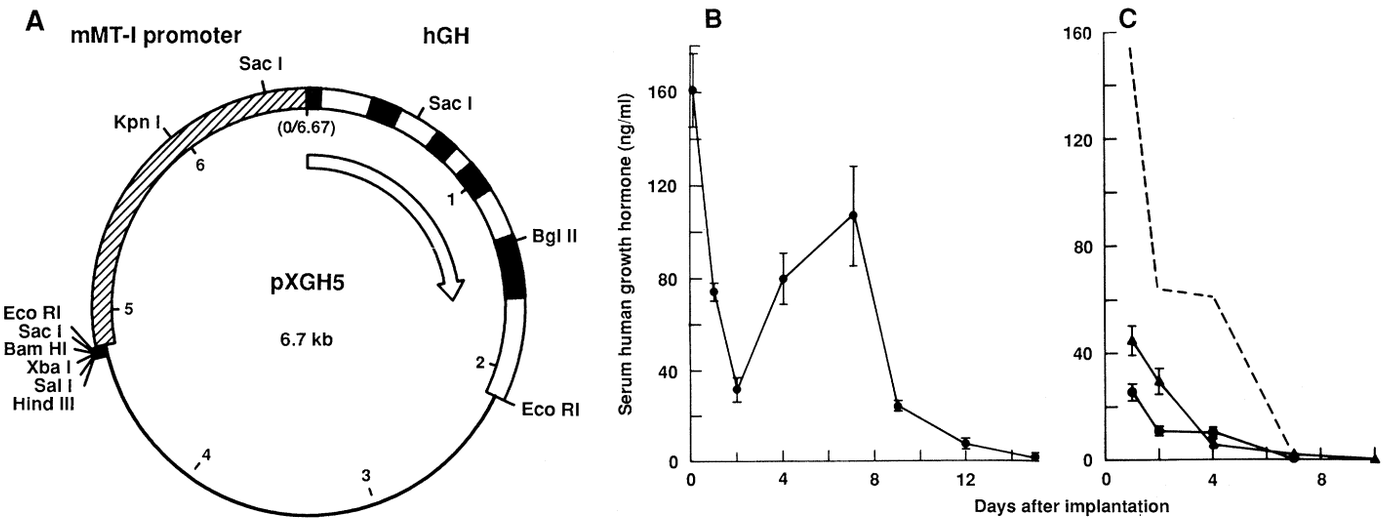

Fig. 1. Intraperitoneal, subcutaneous, and subcapsular transkaryotic implantation. (**A**) Structure of the mMT-I/hGH fusion gene. To generate a stable hGH-secreting cell line, Ltk$^-$ cells were co-transfected with pXGH5 (*8*), a mouse metallothionein I/hGH fusion gene, and pTk1 (*9*), which is a pBR322 derivative containing the herpes virus thymidine kinase gene, by calcium phosphate precipitation (*18*). Stable transformants were isolated by HAT selection and screened for hGH secretion into the medium. The line selected for implantation was designated Ltk$^+$GH (2×10^7 Ltk$^+$GH cells secrete hGH at 1 to 10 μg per day). (**B**) Nine adult C3H mice (Jackson Laboratory) received intraperitoneal injections of mouse Ltk$^+$GH cells. For each animal, 2×10^7 Ltk$^+$GH cells were washed with phosphate-buffered saline (PBS), trypsinized for 10 minutes at 37°C, suspended in 10 percent calf serum in Dulbecco's modified Eagle's medium, and centrifuged for 5 minutes at 1000 rev/min. The medium was then aspirated, and the cells were resuspended in 1 ml of PBS and injected. Mice were bled at indicated times and serum hGH was measured by solid-phase two-site radioimmunoassay (a kit from Nichols Institute or Hybritech, Inc.). Mouse growth hormone is not detectable with this assay, and control mice intraperitoneally implanted with nontransfected Ltk$^-$ cells do not contain hGH in their sera. Each point represents the serum hGH (mean ± SE). Variability in hGH between experiments is about twofold, but has been as high as fourfold; the shape of the curve does not vary widely. Six of the nine mice that received stably transfected cells survived the experiment. The primary cause of death was related to the multiple bleedings in a comparatively short time (the transkaryotic and control mice were equally susceptible to repeated bleedings). The number of bleedings was reduced by omitting the 3-hour time points for the remainder of the experiments presented here, and the interval between time points was increased when possible. (**C**) Subcutaneous and subcapsular implantation of Ltk$^+$GH cells. Each of ten adult C3H mice received subcutaneous implants of 2×10^7 Ltk$^+$GH cells, prepared as described above (solid line and triangles) and ten mice received implants beneath a renal capsule with approximately 3×10^6 cells per animal (solid line and circles). For subcapsular implantation, the cells were trypsinized and centrifuged as above, the medium was aspirated, and the cell pellet was implanted via a cannula. Mice were bled at indicated times for determination of serum hGH; the average serum hGH and standard error bars are shown. Ten mice survived the subcutaneous implants and nine survived the subcapsular implants. The dashed line shows the data for the subcapsular transkaryotic animals normalized as if 2×10^7 cells were implanted (that is, the mean value of serum hGH at each time point after subcapsular implantation multiplied by six). Several mice receiving subcapsular and intraperitoneal implants were observed for 3 to 6 months and then killed; no abnormalities were found at autopsy.

recipients. Under certain conditions, the survival of the implant can be maintained for more than 3 months. We propose that this technique be called "transkaryotic implantation," with "transkaryotic" suggesting that the nuclei of the implanted cells have been altered by the addition of DNA sequences by stable or transient transfection.

Once genetically engineered cells are implanted into an animal, it can be relatively difficult to recover and characterize them. To circumvent this problem, we have developed a functional assay to monitor the fate of the implanted cells based on their ability to synthesize and secrete hGH. Previously we have shown that hGH is well suited to serve as a reporter molecule in gene transfer experiments, partly as a result of the stability of the hGH messenger RNA (mRNA) and protein and the ease with which hGH can be assayed (*8*). Cultured mouse Ltk$^-$ fibroblasts were therefore co-transfected with (i) pXGH5, a plasmid containing a fusion gene composed of mouse metallothionein-I (mMT-I) promoter and hGH structural sequences (*8*), and (ii) pTk1, a plasmid containing the herpes virus thymidine kinase gene (*9*), and HAT (hypoxanthine, aminopterin, and thymidine)–resistant cell lines were selected and analyzed for hGH expression. Cells from one such line, Ltk$^+$GH, were then introduced intraperitoneally into mice of the same strain (C3H) as the original donor of the cultured L cell line. We expected that if the cells survived, they would secrete hGH into the peritoneal fluid and the hGH would be taken up by lymphatics and ultimately reach the bloodstream, which could then be assayed for its hGH content.

Ltk$^+$GH cells were injected intraperitoneally (2×10^7 cells per animal) into each of nine C3H mice, and serum hGH concentrations were determined at various times after the cells were implanted (Fig. 1B). The time course of hGH expression by the implanted cells could be divided into three phases in this experiment. At 3 hours, the mean hGH concentration was 161 ng/ml; during the next 2 days the values declined rapidly (trauma phase). From day 3 until day 7, serum hGH increased dramatically (acclimation phase) and then declined so that it was barely detectable by day 15 (elimination phase).

This initial experiment demonstrated that transkaryotic implantation of stably transfected cells can deliver a protein product to the bloodstream for approximately 2 weeks after implantation. These data suggested the following model for the fate of the implanted cells: during the initial days after implantation, hGH expression decreased as a result of Ltk$^+$GH cell death, presumably because of a combination of the damaging effects of handling the cells and of growth conditions in the peritoneum; some of the transfected cells recovered and, during acclimation, the surviving cells thrived, as was reflected by hGH production; the implanted cells ultimately stopped producing hGH, either because the animal has some means to remove or inactivate the cells, or because the peritoneal fluid is an inhospitable location for L cell growth. In order to test certain aspects of this model and to analyze the utility of transkaryotic implantation, we implanted Ltk$^+$GH cells into mice under various experimental conditions.

The utility of transkaryotic implantation as a gene therapy system would be significantly enhanced if the transfected cells could be targeted to any organ. It seems unlikely that every organ is capable of responding to implanted cells in an identical manner, however, and to test this hypothesis, we placed the transfected mouse L cells into various locations in C3H mice. Subcutaneously implanted Ltk$^+$GH cells (2×10^7 cells per animal) produced hGH for up to 10 days (Fig. 1C). Serum hGH falls steadily from day 1 after implantation, and the acclimation phase noted with intraperitoneal implantation is not seen with subcutaneous implantation. A similar pattern is found for cells (3×10^6 cells per animal) implanted under the renal capsule (Fig. 1C); by day 7 after implantation, little serum hGH was detected. Approximately one-sixth the number of cells was used for subcapsular implantations as for intraperitoneal and subcutaneous implantations because of the limited capacity of the subcapsular space. If the serum hGH observed during the subcapsular experiments is normalized to cell number (Fig. 1C), the subcapsular route of administration appears more efficient than the subcutaneous route (*10*).

Several factors may contribute to the differential ability of the implanted cells to produce hGH in various locations in the body. The perfusion of the implant site could provide the cell with vital nutrients and influence both the viability of the cells and the amount of hGH taken up into the circulation. The hydrostatic pressure to which the cells are subjected could also influence the functioning of the implant—cells under the renal capsule, for example, are presumably under significantly higher hydrostatic pressure than those in the peritoneum. The cells may attach better in certain locations, and finally, the relative surveillance of the implant by cells of the immune system may influence the amount of hGH produced. We have also effected transkaryotic implantations intrahepatically, intraportally, and into the inferior vena cava, with hGH expression generally stopping after approximately 7 to 11 days (*11*).

The mMT-I promoter is inducible by zinc, and we have previously shown that pXGH5 expression in transiently transfected Ltk$^-$ cells is stimulated 10- to 20-fold by zinc (*8*). To determine whether the stably transfected Ltk$^+$GH cells were similarly responsive, cells were implanted intraperitoneally in mice, some of which were then given 76 m*M* $ZnSO_4$ in their drinking water. Those animals treated with zinc (Fig. 2A) expressed ten times more hGH in their sera than did those with no treatment. This induction is comparable to previously reported results for metallothionein fusion genes (*8, 12*) and indicates that the cells remain responsive in the intraperitoneal milieu. Furthermore, RNA prepared from cells recovered from subcapsular implants contains the correct mMT-I/hGH fusion mRNA (Fig. 2B). Taken together, these results suggest that the implanted cells are viable and behave predictably. Of equal importance is the fact that the cells, once in place in the animal, can still be modulated by external means.

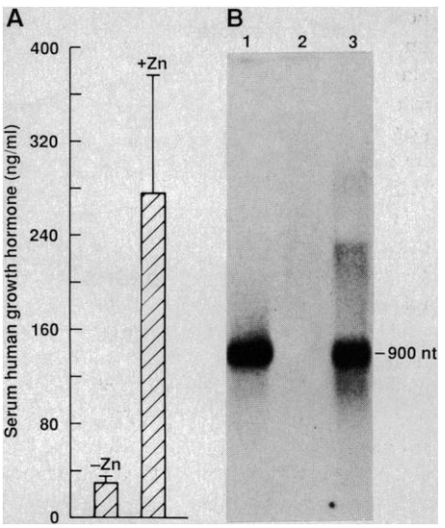

Fig. 2. Characterization of implanted Ltk$^+$GH cells. (**A**) Effect of zinc on pXGH5 expression in transkaryotic mice. Ten adult C3H mice received intraperitoneal implants of 2×10^7 Ltk$^+$GH cells. On the following day, drinking water of five of these mice contained 76 m*M* $ZnSO_4$. Four days after implantation, serum hGH was determined. Shaded bars represent average serum hGH levels for each set of five mice and standard errors are indicated. (**B**) Detection of the mMT-I/hGH mRNA in cells recovered from a subcapsular implant. A kidney containing subcapsular Ltk$^+$GH cells was removed from an adult C3H mouse and total cellular RNA was prepared and subjected to Northern blot analysis, with an hGH complementary DNA probe (*19*) as described (*8, 20*). (Lane 1) Total cellular RNA (125 ng) from Ltk$^+$GH cells in culture; (lane 2) total cellular RNA (10 μg) from a control C3H kidney; (lane 3) total cellular RNA (10 μg) from kidney containing subcapsular Ltk$^+$GH cells. The samples in lanes 1 and 3 contain the same hybridizing band of approximately 900 nucleotides, which is predicted to contain 64 nucleotides of mMT-I sequences and 817 nucleotides of hGH sequences, in addition to a polyadenylate tail; this indicates that the same fusion mRNA is synthesized in implanted cells as in cultured cells.

For example, from days 1 through 7 after implantation, hGH-producing cells could be visualized (either with the naked eye or by light microscopy) under the renal capsule. In all our experiments, once hGH disappeared from the serum, it never reappeared. In addition, if the kidney that received the subcapsular implant is removed from a transkaryotic mouse expressing hGH, all traces of such expression disappear within several hours after the nephrectomy. The transfected cells also appear to remain localized at the site of intraperitoneal and subcutaneous implantation.

Since serum hGH dropped precipitously

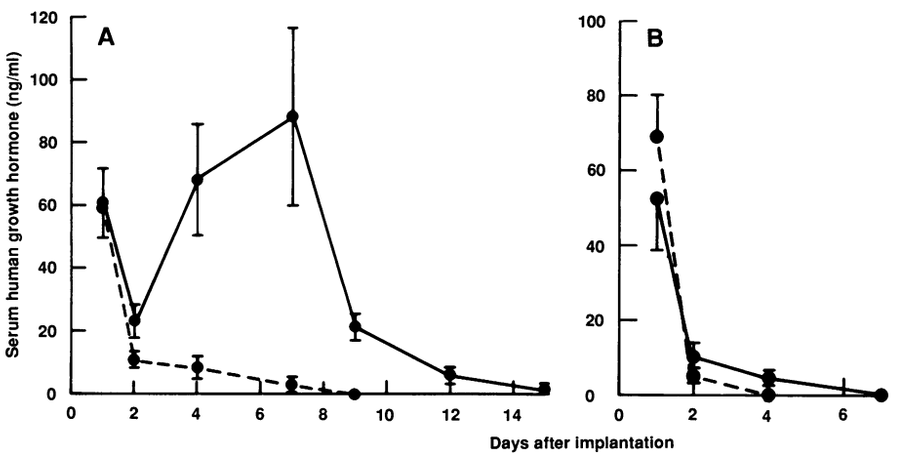

Fig. 3. The role of the immune system in transkaryotic implantation. (**A**) Six adult C3H mice were injected intraperitoneally with Ltk$^+$GH cells and were bled at the indicated times after implantation for serum hGH determinations (solid line with standard error bars indicated; this is a replotting of the data from the six mice that survived the experiment presented in Fig. 1). Six weeks later, the same mice, which showed no trace of serum hGH expression, received a second intraperitoneal injection of 2×10^7 Ltk$^+$GH cells per animal and were bled as above (dashed line with standard error bars indicated). (**B**) Ten adult C57BL/6 mice (Jackson Laboratory) were injected intraperitoneally with Ltk$^+$GH cells as above (solid line with standard error bars indicated). Six weeks later the same mice (which showed no trace of serum hGH expression) received a second intraperitoneal injection of Ltk$^+$GH cells as in (A) (dashed line with standard error bars indicated). For each implanation 2×10^7 cells were used.

between 7 to 15 days after intraperitoneal implantation, it seemed possible that this elimination phase was mediated by the immune system. As a first step in studying the potential role of the immune system in transkaryotic implantation, we again challenged, with stably transfected cells, six C3H mice (of the nine presented in Fig. 1B, solid line) that had previously received intraperitoneal implants of stably transfected cells. When these six mice were first exposed to Ltk$^+$GH cells, hGH expression continued for 15 days, but after a second challenge, hGH was barely detectable by day 7 and absent by day 9 (Fig. 3A). The hGH profile in the sera of the mice that were challenged a second time lacked the acclimation phase. These results are consistent with an anamnestic response of the host against the Ltk$^+$GH cells. Although Ltk$^+$ cells were originally derived from C3H mice, it is likely that either the cells or the mice had undergone some genetic alterations over the course of the almost four decades since the cell line was originally established (*13*) so that the cells and mice are no longer syngeneic with one another.

When frankly allogeneic mice (C57BL/6; representing both H-2 and non–H-2 determined incompatibilities) received Ltk$^+$GH cells intraperitoneally, their pattern of hGH expression resembled that of C3H mice challenged a second time. After a second challenge, no hGH was detectable in allogeneic recipients after day 4 (Fig. 3B). From these experiments, we conclude that the primary cause of the cessation of hGH expression after intraperitoneal implantation is cell death due to the host's immune system.

If the Ltk$^+$GH cells are destroyed by transplant rejection, immunosuppression should prolong the functional life of the implant. Transkaryotic mice were subjected to three immunosuppressive regimens: rabbit antiserum to mouse thymocytes (RAMTS) (*14*), dexamethasone, and a combination of the two. Ltk$^+$GH cells were implanted intraperitoneally into C3H mice, which were then immunosuppressed and monitored for serum hGH. The RAMTS-treated mice showed higher serum hGH and expressed hGH for approximately 2 weeks longer than untreated mice (Table 1). Dexamethasone-treated mice (daily doses) also showed elevated and prolonged expression, with serum hGH detectable until 48 days postimplantation. Dexamethasone may act to increase hGH production by two distinct mechanisms: in addition to its effect on the immune system, we have noted (*8*) that Ltk$^-$ cells transiently transfected with pXGH5 secrete three to four times more hGH when cultured in the presence of dexamethasone.

Table 1. Effects of immunosuppression on intraperitoneally implanted Ltk$^+$GH cells. Experimenta methods were as described in the legend to Fig. 1. Adult C3H mice received Ltk$^+$GH cells in traperitoneally with or without immunosuppression and blood samples were obtained on the indicatec days for hGH assays. For each time point, average hGH and standard errors are indicated (figures ir parentheses represent the number of surviving mice in the group). Nine mice received no immunosup pression, seven mice were treated with rabbit antiserum to mouse thymocytes (RAMTS) (0.25-m injections on days −1, 0, 1, and 3 with respect to implantation—the day 0 injections were intramuscula and the remainder were intraperitoneal) and eight mice were treated with dexamethasone (twice daily intramuscular injections of 100 μg beginning the day of implantation).

Days after implantation	Serum hGH (ng/ml)		
	No immunosuppression	RAMTS	Dexamethasone
1	74.1 ± 3.8 (9)	89.7 ± 18.6 (7)	94.8 ± 9.2 (8)
2	31.5 ± 5.4 (9)		86.6 ± 10.5 (8)
3		84.7 ± 20.4 (7)	
4	80.0 ± 11.4 (9)		54.3 ± 14.2 (8)
5		173.1 ± 34.6 (7)	
7	107.5 ± 21.6 (9)	160.8 ± 75.4 (7)	
8 to 10	24.3 ± 2.1 (7)	38.3 ± 5.4 (7)	99.3 ± 48.0 (8)
12 to 13	7.4 ± 2.1 (7)	28.3 ± 5.6 (7)	32.0 ± 15.1 (7)
15 to 17	1.5 ± 0.7 (6)	28.8 ± 7.5 (7)	28.3 ± 18.1 (7)
21	0.0 (6)	13.9 ± 3.7 (5)	28.0 ± 13.6 (7)
27	0.0 (6)	10.4 ± 6.6 (4)	13.9 ± 6.9 (6)
34		0.0 (4)	17.3 ± 11.7 (3)
41			7.5 ± 6.1 (3)
48			2.7 ± 2.2 (3)

For dual immunosuppression, C3H mice received RAMTS and dexamethasone after Ltk$^+$GH cells were intraperitoneally implanted. Two of these mice expressed hGH for more than 3 months (Table 2). In one of the mice, hGH rose to 500 ng/ml at 66 days after implantation and remained elevated until death occurred approximately 10 days later. As mentioned above, high levels of serum hGH for an extended period often prove to be lethal to mice. Laparotomy of this mouse disclosed large collections of Ltk$^+$GH cells in the form of plaques of new tissue distributed widely on many peritonea surfaces. Viable cells were also suspended ir ascitic fluid. The primary cause of death wa most likely the extremely elevated hGH rather than the increased peritoneal cel mass; it is also possible that the immunosuppressive regimen itself contributed to thi death. In approximately 20 percent of control C3H mice injected with nontransfected mouse Ltk$^-$ fibroblasts and treated with dual immunosuppression, similar cell proliferation was noted, but these mice survived for more than 100 days after implantation.

Table 2. Effects of dual immunosuppression (RAMTS and dexamethasone) on intraperitoneal and subcapsular implants. Experimental methods were as described in the legend to Fig. 1. Seven adult C3H mice received intraperitoneal implants of 3×10^6 Ltk$^+$GH cells and ten mice received renal subcapsular implants of 3×10^6 cells. All animals received RAMTS and, twice daily, intramuscular injections of 100 μg dexamethasone for 7 days after implantation (as described in the legend to Table 1). The average increase in hGH observed on days 66 and 73 for the mice receiving intraperitoneal implants reflects high hGH levels (in excess of 500 ng/ml) in one of the five surviving mice in the group.

Days after implantation	Serum hGH (ng/ml) with RAMTS + dexamethasone	
	Intraperitoneal	Subcapsular
1	9.4 ± 2.9 (7)	25.8 ± 4.5 (10)
2	2.9 ± 0.8 (7)	11.4 ± 1.6 (10)
4	5.8 ± 1.7 (7)	17.5 ± 2.2 (10)
7	7.5 ± 3.6 (7)	19.4 ± 2.4 (10)
10	9.6 ± 3.9 (7)	53.4 ± 16.2 (9)
13	43.7 ± 19.3 (7)	26.2 ± 7.2 (9)
16	22.0 ± 8.5 (7)	7.5 ± 2.1 (9)
21	18.3 ± 7.5 (7)	6.1 ± 1.7 (9)
28	14.1 ± 5.9 (5)	12.2 ± 6.5 (9)
35	13.1 ± 6.9 (5)	9.2 ± 2.8 (8)
42	9.7 ± 4.9 (5)	9.7 ± 5.0 (7)
50	12.2 ± 6.4 (5)	7.4 ± 4.6 (6)
58	8.6 ± 4.1 (5)	2.7 ± 1.8 (4)
66	144.2 ± 84.4 (5)	4.0 ± 2.6 (4)
73	132.2 ± 105.8 (5)	3.7 ± 2.4 (3)
80	31.7 ± 24.0 (4)	3.0 ± 2.4 (3)
87	25.4 ± 17.9 (2)	4.0 ± 3.3 (3)
94	21.1 ± 14.9 (2)	6.2 ± 4.3 (2)

The prolonged survival of intraperitoneal implants as a result of immunosuppression led us to attempt similar experiments with mice receiving cells beneath a kidney capsule (Table 2). Treatment with a combination of RAMTS and dexamethasone resulted in hGH expression for more than 3 months in mice receiving subcapsular implants. Serum hGH peaked at approximately day 10 after implantation, declined over the next 6 days, and then remained at approximately 5 to 10 ng/ml for more than 2 months.

We have demonstrated that cultured murine cells can be engineered to produce a protein that they normally do not synthesize and that, when implanted into mice, the cells function in a reproducible and predictable fashion and show normal responsiveness to stimulation. The transfected cells function when placed in several locations within the animal, with the viability of the cells dependent, in part, on the site of implantation. The destruction of the implanted cells involves immune mechanisms and can be delayed by appropriate immunosuppressive agents.

Our results represent the initial development and characterization of an animal model system for gene delivery—the first of many steps toward the formulation of what may become a practical approach to gene therapy. Mouse L cell fibroblasts have been in culture for years and are, by definition, transformed. They are useful in these early stages because they are easily maintained and transfected and, as such, have enabled some of the important variables affecting implantation to be elucidated. When these cells are engineered to secrete hGH, their presence can be detected once implanted into the animal and their functional status can be readily monitored. The use of Ltk^+GH cells, therefore, allowed us to make observations that would have been difficult to make with many other available markers, such as the neomycin resistance gene. In addition to its role as a marker for implanted cells, hGH may have practical significance in the growth of transkaryotic animals.

The major advantage of this initial system of transkaryotic implantation is that it permits rapid and quantitative evaluation of the fate of cells after they are introduced into an animal. The use of Ltk^+GH cells, however, which ultimately are destroyed by C3H mice, necessarily led to the use of immunosuppression to extend their survival after implantation into the animal. Although it is both feasible and helpful to develop immunosuppressive regimens that can be tolerated by the transkaryotic host, the major limitation of the system as developed thus far is that these cells are not host-derived. In fact, it is remarkable that cells maintained in culture for several decades are able to function so efficiently when reintroduced into mice. At present, it seems that the ideal gene delivery system would utilize primary cells obtained from the subject to be treated rather than from established cultures of transformed cells. Primary fibroblasts, which are both readily accessible and readily cultured, seem to be suitable candidates for adaptation to transkaryotic implantation. As progress in culturing and transplanting other types of primary cells continues to be made (*15, 16*), the use of these cells for transkaryotic implantation should also be considered.

Important advances toward the application of our approach to gene therapy would require the development of conditions that permit the rapid transfection of primary cells with a gene construct that is regulated in a physiologically relevant fashion. What the fate of these cells would be when reintroduced into their host is not clear. Should the cells suffer prompt antigenic shifts while in culture, immunosuppression would become unavoidable. If it is feasible to generate clonal lines of transfected primary cells from the subject to be treated, suitable numbers of stably transfected primary cells producing a new or altered gene product could then be reimplanted into the individual (with or without immunosuppression) from which they were derived. Our data suggest that several sites for the implantation of genetically engineered cells may prove satisfactory for this purpose, and that long-term, vigorous function of transfected cells can be achieved after they are transferred.

Transkaryotic implantation may have several applications in studies of the immune system. We have shown that antiserum to thymocytes and dexamethasone have pronounced effects on the fate of implanted cells. When considered from an alternative perspective, transkaryotic implantation of Ltk^+GH cells allowed the effects of dexamethasone and the antiserum to thymocyte to be analyzed quantitatively. It is likely that this approach will be applicable to the quantitative evaluation of the efficacy and mode of action of a variety of other immunosuppressive agents (*17*).

Transkaryotic implantation as a gene therapy system can best be evaluated with reference to the most widely used gene therapy system, retroviral-mediated genetic transformation of marrow cells followed by transplantation of the marrow into lethally irradiated mice. The major practical problems with the marrow system naturally followed as a consequence of the need to use retroviruses as the means to transform marrow cells at essentially 100 percent efficiency. Our approach differs in that a single, well-characterized clonal line of transfected cells is implanted, as opposed to millions of retroviral-infected marrow cells, each of which is genetically distinct yet genetically uncharacterized. In addition, the use of nonretroviral-mediated transfection circumvents known difficulties with gene expression in retroviral vectors and potential problems in the recipient related to the use of retroviruses. These comparisons are not meant to imply that the marrow system is unworkable, but rather to indicate that it was (and is) appropriate to search for other gene therapy systems; it is unlikely that such a system will be entirely without flaws in its initial form. Transkaryotic implantation has several strengths that give it the potential to become a practical system for human gene therapy.

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10. A major technical advantage of the subcapsular route is that implants can easily be recovered for histochemical studies. Cells expressing hGH were observed within the subcapsular implant by immunoperoxidase staining, and the morphology of the adjacent renal parenchyma is normal. To ascertain the effect, if any, of hGH on the survival of implanted cells, nontransfected Ltk^- cells were placed subcapsularly in C3H mice. The fate of these cells as visualized by hematoxylin-eosin staining was identical to that of subcapsularly implanted Ltk^+GH cells, demonstrating that hGH expression does not have a major effect on the fate of the implanted cells.
11. The host animals tolerate excess hGH quite well, and long-term exposure to low to moderate levels (approximately 0 to 400 ng/ml) seems to have no ill effects on the mice. If more than 400 ng/ml persists in an animal for more than 1 to 2 weeks, however, the mouse begins to weaken, presumably due to the metabolic effects of hGH. In contrast, transgenic mice expressing more than 400 ng/ml of hGH show no sign of growth hormone–related abnormalities. This difference between the transkaryotic and transgenic mice may be attributable to the continuous presence of hGH in the transgenic mice beginning early in development.
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