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BRIEF COMMUNICATIONS

METHODOLOGY FOR THE ASSESSMENT OF LUNG PROTECTION

HUMAN PULMONARY ARTERY ENDOTHELIAL CELL PRESERVATION USING HAEMACCEL

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This investigation was designed to show an original methodology for the assessment of lung preservation and to analyze the efficacy of a low potassium polygelin solution (haemaccel [HM]) on isolated human pulmonary artery endothelial cells. The effects of HM were compared with those of low potassium dextran (LPD), Belzer (University of Wisconsin [UWS]), and Euro-Collins solutions. The viability of the endothelial cultures was assessed by means of both total protein content and recovery of metabolic cellular function expressed as the protein synthesis rate after 6 hr and 16 hr of incubation at 10°C. Our results failed to show any significant difference in the total protein content for HM, LPD, and UWS, both after 6 and 16 hr of incubation; however, the Euro-Collins-preserved sample revealed a significant drop in this parameter as early as 6 hr after the start. This finding was regarded as a clear indication of cellular cytotoxicity. In contrast, the metabolism recovery capacity of the cells varied significantly between HM and UWS at 6 hr and among HM, LPD, and UWS at 16 hr; at 6 hr, however, no significant difference was observed between HM and LPD. In conclusion, HM appears to exert a more significant effect on human pulmonary artery endothelial cell metabolism recovery than do the other fluids, thus suggesting its suitability as a long-term pulmonary perfusate.

Attempts have been made to use various isolated parenchymal cells in organ preservation screening methods (*1-3*); however, since the first site of contact with preservation solutions is the vascular endothelium, this cellular compartment is considered the most susceptible to preservation damage. Endothelial cells have been widely used as a preservation screening method (*4-9*); nevertheless, the conclusions resulting from these investigations should not be applied to all organs. First, endothelial cells used in research are frequently harvested from sources as varied as the peripheral venous vessels, umbilical cord, and aorta. Second, although the donor is often human, several surveys have reported that, due to greater availability, their cells had been harvested from a variety of mammalian species. Arterial endothelial cells are known to differ from venous and fetal endothelial cells, and investigations are moreover referred to possible morphological and biochemical differences in humans cells derived from different vascular beds (*10-12*). In addition, when the endothelial cells are derived from animal donors, interspecies differences may also arise (*13*). Another important factor that limits the homogenization of the data is the variety of methodologies for the assessment of cultured endothelial cell protection after incubation in solid-organ preservation solutions. The methods used range from morphological and morphometric evidence of cellular damage to biochemical analyses of viability. Microscope evaluation methods (phase contrast microscopic appearance of monolayer cellular cultures, trypan blue dye exclusion, morphological and morphometrical ultramicroscope evaluation) are characterized by the subjective limitations of the investigation and by the lesions determined by the fixation liquid. Most studies based on biochemical techniques (release of cytoplasmic enzymes, nuclear incorporation of radioactively labeled precursor) fail to differentiate between cytotoxic and cytostatic effects (*6*). At present, the only sensitive vitality test for cellular damage (real cytotoxic effect) is the reduction of 3-(4,5-dimethyltiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to an insoluble purple dye crystal (1-[4,5-dimethyltiazol-2-yl]-3,5-diphenylformazan) by mitochondrial dehydrogenase (*6*).

All of these vitality methods follow the "all or nothing" principle. Cytolysis evaluation parameters ignore the discrepancies in the metabolic function recovery of cells submitted to different types of stress. It is now generally accepted that the main target in organ preservation studies should be to identify a perfusate able to ensure proper organ vitality and prompt functional recovery immediately after transplantation. Moreover, it is agreed that a solution unable to guarantee prompt resumption of the organ's functions is unsuitable for preservation. If these parameters are applied to cellular cultures, researchers here too should strive to identify a solution capable of simultaneously maintaining cellular

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vitality and guaranteeing a prompt and rapid recovery of the cultures' metabolic function. One of the main parameters used to assess the capacity for cellular metabolic function recovery is protein synthesis, or rather, the speed with which cells fulfill this task. Protein synthesis evaluation is performed by incorporating a labeled protein precursor in the newly produced protein. Then, the amount of marked amino acid present in proteins produced over a given period is noted, indicating the speed with which proteins are produced and providing an index of cellular functioning. There is only one drawback: data regarding protein synthesis speed are comparable only if the subcultures involved have an identical concentration of structural cell proteins (in other words, an identical number of cells). This test has proved of great assistance in enabling our group to assess this type of recovery. For example, we have often used it to evaluate recovery after thermal (heat shock) (*14*) and osmotic stress (*15*).

Focusing our interest on lung preservation, we have chosen as a lung preservation screening method the endothelium of the pulmonary artery harvested from human donors. To our knowledge, this cellular population has hitherto never been used for metabolic studies. The present research was designed to analyze the efficacy of a low potassium polygelin solution (haemaccel [HM*]) in the prevention of ischemic lesions after prolonged preservation using cultured human pulmonary artery endothelial cells (HPAEC) as a suitable lung preservation screening method; the results were then compared with those obtained using low potassium dextran solution (LPD), University of Wisconsin solution (UWS), and Euro-Collins solution (ECS).

Endothelial source. Cryopreserved tertiary cultures of HPAEC (CC-2530; Clonetics Corp., San Diego, CA) in a screw-cap vial containing approximately 5×10^5 cells were used. Each cell strain was isolated from a single donor with no pooling.

Preparation of endothelial cell growth medium and thawing of cells. Endothelial cell growth medium (EGM) (CC-3024; Clonetics) is a complete medium based on the MCDB 131 medium formulation and supplemented with 10 ng/ml human recombinant epidermal growth factor (CC-4017; Clonetics), 1 μg/ml hydrocortisone, 2% fetal bovine serum (CC-4101; Clonetics), 50 μg/ml gentamicin, 50 ng/ml amphotericin B, and bovine brain extract (CC-4092; Clonetics) 12 μg/ml final protein concentration with 10 μg/ml heparin.

EGM was equilibrated in a 37°C 5% CO_2 humidified incubator for 30 min. A vial containing isolated cells was placed in a 37°C water bath until the contents had almost thawed. The contents were then placed in the equilibrate culture 25-cm^2 flask at a seeding density of 2500 cells/cm^2 before they were transferred to an incubator. Twenty-four hours later, the growth medium was changed to remove any residual dimethylsulfoxide present into the thawed medium.

Protocol for subcultures. Since confluence arrests mitosis and reduces cells viability, subcultures were prepared before proliferating cultures became confluent (approximately 85% of confluence).

The culture medium was removed from the HPAEC stock flask and the cells were covered with 3 ml of the supplied trypsin/EDTA solution (CC-5012; Clonetics). The cell monolayer was examined using a microscope and the culture was left in trypsin solution until the cells had detached themselves (3–4 min). The detached cells were then rounded up while the flask was rinsed with 2 ml of HBSS (CC-5022; Clonetics). The cells were next transferred into a sterile centrifuge tube containing 3 ml of trypsin neutralizing solution (CC-5002; Clonetics). The cells thus harvested were centrifuged at 220×*g* for 5 min to produce a cell pellet that was subsequently resuspended in 2 ml of EGM and counted with a Burker hemacytometer. At this point, the new subcultures were inoculated at a density of 5000 cells/cm^2 in 9-cm^2 well plates. The EGM was changed every 3 days. A week later, the subcultures were observed to have attained a 80% confluence. The subcultures were now considered ready for the preservation experiments.

Preservation. The HPAEC cultures were incubated for 6 hr and 16 hr at 10°C in 2 ml/well of HM (Behring, Marburg, Germany), LPD (Sigma Chemical Co., St. Louis, MO), UWS (Du Pont Pharmaceuticals, Wilmington, DE), and ECS (Baxter, Deerfield, IL). A warm control (37°C) was performed with EGM. After incubation, two tests were performed to assess the effects of the solutions. The first was designed to evaluate the amount of protein in the subcultures and the result was considered an indication of the number of cells present in the subcultures. Since cells were seeded at the same density and each one is endowed with an identical protein structure, while any protein produced in 1 hr is negligible compared with structural protein, we decided that variations in this parameter could be regarded as an index of culture cell loss. In other words, changes in the amount of protein were indirect proof of cell death. The second test was a metabolic test set up to calculate cellular metabolism recovery after physical stress (hypothermic metabolic ischemia). The test was assessed according to the protein synthesis rate. This test measured the speed of cell protein production after cell incubation in a growth medium supplemented with a labeled amino acid.

Technique for the assessment of total protein content and cellular metabolism recovery. Cells were preserved in each solution and then incubated in [^{3}H]leucine (Amersham, Bucks, UK) growth medium for a 1 hr at 37°C to finally measure the cellular metabolic activity. The growth medium was composed of F12 (HAM's nutrient mixture F-12; GIBCO, Grand Island, NY) and minimal essential medium (GIBCO) with a 1:1 ratio without leucine (final leucine concentration = 0.05 mM), supplemented with 5% fetal calf serum with labeled leucine ([^{3}H]leucine, 3 μCi/ml). This radioactive medium was then removed and the cells underwent 3 rapid washings with glucose supplemented with Earle's salt solution at 4°C, after which 10% trichloroacetic acid cold solution was finally added. The precipitate was solubilized in 0.2 N NaOH and the radioactivity of the acid-insoluble fraction was measured with a liquid scintillation counter. The total protein content was measured using a dye fixation method (Bio-Rad, Richmond, USA), with bovine serum albumin as standard. Finally, dye absorption was measured using a spectrophotometer.

Statistical analysis. The results are expressed as micrograms of protein per cm^2 and as nanomoles of [^{3}H]leucine per milligram of protein per 60 min, and are presented as mean

* Abbreviations: ECS, Euro-Collins solution; EGM, endothelial cell growth medium; LPD, low potassium dextran solution; HM, haemaccel; HPAEC, human pulmonary artery endothelial cell; UWS, University of Wisconsin solution.

± SD of 3 wells, at a 5000/cm^2 density for each solution studied. Student's t test was used for statistical comparison. $P<0.05$ was considered significant.

The results of the research are reported in Table 1. Regarding the total protein content after 6 hr of incubation, the subcultures preserved with HM, LPD, and UWS failed to reveal any significant difference from the control. In other words, the number of cells present in the subcultures adhering to the substratum that determine the total protein content was practically identical, with no significant instances of cell death. However, the subcultures preserved with ECS showed a significant drop in the number of proteins, suggesting a clear indication of its cytotoxicity. At the second time interval (16 hr), a significant difference was observed between the warm control and HM, LPD, and UWS, while no significant difference was observed among the solutions. This demonstrates that prolonged hypothermic preservation determines a significant loss of endothelial cells that, in our experiment, is not dependent on the solutions used.

Although the cytotoxic effect of HM, LPD, and UWS was identical at 6 and 16 hr, significant differences were identified for all three solutions regarding the recovery of cellular metabolic activity.

After 6 hr of preservation and 1 hr of warm (37°C) simulated reperfusion with growth medium supplemented with labeled leucine, no significant difference was observed between HM- and LPD-preserved subcultures, but such a discrepancy existed between HM- and UWS-incubated subcultures. At the second time interval and after the same period of simulated reperfusion, the HM-LPD difference became significant and the HM-UWS discrepancy remained so. Although HM-induced cytotoxicity is similar to that of other solutions (with the sole exception of ECS), HM appears to exert a less inhibitory effect on cellular metabolism, making possible a rapid recovery of cellular metabolic function after a prolonged ischemic stress.

During solid-organ transplantation, the physical condition of preservation (pressure used during vascular flush-out, temperature, humidity, O_2 and CO_2 concentration, etc.), the mechanical manipulation of the organ, and, above all, the composition of preservation solutions (pH, osmolarity, high or low doses of potassium, oncotic agents, drugs etc.) are known to modify the endothelium to produce cell dysfunction and disruption. The resulting damage to the endothelial surface exposes the thrombogenic basement membrane and leads to heightened vascular permeability and alterations in the vascular tone, with immediate effects in the form of organ edema and graft dysfunction (*16*).

Although for a considerable time intracellular solutions have been considered standard for solid-organ preservation and theoretically regarded as superior to extracellular solutions (*17*), several recent surveys have suggested that the latter may be more effective, at least in regard to lung protection; this is due in part to the milder vasoconstriction induced by low potassium-ion concentration and in part to the direct high potassium concentration-related injuries produced during flush and storage periods. (*18*, *19*). However, a low potassium solution must be added with colloid-osmotic agents to counteract the cell swelling caused by hypothermic storage and by the solution itself.

Haemaccel is an extracellular-type fluid containing an oncotic agent (polygelin) with an average molecular mass of 35 kDa. HM is iso-oncotic with plasma; therefore, the risk of cell dehydration and swelling is reduced to a minimum. This

TABLE 1. Total protein content and rate of protein synthesis of HPAEC subcultures after 6 hr and 16 hr of preservation at 10°C

	Total protein content (μg of proteins/cm^2)		
	Time 0	6-hr P37[a]	16-hr P37
Warm control	7.56±0.11[b]	7.53±0.65[d]	7.54±0.75[e]
	Time 0	6-hr P10	16-hr P10
HM	7.52±0.29[b]	7.48±0.87	6.15±0.33[f]
LPD	7.49±0.41[b]	7.23±0.79	6.08±0.2
UWS	7.54±0.37[b]	7.14±0.36	5.57±0.62
ECS	7.51±0.46[b]	5.80±0.22[c]	Not performed
	Rate of protein synthesis (nmol [^{3}H]leucine/mg of proteins/min)		
	Time 0	6-hr P37 + 1-hr R37	16-hr P37 + 1-hr R37
Warm control	7.70±0.56[b]	7.71±0.88	7.69±0.66
	Time 0	6-hr P10 + 1-hr R37	16-hr P10 + 1-hr R37
HM	7.68±0.44[b]	6.05±0.74	4.88±0.40
LPD	7.74±0.13[b]	5.35±0.47[g]	3.87±0.23[i]
UWS	7.72±0.72[b]	2.99±0.23[h]	2.08±0.20[l]

[a] Abbreviations used in table: P37, preservation at 37°C; P10, preservation at 10°C; R37, simulated reperfusion with growth medium with labeled amino acid at 37°C. The results are expressed as mean ± SD. Student's t test was used for statistical comparison.

[b] Data at time 0 confirm that the subcultures are seeded with the same number of cells (total protein content) and present the same metabolic function recovery (rate of protein synthesis) at the beginning of preservation; [c] warm control vs. ECS: $P<0.05$; [d] warm control vs. HM, vs. LPD, and vs. UWS: $P>0.05$; [e] warm control vs. HM, vs. LPD, and vs. UWS: $P<0.05$; [f] HM vs. LPD and UWS $P>0.05$; [g] HM vs. LPD, $P>0.05$; [h] HM vs. UWS, $P<0.05$; [i] HM vs. LPD, $P<0.05$; [l] HM vs. UWS, $P<0.05$. HM seems to exert a more significant effect on cell metabolism-recovery than do the other fluids, thus suggesting its suitability as a long-term pulmonary perfusate.

TABLE 2. Main characteristics of LPD and HM solutions

	LPD	HM
Na (mmol/L)	168	140
K (mmol/L)	4	5.1
pH	7.4	7.3
Osmolarity (mOsm/L)	290	285
Colloid-oncotic agent	Dextran 40 (2%)[a]	Polygelin (35%)[b]
Oncotic pressure (mmHg)	15–19	25.7–28.6

[a] Dextran 40: low molecular mass dextran, average molecular mass 40 kDa.

[b] Small polypeptides (molecular mass 5–15 kDa) derived from gelatine hydrolase and cross-linked with hexamethylene di-isocyanate to form molecules of polygelin (molecular mass 35 kDa).

solution was widely used in the past in all types of shocks, in hemodilution procedures during extracorporeal circulation, and as a carrier solution during intravenous insulin infusion. Nevertheless, even though it was tested in some experimental organ preservation research studies (*20–22*), in the last few years it has largely been ignored. To date, this solution has never been used for the investigation of lung preservation. Our research shows that HM exerts a less depressing effect on cellular metabolism and enhances functional recovery of cultured HPAEC. There exists no immediate explanation for this phenomenon. Although the compositions of HM and UWS vary, no single element can be held responsible for UWS's greater inhibition of metabolism. The differences produced by HM and LPD are more easily justified, given the less complex nature of the two solutions. Table 2 shows that the most obvious difference lies in the lesser colloid-osmotic pressure exerted by LPD; this diminished pressure may determine a greater cellular swelling of LPD-incubated subcultures, especially after a prolonged hypothermic preservation.

In conclusion, HPAEC can be used successfully as a lung preservation screening method. Preliminary data regarding the use of HM in endothelial cell preservation suggest its suitability as solution for prolonged lung protection.

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CRYOPRESERVATION OF PURIFIED BOVINE ISLETS OF LANGERHANS[1]

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In this study, bovine islets were isolated by collagenase digestion and density gradient purification, equilibrated stepwise with 3 M dimethylsulfoxide at 24°C, nucleated at −150°C, slow cooled at 0.25°C/min down to −40°C, and finally stored at −150°C. After variable periods of time, the islets were quickly thawed at 37°C, and dimethylsulfoxide was removed by 0.75 M sucrose. Postthawing recovery was 86±6% islet equivalents. Histology confirmed the identity and morphological integrity of the islets. Insulin release from the frozen-thawed islets was 0.13±0.03 μU/is/min at 3.3 mmol/L glucose and increased significantly (0.27±0.04 μU/is/min, $P<0.05$) at 25 mmol/L glucose. Encapsulated, cryopreserved islets reversed hyperglycemia in diabetic mice after 6–8 days following implantation. Therefore, the method described in this paper permitted successful cryopreservation of bovine islets of proven in vitro and in vivo viability.

Transplantation of the pancreas in addition to kidney has a major role in the treatment of uremic or posturemic patients with type 1 insulin-dependent diabetes mellitus (*1, 2*). More recently, there have been several reports of insulin independence following allotransplantation of isolated pancreatic islets (*3, 4*). This latter procedure seems to offer a number of advantages, including the simplicity of the implantation procedure, and the possibility of reducing islet immunogenicity by immunoalteration and/or immunoisolation techniques (*5, 6*). However, the limited availability of human donors makes the search for nonhuman islet sources mandatory for future developments in pancreatic islet transplantation. From this point of view, porcine islets are being evaluated extensively (*7–10*). An additional, alternative animal source of islets of Langerhans could be the bovine pancreas (*11*). Bovine pancreases are as readily obtainable as porcine pancreatic glands, bovine insulin has long been used to treat human diabetes, and, potentially, beef islets could be less fragile than porcine islets. In view of the potential use of bovine islets for transplantation studies, techniques are needed for long-term storage of the islets from this animal species. At the present time, successful cryogenic procedures have been developed for sub-zero preservation of rodent, dog, pig, and human islets (*12, 13*). In this report, we describe a method for the cryopreservation of purified bovine islets, and show that frozen-thawed pancreatic islet tissue maintains morphological integrity and in vitro and in vivo viability.

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Bovine pancreases were obtained at a local slaughterhouse from animals approximately 11–18 months old, and processed as described previously in detail (*11*). The glands were transported to the laboratory in Eurocollins solution (Fresenius, Hamburg, Germany) at 4°C. Warm and cold ischemia times were less than 15 min and 120 min, respectively. The enzyme collagenase (Type XI, Sigma Chemical Co, St. Louis, MO) was used for digestion of the pancreases. The pancreatic duct of the glands was cannulated and the digestion solution (2 mg/ml dissolved in 300 ml of HBSS, containing 2% bovine serum) was injected (3-fold in volume the weight of the pancreas) to distend the tissue. After the distension, the gland was placed into a 500-ml glass beaker, and the solution not used for distension was added to the beaker. This was loaded into a shaking water bath at 37°C, activated at 120 revolutions per min. After 20, 30, and 40 min, the tissue was shaken with forceps for 30 sec; then, the digestate was filtered through 300-μm and 90-μm mesh stainless steel filters, in sequence. The solution that passed through the filters and the tissue entrapped on the 300-μm mesh filter was again placed into the water bath, for further digestion. The tissue that remained on the 90-μm mesh filter was washed with 250 ml of Eurocollins solution at 4°C and was left to settle for 20 min. For the purification, the digested tissue was pelletted at 400×*g* for 2 min at 4°C. Then, 1-ml aliquots were loaded into 50-ml plastic conicals and resuspended in 13 ml of Histopaque 1077 (Sigma). This layer was topped with 10 ml of HBSS. After centrifugation at 800×*g* for 5 min at 4°C, the islets were recovered at the interface between the Histopaque and the HBSS layers. The purified islets were cultured free-floating for 7 days in tissue culture medium M199 (Sigma), pH 7.4, containing 10% bovine serum (Sigma), 0.2 mmol/L L-glutamine, 25 mmol/L HEPES, 100,000 U/L penicillin, 100,000 μg/L streptomycin, 50,000 μg/L gentamycin, and 1,000 μg/L amphotericin B, at 37°C in 5% CO_2 and 95% humidified air.

After 7 days of culture, the islets were transferred to 50-ml conical tubes and centrifuged at 600×*g* for 3 min at 24°C. The supernatant was removed and the pellet was resuspended in 0.5 ml of M199 culture medium. Aliquots of approximately 1000 islets were then transferred into 2.5-ml cryotubes (Nalgene, Hereford, UK). Successively, 0.25 ml of 2 mol/L dimethylsulfoxide (DMSO*) (Sigma) was added at 24°C, and, after a 5-min interval, a second addition of 0.25 ml of 2 mol/L DMSO was performed. After 25 min of equilibration at 24°C, 1 ml of 3 mol/L DMSO was added to each cryotube. The cryotubes were then placed in an ice bath for 15 min, and successively moved to a climatic chamber (Angelantoni Climatic Systems, Perugia, Italy) equipped with a programmable system (ASCON, Milano Italy) and set at −7.0°C. After 5

* Abbreviations: DMSO, dimethylsulfoxide.

min at −7.0°C, the islets were nucleated at −150°C for 5 min and then placed again at −7.0°C for 10 min to permit the release of the latent heat of fusion. After that, the islets were cooled at 0.25°C/min to −40°C, and finally stored at −150°C.

After variable periods of storage (from 1 day to 2 months), the islets were rapidly thawed in a 37°C water bath. Then the cryotubes were centrifuged at 600×*g* for 2.5 min at 4°C, and the supernatant was removed. Half a milliliter of 0.75 mol/ sucrose in M199, containing 2% Trasylol, was then added to each tube to remove the intracellular DMSO. After 30 min of incubation at 4°C, 0.5 ml, 0.5 ml, and 1 ml of medium M199 was added at 5-min intervals at 24°C to dilute the sucrose. Finally, the islets were centrifuged at 600×*g* for 2.5 min, resuspended in M199 culture medium, and transferred to culture flasks (approximately 3000 islets in 40 ml of culture medium per flask) at 37°C in 5% CO_2 and 95% humidified air.

Islet aliquots were removed for count, histological staining, and viability assessment immediately before freezing and 2 days after thawing. In vitro islet function was evaluated by incubation at varying glucose concentrations, as described previously (*11*). In vivo viability was assessed by transplanting 7-day cultured or cryopreserved islet tissue into BALB/c mice with streptozotocin-induced diabetes (blood glucose values higher than 22 mmol/L). The animals received approximately 2000 islets intraperitoneally, either nonencapsulated or coated with polyvinyl alcohol/polyacrylic acid membranes (1000 islets per macrocapsule). The procedure to prepare the membranes and their characterization have been detailed previously (*14*).

Results are given as mean ± SEM. Student's *t* test for unpaired data was used to evaluate differences between means.

To develop the method, 15 cryopreservation experiments were performed, with islets prepared from 6 different pancreases. The data in this paper show the results of the last 6 consecutive cryopreservation experiments. We used 13,500±3,156 actual islets (12,150±2,834 islet equivalents [IE]) per cryopreservation experiment. Post-thaw recovery was 13,354±2,319 actual islets (98±8%) and 11,929±2,089 IE (86±6%). Hematoxylin-eosin staining and immunocytochemical characterization (Fig. 1) of the cryopreserved bovine islets demonstrated the identity and morphological integrity of the tissue. Neutral red staining (performed by using a final staining concentration of 3.2 μg/L) (*15*) showed that >90% of islets were viable. Insulin release from cryopreserved islets was 0.13±0.03 μU/is/min at 3.3 mmol/L glucose, and increased to 0.27±0.04 μU/is/min at 25 mmol/L glucose ($P<0.05$). The corresponding figures with noncryopreserved bovine islets were 0.06±0.01 μU/is/min at low glucose ($P<0.05$ vs. cryopreserved islets) and 0.21±0.01 μU/is/min at high glucose.

As shown in Figure 2, intraperitoneally transplanted, nonencapsulated, noncryopreserved islets did not cause any change of plasma glucose in diabetic mice (n=5). Noncryopreserved, encapsulated bovine islets promptly restored normoglycemia in the transplanted animals (n=4). When cryopreserved, encapsulated islets were transplanted into diabetic mice (n=5), a reduction of blood glucose concentrations toward normal values was observed after 4–7 days following implantation.

In the past few years, several studies have been published on the long-term cryogenic storage of rodent, dog, pig, and human pancreatic islets (*12*, *13*). In the present paper, for the first time, we describe a technique to cryopreserve islets of Langerhans from the bovine pancreas. Bovine islets are being considered as a potential alternative source of pancreatic endocrine tissue for transplantation studies (*11*).

Our results show that, by using a modification of a freezing-thawing technique developed previously for porcine islets

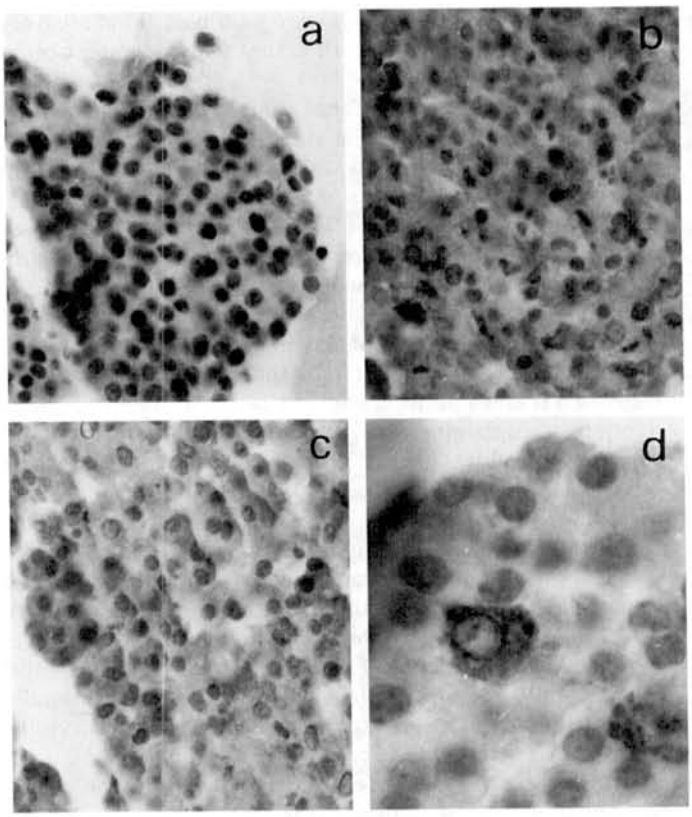

FIGURE 1. Histology of frozen-thawed bovine pancreatic islets: (a) hematoxylin-eosin staining; (b) insulin immunocytochemical staining; (c) glucagon immunocytochemical staining; (d) somatostatin immunocytochemical staining.

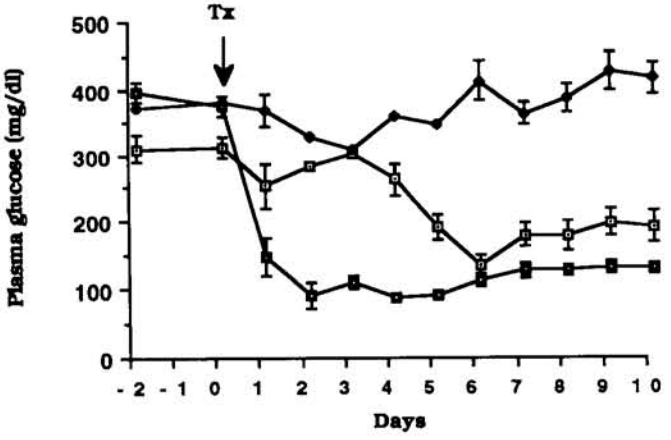

FIGURE 2. Plasma glucose of mice with streptozotocin-induced diabetes that had received either noncryopreserved, nonencapsulated bovine islets (♦), noncryopreserved, encapsulated islets (□), or cryopreserved, encapsulated islets (■).

(*13*), it is possible to achieve a very good post-thaw recovery of bovine islets in terms of both actual and equivalent numbers.

The histological and functional studies we performed demonstrated the identity and the viability of the cryopreserved islets. Although neutral red evaluation is not the ideal technique to test islet viability in vitro, it gives quick and sufficiently reliable information (*15*). Similar to what has been reported previously with porcine and human islets (*12*, *13*), some functional differences were observed between noncryopreserved and cryopreserved bovine islets. In vitro insulin release studies showed that, compared with controls, the frozen-thawed islets secreted more insulin at low glucose and exhibited a somewhat decreased glucose-stimulated hormone secretion at high glucose. This suggests that, during the cryogenic procedure, some damage may occur, leading to a partial loss of the feedback controls in the β-cells, or a leaky membrane system. Also, our in vivo studies confirm that more cryopreserved islets are needed to achieve normoglycemia in laboratory animals, compared with noncryopreserved tissue. Indeed, in our experiments, a mass of 2000 noncryopreserved, encapsulated islets reverted diabetes in mice, whereas a similar amount of frozen-thawed bovine islets did not completely normalize plasma glucose in such animals. Further studies are needed to define dose titration curves to cure diabetes with cryopreserved bovine islets.

In conclusion, this study demonstrates that cryogenic storage of purified bovine pancreatic islets is feasible, and that the islets maintain in vitro and in vivo viability following the cryopreservation procedure.

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LAPAROSCOPIC LIVE DONOR NEPHRECTOMY

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A laparoscopic live-donor nephrectomy was performed on a 40-year-old man. The kidney was removed intact via a 9-cm infraumbilical midline incision. Warm ischemia was limited to less than 5 min. Immediately upon revascularization, the allograft produced urine. By the second postoperative day, the recipient's serum creatinine had decreased to 0.7 mg/dl. The donor's postoperative course was uneventful. He experienced minimal discomfort and was discharged home on the first postoperative day.

We conclude that laparoscopic donor nephrectomy is feasible. It can be performed without apparent deleterious effects to either the donor or the recipient. The limited discomfort and rapid convalescence enjoyed by our patient indicate that this technique may prove to be advantageous.

Recent improvements in video technology and surgical instrumentation have resulted in the application of minimally invasive techniques to a myriad of surgical procedures. In many instances, laparoscopic procedures have proven more advantageous when compared with the standard open approach. Laparoscopic nephrectomy was first successfully performed clinically in 1990 by Clayman and associates (*1*). It is now offered routinely to patients in several institutions for both benign and malignant diseases. It has proven efficacious with minimal morbidity, decreased postoperative pain, shorter convalescent time, and decreased length of hospital stay (*2, 3*). Gill et al. (*4*) have demonstrated the feasibility of laparoscopic donor nephrectomy in a porcine model. We report the first case in which a live-donor laparoscopic nephrectomy was performed. The clinical course, operative technique, and potential advantages are discussed.

J.S. was a 41-year-old woman (HLA: A1,2 B8,39 DR13,17) with end-stage renal disease secondary to glomerulonephritis. Her brother, J.G., a 40-year-old man (HLA: A2,3 B18,39 DR1,17), volunteered to donate his kidney. Laparoscopic donor nephrectomy was discussed at length with both the donor and recipient and informed consent for the procedure was obtained. The surgery was performed on February 8, 1995.

The donor was placed in a modified left lateral decubitus position. Pneumoperitoneum was established via a Veress needle. Four 12-mm ports were placed in the abdomen (at the umbilicus, 2 cm medial to the left anterior superior iliac spine, 2 cm anterior to the left eleventh rib, and in the midaxillary line at the level of the umbilicus; Fig. 1). The videoendoscope was inserted via the umbilical port. After the descending colon was mobilized medially by incising the lateral peritoneal reflection, Gerota's fascia was identified and entered. The lateral aspect of the kidney was freed. Mobilization of the lower pole enabled identification of the ureter. The ureter and a generous mesoureter were dissected from surrounding structures to the level of the iliac bifurcation. The ureter was divided approximately 12 cm distal to the lower pole of the kidney, after the distal limb was occluded with a 9-mm endostapler. By utilizing sharp dissection, the upper pole was isolated from adjacent structures. The kidney was then reflected medially and posterior attachments were divided. At this point, the kidney was free from all attachments except the renal vessels.

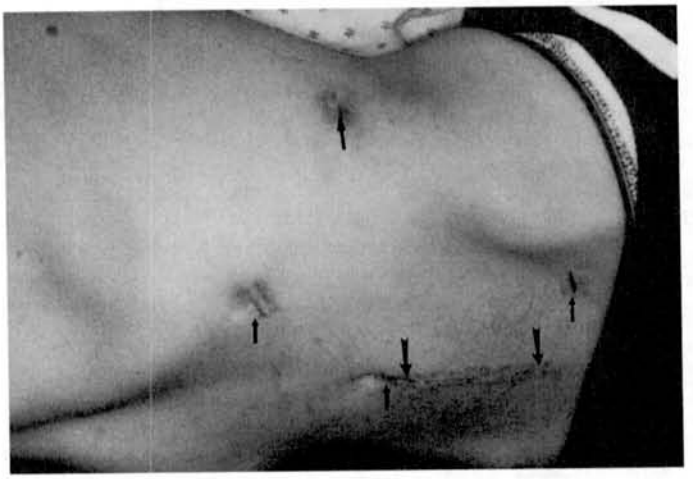

FIGURE 1. The small arrows identify the location of the instrument ports. The large arrows indicate the extent of the 9-cm lower midline incision used for extraction of the kidney.

The renal vein was identified in the hilum and dissected medially. This allowed for identification of the gonadal and adrenal veins, which were clipped and divided. With the vein retracted anteriorly, the renal artery was recognized and dissected free to its origin at the aorta (Fig. 2). In preparation for removal of the kidney, the umbilical trocar incision was extended inferiorly to create a 9-cm midline incision. This was carried down to, but not through, the peritoneum. The renal artery was occluded proximally with two 9-mm vascular clips and divided. The renal vein was transected anterior to the aorta utilizing an endovascular-GIA stapler. The peritoneum was then incised and the kidney was removed manually from the peritoneal cavity. Direct camera vision from the perspective of the iliac trocar facilitated localization of the kidney for extraction. The kidney was subsequently flushed with an iced modified Euro-Collins solution. Total warm ischemic time was limited to under 5 min. Operative time for the donor nephrectomy was 3.5 hr. No intraoperative complications occurred. Blood loss was minimal.

Oral intake was tolerated the evening of surgery and the donor was discharged home on the first postoperative day. He experienced minimal postoperative pain. Total parenteral

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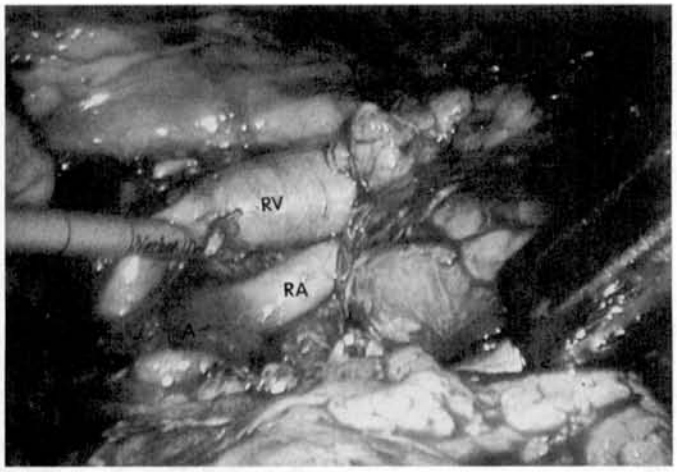

FIGURE 2. Laparoscopic video magnification aids in the dissection of the renal vessels. The renal vein (RV) is elevated by the suction/irrigator, thus exposing the renal artery (RA). The origin of the renal artery at the aorta (A) is clearly visualized.

analgesic requirements consisted of 20 mg of morphine sulfate in the recovery room and 100 mg of meperidine on the morning of the first postoperative day. Within 7 days of surgery, the donor had no further pain. He returned to work as a welder 2 weeks after surgery.

The recipient operation was also uneventful. Adequate lengths of renal artery, vein, and ureter were provided (Fig. 3). Urine production commenced immediately upon revascularization. By the second postoperative day, the recipient's serum creatinine level had decreased to 0.7 mg/dl. She was discharged home on the ninth postoperative day with a creatinine level of 0.6 mg/dl on triple immunosuppression consisting of azathioprine, prednisone, and FK506.

This case demonstrates that laparoscopic live-donor nephrectomy can be performed without detriment to either the donor or the recipient. By placing the incision in a low midline position, we believe we markedly decreased the morbidity usually associated with the traditional flank incision. Laparoscopic visualization affords excellent exposure to the kidney and the hilar structures. Video magnification facilitates dissection of the renal vessels. The laparoscopically retrieved kidney had sufficient length of renal vein, renal artery, and ureter for standard allograft implantation. Studies done in a porcine laparoscopic donor nephrectomy model demonstrated that the lengths of the renal vessels and the ureter were similar for those kidneys harvested laparoscopically and those removed by the conventional approach (*4*). Warm ischemic time in the porcine model averaged 9.8±7.2 min (*4*). However, we were able to limit the warm ischemia to under 5 min. Although this is acceptable, we are confident that, with increased experience, warm ischemic time can be further decreased. Similarly, as has been seen with laparoscopic cholecystectomy, total operative time should approach that expected for an open procedure.

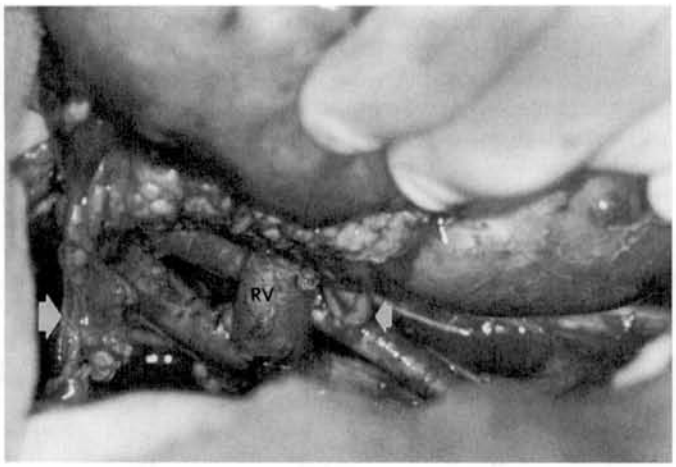

FIGURE 3. Adequate lengths of allograft ureter (large arrow), renal vein (RV), and renal artery (small arrow) were obtained. This is exhibited here with the kidney in situ after transplantation.

The laparoscopic approach to live-donor nephrectomy that we performed was transperitoneal. The open anterior transperitoneal procedure is an accepted method for performing live-donor nephrectomies (*5*, *6*) and results in minimal morbidity comparable to that of the extraperitoneal flank approach (*7*). The small lower midline incision that we used for extraction of the kidney was well tolerated. However, Yang et al. (*8*) have described a minimally invasive extraperitoneal open flank approach utilizing endoscopic visualization and purpose-built retractors, which may be an acceptable alternative.

This case has demonstrated not only that the laparoscopic approach to live-donor nephrectomy is technically feasible, but that it may provide significant advantages to the patient. In a retrospective review of open live-donor nephrectomies, Clayman et al. (*9*) have reported that the average dose of parenteral analgesia was 66 mg of morphine sulfate, the mean length of hospital stay was 6.4 days, and the average delay until resumption of full activity was 80 days. In other series of live-donor nephrectomies, lengths of stay ranging from 6 to 10 days have been reported (*7*, *10*, *11*). We retrospectively reviewed the previous 21 consecutive live-donor nephrectomies performed at our institution from January 1991 to January 1994 and found the mean length of hospitalization to be 5.6±1.8 days. The 1-day hospital stay required by our patient compares favorably with our open-donor nephrectomy experience. Similarly, a low analgesic requirement was seen with this procedure. The expectation that postoperative pain and discomfort may be minimized by the laparoscopic approach is further supported by the series of 10 laparoscopic nephrectomies in which the average patient received only 19 mg of morphine sulfate and had returned to normal activity within 1.7 weeks (*2*).

Despite the extreme organ shortage, in 1993, live-donor transplantation accounted for only 24.8% of all renal transplants performed in the United States (*12*). The application of minimally invasive techniques to the donor nephrectomy operation, with its potential advantages of decreased pain and shorter convalescence, may make live donor renal transplantation more attractive. Conceivably, these advantages could increase the number of live-donor renal transplants performed and may help to alleviate the critical deficit of transplantable organs.

In the present climate of fiscal concern, the potential reduction in hospital stay offered by the laparoscopic approach may serve to lower donor costs. However, one caveat is that the use of relatively expensive disposable laparoscopic instruments and longer operating room times may partially

offset any savings. The potential benefit of more rapid return to normal activities and employment should not be minimized. One can predict that, as transplantation becomes the focus of more sophisticated outcomes research, shorter convalescence will tilt the economic balance in favor of the laparoscopic approach, even if savings in hospital charges are limited.

For live-donor nephrectomy, the reported mortality is 0.07% (*13*) and the incidence of major complications is 1–8% (*7*, *10*, *13*). To obtain similarly acceptable results with the laparoscopic operation, we are presently utilizing rather stringent selection criteria. Currently, thin individuals without a history of previous abdominal surgery are being considered as candidates. Additionally, normal renovascular and ureteral anatomy are required. However, it is anticipated that the laparoscopic approach will eventually be considered appropriate for most live donors as our comfort level increases with the technical aspects of the operation.

In the case reported here, the operation was performed by an experienced laparoscopic surgeon (L.R.K.) who routinely performs complex laparoscopic cases such as nephrectomies. We caution that a surgeon must possess a high degree of laparoscopic proficiency before attempting this procedure. Additionally, we recommend that there be a low threshold for open conversion if one encounters such difficulties as significant bleeding, or unexpected anatomic variants not diagnosed prospectively by angiography. Additional experience is required in a large number of patients before one can conclude that the laparoscopic approach should replace the standard open technique of live-donor nephrectomy. However, we believe that the potential benefits of the minimally invasive procedure justifies its continued careful application and assessment.

In summary, this case demonstrates that laparoscopic live-donor nephrectomy is technically feasible. The operation was performed safely. The integrity of the donated kidney was not compromised. Ureteral and vascular length were adequate and warm ischemic time was within acceptable limits. Additionally, the laparoscopic operation may offer advantages to the donor in terms of comfort and convenience. The potential decrease in hospitalization and convalescence observed with this operation may prove to be financially advantageous. Based on the excellent results achieved with this case, we believe that further careful study of this procedure is warranted.

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HEPATITIS C-ASSOCIATED CRYOGLOBULINEMIA AFTER LIVER TRANSPLANTATION

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Mixed cryoglobulinemia is well known to be associated with hepatitis C virus (HCV) infection. We report two cases in which cryoglobulinemia appeared or became grossly exacerbated after orthotopic liver transplantation. In both cases, there was co-appearance of cryoglobulinemia with the reinfection of the grafted liver with HCV. It is postulated that the cryoglobulinemia might be related to secondary HCV infection in these patients.

Essential mixed cryoglobulinemia (EMC*) is a disorder characterized clinically by purpura arthralgia, neuropathy, glomerulonephritis, and systemic vasculitis (*1–3*). There are three different types. Type II is the result of precipitation of polyclonal IgG and monoclonal IgM rheumatic factor and has been found to be associated with hepatitis C virus (HCV) infection (*4*). Anti-HCV antibodies were found in the serum of 36–98% of patients with EMC (*5*). The composition of the precipitate extracted from HCV-infected patients was found to contain concentrated HCV virions and HCV antigen-antibody complexes (*4, 6, 7*). Patients who are HCV carriers were found to suffer from more severe clinical manifestations of cryoglobulinemia than anti-HCV-negative patients (*8*).

HCV infection is the cause of chronic liver disease in a large number of patients, at times necessitating liver transplantation (*9*). Reinfection of the transplanted liver with HCV commonly occurs after transplantation for HCV cirrhosis (*10*), causing changes ranging from mild inflammation to severe hepatitis with consequent cirrhosis of the grafted liver (*11*). No report has been published to date linking EMC with grafted liver HCV reinfection. We present a patient in whom EMC appeared de novo after HCV-grafted liver infection, and a patient in whom mild pretransplant EMC reappeared after surgery in a more severe form.

Case 1 was a 39-year-old Caucasian male suffering with chronic active HCV hepatitis for 12 years, with advancing cirrhosis throughout the years, who finally underwent liver transplantation. Before transplantation, cryoglobulin was not detected in the serum and the patient had no clinical manifestations of EMC. Complement levels were normal, and both antinuclear factor and rheumatoid factor were negative. Early rejection was noted in the immediate postoperative period that was treated successfully with FK506. About 3 months after transplantation, liver enzyme levels were noted to be elevated, and a biopsy was performed that demonstrated chronic active hepatitis. Serological testing proved the patient to be anti-HCV positive and HCV RNA was positive by reverse transcriptase polymerase chain reaction. Antiviral treatment with ribavirin (up to 15 mg/kg/day) was initiated, but this was not found useful for eliminating HCV RNA from serum. Before the transplantation, the patient had no clinical or laboratory signs of cryoglobulinemia, but 7 months after surgery, pedal purpura was noted. Biopsy of the purpura demonstrated perivascular, lymphocytic inflammatory cell infiltrate. The patient's serum was found to have 3% of cryoglobulin and HCV RNA was detected in the cryoglobulin.

Case 2 was a 47-year-old Caucasian woman known to be suffering with liver cirrhosis due to chronic HCV infection for 8 years. Pedal purpura developed and mild proteinuria appeared. Total urine protein was 1 g/L and clinical and laboratory tests were not compatible with nephrotic syndrome. Cryoglobulin (cryocrit) was found to be 2% of the serum. An unsuccessful attempt at treatment with interferon-α was initiated. Two years later, after severe weakness and intractable ascites, she underwent orthotopic liver transplantation. Postoperative complications included early rejection treated with corticosteroids and common bile duct stenosis treated with endoscopic bile duct stent placement. Additionally, nephrotic syndrome developed. The patient's serum was retested and found to be positive for anti-HCV antibody and HCV RNA. Cryocrit was found to rise from the 2% pretransplant level to 10–20% and immune electrophoresis demonstrated a peak of IgM/K and IgG/K. HCV RNA was detected in the cryoprecipitate.

The patient was treated using plasmapheresis. There was a consequent reduction in the cryocrit, but no improvement in her nephrotic syndrome. Viral genotypes and viremia levels were not tested in either case.

We have described two patients in whom HCV-associated mixed cryoglobulinemia appeared or reappeared after liver transplantation. In the first patient, a rather slow preoperative chronic active hepatitis course converted to a rapidly progressive course of HCV hepatitis, which led to cirrhosis of the grafted liver despite ribavirin treatment. This change in the clinical course was accompanied by an appearance of cryoglobulin not found before surgery. In the second patient, cryoglobulinemia symptoms as well as serum cryoglobulin levels aggravated significantly after transplantation, concurrent with the return of the HCV infection.

There are several possible explanations for these observations. The first is that cryoglobulinemia is the result of the initial disease and not related to the transplantation. Cryoglobulinemia may develop during the clinical course of patients suffering with HCV hepatitis. The appearance of cryoglobulinemia in these patients might in fact have been related to the usual disease course unrelated to the transplantation. This is, however, a less possible explanation, as one would expect that the EMC syndrome will develop during longer periods of time. It is also noteworthy that liver-transplanted patients are treated with a variety of medications,

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* Abbreviations: EMC, essential mixed cryoglobulinemia; HCV, hepatitis C virus.

some of which might theoretically induce the appearance of cryoglobulinemia through their immune-modulating properties. Corticosteroids, cyclosporine or FK506, azathioprine, and ribavirin were used to treat these patients. Corticosteroids are usually used as treatment for cryoglobulinemia, although at much higher doses, so we would even anticipate improvement rather than aggravation of the cryoglobulinemia. We have found no report linking cyclosporine, FK506, or azathioprine with the appearance of cryoglobulinemia. Ribavirin, an antiviral drug, should have, if at all, lowered the viral load and thus acted as a treatment for cryoglobulinemia, as is the case with interferon (*12*).

The most possible explanation for the above findings is that the appearance of cryoglobulinemia is related to the reappearance of HCV in the patient who has undergone orthotopic liver transplantation. It has been postulated that the HCV-associated mixed cryoglobulinemia response to interferon treatment is in close correlation with the disappearance of HCV RNA from the serum (*12*). As the main reservoir for HCV is the liver, transplantation abolishes this source, rendering some of the patients HCV free (*11*). When the grafted liver is reinfected, HCV RNA levels most probably rise, with an excess of viral load, resulting in the return, or the de novo appearance, of cryoglobulinemia. It is conceivable that graft reinfection is followed by an enormous viremia within a short period of time, thus triggering the appearance of cryoglobulinemia, even in patients who had no evidence of cryoglobulinemia before transplantation. As the evidence for reinfection of the grafted liver with HCV is growing, we will probably see more cases of, or exacerbation of, cryoglobulinemia after liver transplantation.

Detection of cryoglobulins in the serum, even at a very low percentage, should be taken into consideration before performing orthotopic liver transplantation in an HCV-infected patient, and these patients should be followed carefully for exacerbation of cryoglobulinemia-associated symptoms. Larger series of patients with hepatitis C infection, both with and without cryoglobulinemia, should be studied in order to reach a conclusion concerning the interrelationship of cryoglobulinemia and graft injury.

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FULMINANT HEPATIC FAILURE FOLLOWING VARICELLA-ZOSTER INFECTION IN A CHILD

A Case Report of Successful Treatment with Liver Transplantation and Perioperative Acyclovir

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Fulminant hepatic failure (FHF*) in children is an infrequent illness that has a high mortality rate despite intensive medical treatment. Common etiologies for this disorder include hepatitis A, B or C, non-A, non-B and non-C hepatitis, drug-induced reactions, Budd-Chiari syndrome, and Wilson's disease (*1*). The varicella-zoster virus (VZV) is not generally linked to severe hepatitis or FHF at any age. To our knowledge, only 1 case of fatal FHF caused by VZV infection has been reported in a child (*2*). We report below a case of FHF caused by VZV infection associated with steroid therapy for asthma in a child who was treated successfully using liver transplantation and perioperative acyclovir therapy. The patient was a previously healthy 7-year-old, 20-kg, white male who was hospitalized for asthma at a community hospital from May 20 to May 24, 1993. His asthma was sufficiently severe to require intravenous methylprednisolone (30 mg/day) for 4 days. He was discharged home on 20 mg of methylprednisolone orally for 3 days. On May 27, he developed a typical chickenpox rash with reddish vesicles scattered over the body. Three days later he was admitted to the hospital with severe periumbilical abdominal pain. Liver enzymes at that time were slightly elevated (aspartate aminotransferase [AST]: 218 U/L, alanine aminotransferase [ALT]: 211 U/L). His mental status was normal. As his condition worsened, he was transferred on June 1, 1993, to Oakland Children's Hospital. Upon arrival, he was noted to have many intact skin vesicles over his upper chest. His liver functions had worsened (AST: 1628 U/L, ALT: 1036 U/L). His mental status waxed and waned. He was started on intravenous acyclovir at a dosage of 500 mg/m^2 every 8 hr. Over the next 3 days, he deteriorated rapidly, with decreasing levels of consciousness (stage III-IV coma), worsening coagulopathy, and increasing hemorrhagic varicella lesions on his skin. He was then transferred to our institution for consideration for liver transplantation.

Physical examination showed a blood pressure of 110/70 mmHg, a 36°C temperature, a regular pulse of 135, respiration of 20/min. He was intubated and ventilated. His pupils were equal and his neck was supple without nodes. Chest examination revealed no abnormality. Cardiac auscultation revealed normal heart tones without any gallop. There were decreased bowel sounds and no hepatosplenomegaly on abdominal examination. Multiple hemorrhagic, nonvesicular skin lesions were noted in the trunk and extremities. Laboratory tests on admission were: hematocrit, 0.35; white blood cell count, 19,400/mm^3; platelet count, 91,000/mm^3; serum glucose, 248 mg/dl; cholesterol, 87 mg/dl; total bilirubin, 2.2 mg/dl; total protein, 4.4 g/dl; albumin, 2.4 mg/dl; lactate dehydrogenase, 9,580 U/L; alkaline phosphatase, 96 U/L; AST, 7,620 U/L; and ALT, 4,490 U/L. Prothrombin time was 27.4 sec, and the fasting venous blood ammonia level was 451 μg/dl. Hepatitis B surface antigen and antibody, hepatitis A antibody, and hepatitis C antibody were negative. Stool samples were positive for occult blood. Blood, urine, and stool cultures were negative for cytomegalovirus and Epstein-Barr virus, but VZV was isolated from blood and skin vesicle fluid collected on June 3.

On June 4, 1993, the patient underwent an urgent liver transplantation. At operation, the native liver was almost black in color, with areas of hemorrhage that included the gallbladder. There was a great deal of blood in the lumen of the small and large intestines. The spleen also appeared hemorrhagic. Histopathologic examination of the explanted liver demonstrated extensive necrosis. Much of it was centrilobular, but it also appeared as small foci scattered randomly through the small amounts of residual hepatic parenchyma (Fig. 1). Viral inclusions were not demonstrable, nor were VZV antigens convincingly identifiable by direct immunofluorescence assay, although these studies were hampered by extensive necrosis and high background staining.

During the postoperative course, the patient continued to be treated with intravenous acyclovir at a dosage of 500 mg/m^2 every 8 hr for 15 days. He received cyclosporine,

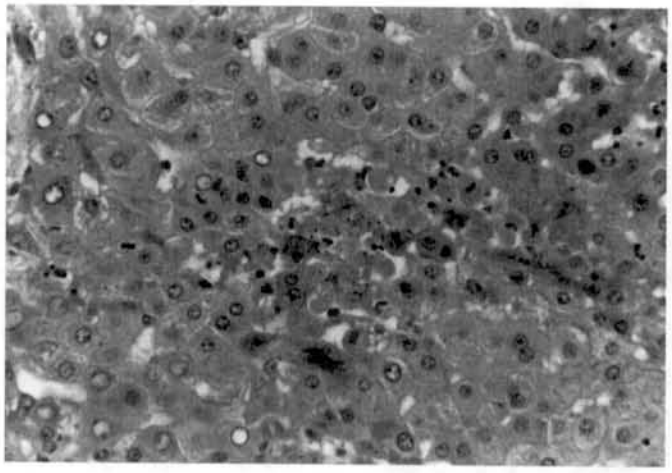

FIGURE 1. Typical small focus of bland hepatocyte necrosis with smudgy cells and minor reaction. Most of the liver parenchyma showed extensive necrosis (hematoxylin and eosin, ×200).

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* Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; FHF, fulminant hepatic failure; VZV, varicella-zoster virus.

methylprednisolone, and antithymocyte immunoglobulin for immunosuppression.

The patient recovered well and was discharged 36 days after the transplantation. He had no residual neurological deficit and no recurrence of varicella. Hepatic involvement has been described in childhood varicella, but it is most often subclinical (*3*). Although immunocompromised patients have a higher incidence of disseminated varicella, including pneumonitis, meningoencephalitis and hepatitis, FHF is rare. Four cases of fatal FHF as a result of VZV infection have been reported. All were in patients who either were receiving immunosuppression therapy or had been splenectomized.

Only one of the reported cases of fatal FHF from primary varicella was a pediatric patient (*2*). The child had been treated with azathioprine and corticosteroids after renal transplantation with simultaneous splenectomy. Three adult cases of fatal hepatic necrosis caused by VZV have been reported in the literature. One patient had reactivated VZV infection after a splenectomy (*4*). The other two adults had primary varicella infection, one following renal transplantation (*5*) the second after bone marrow transplantation (*6*).

Corticosteroids have been implicated as a causative factor in the increased severity of varicella. By contrast, Falliers and Ellis (*7*), from their experience with varicella in asthmatic children receiving corticosteroids, concluded that the underlying basic disease (e.g., cancer, leukemia) rather than the therapy contributed to the severity of varicella. Our patient did not have an underlying debilitating disease such as cancer or leukemia and he had been treated only with a short course of corticosteroids for asthma.

In children, the differential diagnosis of FHF includes viral hepatitis, drug-induced injury, Budd-Chiari syndrome, and Wilson's disease (*1*). These were ruled out in our patient, but his clinical presentation fulfilled the criteria for FHF set forth by Trey and Davidson (*8*) and Bernuau et al (*1*). Reye's syndrome, which could resemble the clinical presentation of our patient, is characterized by a diffuse fatty microvacuolization histologically. These findings were not observed in the explanted liver. Although viral inclusion bodies were not identified from his explanted necrotic liver after 7 days of intravenous acyclovir therapy, the clinical and laboratory data strongly suggest that this patient developed FHF from varicella hepatitis.

An important consideration in patients transplanted for viral hepatitis is the risk of recurrence in the liver allograft. The effects of oral acyclovir for treatment of VZV infection in otherwise healthy children have been reported (*9*). In our case, perioperative intravenous administration of acyclovir might have contributed to the prevention of recurrent VZV hepatitis.

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IMPORTANCE OF SURVEILLANCE MYCOBACTERIAL CULTURES AFTER LIVER TRANSPLANTATION

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The routine use of isoniazid prophylaxis after liver transplantation is a controversial issue because the benefits must be weighed against the risk of hepatotoxicity. We decided not to institute isoniazid prophylaxis but to study the efficacy of a surveillance mycobacterial program. One hundred patients were included in the protocol. Sputum and urine samples were processed before transplantation and on days 15, 30, 60, 90, 120, 150, and 180 for acid-fast stain and culture. One case of tuberculosis was promptly identified and successfully treated. Cases of tuberculosis with negative surveillance cultures were not identified. Our approach indicates that surveillance mycobacterial cultures can permit rapid identification of tuberculosis after liver transplantation and it is an alternative for groups who questioned isoniazid prophylaxis.

One of the more controversial issues in transplantation is the approach to tuberculosis including the routine use of isoniazid (INH*) prophylaxis. The incidence of tuberculosis after solid organ transplantation has been reported to be between less than 1% to more than 4% (*1*, *2*). Moreover, in some endemic areas, such as Spain, the incidence can be higher (*3*). Of the first 85 liver transplants performed in our hospital, tuberculosis developed in 2 patients (2.3%) and one of them died. In addition, the infection often presents with atypical clinical features, for example, disseminated disease, and a fatal outcome occurs more frequently (*4*).

The American Thoracic Association have recommended a minimum of 6 months of INH prophylaxis for individuals with a positive tuberculin test or who are anergic and who receive immunosuppressive therapy (*5*). Nevertheless, the use of INH prophylaxis must be weighed against the significant risk of hepatotoxicity. In the largest report of 139 kidney transplant patients treated prophylactically with INH, 13 patients (11%) had important hepatocellular enzyme elevation and INH hepatitis developed in 3 patients (2.5%) (*6*). The issue is more controversial in liver transplantation because moderate hepatocellular enzyme elevations secondary to INH can simulate liver rejection. Some authors (*1*, *2*, *7*) have concluded that the risk of INH hepatotoxicity is greater than the benefits in liver transplant patients with no other risk factors for tuberculosis. Risk factors that can lead to institution of INH prophylaxis include recent tuberculin converters, history of inadequately treated tuberculosis, and significant abnormalities on chest radiographs. Finally, patients who require therapy with antilymphocyte preparations are also candidates for INH prophylaxis.

Nevertheless, it is important to emphasize that tuberculosis after transplantation can be treated effectively if it is identified promptly. Consequently, after the high incidence of tuberculosis observed in our 85 liver transplant patients, we decided not to institute INH prophylaxis but to perform surveillance mycobacterial cultures. Three sputum and urine samples, each of them collected over successive days, were processed before transplantation, every 2 weeks in the first month, and monthly in the first 6 months after transplantation. Stains for acid-fast bacilli were performed using standard methods. A radiometric system (BACTEC, Johnston Laboratories) was used for mycobacterial cultures. The cost of routine culture is $700 per patient.

One hundred liver transplant patients have been enrolled in the protocol. The median age was 34-years and the median follow-up was 12 months. The only observed case of tuberculosis was identified when the patient was asymptomatic. The patient was treated successfully. The patient was 39 years old and underwent liver transplantation for alcoholic cirrhosis. He received immunosuppression with cyclosporine, prednisone, and azathioprine. Ten days after transplantation, he was treated with three 1-g boluses of methylprednisolone for rejection. Although stains for acid-fast bacilli were negative, the cultures of the sputum obtained on day 30 grew *Mycobacterium tuberculosis* after 10 days of incubation. It was resistant to rifampin and pyrazinamide. The patient was asymptomatic and the chest x-ray was normal. In the first 2 months, the patient received INH (300 mg/day), ethambutol (25 mg/kg/day, 1600 mg/day), and streptomycin (1 g/day). After 2 months, streptomycin was stopped and he continued on INH (300 mg/day) and ethambutol (15 mg/kg/day, 800 mg/day) for 10 months. At the end of treatment, the patient became ZN and culture negative. He remained asymptomatic without significant toxicity.

In summary, our approach indicates that surveillance mycobacterial cultures can permit the prompt identification of tuberculosis after liver transplantation. Nevertheless, the possibility that clinically significant tuberculosis might occur in spite of negative cultures should be considered. The sensitivity of the surveillance approach taken one sample at a time should be studied in an effort to reduce the cost. More sensitive techniques, such as polymerase chain reaction, may be even more effective than cultures and should be investigated. This strategy can represent an alternative for groups who have questioned the INH prophylaxis after transplantation. A prospective multicenter trial comparing INH prophylaxis with our approach is the only method to test these recommendations.

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* Abbreviations: INH, isoniazid.

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VARICELLA VACCINE IN CHILDREN REQUIRING RENAL OR HEPATIC TRANSPLANTATION

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In immunocompromised children, chickenpox is a serious disease with high morbidity and mortality (*1-5*).

The severity, as well as the high cost of treating varicella zoster virus (VZV*) infection in these patients, necessitates vaccination in all pediatric renal and liver transplant candidates, where natural immunity is lacking and when the reality of pharmacologically induced immunosuppression looms in their future (*6*). For this reason, since 1989 we have been vaccinating all of our pediatric transplant candidates without natural immunity against VZV infection.

We studied 68 children. Their ages ranged from 18 months to 15 years (average age, 5 years, 3 months), and there were 30 males and 38 females. Eighteen patients had hepatic failure and 50 patients had end-stage renal disease.

Twenty-five of the renal transplant patients had been in hemodialysis for at last 2 years, and 15 were undergoing peritoneal dialysis. The remaining 10 patients were receiving conservative and dietary therapies. All patients gave negative histories for chickenpox and no specific VZV antibodies were detected. No patient was receiving immunosuppressive therapy.

A live attenuated OKA strain of VZV was used. The vaccine was obtained via virus propagation in W-138 and and MRC-5 human diploid cell cultures. A 0.5-ml dose of the vaccine was administered subcutaneously in the deltoid region. Each dose contained not less than 2000 plaque-forming units.

Seroconversion was evaluated at 30- and 90-day intervals after vaccination (43 patients at 30 days, 15 patients at 60 days, and 10 patients at 90 days). Serum antibody detection was carried out by ELISA (IgG and IgM specific; Boehringer). The technique used was that of Berger (*7*).

Fifteen frozen serum samples from patients who did not seroconvert (nonresponders) by ELISA methodology were tested against the more sensitive immunofluorescence antibody to membrane antigen method (FAMA laboratory, Smith, Kline and French, Brussels) according to Williams (*8*) with the modification of Iltis (*9*) (positive titer >1:4) (*10-12*).

Nonresponders received a second dose of anti-VZV vaccine. Among revaccinated patients, the interval between the first and second dose of the vaccine ranged from 2 to 17 months.

Of the 68 vaccinated children, 34 (50%) had positive antibody titers to VZV, as verified by ELISA testing, 30 days after the dose was administered. The response rate was 50% (25 patients) in the end-stage renal disease group. The same percentage was seen among patients with hepatic failure (50%, i.e., 9 patients).

Using the FAMA method on frozen serum samples, antibody titers could be determined in only 15 patients of the nonresponder population (34 patients). Of the 15 patients, 10 demonstrated a titer of >1:4. Thus, by adopting a more sensitive antibody determination technique, the percentage of responders to one dose of vaccine was 64.7%.

Of the nonresponder patients (by ELISA methodology after first dose), 9 patients received organ transplants. Twenty-five patients received a second dose of anti-VZV vaccine; an ELISA assay confirmed seroconversion in 16 of these patients. Therefore, the percentage of ELISA-tested responders increased to 73.5%.

There were no local or generalized side effects reported or diagnosed. Particularly, there was no exanthem or herpes zoster. Compliance with the nonobligatory vaccine regimen was 100%. No intrafamiliar VZV infection was reported.

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* Abbreviation: VZV, varicella zoster virus.

In our experience, OKA strain anti-VZV vaccine is safe, innocuous, and efficient. By adopting a sensitive serum antibody determination technique and by administering a second dose of the vaccine when possible, the percentage of responders could be increased to 84.7%: the same percentage is seen in the healthy population (*13-15*).

In our ongoing 6-year follow-up, the vaccinated patients maintained their immune state, as demonstrated by the persistence of antibody titer and the clinical absence of disease. This was also true in the patients who received transplants.

From this study, the following points emerge: (1) Considering the cost/benefit relationship, a vaccination program in a high-risk pediatric population (*16*) is necessary and valid. (2) An economical yet more sensitive testing technique, available in all laboratories, is needed. (3) Timely initiation of an anti-VZV vaccination, before the patient's clinical condition, especially nutrition status, begins to deteriorate, may influence the immune response. (4) After the first dose, all ELISA-negative patients should be given a second dose of anti-VZV vaccine.

In conclusion, given the morbidity and mortality of VZV infection, as well as the cost/benefit relationship in these pediatric patients immunocompromised for life, vaccinations remain the only valid prophylaxis against chickenpox.

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METABOLIC COMPLICATIONS AFTER LIVER TRANSPLANTATION

DIABETES, HYPERCHOLESTEROLEMIA, HYPERTENSION, AND OBESITY

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We retrospectively studied the incidence of diabetes, hypercholesterolemia, hypertension, and obesity in 123 consecutive adult liver transplant recipients (61 men and 62 women) who were alive at least 1 year after transplantation. We also studied the change in these metabolic complications in 61 patients who subsequently were able to be tapered to 5 mg prednisone per day. One year after transplantation—a point at which almost all patients were on maintenance immunosuppression and had stable graft function—the incidence of diabetes was 13% and hypertension was 69.1%. The overall incidence of hypercholesterolemia (serum cholesterol >240 ng/ml) was 31% and was more frequent in women than in men (38.7% vs. 23.0%, $P<0.06$). The incidence of obesity at 1 year was 41.9% in women and 39.3% in men. With tapering of prednisone from 10 mg to 5 mg per day in 61 patients, the mean serum cholesterol decreased from 224.6±65.2 mg/dl to 203.3±65.5 mg/dl, $P<0.005$. With steroid tapering, 8 patients were able to discontinue antihypertensive medications and 4 were able to discontinue insulin treatment for diabetes. Five patients became obese during the steroid-tapering period. No patient developed irreversible rejection with steroid tapering and no immunologic graft losses occurred more than a year after transplantation. Nine patients who lived a year subsequently died. Of these, 7 patients were diabetic and 2 died of cardiac disease. We conclude that metabolic complications such as diabetes, hypertension, and hypercholesterolemia are common late after liver transplantation and that these may contribute to patient morbidity and mortality. In addition, we conclude steroid tapering to 5 mg/day does not lead to graft loss and may decrease the incidence and severity of late metabolic complications.

The long-term complications of renal transplantation are well known, but little data exist regarding the prevalence of complications after liver transplantation. With the increasing survival of liver transplant recipients, the long-term outcomes of these patients are becoming more important to transplant physicians. The first aim of this study was to determine the incidence of some of the common metabolic abnormalities, such as diabetes, hypertension, hypercholesterolemia, and obesity, in liver transplant recipients 1 year after transplantation. One year was chosen because at this point most patients are on stable immunosuppressive regimens and have stable graft function.

The second aim was to study the changes in these complications with a gradual tapering of prednisone from 10 mg/day to 5 mg/day. Finally, we studied the causes of late death after liver transplantation to begin to assess the effect of these metabolic complications on patient survival.

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It is our hypothesis that steroid tapering would be well tolerated immunologically by liver transplant recipients and that such tapering to physiologic levels even years after transplantation might ameliorate the metabolic complications often associated with steroids.

We retrospectively studied 123 consecutive adult liver transplant recipients (age >20) transplanted from December 1988 to June 1993 who lived for at least 1 year after transplantation. Four patients with hepatitis B were excluded from this analysis because they never received steroids for immunosuppression. The analysis was concluded in November 1994.

Induction immunosuppression included high dose prednisone with tapering to 20 mg/day by 1 week and then to 10 mg/day by 6 months after transplantation. Cyclosporine was administered intravenously for 14 days after transplantation until the T-tube was clamped. Cyclosporine was then given by mouth to attain a level of 200–350 ng/dl. Azathioprine was not used for maintenance immunosuppression. OKT3 was used as a first-line drug to treat more than 50% of the early acute rejection episodes, although a few patients received high dose prednisone.

Diabetes was defined as a fasting glucose greater than 140. Diabetic patients were treated with either twice daily injections of insulin or oral hypoglycemic agents, in addition to a carbohydrate-restricted ADA diet. Serum C-peptide and glycosylated hemoglobin levels were measured in diabetic patients to determine the type of diabetes and the level of glucose control, respectively.

Hypercholesterolemia was defined as a fasting serum cholesterol greater than 240 mg/ml. Serum triglyceride levels will not be reported in this study because their levels varied widely from blood draw to blood draw in patients not on defined diets. All patients were instructed to follow a low-fat diet after transplantation. No patient was treated with cholesterol-lowering agents at 1 year after transplantation.

Hypertension was defined as a diastolic blood pressure greater than 90 mmHg and/or a systolic blood pressure greater than 160 mmHg. The first antihypertensive drug used in all patients was a calcium channel blocker. No patients were treated with diuretics except for short courses to relieve pedal edema.

Obesity was defined as a body mass index (BMI*) greater than 28 in men and 26 in women. BMI is defined as the weight in kilograms divided by the height in meters squared.

Of the 123 patients, 113 were on 10 mg of prednisone at 1 year. After 1 year, patients with stable graft function were gradually, but persistently, tapered to 5 mg of prednisone per day over a period of 1–2 years. In comparing the prevalence of metabolic complications with steroid tapering, 3 measure-

* Abbreviation: BMI, body mass index.

ments at 1, 2, and 3 months after achieving the stable steroid dose were used. For determining the mean serum cholesterol of the groups, the fasting serum cholesterol 2 months after achieving the steroid dose was used. The mean time after transplantation to achieve 10 mg/day was 10.1±4.5 months and 29.4±9.5 months for 5 mg/day.

Rejection episodes occurring during this time were treated with either high dose prednisone or OKT3. After a rejection episode, the maintenance prednisone dose was then set at 20 mg/day with a gradual tapering over the ensuing 3 months to 10 mg/day. If the patient tolerated this without rejection, the prednisone dose was lowered to 7.5 mg/day for approximately 3 months and then 5 mg/day.

Data were analyzed using the SAS statistical program. Continuous variables were evaluated using the two-tailed *t* test. Paired data (10 mg to 5 mg data points) were evaluated using the paired *t* test.

Table 1 shows the incidence of diabetes both before and after transplantation. Prior to transplantation, 9 patients were diabetic (5 were insulin dependent, 2 were on oral hypoglycemic agents, and 2 were diet controlled). Twelve more patients became diabetic after liver transplantation. Six patients, 3 with pretransplant diabetes and 3 with posttransplant diabetes, died in the first year after transplantation. These patients died of either sepsis or primary nonfunction of the liver graft.

Sixteen patients, 6 with pretransplant diabetes and 10 with posttransplant diabetes, lived for at least 1 year after transplantation. All but one of these patients required insulin therapy at 1 year after transplantation. The non-insulin-requiring diabetic was diet controlled. Fifteen of these 16 patients were type II diabetics as determined by normal or elevated fasting C-peptide levels (mean 4.6±1.8 pmol/ml). Glucose control was generally good, with glycosylated hemoglobin levels averaging 6.6±1.8% (normal <5%). The mean steroid dose in diabetics was the same as in nondiabetics (10 mg/day) at 1 year.

Seven of these diabetic patients who lived a year subsequently died, 2 of cardiac disease. Nine are still alive; 5 of these are still on insulin while 4 have been able to be discontinue insulin treatment and are now diet controlled. All 4 of these patients discontinued their insulin only when their prednisone dose was tapered to 5 mg/day.

TABLE 1. Characteristics of patients with diabetes after liver transplantation

Before transplantation		After transplantation
	Patients with diabetes before Tx	
n=9	5 insulin dependent	8 insulin dependent
	2 oral hypoglycemic agent	1 diet controlled
	2 diet controlled	
	Patients with diabetes beginning after Tx	
n=13	13 insulin dependent	
Patients with diabetes who lived at least a year after liver Tx		
n=16	7 subsequently died	
	9 still alive	
	4 tapered off insulin, now diet controlled	
	5 insulin dependent	

The overall incidence of hypercholesterolemia 1 year after transplantation was 31% (Table 2). Hypercholesterolemia was more common in women than in men (38.7% vs. 23.0%, $P<0.06$). In addition, the mean cholesterol level was higher in women than in men (229.9±67.6 vs. 210.1±49.5, $P<0.09$). The mean serum cholesterol decreased from 224.4±65.4 mg/dl to 203.3±65.5 mg/dl ($P<0.005$), with tapering of steroids from 10 mg/day to 5 mg/day (Table 3). Steroid tapering decreased the serum cholesterol in 42 of the 61 patients and 19 patients had more than a 30-point decrease in cholesterol levels. Patients were tapered to 10 mg prednisone per day by 10.1±4.5 months after transplantation (range 6–24 months) and then to 5 mg/day by 29.4±9.5 months (range 12–48 months).

Sixty-nine percent of the patients were hypertensive 1 year after transplantation (Table 2). The incidence was more similar in men (72.1%) than in women (66.1%).

Eight hypertensive patients who had their prednisone dose tapered to 5 mg/day between 2 and 3 years after transplantation were able to discontinue antihypertensive medicines (Table 4). All 8 of these patients had been maintained on a single agent, a calcium channel blocker.

Given the incidence of ascites, the true incidence of obesity prior to transplantation is difficult to ascertain. Based solely on BMI that should overstate the incidence of obesity, 26.2% patients were obese before transplantation. One year after transplantation, 40.7% of the patients were obese, including 41.9% of the women and 39.3% of the men (Table 2). Only 1 obese patient was able to lose weight in subsequent years and 5 more patients became obese after 1 year despite steroid tapering, for a net increase of 4 obese patients (Table 4). Subsequent follow-up has shown that no other patient has been able to become nonobese even 6 months after steroid tapering.

Nine of the 16 patients with diabetes after transplantation were hypercholesterolemic. All of these 9 patients were hypertensive and 6 were women. This group seems to comprise a high risk category for cardiovascular complications.

Nine patients who lived for a year subsequently died (Table 5). Two patients died of malignancy. Three patients died of infection. Two diabetic patients died of cardiac disease. One patient died of hemorrhagic pancreatitis and 1 patient died of liver failure due to noncompliance with immunosuppressive medicines. Six diabetics died after surviving a year; however, only 4 patients died of causes commonly associated with diabetes (cardiovascular disease and infection). Of note, immunologic graft loss was not a cause of death in any compliant patient.

Our data suggest that the incidence of metabolic complications after liver transplantation is similar to the incidence seen in renal transplant recipients and that these metabolic complications can be decreased with steroid tapering. The 13% incidence of posttransplant diabetes after liver transplantation in our population is similar to that of renal transplant recipients (*1*). Unlike the experience reported by others (*2*), none of our diabetic patients before transplantation were "cured" of their diabetes by liver transplantation. The increasing number of insulin-dependent diabetics shows that diabetes became more severe in 8 of the 9 pretransplant diabetic patients after liver transplantation and this is probably due to treatment with corticosteroids. Four diabetics patients in our group have been able to discontinue insulin

TABLE 2. Incidence of diabetes, hypertension, hypercholesterolemia, and obesity at 1 year after liver transplantation

	All	Men	Women
Total	123	61 (49%)	62 (51%)
Diabetic	16 (13.0%)	10 (9.7%)	6 (16.4%)
Hypercholestolemia	38 (31.0%)	14 (23.0%)	24 (38.7%) $P<0.06$
Hypertension	85 (69.1%)	44 (72.1%)	41 (66.1%)
Obesity	50 (40.7%)	24 (39.3%)	26 (41.9%)

TABLE 3. Change in serum cholesterol level with tapering of prednisone from 10 mg to 5 mg/day after liver transplantation

Prednisone dose	Time after Tx (mo)	n	Mean serum cholesterol	P^a
10 mg/day	10.1±4.5	61	224.4±65.4	
5 mg/day	29.4±9.5	61	203.3±65.5	<0.005

[a] Paired t test.

therapy when prednisone was tapered to 5 mg/day. Similarly, Hricick et al. (*3*) have shown that steroid withdrawal can reverse posttransplant diabetes in renal transplant recipients. The survival of diabetic patients was less than that of nondiabetic patients—both in the first few months after transplantation and after 1 year.

Most patients with diabetes after liver transplantation require insulin therapy. Our data suggest that the best long-term treatment for posttransplant diabetes is to minimize the steroid dose.

The distribution of cholesterol levels in the general population is bell shaped, with the upper 5–10% considered hypercholesterolemic. In general, hypercholesterolemia is diagnosed if the plasma fasting cholesterol level exceeds 200–240 mg/dl. We have used the more stringent criterion of hypercholesterolemia of greater than 240 mg/dl. The 31% incidence of hypercholesterolemia in our patients 1 year after liver transplantation is slightly lower than the 40–50% incidence reported by others. However, both McDiarmid et al. (*4*) and Munoz et al. (*5*) reported the incidence at earlier points after transplantation when the prednisone doses were higher. The incidence of hypercholesterolemia is slightly greater than the 15–25% incidence observed in renal transplant recipients (*6*, *7*). The higher incidence in women, however, also has been reported in renal transplant recipients (*1*). A decrease in mean serum cholesterol with steroid tapering also occurs in renal transplant recipients (*8*). Tapering prednisone lowers cholesterol levels in the majority of liver transplant recipients, with the benefit greater in some patients than in others. Currently, our first line of treatment for hypercholesterolemia is steroid tapering, followed by cholesterol-lowering agents in patients with serum cholesterol levels greater than 300 mg/dl.

The 69% incidence of hypertension in our liver transplant recipients is just slightly less than that of renal transplant recipients receiving similar cyclosporine-based immunosuppression (*9*). Textor (*10*) has observed a 65% incidence of hypertension in his liver transplant recipients. Although the incidence of hypertension remained high in the 61 patients tapered to 5 mg/day (55.7%), 8 patients were able to discontinue antihypertensive medications with tapering.

The incidence of obesity after liver transplantation has never been reported. The high rate of weight gain in these patients is worrisome for many reasons. First, it may contribute to cardiovascular risk factors, such as hypertension, diabetes, and hypercholestrolemia. Although obesity has been cited as a risk factor for immunologic graft loss in renal transplantation (*11*), it does not seem to increase the rate of liver graft loss. In addition to increasing cardiovascular risk factors, the increased weight in liver recipients may make this high risk group even more prone to pathologic fractures.

Most patients were tapered from 10 mg prednisone per day to 5 mg/day over an 18-month period between 10 and 29 months after transplantation. During this period, patients were taking more than 5 mg/day of prednisone and gained weight. Weight gain might be reversible with steroid tapering, but it will certainly be more difficult to reverse than other complications, such as diabetes, hypercholesterolemia, and hypertension.

In our group, 9 patients who lived for at least a year later died. Of these patients, 2 died of cardiac disease. Both of these patients were hypertensive and diabetic. Five of the remaining 7 deaths occurred in diabetics, 2 secondary to infection. These data support our assumption that metabolic complications such as diabetes and hypertension are risk factors for late death after liver transplantation.

Finally, our experience agrees with that of others that rejection is rarely a cause of graft loss late after liver transplantation. No compliant patient in our series lost their graft to rejection more than a year after transplantation. In another series, the incidence of rejection after 1 year was 4% (16 rejection episodes in 375 patients) (*12*). Fifteen of these 16 rejection episodes resolved with treatment, while 1 episode progressed to chronic rejection and death. These results support our assumption that late rejection after liver transplantation is rare and is usually mild and treatable.

It is currently our policy to taper prednisone to 10 mg/day by 1 year after transplantation in patients with stable graft function. We then decrease the prednisone to 7.5 mg/day at 15 months and then 5.0 mg/day by 18 months. If a rejection occurs during this period, the prednisone is increased to 20 mg/day after treatment with either high dose steroids or OKT3. The prednisone dose is again tapered to 5 mg/day over the next year. In patients with diabetes, obesity, severe hypertension, or hypercholesterolemia, the prednisone dose may be decreased below 10 mg/day before 1 year.

We conclude that metabolic complications such as diabetes, hypertension, hypercholesterolemia, and obesity are common after liver transplantation and seem to contribute to patient morbidity and mortality. The incidence of these complications is similar to or greater than that of renal transplant recipients. In contrast to the case in renal recipients, late rejection is rare in liver transplant recipients, and when it occurs, it is usually mild and rarely leads to graft loss. In addition, these data show that tapering of steroids to 5 mg/day with the maintenance of therapeutic levels of cyclosporine does not lead to graft loss and decreases the incidence

TABLE 4. Changes in metabolic complications with tapering of prednisone from 10 mg to 5 mg/day after liver transplantation

Prednisone dose	Time months	n	Diabetes	Hypertension	Obesity
10 mg/day	10.1±4.5	61	14.8% (9)	68.8% (42)	41.0% (25)
5 mg/day	29.4±9.5	61	8.2% (5)	55.7% (34)	47.5% (29)
P^a			<0.045	<0.01	<0.2

[a] Paired t test.

TABLE 5. Deaths in patients more than 1 year after liver transplantation

Time (mo)	Cause	Complication
35	Colon cancer	—
23	Hodgkin's lymphoma	DM
42	Pneumocystis	—
18	Candida sepsis s/p CABG	DM
24	Pancreatitis	DM
37	Noncompliance liver failure	DM
36	Congestive heart failure	DM, HTN
60	Myocardial infarction	DM, HTN, obese
36	Sepsis/MSOF	DM

and severity of late metabolic complications. We conclude that the gradual tapering of prednisone to 5 mg/day is safe and should be aimed for in all liver transplant recipients with stable graft function.

Finally, other groups have shown that complete steroid withdrawal may be tolerated immunologically by liver transplant recipients (*13*, *14*). It is possible that complete withdrawal of steroids may be possible in our patients with further improvement in metabolic complications.

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INTRATHYMIC INJECTION OF ALLOANTIGEN MAY LEAD TO HYPERACUTE REJECTION AND PROLONGED GRAFT SURVIVAL OF HEART ALLOGRAFTS IN THE RAT

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The recent reports describing the beneficial effect of intrathymic (IT*) inoculation of donor antigen on subsequent allograft survival (*1, 2*) prompted us to study this phenomenon in the high responder WAG to BN rat strain combination as well as in the low-responder BN to WAG combination. Previously in the WAG to BN model, we found that a donor-specific blood transfusion (DST) given 1 week before heterotopic heart transplantation led to accelerated rejection in 5 days, whereas in the reverse model, a DST consistently led to marked prolongation of graft survival (*3, 4*). These opposing phenomena led us to determine whether the effects of DST in the two rat strain combinations mentioned above would also occur after IT injection of donor-type cells.

Male rats of the inbred WAG ($Rt1^u$) and BN ($Rt1^n$) strains were used (HSD, Zeist, The Netherlands). The animals were 8–12 weeks old and weighed between 200 and 250 g. The experimental protocols adhered to the rules in the *Guidelines on the Protection of Experimental Animals* by the Committee on Animal Research of The Erasmus University, Rotterdam, The Netherlands. Heterotopic intra-abdominal heart transplantation was performed as described by Ono and Lindsey (*5*). Graft function was assessed by daily palpation under ether anesthesia. Absence of heart contractility was taken as the endpoint of graft survival. Rabbit anti-rat antilymphocyte serum (ALS) was prepared by repeated subcutaneous immunization of New Zealand White rabbits with rat thymocytes as described before (*6*). The serum was decomplemented for 1 hr at 56°C. Treatment of BN recipients with 4 ml/kg ALS on days 0, 1, and 2 after transplantation prolonged the survival of WAG hearts from 8 to 23±4 days.

Spleen cell suspensions were prepared by passage of minced spleens through a steel mesh. Cells were washed 3 times in RPMI medium (Gibco, UK), and erythrocytes were lysed using a hemolytic buffer. Viability of the spleen cells was assessed by trypan blue exclusion and was always greater than 90%. The suspension was adjusted to 25×10^7 cells/ml. IT inoculation was performed under ether anesthesia after exposure of the thymus via a median incision in the manubrium. A 0.4×12-mm needle connected to a pipette (Multipette 4780, Eppendorf, Hamburg, Germany), was used to inoculate 5 fractions of 10 μl of suspension into each lobe. Consequently, a total dose of 25×10^6 splenocytes in a volume of 0.1 ml was given. Directly after IT injection, 1 ml of ALS was given intraperitoneally.

Histopathology and immunohistochemistry were performed routinely as described earlier (*7*). The following monoclonal antibodies were used: MARM-4 (mouse anti-rat IgM), MARG-G2a (mouse anti-rat IgG) (both obtained from H. Bazin, University of Louvain, Belgium), mouse anti-total complement, mouse anti-C1q (classical pathway of complement activation), and mouse anti-factor B (alternative pathway of complement activation) (all obtained from the Central Laboratory of the Netherlands Red Cross, Amsterdam, The Netherlands). Statistical analysis of the survival data was performed using the Kruskal-Wallis analysis of variance and Wilcoxon's rank-sum test.

In the BN to WAG combination, IT injection of donor splenocytes and treatment with ALS 7 days before transplantation led to prolonged graft survival (mean graft survival ± SD, 55.3±43.7 days); 3 of 7 grafts survived for more than 100 days (Table 1). Controls that received syngeneic WAG splenocytes and ALS rejected their grafts in 14.4±11.7 days. The use of ALS appeared mandatory to obtain graft prolongation as injection of spleen cells resulted in only normal graft rejection (group 2).

In the high responder WAG to BN combination, IT injection of WAG splenocytes alone 7 days before transplantation led to accelerated graft rejection in 5 days (Table 2, group 7). Concomitant injection of WAG spleen cells and ALS led to very acute and hyperacute rejection. In group 8, 3 of 5 grafts were found rejected the day after transplantation. To investigate the kinetics of graft rejection, this experiment was repeated and graft function was assessed every 30 min during the first 4 hr. It appeared that grafts were rejected in 30, 90, and 120 min and 1 and 5 days (group 9). The occurrence of hyperacute rejection apparently was unique to the IT route of administration, since intravenous injection of splenocytes did not result in hyperacute rejection (group 10), although it did lead to accelerated rejection. Grafts in this group were rejected in a similar accelerated fashion as observed earlier following a single DST. Histology revealed that most of the vessels from hyperacutely rejected hearts were thrombosed; there was moderate edema but virtually no interstitial hemorrhage or cellular infiltrate. All hearts from group 9 showed endothelial deposition of IgM but no IgG. There was positive staining for C1q, which was more pronounced than in acutely rejected BN hearts from group 7. Staining for factor B was weak and could be detected only incidentally. These observations confirm the hyperacute nature of the rejection process, by revealing the classical features of IgM antibody- and complement-mediated rejection.

Three different strategies were followed to prevent hyper-

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* Abbreviations: ALS, antilymphocyte serum; DST, donor-specific blood transfusion; IT, intrathymic.

TABLE 1. Heart graft survival after IT injection of splenocytes in the BN to WAG combination[a]

Group	Treatment	Survival time (days)	Mean ± SD
1	None	8, 8, 8, 8, 9	8.2±0.5
2	BN spleen cells day −7	7, 8 (3×)	7.8±0.5
3	BN spleen cells + ALS day −7	9, 14, 16, 48, >100 (3×)	55.3±43.7
4	WAG spleen cells + ALS day −7	7, 8, 10, 12, 35	14.4±11.7

[a] Spleen cells (25×10^6) and 4 ml/kg ALS were given 7 days before heart transplantation. Group 3 versus group 4: $P=0.05$.

TABLE 2. Heart graft survival after IT injection of splenocytes in the WAG to BN combination[a]

Group	Treatment	Survival time (days)	Mean ± SD
5	None	8, 8, 8, 8, 8	8.0
6	BN spleen cells + ALS day −7	12, 12, 12, 12	12.0
7	WAG spleen cells day −7	5, 5, 5, 5, 5	5.0
8	WAG spleen cells + ALS day −7	1, 1, 1, 2, 3	1.8±1
9	WAG spleen cells + ALS day −7	30, 60, 90 min, and 1, 5 days	
10	WAG spleen cells iv. + ALS day −7	4, 4, 5, 5, 6	4.8±0.8
11	WAG spleen cells day −7 + ALS day −14	4, 4, 5, 6 (3×), 8	5.6±1.4
12	Splenectomy day −12 WAG spleen cells + ALS day −7	2, 5, 5, 6, 7, 8	5.5±2.7
13	WAG spleen cells + ALS day −7 CVF days −1, 1, 3, 5	4, 6, 7, 8, 8	6.6±1.7

[a] Spleen cells (25×10^6) and 4 ml/kg ALS were given on day −7 or as indicated. Cobra venom factor (CVF) was given intravenously in a dose of 100 μl/day.

acute rejection in the WAG to BN model. First, since we demonstrated earlier that immune complexes can be highly immunogenic (*8*), an experiment was performed in which ALS was given 7 days before IT antigen injection, thus preventing ALS-antigen complex formation (group 11). In this group, hyperacute rejection did not occur, but hearts still were rejected in an accelerated fashion. In a second attempt to prevent hypersensitization, splenectomy to reduce antibody formation was performed 5 days before intrathymal inoculation (group 12). Again, hyperacute rejection was omitted but prolonged graft survival was not observed. Finally, serum complement was depleted by administration of 100 μl of cobra venom factor (Imutran, Cambridge, UK; activity: 9000 CH_{50} Units measured against sheep erythrocytes) on days −1, 1, 3, and 5 relative to transplantation (group 13). This strategy also resulted in prevention of hyperacute rejection, but no prolongation of graft survival was noticed.

Previous studies in our laboratory have demonstrated the opposing effects of DST in the BN to WAG and the reverse donor-host combination. The present study shows marked similarities but also demonstrates differences between DST and IT. In the strong WAG to BN combination, the similarities were striking: sensitization was achieved regardless of the route by which donor-type antigen is introduced. The intrathymic route even gave rise to hyperacute rejection if ALS was given on the same day. It is difficult to understand why hyperacute rejection was provoked by this combined treatment. If immune complexes were responsible, a similar hyperacute response should have occurred after the intravenous injection of spleen cells; however, this was not the case (group 10). Since splenectomy resulted in abrogation of hyperacute rejection, it is likely that an extreme antibody reponse by spleen cells was induced by processed alloantigen migrating from the thymus. In addition, this reasoning starts from the assumption that ALS has a peculiar effect on the BN thymus. Interestingly, all strategies to overcome hyperacute rejection were equally effective, ultimately leading to a similar accelerated type of rejection as following intrathymic or intravenous injection of donor-type cells alone (groups 10–14). Hyperacute rejection following IT injection of spleen cells has not been described earlier in an allogeneic combination, but has been reported to occur in the hamster to rat model (*9*). In this concordant combination, where hearts normally are rejected in 3–4 days, IT injection of hamster spleen cells plus treatment with ALS provoked accelerated graft rejection in 20 min to 1 day.

In the low responder BN to WAG combination, the effect of IT injection of spleen cells was less profound than that of a single DST. The prolongation of allograft survival achieved thus far by IT spleen cells and ALS contrasts unfavorably with the permanent survival, which in this model can be induced by a single DST, without additional immunosuppression (*4*). However, it should be noted that the optimal cell dose and timing of IT injection and ALS administration relative to heart transplantation have yet to be determined. IT injection of donor-type cells without ALS had no effect at all, which contrasts with the immunizing effect of IT injection in the reverse combination. This finding again illustrates the marked differences between the two models used in the present study, and underscores the notion that a unifying theory on tolerance induction by DST and IT injection is hard to conceive.

In conclusion, we demonstrated that, similarly as following DST, the ability to induce prolonged acceptance of heart allografts by IT injection of donor-type cells is highly dependent on the donor-host combination used. The remarkable finding that IT injection can also lead to sensitization and even hyperacute rejection indicates that caution should be exercised with this ambiguous procedure.

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