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IMMUNOINTERVENTION

Drug Development and Testing in Relation to Cell Migration and Chimerism

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WE will focus first on the meaning of the graft acceptance that is the transplanters' main reason for immunosuppressive drug development, and second on how to achieve graft acceptance after xenotransplantation.

CELL MIGRATION AND CHIMERISM

Graft acceptance, at least as we now see it, is a process that begins within a few minutes after the revascularization of any whole organ with a brisk two-way cell traffic in which dendritic and lymphoid cells from the recipient and those from the graft are exchanged with consequent graft and patient chimerism¹⁻³ (Fig 1), providing there is effective immunosuppression.

Part of the evidence for this concept was obtained by studying liver recipients who were alive 10% to nearly 23 years posttransplantation (Table 1). Six of these 43 survivors stopped their medication 1 to 11 years after transplantation and have remained drug free for 5 to 13 years. The lymphocytes of all these treated or untreated patients

Table 1. Liver Transplantation January 1970 to January 1982

Total	206
Surviving	43 (21%)
Off medication	6*

*Stopped 1 to 11 years postoperatively; off drugs 5 to 13 years.

reacted vigorously to the lymphocytes of third-party donors. We believe that they are tolerant.

This spring we performed multiple biopsies on six of the drug-free patients and on 16 more still under therapy looking for evidence of chimerism with HLA markers or with sex karyotyping in a subgroup of nine women who received male livers (Table 2). Chimerism was detected with immunostaining or with polymerase chain reaction (PCR) in every case. Biopsy samples were taken of the liver, skin, and a convenient lymph node; also, blood was examined via PCR. In situ hybridization by Jake Demetris using γ probes identified the hepatocytes, ducts, and endothelial cells as male (bright spots) in the female recipients. Male cells in these women also were found in the lymph nodes and skin. In the other patients who had donors of the same sex, donor cells have been demonstrated by PCR or immunostaining in the intestine, heart, bone marrow, blood, or even aorta. The same process occurs with the successful transplantation of all other kinds of whole organs, including the kidney, although in smaller numbers.

Cell migration is the secret of graft acceptance and the explanation for two phenomena described nearly 30 years

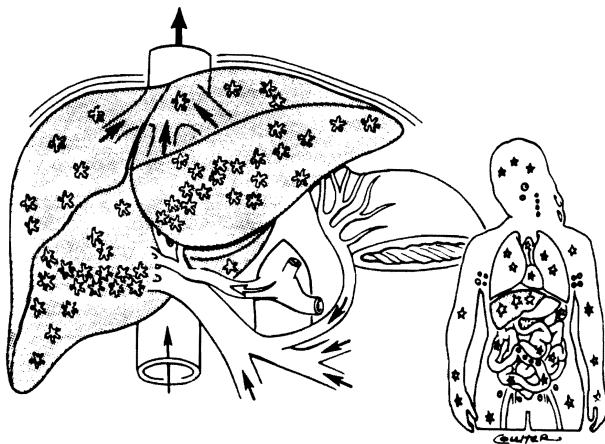


Fig 1. Cell migration and repopulation after liver transplantation.

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Table 2. Liver Transplantation: Studies of Chimerism (of 43 After 10½ to 21 Years)

Total studied	22*
Positive chimerism	All studied
Male to female chimerism	9/9†

*Immunocytochemistry, PCR.

†In situ hybridization, PCR.

ago: the reversal of kidney rejection by steroids and the subsequent ability to reduce the intensity of immunosuppression (referred to as tolerance).⁴ Why this happened was a mystery in 1963. In retrospect, the solution to the mystery was there all along in exhaustive skin test studies on these early Colorado recipients and their donors. Seventy-seven percent of the skin reactions that were positive in the donor but not the recipient crossed over to the previously negative recipient, along with the transplanted kidney. When this did not occur (in the other 23%), it meant that the kidney transplant had failed. Our immunologists, Kirkpatrick and Wilson, speculated (correctly) that the migration of the skin tests was "caused by adoptive transfer of donor cellular immunity by leukocytes in the renal graft vasculature and hilar lymphoid tissue."⁵

Thus, in retrospect the observations of rejection reversal and what was called tolerance were reflections of cell migration and repopulation.^{1,2} Without understanding why, the observations led to the empiric therapeutic dogma upon which our specialty of whole-organ transplantation is based, and with which new drugs are tested clinically (Table 3). The dogma calls for daily baseline treatment (in these early days with azathioprine) plus intervention with the highly dose-maneuverable adrenal cortical steroids (or later antilymphoid agents) to whatever level is required to maintain stable graft function. This creates a trial and potential error situation for every patient as drugs are weaned. Although the new drugs that have been added through the years have been increasingly potent, they can be viewed as traffic directors—allowing cell movement (Fig 1) but preventing the immune destruction that is the natural purpose of the traffic. It does not matter exactly how the immune reaction is disrupted, only that this be achieved without killing all of the migratory cells. The emasculated but living cells that normally cause graft immunogenicity and rejection become instead the

missionaries subserving chimerism, graft acceptance, and ultimately tolerance.

Disruption of the function of the lymphocyte can be at the level of antigen processing (claimed at one time for deoxyspergualin), at an early stage in T-cell activation as occurs with cyclosporine and FK 506, or distal to this with rapamycin, which does not inhibit the secretion of cytokines including interleukin-2 but blocks their action. The so-called antiproliferative drugs (of which azathioprine was the prototype) work even more distally.

XENOTRANSPLANTATION

Ironically, drugs of this latter class that have been in our hands for more than 30 years hold the key that can unlock the door to xenotransplantation.⁶ These antimetabolite drugs block enzymes required for the synthesis of ribonucleotides. Consequently, they inhibit the DNA synthesis without which the final step of clonal lymphocyte expansion cannot proceed normally. All of these agents affect both T and B lymphocytes, but with some specificity. Azathioprine is more T cell directed and cyclophosphamide has a greater B-cell effect.

Although the duality of humoral and cellular mechanisms of xenograft rejection has been common knowledge, the antibody component has been refractory to treatment. For example, a hamster organ is confronted in the rat by a moderate titer (1:16 to 1:32) of preformed heterospecific cytotoxic antibodies and subsequently by a rapidly gathering antibody storm that destroys abdominally placed cardiac grafts within 3 days in untreated recipients, before there is a trace histopathologically of immunocyte infiltration. By itself, FK 506, which prevents T-cell activation and cytokine secretion, could in doses of 2 mg/kg/d prolong survival by only 1 day (Table 4). Monotherapy with either of two experimental antiproliferative drugs that suppress purine (RS 61443) or pyrimidine (Brequinar) synthesis tripled or quadrupled survival (Table 4) but did not permit consistent chronic survival. However, when either of these two antimetabolite drugs or when the conventional anticancer drug cyclophosphamide was added to FK 506 for the first 2 postoperative weeks, extended survival using continued FK 506 alone became routinely possible. It was particularly noteworthy that a single large dose of cyclophosphamide 10 days before transplantation permitted 100% success with daily FK 506 (Table 4).

A complete report of these studies has been published elsewhere.⁶ The conclusion was that prevention or mitigation of heterospecific antibody rejection by interdiction of the B cell-proliferative response with a variety of antimetabolite drugs (including some not shown in Table 4) for a surprisingly short period after transplantation or even beforehand is the essential first step to successful xenotransplantation, and unmasks the potential of continuous therapy with T cell-directed immunosuppressants such as FK 506. Such combination therapy should be

Table 3. Central Therapeutic Dogma

Baseline therapy with one or two drugs:
Azathioprine
Cyclophosphamide
Cyclosporine
Cyclosporine + azathioprine
FK 506
FK 506 + azathioprine
Secondary adjustments with steroids or antilymphoid agents
Case-to-case trial (and potential error) of weaning

Table 4. Graft Survival

Graft Type	Group	Treatment*	Without FK 506		With FK 506	
			n	Survival >30 days	n	Survival >30 days
Heart	1	None	6	0	—	—
	2	FK 506	6	0	—	—
	3	RS-61443	4	0	6	5
	4	Brequinar	6	1	5	5
	5	Cyclophosphamide	5	0	5	5
	6	Cyclophosphamide	5	0	5	5
Liver	7	None	8	0	—	—
	8	FK 506	10	5	—	—
	9	RS-61443	5	0	10	9
	10	Brequinar	7	1	7	6
	11	Cyclophosphamide	5	0	10	9
	12	Cyclophosphamide	5	0	15	12

Animals alive at 30 days survived as long as FK 506 was continued for 100 days, no matter what the adjuvant induction drug.

*Daily dose (mg/kg): FK 506, RS-61443, Brequinar, cyclophosphamide, 2.0 × 6, 1.0 × 25 (heart) or 1.0 × 30 (liver), and 0.5 on alternate day thereafter; 20.0 × 15 (14) starting day before transplant (heart) or day of transplant (liver); 4.0 × 3 and 3.0 × 12 starting day before transplant (heart) or 3.0 × 14 starting day of transplant (liver); 7.5 × 15 (14) starting day before transplant (heart) or day of transplant (liver), except groups 6 and 12, who received one dose of 80 mg 10 days preoperatively.

clinically applicable as long as the humoral antibodies do not act so rapidly that they cause hyperacute rejection in a matter of a few minutes or hours. This condition has been demonstrated empirically with baboon to human kidney⁷ and heart⁸ xenotransplantation.

The effectiveness of cyclophosphamide in these drug cocktails was extremely encouraging because cyclophosphamide had a firmly established track record for clinical allotransplantation (kidney, liver, or heart) in extensive trials more than 2 decades ago.⁹ In those trials in which the barrier was cellular (not humoral) immunity, cyclophosphamide had about the same potency in drug cocktails as azathioprine in head to head comparisons. It could be given daily for years.

After breaking through the antibody barrier, the process of xenograft acceptance involves the cell migration and consequent systemic chimerism that were recently delineated for allografts.¹⁻³ With a monoclonal antibody that recognizes LEW rat but not hamster cells,¹⁰ we already have shown that rat recipient dendritic and lymphoid cells

are incorporated into hamster heart or liver xenografts examined 3 to 100 days after transplantation.¹⁰ The displaced cells leaving these organs could be detected with polyclonal antihamster antisera (unpublished observations) and confirmed with PCR techniques (Fig 2).

For the molecular examination of the rat tissues, 1 µg of genomic DNA extracted from each tissue was PCR amplified for 30 cycles with hamster-specific oligonucleotides. One fifth of the volume of each reaction was size separated on agarose gel, transferred onto a nylon membrane (Hybond-N+, Amersham, Arlington Heights, Ill), and probed with a hamster hypoxanthine phosphoribosyltransferase (HPRT) exon 9 probe.^{11,12} The transplanted organs were also analyzed, but their amplifications were not included in the experiment shown in Fig 2, in order to allow a clear visualization of the fainter signals from the tissues of the recipients. Genomic DNAs extracted from hamster and rat spleen were amplified as positive and negative controls, respectively.

The chimerism was most obvious and frequent in the spleen and/or heart of the recipients, being unequivocal after liver transplantation and of low level if present at all after heart transplantation. These findings mean that successful clinical xenotransplantation must be visualized along the same lines of donor–recipient cellular intimacy as I described at the outset to be the fundamental means of allograft acceptance. It also means that this is an achievable objective with drugs currently available. The experimental background that I have described was the justification for the baboon-to-human liver transplant that took place in Pittsburgh in June 1992.

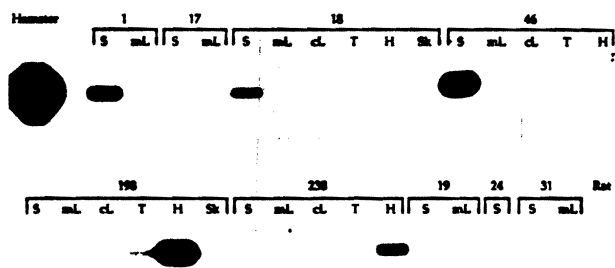


Fig 2. Specific amplification of exon 9 of the HPRT gene. S, spleen; mL, mesenteric lymph node; cL, cervical lymph node; T, thymus; H, heart; S, skin. Rats 1, 17, 18, 46, 198, and 238 received hamster livers 104 to 141 days previously; rats 19, 24, and 31 received hearts 111 to 135 days earlier.

REFERENCES

- Starzl TE, Demetris AJ, Murase N, et al: Lancet 339:1579, 1992
- Starzl TE, Demetris AJ, Trucco M, et al: Lancet 340:876, 1992

3. Starzl TE, Demetris AJ, Trucco M, et al: *New Engl J Med* 227:1992
4. Starzl TE, Marchioro TL, Waddell WR: *Surg Gynecol Obstet* 117:385, 1963
5. Wilson WEC, Kirkpatrick CH: In: Starzl TE (ed). *Experience in Renal Transplantation*. Philadelphia, PA: Saunders, 1964, p 239
6. Murase N, Starzl TE, Demetris AJ, et al: *Transplantation* 55:1993
7. Starzl TE, Marchioro TL, Pteres GN, et al: *Transplantation* 2:752, 1964
8. Bailey L, Nelson-Cannarella S, Concepcion W, et al: *JAMA* 254:3321, 1985
9. Starzl TE, Putnam CW, Halgrimson CG, et al: *Surg Gynecol Obstet* 133:981, 1971
10. Valdivia LA, Demetris AJ, Langer AM, et al: *Transplantation* (in press)
11. Rossiter BJB, Fuscoe JC, Muzny DM, et al: *Genomics* 9:247, 1991
12. Chiaverotti TA, Battula N, Monnat RJ Jr: *Genomics* 11: 1158, 1991