1827

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DETECTION OF ANTI-IDIOTYPIC ANTIBODIES AFTER OKT3 TREATMENT BY FLOW CYTOMETRY

We read the interesting article by McIntyre and Higgins (*1*) describing a rapid assay to detect anti-idiotypic anti-OKT3 antibodies generated after treatment with OKT3 with the help of the Jurkat cell line. We have developed an analogous method and measured the amount of anti-idiotypic antibodies in heart and kidney transplant recipients after OKT3 therapy, using the human T cell line HPB-ALL (*2*, *3*). The HPB-ALL cell line is, like Jurkat, easy to grow in suspension, but it generates a more homogenous population in a flow cytometer analysis in terms of forward and side scatter. In addition, the HPB-ALL cell line has a higher OKT3 binding capacity (185×10^3 vs. 73×10^3 CD3 determinants/cell), providing a more intense fluorescence signal with FITC-conjugated OKT3 (*4*). We agree with the authors, that this method is specific for measuring anti-idiotypic antibodies against the therapeutically used antibody. The specificity of our test was confirmed with two other anti-CD3 antibodies, i.e., BMA 030 and BMA 033, mouse monoclonal antibodies of an identical ($IgG2_a$) and a different isotype (IgG3), respectively.

We eliminated the interference of common anti-mouse antibodies by preincubating patient plasma with normal mouse serum before adding FITC-OKT3. We have calculated the amount of OKT3 captured by anti-idiotypic antibodies in the plasma samples. Therefore, we measured the fluorescence of HPB-ALL-bound FITC-OKT3 in the presence of different quantities of FITC-OKT3 in the absence of plasma and we compared this curve with the one generated in the presence of patient plasma. The shorter procedure of McIntyre and Higgins results in a titer of anti-idiotypic antibodies without an indication of the influence of these anti-idiotypic antibodies on the amount of available OKT3 in the plasma in case of retreatment with OKT3.

In conclusion, we agree that this method is suitable for monitoring patients.

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DONOR-RECIPIENT MICROCHIMERISM AND TOLERANCE INDUCTION

The fully allogeneic mouse strain combination of C57BL/10 (B10) donor → C3H/He recipient (MHC class I, II, MHC disparate) allows routine spontaneous acceptance of liver allografts but not of abdominally placed hearts that are rejected after 8 days (*1*). Bushell et al. (*2*) described prolongation of B10 heart survival determined by palpable graft contraction and/or EKG activity, following 2 kinds of donor-specific (DS) blood priming 24–28 days before surgery. However, to be effective, the priming had to be done under a two-dose cover of depleting anti-CD4 monoclonal antibody. Aided by the antibody treatment (which had no effect alone), single unmodified DS transfusion allowed 70% >100-day graft survival versus 60% if 4 daily transfusions of irradiated DS blood were given. At face value, the conclusion from this complex experiment is that both live donor leukocytes and dead donor antigen can be tolerogenic but with very different efficacy. This conclusion was the same one as that reached in principle by Billingham, Brent, and Medawar (*3*) four decades ago and verified frequently since then.

The question raised by Bushell et al. (*2*) is whether leukocyte chimerism is required for permanent tolerance. The answer was yes from the studies of Russell (*4*), who showed that chimerism-associated tolerance to stable skin grafts in the Billingham-Brent-Medawar mouse model was abolished by elimination of the chimeric cells (with isoantibodies). Similarly, Lubaroff and Silvers (*5*) demonstrated in classical experiments with such isoantibodies that the loss of established skin allografts in mice usually required many days or weeks, reflecting the gradual decay of tolerance. The experiments of Bushell et al. (*2*) are less easy to interpret. DS

nonreactivity, whether induced by leukocytes or dead antigen (with obviously different degrees of efficacy), can create a recipient environment conducive to the acceptance of a subsequently transplanted allograft which brings its own supply of chimeric cells. However, quantification of the priming effect by the endpoints of either cardiac contraction or "lack of electrical activity" of the graft is imprecise. The use of chronic rejection for an endpoint in such mouse heart transplant models as described by Orosz et al. (*6*) would have been definitive. In analogous rat cardiac transplant models done in our laboratory, the slow disappearance of chimeric leukocytes correlated closely with chronic rejection of the heterotopic allografts which continued to beat (by palpation) for at least 2 months after chimerism could no longer be detected with immunocytochemical technology (*7*). Subsequent polymerase chain reaction studies revealed scattered foci of donor DNA (unpublished observations).

Thus, the elegant experiments of Bushell et al. (*2*) are entirely congruent with, rather than being contradictory of, our hypothesis ascribing a seminal role to microchimerism in graft acceptance (*8*). Viable migratory donor leukocytes (or the white cells contained in a nonirradiated DS transfusion) are a potentially continuous source of tolerizing antigen. However, because these cells have immunologic capability, they introduce complex mechanisms of peripheral tolerance induction in addition to the presence of antigen as discussed elsewhere (*8–10*). We have shown that the peripheralized chimeric leukocytes exhibit qualitatively similar initial patterns of chimerism after liver and heart transplantation (*11*). In our mouse and rat studies, establishment of progenitor (and possibly stem) cells (*12, 13*) as well as the presence of mature cells of various lineages (*1, 7, 11*) correlated with permanent acceptance of livers (*2, 11*). In contrast, the smaller dose of migratory leukocytes from heart grafts, less favorable lineage composition, or perhaps other factors precluded achievement of a self-perpetuating chimeric state in these rodent recipients (*13*). The heart recipients in both experiments performed by Bushell et al. (*2*) were given a "booster" toward (but presumably still short of) permitting permanent donor leukocyte self-renewal, but to what extent is not analyzable without histopathologic data.

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DONOR-RECIPIENT MICROCHIMERISM AND TOLERANCE INDUCTION (REPLY)

The central point of our paper published recently in *Transplantation* (*1*) is that, when given on 4 consecutive days under the cover of anti-CD4 monoclonal antibody therapy, nonviable irradiated cells were *as effective* as their freshly isolated counterparts given on a single occasion. Thus, viable cells (and hence the potential for microchimerism) were not required to induce the long-term survival of a subsequent allograft in this model.

The success of the anti-CD4/DST protocol developed in this laboratory depends on interactions between donor cells and recipient T cells during a 3-day period of CD4 occupation by antibody (1a). The finding that a *single* dose of irradiated cells given under the cover of anti-CD4 antibody led to acute graft rejection is entirely consistent with our immunocytochemistry studies, which show that while donor cells from a normal DST can be detected in recipient spleens for at least 3 days after injection, irradiated cells are undetectable after about 24 hr. Irradiated cells are thus unable to persist long

enough for the required interactions with antibody-positive $CD4^+$ T cells to occur. We speculated that if the persistence of nonviable cells within the spleen could be enhanced during the period of anti-CD4 antibody occupation, graft prolongation rather than acute rejection might result. As shown in our Figure 3 (*1*), this speculation proved to be correct. Administration of 3 additional doses of irradiated cells led to graft prolongation, which was indistinguishable from that obtained with the basic anti-CD4/DST protocol. These data confirm that, in this model, viable cells are not an absolute requirement for the induction of unresponsiveness. It is worth noting that other studies have also shown that transplantation tolerance can be induced using a variety of nonviable donor antigen preparations (*2, 3*).

Starzl et al. allude to the possibility that migratory cells from the graft may play a role in the induction or maintenance of the unresponsive state. While we cannot formally rule out this possibility, we think it unlikely for a number of reasons. First, recipients pretreated with either anti-CD4 antibody alone or DST alone 28 days before transplantation show only slight graft prolongation (*4, 5*), which indicates that, even in modified hosts, the heart itself is unable to provide sufficient migratory cells to make a major contribution to the development of unresponsiveness. Second, in a series of experiments designed to explore the importance of the graft itself on the maintenance of tolerance, Hamano et al. (*6*) transplanted B10 hearts into C3H recipients under the cover of perioperative anti-CD4 antibody. Fifty days after transplantation, the primary grafts were removed and the animals were transplanted with second donor-specific hearts 60, 130, or 200 days later. While second hearts transplanted 60 or 130 days after removal of the primary graft were accepted (median survival time [MST] >100 days), those transplanted after a delay of 200 days were rejected with a median survival time of 35 days. Significantly, when the primary graft remained in situ for >250 days, second donor-specific grafts survived indefinitely. These data confirm that, in this model, continued unresponsiveness is dependent on the presence of the graft itself rather than on cells which may leave the graft and migrate to peripheral sites. It is interesting to note in this context that there has been at least one clinical example in which evidence of donor-derived cells could be found in several recipient tissues after transplantation, but the cells were no longer detectable after graft rejection (*7*). Such observations suggest that in clinical transplantation, which has been the focus of most of the interest in microchimerism, the continued presence of the graft might explain the evidence for donor-derived cells at tissue sites distant from the graft itself.

Starzl et al. express the view that the way in which we assess graft function in our cardiac allograft models might be regarded by some as rather imprecise. In our laboratory, we define rejection as either the loss of palpable cardiac contraction or the loss of electrical activity as defined by ECG (*8*). Grafts are therefore classified as functioning *only* if they have both palpable contraction *and* ECG. We accept that, as with all animal transplant models, the mouse heart model may have limitations, and we fully support the view that detailed histological analyses are required to determine whether long-term surviving hearts are completely free from immune-mediated damage. Such studies are currently in progress in our laboratory. However, we believe that continued allograft function for greater than 100 days provides a robust demonstration of operational tolerance. The grounds for this conclusion are as follows: (1) the B10 to C3H strain combination used in our experiments represents a mismatch for major plus multiple minor transplantation antigens; untreated animals reject their grafts in 8–12 days. (2) At 100 days after transplantation, C3H recipients of B10 grafts transplanted in the anti-CD4/DST protocol frequently have ECG frequency ratios (graft/native) that approach or even exceed those obtained in syngeneic control animals at the same postoperative time point. (3) Animals with hearts surviving beyond 100 days are truly tolerant, as judged by the stringent test of acceptance of second donor-specific heart grafts (MST>100 days); third-party hearts are rejected acutely (MST=16 days) (*9, 10*). (4) Adoptive transfer of 5×10^7 unfractionated splenocytes from C3H recipients with long-term B10 heart allografts leads to a significant prolongation of B10 hearts transplanted into *unmodified* naive C3H recipients (MST=82 days).

It seems likely that much of the controversy surrounding the possible role of chimerism/microchimerism in clinical transplantation relates to the fact that these terms have been used to describe a number of rather disparate situations, few of which satisfy the most widely accepted definition of chimerism. In the classical sense, chimerism refers to the presence of allogeneic pluripotent stem cells which are capable of self-renewal and give rise to populations of multilineage differentiated cells. If the criterion for chimerism was simply the presence of allogeneic cells of any type, surely every patient with a surviving allograft could be described as chimeric. We believe that the terms chimerism/microchimerism should be reserved for situations in which classical chimerism can be clearly demonstrated, and we suggest that antigen persistence be used in all other cases. A valuable contribution to this discussion has recently been provided by Kawai et al. (*11*), who administered donor bone marrow to irradiated, anti-thymocyte globulin-treated cynomolgus monkeys then examined the recipients for *multilineage* chimerism using flow cytometry. Perhaps the use of the term *multilineage chimerism* by other authors might help to clarify this rather confused area of debate.

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THE USE OF *N*-ACETYLCYSTEINE IN SOLID-ORGAN PRESERVATION SOLUTIONS

We read with interest the article of Fukuzawa et al. (*1*) concerning the reduction of reperfusion damages by *N*-acetylcysteine (NAC) after warm hepatic ischemia.

In this report, they conclude that NAC, intervening in glutathione (GSH) homeostasis, might limit the reperfusion injuries and improve graft survival.

Recently, we have developed an original lung preservation solution (SPAL UP) in which the scavenger system is composed of GSH and NAC (Table 1). This solution has been tested in vitro on different pulmonary parenchymal cells, such as alveolar type II cells (personal communication), fetal fibroblasts (*2*, *3*), and, more recently, human pulmonary artery endothelial cells (data still not published). The effects on cellular viability were compared with those of other solid organ preservation solutions. Our research showed that SPAL UP significantly influenced pulmonary cell viability. Using isolated parenchymal cells as a lung preservation screening model, we are, at present, not able to explain these findings; nevertheless, regarding NAC as a component of our solution, we postulated different mechanisms of action. The -SH radicals of NAC might regenerate glutathione -SH groups oxidized by oxygen-derived free radicals during both ischemia and reperfusion phases and it might also take part directly in the reduction of free radicals. Moreover, NAC is an important intracellular precursor of biochemical synthesis of GSH and readily traverses cellular membrane. When the temperature of preservation is about 10°C (as in our in vitro studies), cellular metabolism could still be active in part; thus, NAC might take part in intracellular regeneration of GSH. Nowadays, considering the indispensable presence of GSH in preservation solutions, we believe that, according to Fukuzawa et al. (*1*), the adjunctive use of an adequate NAC concentration (300 mg/L in our solution) might improve both intracellular and extracellular GSH homeostasis, and thus possibly strengthen the oxygen free radical scavenger system.

TABLE 1. SPAL UP composition

Na^+	125 mEq/L
K^+	4.2 mEq/L
Cl^-	105 mEq/L
Mg^{++}	2 mEq/L
HCO_3	25 mEq/L
Ca	8.9 mg/dl
Fe	133 μg/dl
Glucose	4.4 g/L
Albumin	4.3 g/dl
Methylprednisone	20 mg/L
N-Acetylcysteine	300 mg/L
Glutathione	300 mg/L
Osmolarity	275 mOsm/L pH 7.2

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