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New Immunosuppressive Drugs: Mechanistic Insights and Potential Therapeutic Advances

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INTRODUCTION

It is our objective in this paper to highlight recent discoveries and developments concerning "new" immunosuppressive drugs. Our intention it not to provide an all-encompassing, detailed review of the overwhelming number of experimental findings concerning these biochemically diverse agents which have recently been published. Many of these observations have been surveyed recently under the title of individual drugs (see Table I for references) and have also been reviewed in this volume. Rather, we have chosen, albeit subjectively, to illustrate a number of important recent contributions which research on these agents has made, *first*, to our understanding of lymphocyte cell biology, *second*, to the improvement of immunosuppressive therapy, particularly in the context of organ and tissue transplantation and, *third*, to our awareness of immunological phenomena which underlie and may be crucial for the acceptance of organ transplants.

MECHANISMS OF ACTION OF NEW IMMUNOSUPPRESSIVE DRUGS

What CsA, FK 506 and rapamycin have told us about molecular events underlying lymphocyte activation and degranulation: calcineurin and protein kinases as important signaling mediators

Whilst our understanding of the molecular events that lead to immune cell activation has improved enormously in recent years, we remain quite ignorant of

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the precise sequence of events that leads from antigen recognition to lymphocyte activation and cell proliferation. Recent revelations, however, concerning the molecular actions of cyclosporin A (CsA) and the new immunosuppressive drugs, FK506 and rapamycin, have provided important new information on the biochemical processes which regulate these events. Of special significance has been the realization that none of these T-cell directed immunosuppressants (CsA, FK506 or rapamycin) is, by itself, antilymphocytic. Rather, these molecules are now perceived as "molecular adaptors" which serve to mediate the interaction between their respective intracellular drug-binding proteins (or "immunophilins") and their individual target molecules. Vigorous research on the mode of action of FK506 and CsA (Liu et al. 1991, Liu 1993) has revealed that the heterodimeric, Ca^{2+}/calmodulin-regulated phosphatase *calcineurin* is a major common target of the CsA-cyclophilin A and FK506-FK506 binding protein 12 (FKBP12) drug-immunophilin complexes *in vitro* (cyclophilin A and FKBP12 are the predominant immunophilin isoforms that bind CsA and FK506, respectively). Although rapamycin, which is similar structurally to FK506, also binds to FKBP, it fails to inhibit calcineurin and acts by a quite distinct molecular mechanism (see below).

It has been discovered that calcineurin is a key enzyme in the T-cell signal transduction cascade following T-cell receptor (TCR) activation (Clipstone & Crabtree 1992, 1993, Liu et al. 1992, O'Keefe et al. 1992, Fruman et al. 1992). Furthermore, it is now established that inhibition of calcineurin phosphatase activity, and *not* CsA affinity for cyclophilin A, correlates with the suppression of early T-cell activation events (Nelson et al. 1992). Several of the key immunological findings concerning calcineurin are summarized in Table II. Amongst the most important conclusions from these observations is that interaction of drug-immunophilin complexes with calcineurin and inhibition of its phosphatase activity provides a molecular basis for the inhibitory effect of CsA or FK506 on expression of genes encoding T-cell growth factor (interleukin-2; IL-2) and other cytokines.

Since both CsA and FK506 inhibit specifically the TCR-mediated transcription of the IL-2 gene, its promotor region, and in particular the binding site for the nuclear factor of activated T cells (NFAT), has been the focus of intensive investigation. NFAT is a T-cell specific transcription factor, the activity of which correlates with the extent of IL-2 gene transcription following activation of the TCR. NF-AT has two components – a nuclear (n) subunit (NF-ATn) and a cytoplasmic (c) component (NF-ATc). A key observation reported by Crabtree and his colleagues is that the CsA/FK506 drug-immunophilin complexes, which form pentameric complexes with calcineurin (A and B) and calmodulin (see Fig. 1), block the assembly of functional NF-AT (Flanagan et al. 1991). This appears to be achieved by inhibition of the translocation of the pre-existing NF-ATc from the cytoplasm to the nucleus. NF-ATn is transcriptionally inactive in all cells other than activated T cells and is induced by signals from the TCR. Its appearance is

TABLE I

Diversity and modes of action of "new" immunosuppressive drugs

Effect: drug (structure)	Pharmaceutical company	Human immuno-suppressant use	Recent reviews
Inhibitors of cytokine production:			
CsA and CsG (cyclic peptides)	Sandoz Ltd., Basel, Switzerland	Yes	Di Padova 1989, Schreiber & Crabtree 1992, Fruman et al. 1993
FK506 (macrolide)	Fujisawa Pharmaceutical Co., Osaka, Japan	Yes	Thomson 1989, Schreiber & Crabtree 1992, Thomson et al. 1993b, Peters et al. 1993.
Inhibitors of cytokine action:			
Rapamycin (macrolide)	Wyeth-Ayerst Research, Princeton, NJ	No	Kahan et al. 1991a, Morris 1992, Seghal et al. 1993
Leflunomide (isoxazol derivative)	Hoechst AG, Weisbaden, Germany	No	Xiao et al. 1993, Chong et al. 1993
Inhibitors of DNA synthesis:			
Mizoribine = Bredinin	Sumitomo Chemical Co., Takarazuka, Japan	Yes	Amemiya & Itoh 1993
RS-61443 = Mycophenolate mofetil (morpholinoethylester of mycophenolic acid)	Syntex, Palo Alto, CA	Yes	Sollinger et al. 1991, Allison & Eugui 1993, Platz et al. 1991
Brequinar sodium (quinoline carboxylic acid derivative)	Dupont-Merck Pharmaceutical Co., Wilmington, DE	No	Makowka & Cramer 1992, 1993
Inhibitors of cell activation/maturation:			
Deoxyspergualin (polyamine)	Nippon Kayaku, Tokyo, Japan	Yes	Morris 1991, Suzuki 1993
Inducer of non-specific suppressor cells:			
SK&F 105685 (azaspirane)	Smith Kline Beecham Pharmaceuticals, King of Prussia, PA	No	Kupiec-Weglinski et al. 1993

TABLE II

The immunological significance of calcineurin (see also Fig. 1)

1. Calcineurin is a key rate-limiting enzyme in T-cell signal transduction and mediator of Ca^{++}-dependent events.
2. IL-2 production in T cells activated via the TCR/CD3 complex correlates closely with the level of calcineurin activity.
3. Cells expressing low levels of calcineurin (e.g. T cells) are most sensitive to CsA/FK506.
4. Overexpression of calcineurin overcomes the CsA/FK506-mediated inhibition of NF-AT-dependent cytokine gene transcription.
5. Immunosuppressive activity of cyclosporin analogues correlates with inhibition of calcineurin phosphatase activity.
6. Calcineurin is involved in signalling events that lead to degranulation of cytotoxic T-cells.
7. Calcineurin activity plays a key role in TCR/CD3-mediated induction of apoptosis in T-cell hybridomas.

For further details the reader is referred to recent papers and reviews (Schreiber & Crabtree 1992, Fruman et al. 1993, Clipstone & Crabtree 1992, 1993).

not blocked by FK506 or CsA. Current thinking is that FK506 and CsA block the dephosphorylation by calcineurin of NF-ATc, a step that is required for its translocation to the nucleus. This model has gained credence from the finding of McCaffrey et al. (1993) that NF-ATc is indeed a phosphoprotein in resting T cells and can be dephosphorylated by calcineurin. Moreover, this group has also shown that dephosphorylation of NF-ATc by calcineurin in cell lysates can be inhibited by a specific peptide inhibitor of calcineurin or by pre-treatment of the cells with CsA or FK506. These data suggest both that NF-ATc may be the direct substrate of calcineurin and that it may serve as the ultimate protein messenger of the signal transduction cascade from the cell surface TCR to the nucleus.

A role for calcineurin has also been suggested recently in the degranulation of (murine) cytotoxic T cells (Dutz et al. 1993). Thus, in addition to its role in cytokine gene expression, calcineurin also seems to be involved in T-cell activation processes (e.g. those which precede degranulation) that do not require protein synthesis. Clearly, much insight has been gained into the influence of CsA and FK506 on signal transduction in T cells. It is to the suppressive action of these drugs on IL-2 synthesis by T cells that their capacity to inhibit organ allograft rejection is ascribed. A complementary and/or alternative mechanism, however, has been proposed for the immunosuppressive action of CsA (Erlanger 1993). This is based on the finding that CsA inhibits the chemotactic activity of extracellular cyclophilin for inflammatory leukocytes (Xu et al. 1992). In sharp contrast to these concepts of drug action, much less is known about the molecular mechanisms that underlie the neuro- and nephrotoxic properties of CsA and FK506. It is in this area that improved understanding will be essential for the future design of more specific and less toxic immunosuppressants.

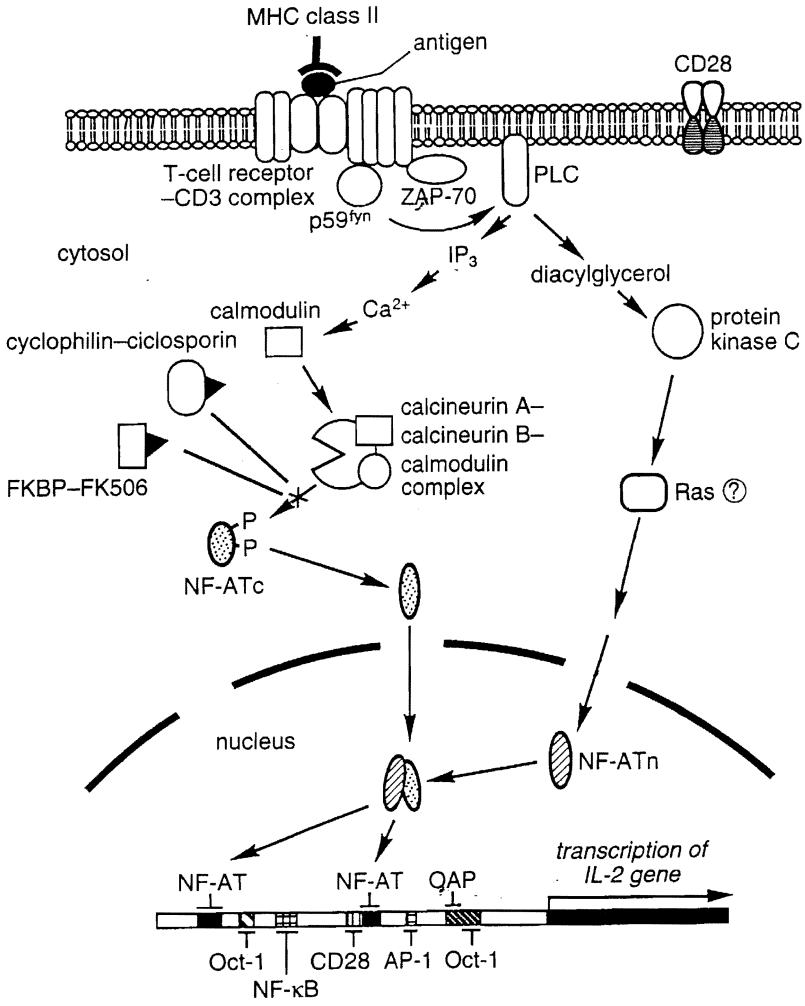

Figure 1. The T-cell receptor-mediated signal transduction pathway leading to interleukin-2 (IL-2) transcription with the recently identified signal-transducing molecules highlighted. PLC: phospholipase Cγl; IP_3: inositol-1,4,5-triphosphate; NF-ATc: the cytoplasmic component of the nuclear factor of activated T cells; NF-ATn: the nuclear component of NF-AT; FKBP: FK506 binding protein. Reproduced from Liu J., *Immunology Today* **14**, 293, 1993 with kind permission of the author and Elsevier Science Publishers Ltd.

In T cells, activation of the genes for both IL-2 and its receptor determines the progression of the cell from the G0 to the G1 phase of the cell cycle. G1-to-S progression is then regulated by IL-2. The powerful immunosuppressant rapamycin is a close structural analogue of FK506 and binds to the same immuno-

philin (FKBP) as FK506, forming a drug-immunophilin (rapamycin-FKBP) complex (Schreiber 1991). This complex, however, does not block calcineurin activity (Liu et al. 1991) or G0-to-G1 progression, as defined by the expression of IL-2 or its receptors. It has recently been discovered that rapamycin potently inhibits the rapid activation of p70S6 kinase following IL-2 stimulation of T cells and, consequently, G1-to-S phase progression (Price et al. 1992, Kuo et al. 1992, Chung et al. 1992). Although this finding has implicated p70S6 kinase activation as important in Ca^{2+}-independent signaling leading to cell proliferation, there is no evidence that p70S6 kinase is involved directly in S-phase entry. On the other hand, recent results obtained using the murine helper T-cell clone D10, have demonstrated that the IL-2-stimulated expression of a serine/threonine kinase p34 cdc2, that is known to be required for cell cycle progression and may be essential for G1→S transition, is a target of rapamycin (Flanagan & Crabtree 1993).

Fig. 2 depicts a model for the action of rapamycin. In this model (for further

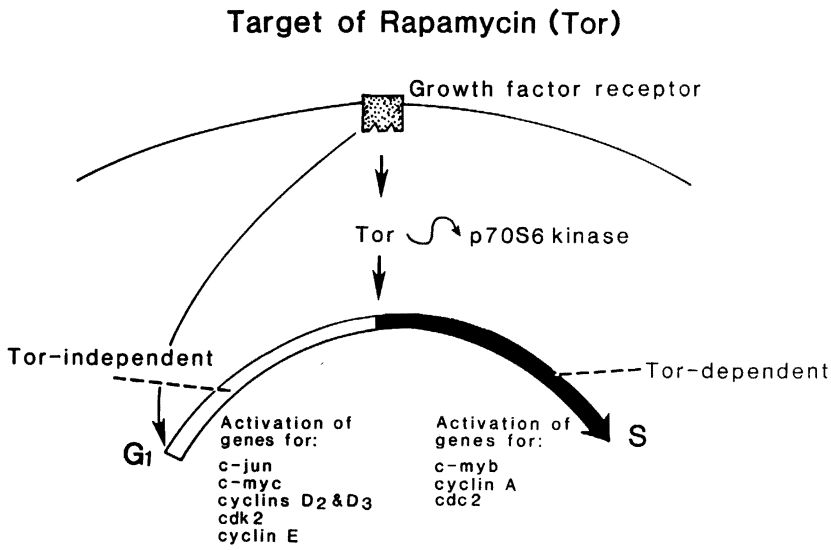

Figure 2. A model for the mechanism of action of rapamycin. IL-2-mediated signalling activates two distinct pathways: a target of rapamycin (Tor)-independent pathway and a Tor-dependent pathway. The Tor-dependent pathway is simply defined as being inhibited by rapamycin. Rapamycin blocks cell-cycle progression at a point where many early to mid-G1 cell-cycle regulatory proteins have accumulated (cyclins D2, D3 and E) yet are unable to execute their function without an additional triggering event(s). The activation of the cyclin E/CDK2 kinase complex, which is blocked by rapamycin, could be the triggering event required for progression into the S phase of the cell-cycle. After Flanagan et al. (1993).

information see Flanagan & Crabtree 1993, and Flanagan et al. 1993), IL-2 signalling via the IL-2 receptor activates both Target of rapamycin (Tor)-independent and Tor-dependent pathways. Rapamycin is thought to block G1→S progression at a point where many early to mid-G1 cell cycle regulatory proteins (cyclins D2, D3 and E) have accumulated, yet cannot fulfil their function without additional triggering events. Activation of the cyclin E/cdk2 complex, which is blocked by rapamycin, may be the triggering event required for progression into S-phase. Undoubtedly, rapamycin provides a valuable tool for analysis and linkage of these autocrine growth factor receptor-induced signalling events to downstream targets which regulate S-phase entry. The reliance of T lymphocytes on a single (IL-2–IL-2R) signalling pathway renders these cells uniquely susceptible to rapamycin.

The discovery of calcineurin and p70S6 kinases as important signaling mediators clearly emphasizes the utility of immunosuppressive drugs as probes of lymphocyte signal transduction.

Leflunomide resembles rapamycin in inhibiting T-cell responses to IL-2

Leflunomide is a novel isoxazol derivative with potent immunosuppressive activity that is unrelated chemically to CsA, FK506 or rapamycin (Chong et al. 1993, Xiao et al. 1993). It prevents acute rejection of kidney, heart and skin allografts in the rat at doses similar to those of CsA. Unlike CsA, however, leflunomide can reverse ongoing acute rat cardiac allograft rejection. *In vitro*, leflunomide is considerably less potent than CsA; 50 μM is the IC_{50} required for inhibition of the mixed lymphocyte reaction, compared with 45 nM for CsA or 1nM for rapamycin (Chong et al. 1993). Since leflunomide is unrelated structurally to any other immunosuppressive drug, its mechanism of action is at present unknown. Recently, leflunomide has been shown to have no effect on the induction of IL-2R expression on activated human T cells. However, it partially reduces IL-2 production by and completely inhibits proliferation of T cells stimulated with either anti-CD3 plus phorbol myristate acetate (PMA) or (unlike CsA or FK506) anti-CD28 monoclonal antibody plus PMA (Chong et al. 1993). Thus, leflunomide resembles rapamycin in that both agents suppress T-cell proliferation, principally by inhibition of T-cell responsiveness to IL-2. It is noteworthy that rapamycin has already been shown to act synergistically with CsA (both at subtherapeutic or minimally effective doses; see below). Leflunomide therefore joins both rapamycin and monoclonal anti-IL-2R antibodies as a promising experimental agent for combination immunosuppressive therapy with CsA, each agent affecting distinct phases of the important IL-2-IL-2R T-cell signal transduction pathway.

OTHER INSIGHTS

Inhibition of cytokine and growth factor action by rapamycin in vivo *may inhibit vascular injury following allostimulation*

Rapamycin exhibits the capacity not only to inhibit the growth of T lymphocytes but also the prolifertion of hepatocytes, endothelial cells, fibroblasts and smooth muscle cells stimulated by "non-immune" growth factors, such as hepatocyte growth factor, epidermal growth factor and fibroblast growth factor beta (Morris 1992, Francavilla et al. 1991, Akselband et al. 1991). Inhibition of paracrine cytokine effects by rapamycin could suppress, indirectly, additional growth factor production. In all types of vascular injury, inflammation and the release of cytokines and growth factors cause luminal narrowing. Recently, Gregory et al. (1993) have made important observations on the influence of rapamycin on arterial intimal thickening (a major complication that limits long-term survival after heart transplantation). In their experimental rat model, vascular injury was caused by either alloimmune stimulation (femoral artery allografting) or mechanical (balloon catheter) damage. They found inhibition of intimal proliferation in arteries by rapamycin. This effect was ascribed to interference by rapamycin with cytokine and growth factor actions that attract and activate macrophages and cause smooth muscle cells to migrate and proliferate. Although mRNAs for growth factors were expressed extensively *in situ*, intimal thickening was reduced compared with injured vessels from untreated control rats. These *in vivo* observations are entirely in line with the *in vitro* selective inhibitory effects of rapamycin on cytokine/growth factor action and may have significant therapeutic implications for the influence of rapamycin on vascularized organ allograft survival.

Macrolide immunosuppressants (FK506 and rapamycin) may exhibit differential interference with cytokine gene expression in vitro *or* in vivo

Two subsets of murine $CD4^+$ T cells (T_{H1} and T_{H2}) have been identified on the basis of their cytokine secretion and functional profiles (Mosmann & Coffman 1989) and there is growing evidence for such human functional subsets. T_{H1} cells produce IL-2, IFN-γ, and TNFβ, whereas T_{H2} cells secrete IL-4, IL-5, IL-10 and IL-13, but not IL-2 or IFN-γ. There is also evidence that, *in vitro*, FK506 may spare expression of the anti-flammatory cytokine IL-10 (cytokine synthesis inhibitory factor) by cloned murine T_{H2} cells, whilst suppressing concomitant IL-4 mRNA production (Wang et al. 1991). Thus, differential interference with cytokine gene expression and crossregulation of T_{H1} cell (IL-2 and IFN-γ production) may be important mechanisms whereby FK506 inhibits immune cell activation and maintains immunosuppression *in vivo*.

There is also evidence from recent studies in the rat that rapamycin administration can result in the divergent transcription of distinct cytokine genes *in vivo*.

The model used was accelerated cardiac allograft rejection in sensitized animals. The findings concern the influence of rapamycin on the early expression of genes encoding cytokines with IL-8-like neutrophil activation/chemotactic properties. In addition, the later expression of T_{H1}- and T_{H2}- specific cytokines, IFN-γ and IL-10 respectively, was examined (Wieder et al. 1993). Quite novel intra-transplant effects of rapamycin were observed. Thus, rapamycin inhibited IL-8-like mRNA/protein production, with subsequent suppression of graft neutrophil infiltration. T_{H1}-specific IFN-γ mRNA was also inhibited. In contrast, there was simultaneous sparing of T_{H2}-specific IL-10 mRNA. These findings suggest that the divergent transcription of distinct cytokine genes may contribute to the immunosuppressive efficacy of rapamycin in sensitized transplant recipients. It is also intriguing that, whereas rapamycin does not inhibit IFN-γ production by activated T cells *in vitro*, it suppresses IFN-γ mRNA/protein production in this *in vivo* model. Taken together, these *in vitro* and *in vivo* studies show that two potent anti-T cell agents (rapamycin and FK506), with distinct molecular actions, spare IL-10 expression whilst suppressing the production of pro-inflammatory cytokines.

Rapamycin inhibits cytokine-induced immunoglobulin production in vitro, *but fails to suppress humoral immunity in presensitized individuals*

It is well-recognized that rapamycin is about 1000-fold more potent than CsA in inhibiting spontaneous or pokeweed mitogen (PWM)-stimulated immunoglobulin (Ig) production by human PBMC *in vitro*. The drug abolishes T-cell help to T-dependent Ig production and also *directly* suppresses Ig production by *pure* B cells stimulated with IL-2 and *Staphylococcus aureus* Cowan 1 (SAC). Luo et al. (1993) have also shown that neither IL-2, IL-4, IL-6 nor IFN-γ was able to reverse the inhibitory effect of rapamycin in unstimulated or PWM-stimulated PBMC. These observations show that *both* T and B cells are direct targets of rapamycin. These *in vitro* studies suggest that rapamycin may be useful in the treatment of presensitized transplant patients with high titers of antibody that normally preclude transplantation. Other *in vivo* studies, however, have shown that rapamycin does not significantly suppress humoral immunity (alloantibody synthesis) in rats with established humoral alloreactivity (Propper et al. 1992).

Synergy between rapamycin and CsA

Mutually synergistic interactions between rapamycin and CsA have been reported both *in vitro* and *in vivo* (Kimball et al. 1991, Kahan et al. 1991b). *In vitro*, rapamycin augments the inhibitory effects of CsA on human PBL activation by PHA, anti-CD3 monoclonal antibody, and the MLR. Rapamycin also enhances the capacity of CsA to suppress cytotoxic T-cell generation and precursor frequency during alloactivation *in vitro*. Moreover, CsA potentiates the inhibitory

effects of rapamycin on the proliferation of IL-2- and IL-6-dependent cell lines (Kahan et al. 1991b). At the same time, antagonism between low doses of FK506 and CsA can be demonstrated, using the same experimental *in vitro* systems.

In vivo, a strong synergistic immunosuppressive effect of low doses of rapamycin and CsA has been reported by Kahan et al. (1991a), using the Buffalo→Wistar Furth rat cardiac allograft model. Thus, minimally effective doses of rapamycin (0.02 mg/kg i.v.) and CsA (2 mg/kg p.o.), when combined, allowed 100% cardiac graft survival beyond 50 days in all graft recipients. Evidence has also been cited (Kahan et al. 1991a) of combined rapamycin/CsA/RS-61443 (mycophenolate mofetil) treatment prolonging mouse heart allograft survival from 18 to 140 days. Morris et al. (1991a) have also reported that the combination of rapamycin with FK506 acts synergistically to prolong mouse heart allograft survival. The latter data are particularly interesting as they are not consistent with reports that rapamycin and FK506 are reciprocal antagonists with respect to T-cell activation *in vitro*. This appearent discrepancy may be explained by differing relative concentration levels of the two drugs. *In vitro*, a 100-fold molar excess of one drug relative to the other is required for exhibition of antagonism.

OTHER DRUGS

SK&F 105685: an inducer of non-specific splenic suppressor cells following experimental cardiac transplantation

SK&F 105685 is a novel azaspirane which suppresses autoimmune disorders in experimental animals. It is also effective, both in short-term pretreatment or treatment, in prolonging cardiac allograft rejection in the rat. Studies by Schmidbauer et al. (1993) have shown that, in this animal model, SK&F 105685 suppresses the graft cellular infiltrate and the induction of IL-2R and abolishes IL-2 and IFN-γ production. Adoptive transfer of spleen cells from SK&F 105685-treated, allografted donors to naive recipients significantly prolonged the survival of donor-specific or third-party cardiac allografts. A search for the identity of the cells conferring this inhibitory effect revealed that, unlike classic $CD8^+$ suppressor T cells, the SK&F 105685-induced cells did not exhibit characteristics of mature, T, B, NK cells or macrophages but may belong to the premyeloid/monocytic lineage (Schmidbauer et al. 1993). They were also similar to "natural suppressor" cells previously generated in experimental animals by total lymphoid irradiation. Significantly, no other class of immunosuppressive drugs with a similar immunopharmacologic profile to SK&F 105685 has been described. It will be of interest to determine whether SK&F 105685 exhibits synergistic interactions with CD4 T cell-directed immunosuppressive therapy using CsA or FK506 in rat allograft models. It is promising that there is already evidence of at least additive immunosuppressive effects with subtherapeutic doses of CsA (Schmidbauer et al. 1993).

RS-61443 (mycophenolate mofetil) facilitates tolerance induction by a CsA-sensitive mechanism

Mycophenolate mofetil (MM) is a pro-drug of the antiproliferative agent mycophenolic acid (MPA). It specifically and reversibly inhibits inosine monophosphate dehydrogenase, thus depleting guanosine and deoxyguanosine nucleotides. Since both T and B cells are more dependent on *de novo* purine biosynthesis than other cells, MPA has a T- and B-lymphocyte-selective antiproliferative effect, both *in vitro* and *in vivo* (for reviews see Sollinger et al. 1991, Allison & Eugui 1993). MM inhibits vascularized organ allograft rejection in animals (Sollinger et al. 1991). The drug may be superior to azathioprine in preventing early human renal allograft rejection because of a higher therapeutic index (Sollinger et al. 1992). Significantly, MM can reverse ongoing rejection, even when OKT3 and high-dose steroids have failed. Interestingly, and in common with many other immunosuppressive drugs with diverse modes of action, short-term treatment of rodents with MM induces tolerance to cardiac allografts (Morris et al. 1991b). MM treatment also facilitates the induction of indefinite, donor-specific tolerance to pancreatic islet allografts in the mouse. Hao et al. (1992) have reported that this latter phenomenon is an active form of tolerance, rather than clonal deletion or anergy and that it can be inhibited by CsA administered in combination with MM. The mechanism underlying this antagonism between CsA and MM remains to be elucidated.

RS-61443 (mycophenolate mofetil) interferes with natural antibody production and adhesion molecule glycosylation

MM seems to be an important new drug for clinical use, because of its low *in vivo* toxicity (unlike CsA and FK506, it is not potentially nephrotoxic). Moreover, even chronic administration of MM in human patients or experimental animals does not appear to increase susceptibility to infections (Platz et al. 1991, Sollinger et al. 1991). Two other recent observations underscore its potential value in the control of organ transplant rejection. *First*, natural antibodies are considered a key element in initiating the hyperacute rejection of discordant organ xenografts. The currently available approaches for the depletion of natural antibodies (such as plasmapheresis or immunoabsorption by organ perfusion) although effective, have only a very temporary effect. Any agent which can effectively control the production of natural antibody may prove valuable in the management of xenograft rejection. It is thus of potential importance that, in addition to inhibiting humoral immune responses to a variety of antigens, MM has now been shown to reversibly inhibit the return of IgM natural antibody to the circulation after its acute depletion (Figueroa et al. 1993). A rebound in antibody production was, however, encountered following cessation of the drug on day 14. Conceivably,

depletion of natural antibody for a significant period after discordant xenotransplantation using MM or other anti-B cell agents may spare the vascular endothelium of the graft from the injurious effects of antibody and complement, enhancing the chances of "accomodation" of the xenografted organ.

One of the important metabolic effects of guanine triphosphate depletion mediated by MPA may be decreases in the transfer of mannose and fucose to glycoproteins, including adhesion molecules with lectin-like domains. These include selectins (e.g. E-selectin), which facilitate the attachment of leukocytes to endothelial cells and to target cells (Allison 1992). It has been postulated that, by this mechanism, MPA could inhibit the recruitment of neutrophils, lymphocytes and monocytes to sites of inflammation, including those of rejecting vascularized organ allografts.

The immunosuppressive action of Brequinar sodium and its potentiation by cytidine

Another important new anti-proliferative drug is Brequinar sodium (BQR) (Makowka & Cramer 1992). In inhibits *de novo* pyrimidine biosynthesis, and consequently both DNA and RNA synthesis, by blocking the activity of dihydroorotate dehydrogenase. Since BQR is known to prevent the synthesis of nucleotides during cell proliferation, we hypothesized that it would be highly effective in controlling strong lymphocyte proliferative responses, but might be less effective in controlling comparatively weak responses that do not necessarily involve new nucleotide synthesis. This question was addressed by us using cultured murine spleen cells in the presence of different stimuli, including Con A, PMA$\pm$ ionomycin, anti-CD3 monoclonal antibodies and anti-Ig.

The addition of BQR (0.001 to 10 μg/ml) at the start of a 72-hour culture period caused dose-dependent inhibition of strong proliferative responses, induced either by Con A (5 μg/ml) or PMA + ionomycin. A residual degree of proliferation persisted, however, even at the highest BQR concentrations. In contrast, no impairment of low concentration Con A (0.5 or 0.1 μg/ml), anti-CD3, or anti-Ig-induced responses was observed. In order to ascertain its role in arresting nucleotide synthesis, we attempted to reverse the inhibitory effect of BQR by adding exogenous uridine or cytidine to lymphocyte cultures. The inhibitory activity of BQR was reversed completely by adding uridine at 0.1 mM (Thomson et al. 1993). In contrast, the combination of BQR and cytidine potentiated the action of BQR and abrogated anti-CD3 or anti-Ig-induced lymphocyte proliferation in a dose-dependent manner. A synergistic inhibitory action between BQR and cytidine was observed when the BQR concentration was higher than 0.1 μg/ml and with cytidine at 0.1 mM (Thomson et al. 1993). It was found that the production of IL-2 and IL-4 was only slightly affected by BQR, but was significantly suppressed by coadministration of BQR and cytidine. Neither BQR on its own, however, nor the combination of BQR with cytidine affected production of

mRNA for IL-2, IL-4 or IFN-γ, as determined by the polymerase chain reaction (Woo et al. 1993a). These novel observations suggest that BQR may not only affect dihydroorotate dehydrogenase activity, but may also inhibit the enzyme cytidine deaminase which convents cytidine to uridine (see Fig. 3 for a proposed model for the sites of action of BQR).

The antimetabolic effects of BQR complement the well-known cytokine synthesis inhibitory actions of FK506 or CsA. The combination of BQR and cytidine, however, offers a further possibility for inhibition of both cytokine production and T- and B-cell proliferation. Indeed, we have also shown recently that BQR inhibits IL-6-induced differentiation of human B cells into IgM-secreting plasma cells (Tamura et al. 1993). This effect was also potentiated by cytidine (but reversed by uridine). Based on these observations, the combination of BQR and cytidine may have potential for the control of graft rejection. Indeed, we have presented preliminary data which show that cytidine potentiates the inhibitory

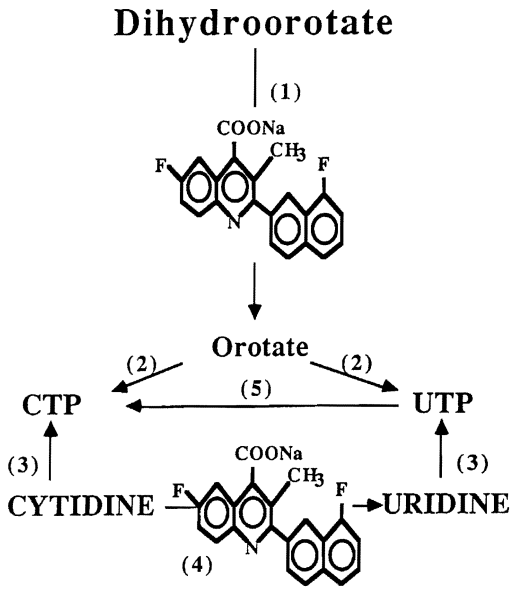

Figure 3. Structure of brequinar sodium (BQR) superimposed on a flow diagram to indicate the probable sites of action of the drug on pyrimidine biosynthesis. Sites of enzyme action are denoted by numbers in parentheses. In addition to inhibiting dihydroorotate dehydrogenase, our recent observations indicate that BQR also inhibits cytidine deaminase. The enzymes involved in the pathway are (1) dihydroorotate dehydrogenase; (2) orotate phosphoribosyltransferase, orotidine 5′ – monophosphate decarboxylase, nucleoside monophosphate kinase and nucleoside disphosphate kinase (in sequence along the pathway); (3) nucleoside kinase; (4) cytidine deaminase and (5) CTP synthetase. CTP = cytidine triphosphate; UTP = uridine triphosphate.

effect on BQR on hamster cardiac xenograft rejection in the rat (Woo et al. 1993b).

Deoxyspergualin (DSG) inhibits cell maturation

The semi-synthetic polyamine 15-deoxyspergualin (DSG) exhibits a novel spectrum of immunosuppressive activity in experimental animals and is effective both in allogeneic and xenogeneic transplantation models in non-human primates (Morris 1991, Suzuki 1993). It is effective (either alone or in combination with other agents) as rescue therapy in human renal transplantation (see below).

Although elucidation of the mode of its action *in vitro* has been slow, it is evident that DSG may affect monocyte/macrophage function predominantly, including inhibition of oxidative metabolism, lyosomal enzyme synthesis, IL-1 production and cell surface expression of MHC class II antigens. Its inhibitory effects on antigen processing/presentation are not reversed by IL-1, IL-6 or anti-CD28, suggesting that neither inhibition of production of these cytokines nor impairment of signalling via the B7/CD28 pathway accounts for the immunosuppressive action of DSG. Apart from influencing monocytes/macrophages, DSG also inhibits the *in vivo* generation of cytotoxic T cells, either directly or indirectly. It has little effect on IL-2 or IFN-γ production. On the other hand, the recovery of secondary cytotoxic T-cell activity (that is susceptible to DSG) following the addition of IFN-γ, suggests that suppression of IFN-γ production may be the main effect of DSG on T-cell populations.

CLINICAL TRIALS WITH NEW IMMUNOSUPPRESSIVE DRUGS (FK506, MYCOPHENOLATE MOFETIL AND DSG)

(i) *FK506*

Clinical trials will be referred to here only briefly. FK506 was first used in clinical organ (liver) transplantation at the University of Pittsburgh Medical Center (UPMC) in February 1989. There have since been numerous reports from this center concerning its efficacy and safety, predominantly in human liver transplantation, and also as primary immunosuppressive therapy in kidney, heart, lung and intestinal transplantation (Starzl et al. 1989, 1991). FK506 also shows a remarkable ability (unlike CsA) to reverse ongoing established liver, kidney or heart rejection. These single-center clinical trials showed FK506 to provide baseline immunosuppressive therapy which was more potent than that achieved with CsA. Recently, the results of multi-center prospective randomized trials comparing FK506 to CsA after primary liver transplantation have been reported. The results of the US multi-center trial have shown that FK506 plus corticosteroids is more effective than CsA-based immunosuppressive regimens for prophylactic immunosuppression (McDiarmid et al. 1993). The FK506-treated group experi-

enced significant reductions in the frequency and severity of rejection with less cumulative intravenous corticosteroid treatment. The side effects of FK506 and CsA are similar, however, with each drug having the capacity to induce nephrotoxicity, diabetogenicity or neurotoxicity. The incidence of post-transplant lymphoproliferative disease and the incidence of CMV infection is similar in FK506- and CsA-treated patients.

One way of assessing the impact of a new immunosuppressive drug is to examine its influence on the transplantation of organs which has proved difficult to achieve using conventional immunosuppressants, including CsA. Thus, human liver transplantation rapidly became a practical clinical service with the advent of CsA. Until recently, death or graft loss after clinical intestinal transplantation was usually the result of failure to control rejection and/or the inability to prevent immunological attack on the host by graft lymphoid tissue (graft-versus-host disease). In animal small bowel transplant models, FK506 has provided results superior to those obtained with CsA (Murase et al. 1990, 1991), prompting a clinical small bowel transplant trial. At the UPMC, small bowel allografts have been transplanted alone, together with liver, or in a small number of cases as part of a multivisceral cluster (Todo et al. 1992). In a study of 28 patients, 82% were alive at a median follow-up of 9 months. Graft survival was 76% after the same period and graft function was satisfactory, with 84% of survivors showing complete enteral sufficiency and the other 16% depending on supplemental parenteral nutrition. Rejection of the intestinal graft was common, with 90% of patients experiencing at least one episode. These patients have been treated with additional corticosteroids and azathioprine. Anti-lymphocyte preparations have also been used occasionally (Fung et al. 1993).

Improved results using FK506 compared to CsA (each in combination with azathioprine) in a small prospective randomized trial of primary adult lung transplantation have also been achieved at the UPMC (Fung et al. 1993). Six-month graft survival and freedom from rejection were significantly greater in the FK506-treated group. Preliminary results have also been obtained using FK506 in human autoimmune diseases (Thomson & Starzl 1992, Thomson et al. 1993a). Ongoing and future clinical trials using FK506 together with other conventional or experimental immunosuppressive agents may permit the further improvement of immunosuppressive drug regimens.

(ii) *Mycophenolate mofetil*

RS-61443 (Mycophenolate mofetil; MM) has been used adjunctively with CsA and prednisone in clinical renal transplantation. The results of two trials, a phase-1 dosage trial and a rescue trial have been reported (Sollinger et al. 1991, 1992). Dosages of MM from 100 mg/day to 3500/day have been used with good tolerance and safely. Less rejection was seen at higher doses. The rescue trial

surveyed 20 cases of refractory kidney rejection under conventional therapy and found an 80% response to MM in doses of 2–3.5 g/day. No evidence of nephro-, neuro-, hepato- or myelotoxicity was seen. In view of the relative safety and efficacy of MM in these preliminary trials, at multicenter randomized trial comparing CsA, MM, and prednisone with CsA, azathioprine, and prednisone has commenced.

(iii) *Deoxyspergualin (DSG)*

DSG has been used in several clinical studies in Japan to treat refractory kidney rejection and is effective either alone or when given with steroids (Amemiya et al. 1990, 1991). A response rate ranging from 70–87% has been reported, and was over 90% when DSG and steroids were used together. DSG has also been used for induction therapy, in combination with CsA-based regimens and has been successful in cadaveric, living-related, ABO-incompatible, and highly sensitized transplant recipients (for references, see Suzuki 1993). Excellent patient and graft survival have been reported, as well as less rejection and nephrotoxicity. Use of DSG in combination immunosuppressive therapy has also proved successful in achieving insulin independence in 2 recently reported cases of human pancreatic islet cell transplantation at the University of Minneapolis (Gores et al. 1993).

THE CHALLENGE OF XENOTRANSPLANTATION

Prolongation of organ xenograft survival with CsA or FK506 together with an antiproliferative agent

Traditional immunosuppressive therapies have had limited success in preventing the rejection of experimental organ xenografts. Thus, drugs such as CsA and FK506 that are highly effective in inhibiting the predominantly cellular responses that are responsible for allograft rejection fail to prolong the survival of concordant hamster-to-rat cardiac xenografts beyond 3–4 days (Gudas et al. 1989, Van den Bogaerde et al. 1991). In this rodent model, antibody and complement have been shown to be the primary mediators of graft rejection. Debilitating therapies, such as the combination of CsA with total lymphoid irradiation and monoclonal anti-CD4 antibody, however, can permit the survival of hamster-to-rat xenografts for several months, but this approach has very limited clinical application. Recently, it has been shown that the combination of cyclophosphamide (Cy) with CsA or FK506 (but not Cy alone) can lead to prolonged (>100 day) cardiac xenograft survival, with associated suppression of anti-hamster antibody production (Hasan et al. 1992, Murase et al. 1993). Serious morbidity, however, due to infection consequent upon excessive immunosuppression, remains a potential problem with the combination of these powerful anti-T and anti-B cell agents. In a recent baboon-to-human liver xenotransplantation, FK506 and Cy were

used as immunosuppressants, together with prednisone and prostaglandin (PGE) (Starzl et al. 1993d). This combination was effective in preventing both the cellular and antibody-mediated rejection described earlier in baboon-to-human xenografts.

IMMUNOSUPPRESSIVE DRUGS AND DONOR-DERIVED CELL CHIMERISM FOLLOWING ORGAN TRANSPLANTATION

(i) *The phenomenon: migration of donor cells from graft to recipient*

Improvements in the immunosuppressive therapy of organ allograft rejection achieved using CsA and FK506 have been attributed to the potent and precise molecular actions of these drugs in inhibiting signal transduction in alloactivated T cells. There is now convincing evidence, however, that following organ transplantation the promotion of peripheral T-cell tolerance to alloantigens by these and other biochemically diverse drugs may be associated, just as significantly, with a permissive effect of each drug on two-way (donor-recipient) leukocyte migration (Starzl et al. 1992, 1993b). This leads to a state of mixed allogeneic cell microchimerism in both graft and host.

It has recently been proposed that this cellular chimerism is a natural consequence of organ transplantation under cover of immunosuppressive drug therapy (Starzl et al. 1992, 1993b). Moreover, it is conceivable that donor-derived leukocytes in the periphery of chimeras may play an important role in achieving and maintaining allotolerance, as many recent reports show that mature T cells can be tolerized after encountering antigen outside the thymus. In humans, the cell chimerism observed following organ transplantation may persist for many years even in patients who have discontinued all forms of immunosuppressive drug therapy (Starzl et al. 1993c). In these studies, there has been no indication of drug specificity as the chimeric state was induced under azathioprine, Cy, CsA or FK506-based protocols, with additional immune modulation by corticosteroids, antilymphocyte antibodies, splenectomy or even thymectomy. Whether they were on or off maintenance immunosuppressive therapy, essentially all of the patients tested using *in vitro* mixed lymphocyte reaction or cell-mediated lymphocytotoxicity methods exhibited some element of donor-specific nonreactivity. The variable ability of different transplanted organs to produce chimerism and, consequently, to induce such narrow nonreactivity, was explained by their comparative content of migratory leukocytes, believed to be greatest in the liver.

These clinical studies indicated that cell migration and subsequent chimerism might be an integral requirement for organ graft acceptance, as well as the seminal step in tolerance induction. There was, however, a paucity of information about the events between the actual transplant and the above observations on chimerism made years later. Information which has helped to fill this gap has been provided by studies in the rat (Demetris et al. 1993).

Although cell migration now appears to be a generic phenomenon following the transplantation of all organs, the rat liver transplant model was selected because the comparatively large volume of cell traffic to and from this organ is ideal for the study of the participating cells. The results have demonstrated that both the acute leukocyte migration and the ensuing long-term chimerism are probably *multilineage*. Donor T and B lymphocytes, as well as macrophages and dendritic cells from the transplanted liver were found to localize quickly in the lymph nodes, spleen and thymus of the recipient. B and T cells homed to appropriate B- and T-dependent areas. The traffic routes thus appeared similar to those utilized by phenotypically identical recipient cells. For the first 3 to 5 days post-transplant, these patterns were similar with or without systemic immunosuppressive drug (FK506) therapy. After a further few days, however, the emigrant donor cells disappeared in the *untreated* recipients.

In contrast, in the rat liver graft recipients immunosuppressed with a 28-day course of FK506, the various donor cells persisted in the sites expected of phenotypically identical recipient lymphoid organs. In addition, a ubiquitous spread was evident after 2 to 4 weeks, with donor cells evident in the skin and heart – in the same way as after bone marrow and allogeneic fetal liver transplantation. Thereafter, the process proceeded and was sustained without the need for intensive maintenance immunosuppressive therapy, or in the absence of all further treatment.

(ii) *The common effect of immunosuppressive drugs: a permissive effect on leukocyte migration*

Both these clinical and experimental studies indicate that after organ transplantation a variety of potentially immunogenic and/or tolerogenic signals may be delivered to all of the lymphoid organs and then throughout the recipient by donor leukocytes. The consequences are dramatically different in untreated versus immunosuppressive drug-treated animals and the ultimate therapeutic effect is obviously not defined by any specific drug action alone. Thus it is common for the introduction of every potent new immunosuppressant to be followed by claims of tolerance induction in experimental animals. This is defined by the permanent acceptance of organ grafts after a short course of immunosuppression and without the need for further treatment or with minimal maintenance therapy.

The precise site of immunosuppressive drug action does not appear to be crucial – whether this be at the level of antigen presentation (DSG), cytokine gene transcription (FK506 or CsA), interdiction of cytokine action (rapamycin), or prevention of clonal expansion by drugs that inhibit DNA synthesis (azathioprine, mizoribine, or BQR) (Fig. 4). Notably, this generalization also applies to

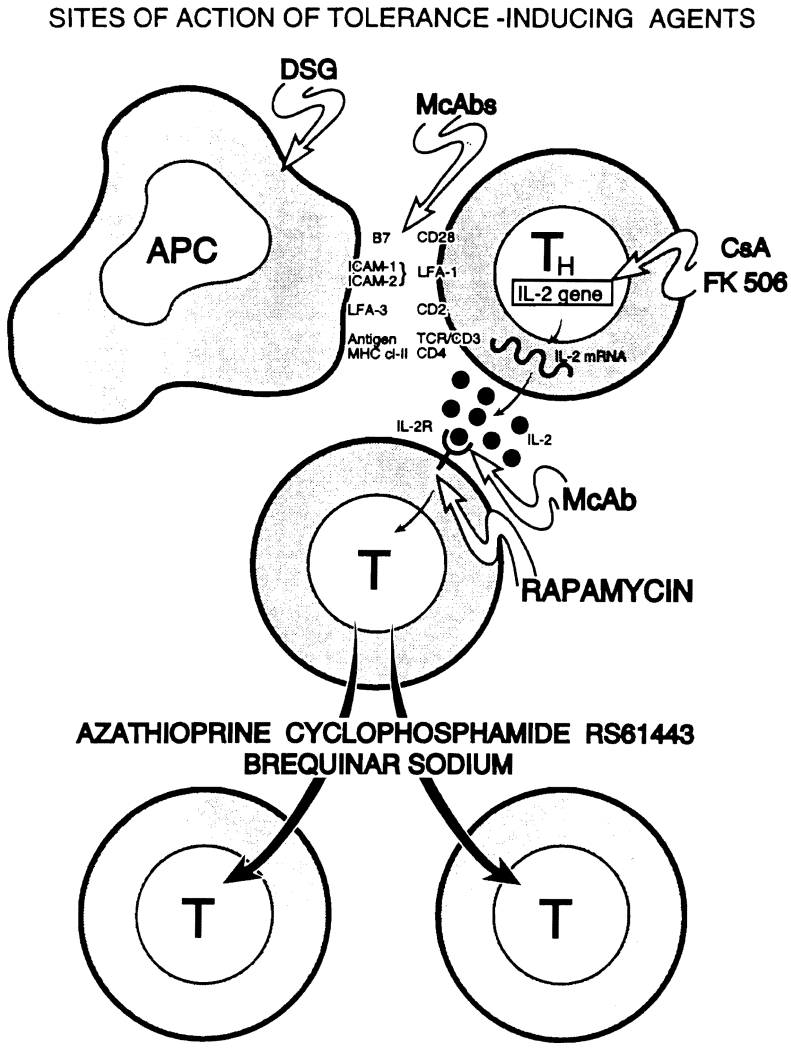

Figure 4. Sites of action of immunosuppressive drugs and monoclonal antibodies (mAb) which have the capacity to induce tolerance to organ allografts in experimental animals. Common to all of the drugs shown is the ability to (ultimately) inhibit T-cell proliferation. Monoclonal antibodies which can induce tolerance in animals include non T cell-depleting anti-CD4 and anti-LFA-1 + anti-ICAM-1 antibodies. DSG = deoxyspergualin; APC = antigen-presenting cell; T_H = T helper cell; IL-2R = interleukin-2 receptor.

monoclonal antibodies directed against cell surface antigens involved in critical cell recognition and interaction events.

We have postulated (Starzl et al. 1993a) that the common influence of each of these diverse agents is a *permissive* effect, allowing the establishment of cell chimerism and the consequent body-wide "engagement" of donor and recipient cells. The chimerism in this hypothesis may be the cause of variable nonreactivity involving the donor/host relationship. The manner by which this nonreactivity occurs remains speculative, but it is clear that the alteration affects both the reactivity of the recipient immune system towards the passenger leukocytes and the converse.

(iii) *Mechanistic concepts*

Generation of a T-cell-mediated immune response leading normally to graft destruction requires, in its initial phase, effective antigen presentation and recognition together with receipt of a second co-stimulatory signal and the response of T helper 1 (T_{H1}) cells to the combined signal (Mueller et al. 1989). Each of these signals is usually delivered to T cells by professional antigen-presenting cells (APC), including (activated) B cells and, above all, by dendritic cells that are prominent in the migration patterns both in the experimental animal studies and the human observations. The dendritic cell (and perhaps other cells, in particular B cells) may be critical because it can modify the expression of MHC, cell interaction and adhesion molecules – all of which determine how antigenic signals are heeded by T cells. It has been speculated that unperturbed dendritic cells present in non-lymphoid organs may have tolerizing potential (i.e. they may express MHC products but not the additional accessory/immunizing functions (Steinman et al. 1993). Unless activated, B cells appear to be unable to provide co-stimulatory signals and can induce transplantation tolerance (Fuchs & Matzinger 1992).

In the absence of co-stimulatory factor activity, there is disruption of the IL-2-IL-2R autocrine pathway. In *in vitro* culture systems, T_{H1}-cell anergy is defined as a defect in IL-2 synthesis and proliferation in response to antigen restimulation. Specific defects in tyrosine phosphorylation pathways required for the induction of IL-2 synthesis may help explain antigen unresponsiveness in tolerant T cells (Cho et al. 1993). Direct interference with the IL-2 induction pathway, using CsA or FK506 (both of which inhibit IL-2 gene transcription) or mAb directed against IL-2 or the IL-2R, is highly effective in inducing immune suppression and has profound implications for the induction of peripheral tolerance. It appears that in animals made tolerant by the infusion of allogeneic leukocytes, there is normal IL-2 gene but low IL-2R gene induction. This may reflect abnormal translation control of IL-2 production (although no such regulation has previously been described for the IL-2 gene).

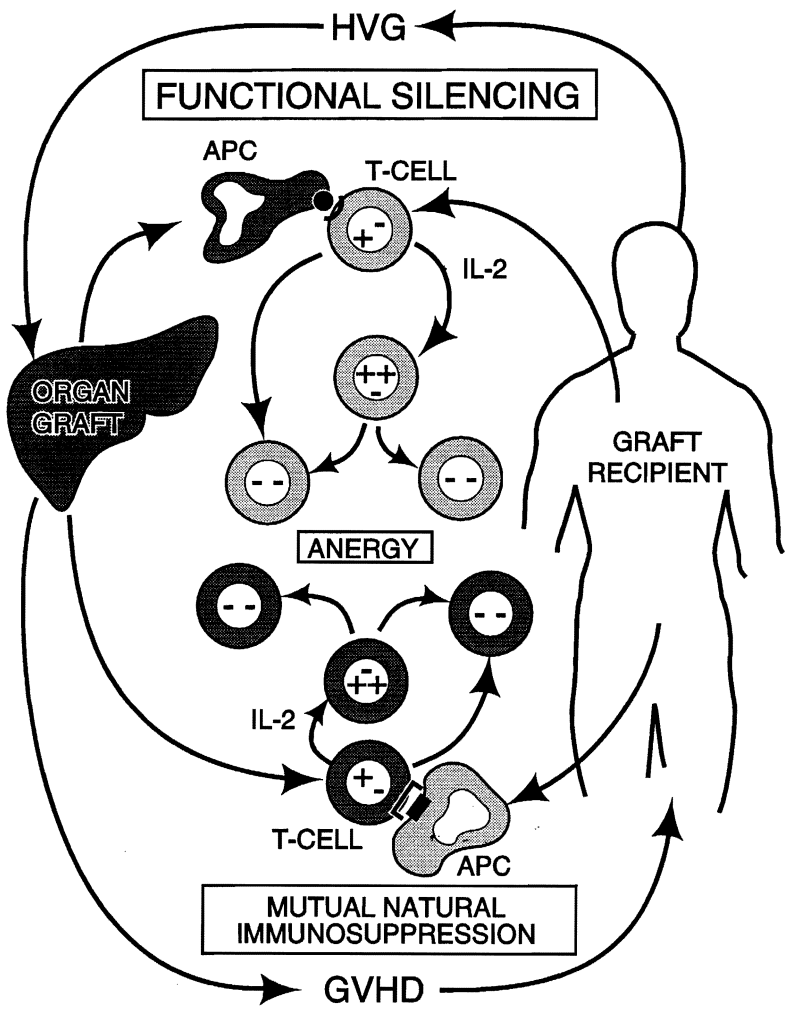

Figure 5. Model of APC-T_{H1}-cell interaction, showing the production within the T-cell nucleus of positive (+) and of negative (−) regulators (anergy proteins) of IL-2 gene transcription. In this conceptual model, anergy relates only to the IL-2 gene and other cytokines (e.g. IFN-γ) may be expressed, albeit at supoptimal levels. In the absence of persistent costimulatory signals (or under the umbrella of immunosuppressive drugs), cell division does not proceed and negative nuclear regulators accumulate, resulting in T-cell anergy. In addition to the action of immunosuppressive agents, chronic antigen stimulation is also envisaged as promoting anergy. In some instances, tolerance can be broken, e.g. by administration of exogenous IL-2. HVG = host versus graft response (allograft rejection); GVHD = graft versus host disease.

It has been proposed (see, e.g. Jenkins 1992) that TCR occupancy leads to the production, through an active metabolic process, of negative regulators ("anergy proteins") that accumulate at later times and repress IL-2 gene transcription, possibly by antagonizing the effects of positive cytokine gene transcription factors. In support of this hypothesis, T-cell specific negative regulation of transcription of cytokine (IL-4) has recently been described. Moreover, a T-cell specific protein which can downregulate IL-4 promoter activity has been identified (Li-Weber et al. 1992). There is also evidence of cellular protein binding to the negative regulatory elements of the IL-2R 2-chain gene (Smith & Greene 1989). Within murine anergic T_{H1} cell clones, this effect on cytokine gene expression may relate only to IL-2, as other cytokines, such as IL-3 or interferon-γ (IFN-γ), may be secreted, albeit at suboptimal levels.

Under cover of potent immunosuppressive drugs which inhibit IL-2 production (CsA or FK506) and in the continuous presence of (graft) alloantigens, it is likely that a chronically-stimulated T cell will continue to make negative regulators, thus reinforcing the state of anergy (Fig. 5). Anergic T_{H1} cells remain viable and can proliferate in response to exogenous IL-2. It is also possible that anergy can be induced in IL-2-producing T cells that receive both TCR and co-stimulatory signals, but are prevented from responding to IL-2 or dividing by, e.g., rapamycin (which blocks IL-2R-induced cell cycle S-phase entry) or leflunomide or inhibitors of DNA synthesis, such as mycophenolate mofetil (RS 61443) or BQR, respectively. Long-lasting antigen-specific anergy may ensue in a manner analogous to that observed in chronic microbial infection (Gaylord & Brennan 1987).

SUMMARY

Together with CsA, the new macrolide immunosuppressants FK506 and rapamycin have proved to be valuable tools in providing new information about key molecular events that underlie lymphocyte activation and degranulation. Studies of their mechanisms of action have pinpointed the phosphatase calcineurin and protein kinases as important signaling mediators in T-cell activation. Other new immunosuppressive drugs, including leflunomide, mycophenolate mofetil, brequinar sodium and deoxyspergualin exhibit diverse inhibitory effects on cells of the immune system and offer considerable promise as adjunctive therapeutic immunosuppressants. FK506 appears to be both a valuable therapeutic alternative to liver or kidney retransplantation and an alternative primary immunosuppressant to CsA in hepatic (especially) and renal transplantation. There is now good evidence that immunosuppressive drugs, both old and new, permit the establishment of donor-derived, multi-lineage cell chimerism following organ transplantation.

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