Drugs of the Future 1998, 23(10): 1091-1098

CCC: 0377-8282/98

**Review Article**

# New anti-CD3 agents for transplantation tolerance induction

***P.L. Mottram[1*], W-R. Han[1], L.J. Murray-Segal[1], I.F.C. McKenzie[2] and G.A. Pietersz[2]***

*[1]University of Melbourne, Department of Surgery, Royal Melbourne Hospital, Victoria 3050, Australia and [2]The Austin Research Institute, Austin Hospital, Heidelberg 3084, Australia. [*]Correspondence*

## CONTENTS



## Summary

Until now the use of monoclonal antibodies to modify T-cell responses in transplantation has had limited success because of the immunogenicity and toxicity of these agents, which restricts their use in clinical transplantation. New reagents have been developed, including modified antibodies and immunotoxins to reduce these side effects and increase T-cell depletion. We have studied ways of using mAbs as inert carriers for cytotoxic drugs, with the aim of killing or inactivating T cells which respond specifically to organ allografts. In our studies, idarubicin (IDA) and aminopterin (AMN) have proven most potent. With anti-CD8 antibodies, which were not immunosuppressive when used alone, we have produced immunosuppressive and indeed tolerogenic conjugates with IDA or AMN coupling. With an anti-CD3, which at immunosuppressive doses was toxic due to cytokine release, the IDA-CD3 conjugate, used at low doses, was tolerogenic and the cytokine release syndrome avoided. Thus, the principle that antibodies "armed" with drugs could be useful in transplantation has been validated in mice. It now remains to apply such studies to primates and ultimately humans – studies which are currently in progress.

## Introduction

While monoclonal antibodies are powerful immunosuppressive agents and can induce significant graft prolongation and tolerance in the mouse (1-3), these agents have been disappointing in human therapy, both in transplantation, where graft prolongation or tolerance is desired, and in tumor immunotherapy (4, 5). In humans, mouse antibodies have been used predominantly. These have a short half-life for two major reasons: firstly, the antibodies are often galactosylated, and as humans have naturally occurring anti-Gal$\alpha$(1,3)Gal antibodies, immune complexes form and these galactosylated antibodies are removed from circulation. Secondly, immune responses to mouse immunoglobulin in humans are difficult to suppress and human anti-mouse antibodies usually appear within 7-10 days. This again shortens the half-life of the antibody, making repeated doses of little value (6). A further problem with murine monoclonal antibodies is that the Fc piece is poor at activating complement in other species. Indeed, even in the mouse, such phenomenon as hyperacute, antibody-mediated graft rejection, Arthus phenomenon and related antibody-mediated effects are difficult to study (5). Thus, mouse antibodies used alone are certainly not the ideal agents to promote graft prolongation in humans. To reduce immunogenicity, antibodies have been selected and engineered as either chimeric or humanized so that the antigenic differences between donor and host antibodies are minimized. A number of such humanized antibodies are now in clinical trials and show some promise (7). Roche has recently launched the first humanized monoclonal anti-IL-2R antibody, Zenapax® (daclizumab). However, at this time OKT3 (anti-CD3) is the only monoclonal antibody which has been widely used for many years to combat rejection in clinical transplantation and has proven effective in reversing graft rejection episodes in humans (8).

This and other anti-CD3 mAbs have been effective in both clinical and experimental studies (9-11). CD3 is essential for T-cell activation following antigen recognition by the T-cell receptor. Anti-CD3 can suppress T-cell responses by modulation of CD3, lysis of T cells and blocking of cell surface CD3. OKT3 is a mouse anti-human mAb which causes rapid T-cell depletion and also activates T cells, stimulating cell division and the release of cytokines, including IFN-γ, TNF-α, IL-4, IL-3 and IL-2. This effect causes "first dose syndrome", with significant morbidity and rarely, mortality. OKT3 is immunogenic, as detailed above, and can only be used for a limited time before the host response renders it ineffective. Both T-cell activation and immunogenicity are dependent on cross-linking of anti-CD3 via the Fc region to $FcR^+$ accessory cells (6). Despite these drawbacks, OKT3 is very effective in treating organ rejection and is routinely used in transplantation as a short-term treatment, either at the time of grafting or to treat rejection episodes. New agents have now been developed in efforts to reduce toxicity and improve the effectiveness of anti-CD3 agents.

## Modification of anti-CD3 to reduce toxicity and immunogenicity

Anti-CD3 mAbs have been studied extensively in rodent models of transplantation and autoimmune disease. The most commonly used agent in experimental transplantation is the hamster anti-mouse mAb 145-2C11 (9). This mAb, directed against the CD3-ε chain, is mitogenic and immunogenic *in vivo*, and is thus the murine equivalent of OKT3. A rat anti-mouse IgG2b, YCD3, also binds to the CD3-ε chain and has a similar ability to activate T cells. It has been used to produce a bispecific monoclonal antibody which binds to cells bearing both CD3 and CD25, the IL-2R (12). This mAb, with one arm from YCD3 and the other from the anti-IL-2R, PC61, binds weakly to unstimulated T cells and does not activate them. It rapidly cross-links and deletes activated ($IL\text{-}2R^+$) T cells. Neither the bispecific mAb nor the T-cell depleting anti-CD3 have been reported to induce tolerance when used alone, although 145-2C11 has been effective at very low doses when combined with other mAbs which reduce the humoral response to the anti-CD3 mAb (13). A nonactivating anti-CD3, G4.18, has induced tolerance in some recipient strains in the rat heart allograft model (11) and the data presented here shows that KT3, a rat anti-mouse mAb with similar activity, induced tolerance in the mouse heart allograft model. KT3, an IgG2a, binds to the same CD3-ε chain sites as 145-2C11 (14), modulates CD3 and activates T cells to produce a range of cytokines, but like the G4.18 antibody, requires the cross-linking agent PMA to induce T-cell proliferation *in vitro*, suggesting that its Fc-FcR links are weaker than those of 145-2C11 (14). Our data and that of others (15) shows that *in vivo* KT3 is less toxic than 145-2C11. Thus, extensive T-cell depletion and production of high levels of cytokines are not required for anti-CD3-mediated immunosuppression and the development of peripheral tolerance. Less toxic anti-CD3 may be tolerogenic in other experimental and clinical systems.

T-cell activation by OKT3 or 145-2C11 is dependent on the presence of the Fc domain. Thus, a number of studies have looked at the use of $F(ab')_2$ preparations from these mAbs for use in immunosuppression. Hirsch *et al*. (16), using a mouse skin allograft model, reported that 145-2C11 $F(ab')_2$ was immunosuppressive when used in repeated doses but was not as effective as the intact mAb. When used to treat graft-*versus*-host disease and inhibit primary antigenic responses to ovalbumin in mice, the anti-CD3 $F(ab')_2$ caused T-cell modulation but not depletion, and immune function recovered rapidly when treatment was discontinued (17). More recently, OKT3 and 145-2C11 $F(ab')_2$ fragments have been used to produce chimeric molecules with low affinity-Fc binding IgGs. These agents were nonmitogenic, but as effective as the native molecule in graft prolongation (18, 19).

## Immunoconjugates designed to increase T-cell depletion

Because anti-CD3 mAbs act by a combination of modulation and target cell depletion, not all CD3 cells are deleted *in vivo*, even in rodents where high doses of anti-CD3 are tolerated (20). Thus, a number of recent studies have used genetically engineered anti-CD3 immunotoxins incorporating diphtheria toxin genes (21, 22). One of these has been used in a rhesus monkey kidney allograft model where it improved host T-cell depletion prior to donor bone marrow infusion, resulting in chimerism and the establishment of central tolerance (23, 24). This immunotoxin used the mutant diphtheria toxin CRM9 and the anti-rhesus CD3-ε FN18 (anti-CD3-IT). It was used with deoxyspergualin to avoid the proinflammatory cytokine release. Graft survival for MHC incompatible kidneys was comparable to that seen with rabbit antithymocyte globulin treatment. T-cell recovery with antibody treatment alone was 50% at 3-4 weeks, while recovery in the immunotoxin-treated animals was 50% at 6-8 weeks. In the first week after treatment and transplantation, nearly complete elimination of T cells from the host lymphoid tissue was achieved in this latter group. This anti-CD3-IT, in contrast to mAb alone, acts by irreversible blockade of protein synthesis and is independent of Fc and complement. A single toxin molecule per T cell should be lethal (24).

## Drugs linked to antibody carriers

The challenge that we undertook was to derive methods whereby antibodies which were ineffective in humans could be made into useful immunosuppressive agents. We turned to the "magic bullet" approach originally described by Paul Ehrlich in 1906 (25), wherein the bullets (antibodies) were armed with either drugs, toxins or

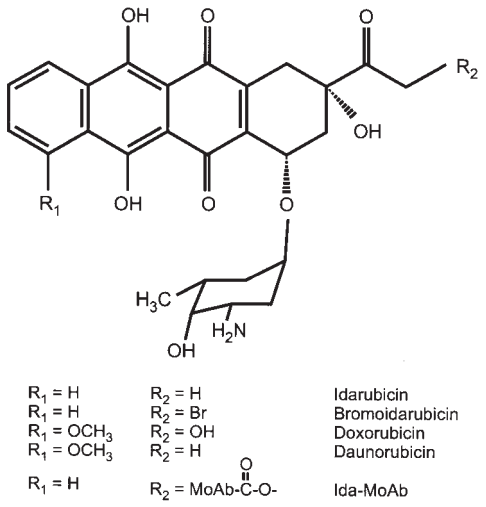


Fig. 1. Structure of idarubicin and related compounds and linkage to monoclonal antibody.

isotopes so when they reached their site of activity they would be more potent. The concept of immunotoxins, using antibodies which attach to cell surface antigens to carry drugs to selected target cells, has become a reality with the advent of monoclonal antibodies and the description of the CD series of cell surface markers. Immunoconjugates using plant and bacterial toxins are now used clinically and for *in vitro* treatment of cells (26-28). In testing drug-anti-T-cell conjugates, which were originally designed for cancer treatment, we have used anthracyclines and an antifolate agent coupled to anti-T-cell mAb for suppression of allograft rejection (29, 30).

*Idarubicin*

This agent belongs to the anthracycline class of drugs, with a broad spectrum of antitumor activity against a large number of cancers (31). Their mechanisms of action are numerous, including DNA intercalation, topoisomerase inhibition and cell surface activity (32, 33). Structure-activity studies have shown that the primary amino group of the sugar moiety is essential for DNA intercalation (34). Nevertheless, a number of active compounds exist with modifications at this site (35). Several strategies have been used for the conjugation of anthracycline anticancer drugs to monoclonal antibodies, with most studies using daunorubicin or doxorubicin (Fig. 1). The various approaches to conjugate linkage are briefly reviewed below.

*Linkage via the sugar moiety*

The early studies of Hurwitz *et al*. (36) used periodate oxidation of the amino sugar for linkage to antibodies. The bond between the C-3 and C-4 of the amino sugar was cleaved with sodium periodate to result in a dialdehyde that was reacted to antibodies via Schiffs bases, which were stabilized by reduction with sodium borohydride. Using this method, conjugates with 2-5 drug residues per antibody molecule could be obtained. However, we and others have shown that these conjugates were devoid of, or had very low, antibody and cytotoxic activity (29, 37). Alternative coupling methods via the sugar moiety used the cross-linking agents glutaraldehyde and water-soluble carbodiimide (36). These methods resulted in conjugates of decreased activity due to homopolymerization of the antibody. Nevertheless, preincubation of cross-linking agent with drug prior to reaction with antibody resulted in conjugates that showed antitumor activity. Our own work with doxorubicin involved modification of the daunosamine amino group with an iodoacetyl group (29). The derivatized doxorubicin was 40-fold less toxic than unmodified doxorubicin. The iodoacetyl doxorubicin was then reacted with thiolated monoclonal antibody. However, these conjugates had poor biological activity and an alternative strategy, based on an acid sensitive linker, was developed by Shen and Ryser and used to produce daunomycin immunoconjugates (38, 39). These conjugates were prepared by first synthesizing a *cis*-acotinyl derivative of daunorubicin from *cis*-acotinic anhydride and then linking this derivative to antibody via a water-soluble carbodiimide. These conjugates released free daunorubicin at pH 4-5.5. Therefore, once conjugates are internalized, exposure to the acid environment of the lysosomes resulted in release of free drug.

Another method, taking advantage of lysosomal release, was described by Trouet *et al.* (40) using peptide linkers between the daunosamine amino group and antibody. These linkers consisted of Leu-Ala-Leu-Ala which were stable in serum but sensitive to lysosomal enzymes and were hydrolized to release free drug.

*Linkage via the methyl ketone side chain*

Reaction with the carbonyl group of the methyl ketone side chain avoids modification of the amino group necessary for interaction with DNA. Several methods have been used for reaction via the carbonyl group using hydrazone linkage. Braslawsky *et al*. made a hydrazone derivative of adriamycin with a pyridyldithio group and reacted this with thiolated antibody (41). These conjugates were shown to be stable at physiological pH, but released adriamycin at pH 4.5. Zunino *et al*. linked daunorubicin to polyamino acids using a 14-bromo derivative (42). The bromoketone reacts with amino groups or carboxyl groups forming amines or esters. Garnett *et al*. used this method to link daunorubicin to a monoclonal antibody (39). Our studies have used the more toxic derivative, idarubicin (29). Conjugates made by reacting bromoidarubicin to antibody were selectively cytotoxic to antibody reactive cell lines and have been used in tumor therapy studies (30, 43) and in the transplantation studies described herein.

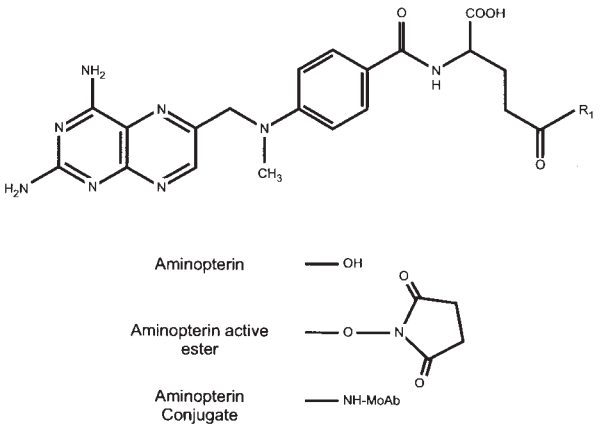


Fig. 2. Structure of aminopterin and active ester, with linkage to moncolonal antibody.

*Aminopterin*

Aminopterin (Fig. 2) is a more toxic analog of the antifolate methotrexate (MTX). These drugs block cell growth by inhibiting the enzyme dihydrofolate reductase. The γ-carboxyl group of AMN can be readily modified without loss of drug activity. AMN and MTX were conjugated to monoclonal antibodies using an active ester derivative. Four to eight drug residues can be linked to antibody without loss of drug or antibody activity. We have previously shown that AMN conjugates are more potent than MTX conjugates, *in vitro* as well as *in vivo* (44).

*Preparation of conjugates*

The immunoconjugates used in our studies were prepared by mixing mAb (2-3 mg/ml) with an excess of aminopterin active ester or 14-bromo-4-demethoxydoxorubicin dissolved in (*N,N*)-dimethyl formamide (10 mg/ml) to covalently couple 2-6 AMI or IDA molecules per mAb molecule (43, 44). Nonconjugated material was removed by gel filtration chromatography and the conjugate quantified by absorbance spectrophotometry and protein determination. The conjugates were used within 48 h, with batches tested for drug and protein concentration and binding efficiency. The antibodies used included mouse anti-mouse CD8 (IgG2a, 1.11) (45), rat anti-mouse CD3 (IgG2a, KT3) (14) and hamster anti-mouse CD3 (IgG2b, 145-2C11) (9). These were isolated from ascites fluid collected from pristane-treated nude rats or mice (46) and purified on a protein-G sepharose column. The IgG concentration was measured by spectrophotometer and the antibody was tested for specificity and reactivity by measuring binding to mouse spleen cells and comparing this with commercial anti-CD3, CD4 and CD8 reagents by flow cytometry. FITC-conjugated antibodies used were 53.6.7 (CD8), H129.19 (CD4) and 145-2C11 (CD3).

**Use of immunoconjugates directed against CD4 and CD8 for drug delivery**

We have used nondepleting doses of mAb or mAb, which have little effect on T-cell function, as drug delivery systems for deletion of activated T cells in specific subsets. Effectiveness against target T cells then relies on the deletion of cells during division following activation by alloantigens expressed on organ grafts. In our initial experiments with idarubicin conjugates using low doses of immunosuppressive mAbs, 53.6.7 (anti-CD8) and H129.19 (anti-CD4), we found IDA conjugated to these mAbs did not increase their effectiveness in prolonging MHC mismatched skin graft survival, although grafts mismatched for point mutations within the MHC could be prolonged with IDA conjugated mAb but not with mAb alone (47). In contrast, the survival of vascularized hearts transplanted across MHC plus background differences could be prolonged, often indefinitely, by immunosuppressive doses of anti-CD4 or anti-CD8 mAbs (48). Coupling IDA or AMN to these mAbs did not improve graft survival or reduce the doses of mAb required (Mottram *et al.*, unpublished data). We found that both of these drugs could, however, be used to increase the effectiveness of a non-immunosuppressive mAb directed against CD8, with IDA more effective than AMN in this context.

We used a haplotype specific anti-CD8, Ly2.1 (1.11) (45), which bound to CBA but not BALB/c or C57BL/6 T cells (Table I). The AMN- or IDA-mAb conjugates were immunosuppressive and prolonged BALB/c to CBA, but not CBA to BALB/c heart survival. This showed clearly that IDA or AMN alone did not prolong graft survival, but had to be attached to the appropriate T cells by mAb binding. IDA-mAb was effective for 10-15 days after treatment. Recovery of T-cell function at this time was probably due to repair of IDA-induced DNA cross-linking, since thymectomized animals also recovered function, indicating that replacement of IDA affected cells was not required (49-51). Dose-response studies showed that low, nontoxic doses of IDA-mAb were effective as immunosuppressive agents, while FACScan studies showed that total CD8 cells were not depleted to any great extent, indicating that only alloreactive cells were deleted (50, 51).

**Use of immunoconjugates directed against CD3 for drug delivery**

Having shown that IDA and AMN conjugates could in principle be used as immunosuppressive agents, with IDA conjugates more effective than AMN, we switched from the haplotype specific anti-CD8 mAb and tested an anti-CD3 (KT3), which had been reported to be less T-cell depleting and thus less toxic than other anti-CD3 agents (14). KT3 was significantly less toxic *in vivo*, as measured by TNF-α release and blood glucose changes, than 145-2C11, a mAb known to induce first dose syndrome (52). Although both doses of KT3 tested caused significant

*Table I: Aminopterin and idarubicin anti-CD8 immunoconjugates prolong mouse heart allograft survival.*

| Treatment[a] | Donor | Recipient | Graft survival (days) | Median | P value[b] |
|---|---|---|---|---|---|
| None | BALB/c | CBA | 12, 12, 14, 14, 17, 17 | 14 | |
| None | CBA | BALB/c | 9, 14, 15, 16 | 14.5 | |
| 1.11 (anti-CD8) 1 mg | BALB/c | CBA | 11, 11, 16, 17, 18 | 16 | |
| AMI-1.11 20 µg/1 mg | BALB/c | CBA | 17, 20, 23, 37, 40, 51, 54, 86 | 38.5 | < 0.05 |
| AMI-1.11 20 µg/1 mg | CBA | BALB/c | 11, 11, 13, 13, 15, 18, 20 | 13 | |
| IDA-1.11 10 µg/1 mg | BALB/c | CBA | >100, >100, >100, >100, >100, >100 | >100[c] | < 0.05 |
| IDA-1.11 10 µg/1 mg | CBA | BALB/c | 9, 9, 10, 11, 12, 12 | 10.5 | |

[a]This is the total dose given. Dose distributed i.p. on days -1, 0, 1, 2 (1.11) with transplantation on day 0. [b]Compared with untreated controls (Mann-Whitney U test). [c]Skin graft challenged. Donor skin survived >50 days, third party skin rejected in < 13 days. (Some of this data was previously published and is reproduced with permission from *Transplantation* 1993, 55(3): 484-90.)

*Table II: Toxic effects of anti-CD3 mAb on CBA recipients.*

| Treatment[a] | Hours after treatment | TNF-α, pg/ml (n) | BG, mmol/l (n) |
|---|---|---|---|
| None or PBS, 0.5 ml i.p. | 3 | 90 ± 5 (2) | 11 ± 1 (4) |
| | 20 | | 11 ± 0.5 (3) |
| KT3, 0.5 mg | 3 | 772 ± 75[b] (3) | 7.6 ± 2 (4) |
| | 20 | 119 ± 36 (3) | 8 ± 0.2[b] (3) |
| KT3, 0.05 mg | 3 | 593 ± 46[b] (3) | 6.9 ± 1.5[b] (7) |
| | 20 | 95 ± 10 (3) | 6.1 ± 1[b] (7) |
| 145-2C11, 0.05 mg | 3 | 2360 ± 11[c] (2) | 8.1 ± 1[b] (13) |
| | 20 | 190 ± 21[b] (3) | 4.3 ± 0.6[c] (10) |

[a]Mice were given a single dose of mAb in 0.5 ml PBS, i.p. [b]Significantly different to controls, $p < 0.05$. [c]Significantly different to controls, and to the equivalent dose of KT3 at this time point, $p < 0.05$. (This data was previously published and is reproduced with permission from *Transplantation* 1997, 64(5): 684-90.)

increases in TNF-α levels compared with phosphate buffered saline-treated mice, these had returned to normal by 20 h (Table II). TNF-α levels induced by 145-2C11 were significantly (up to 4 times) higher than those induced by KT3 and remained above normal levels at 20 h. Significant decreases in blood glucose were seen in all anti-CD3-treated mice at 3 and 20 h, but 145-2C11-treated mice had significantly lower blood glucose than KT3-treated mice at 20 h. Thus, KT3-treated mice, even when receiving 10 times the dose of 145-2C11, show significantly less symptoms of first dose syndrome at 3 h and are recovering well by 20 h, compared with mice treated with 145-2C11 (53).

The primary heart allograft survival data (Table III) showed, however, that KT3 was an excellent immunosuppressive agent in CBA mice. Unlike T-cell activating mAbs (54), KT3 could induce alloantigen-specific tolerance in CBA mice when used for T-cell depletion at the time of transplantation. In mice treated with a total dose of 0.25 or 0.5 mg KT3, BALB/c hearts survived indefinitely in completely MHC mismatched CBA recipients. The 0.5 mg total dose of IDA-KT3 (6.9 µg of IDA) also

*Table III: Idarubicin anti-CD3 immunoconjugates prolong mouse heart allograft survival.*

| Treatment[a] | Donor | Recipient | Graft survival (days) | Median | P value[b] |
|---|---|---|---|---|---|
| None | BALB/c | CBA | 12, 12, 14, 14, 17, 17 | 14 | |
| None | CBA | BALB/c | 9, 14, 15, 16 | 13.5 | |
| KT3 (anti-CD3), 0.5 mg | BALB/c | CBA | >100 x 4 | >100[c] | <0.05 |
| KT3, 0.25 mg | BALB/c | CBA | 15, >100 x 3 | >100[c] | < 0.05 |
| KT3, 0.1 mg | BALB/c | CBA | 15, 17, 17, 18 | 17 | >0.05 |
| IDA-KT3, 0.5 mg | BALB/c | CBA | >100 x 5 | >100[c] | < 0.05 |
| IDA-KT3, 0.1 mg | BALB/c | CBA | 16, >100 x 6 | >100[c] | < 0.05 |
| IDA-KT3, 0.05 mg | BALB/c | CBA | 20, 23, 28, 28, 34 | 28 | < 0.05 |

[a]This is the total dose given. Dose distributed i.p. on days -1, 0 with transplantation on day 0. [b]Compared with untreated controls (Mann-Whitney U test). [c]Skin graft challenged. Donor skin survived >50 days, third party skin rejected in < 13 days. (This data was previously published and is reproduced with permission from *Transplantation* 1997, 64(5): 684-90.)

produced alloantigen-specific tolerance but at this dose the activity of the antibody alone was sufficient to inactivate CD3 cells and allow the development of tolerance. In contrast, the 0.1 mg dose of KT3 was not able to prolong heart allograft survival, while 0.1 mg of IDA-KT3 (1.6 mcg IDA) produced alloantigen-specific tolerance in 6/7 mice. A lower dose of 0.05 mg (0.8 mcg IDA) significantly improved graft survival to 28 days ($p$ <0.05 compared with untreated controls) but did not induce long-term graft survival (53). Thus, attaching IDA to KT3 significantly improved the ability of the antibody to act as an immunosuppressive agent. Immunosuppression with the 0.1 mg IDA-KT3 dose was not due to the action of the antibody alone, since KT3 was ineffective at this dose. Thus, immunosuppression at this dose was due to IDA, carried to the CD3 cells by KT3.

Animals with heart allografts surviving >100 days were tested for specific tolerance by challenge with skin grafts from donor (BALB/c) and third party (C57BL/6) strains (Table III). In all groups of long survivors, donor strain skin was intact at >50 days, while third party skin was rejected by 16-21 days ($p$ <0.05 for donor *vs.* third party skin in each group). Thus, animals treated with these doses of KT3 or IDA-KT3 showed alloantigen-specific tolerance. In the IDA-KT3 0.1 mg group the low dose of mAb, carrying a few molecules of the cytotoxic drug IDA to each T cell, must have been sufficient to delete $CD3^+$ cells undergoing division after the IDA-KT3 treatment, including those mounting the allogeneic response. The addition of IDA to KT3, therefore, improved both the specificity and the immunosuppressive activity of the antibody.

## Conclusions

Thus, the pan-T-cell agent KT3 can be used as a carrier for the cytotoxic drug IDA to induce alloantigen-specific tolerance. Nonimmunosuppressive doses of KT3 were used to carry a few molecules of the cytotoxic drug to each $CD3^+$ cell, cross-linking the DNA (30) and causing destruction of T cells undergoing cell division, which in the presence of the allograft will remove alloreactive cells.

The IDA-KT3 conjugate extends our previous work with anti-CD4 and anti-CD8 by creating a broad spectrum reagent which is useful in an MHC mismatched donor/recipient strain combination in the mouse. This treatment allows the use of lower doses of antibody and drug, with rapid removal of the conjugate from the host. The simple process of coupling drugs such as IDA to mAb can improve their performance in other treatments where target cell depletion of dividing cells is required (30).

Both KT3 and IDA-KT3 treatments used here induce long-term alloantigen-specific tolerance, characterized by the continued survival of grafts after ceasing immunosuppression and primary graft survival after challenge with a second graft of donor tissue (55, 56). This second graft also survived indefinitely, while third party grafts were rejected. Since rechallenge with donor tissue is not possible in human transplantation, animal models with demonstrable tolerance must be used to try to identify new agents which will induce a tolerant state. Tolerance in animal models can be induced by short-term inactivation of T cells by monoclonal antibody treatment, either alone or combined with drugs and/or donor tissue, with tolerance developing as T cells recover from treatment (55, 57, 58) or are replaced by allogeneic bone marrow transplantation (4). Current immunosuppression for clinical transplantation causes long-term nonspecific immune dysfunction, increasing the risk of infection and malignancy (59). Tolerance may have been achieved in some human allografts in the rare individuals who have ceased immunosuppressive therapy and not rejected their grafts (60). Agents such as IDA-KT3, by causing transient, very specific target cell depletion while leaving the rest of the immune system intact, demonstrate that allografts can survive in recipients without sustained destruction of the immune system, and that tolerance can be achieved in these recipients. They may offer an alternative treatment when donor bone marrow is not available.

There have been considerable advances recently in the use of anti-CD3 agents for immunosuppression, with the development of less immunogenic and T-activating bispecific, humanized and chimeric antibodies. The new diphtheria toxin conjugates will no doubt prove useful for the induction of deletional tolerance where allogeneic bone marrow transplantation is used. The anthracycline conjugates will be useful where specific target cell deletion is required. The simple coupling process described here could also be used to attach IDA, or similar anthracyclines, to suitable nonactivating or nondepleting anti-human monoclonal CD3, or to a combination of anti-CD4 and anti-CD8 mAbs. The great advantages of using conjugates such as IDA-KT3, where the mAb acts as a carrier for the cytotoxic drug, will be in the reduced doses of mAb required and decreased toxicity of the drug in coupled form.

## Acknowledgements

This work was supported by the National Heart Foundation of Australia and the National Health and Medical Research Council of Australia, the Victor Hurley Medical Research Foundation and the Potter and Ramaciotti Foundations.

## References

1. Cobbold, S.P., Adams, E., Marshall, S.E., Davies, J.D., Waldmann, H. *Mechanisms of peripheral tolerance and suppression induced by monoclonal antibodies to CD4 and CD8.* Immunol Rev 1996, 149: 5.

2. Chavin, K.D., Qin, L., Lin, J., Yagita, H., Bromberg, J.S. *Combined anti-CD2 and anti-CD3 receptor monoclonal*

*antibodies induce donor-specific tolerance in a cardiac transplant model.* J Immunol 1993, 151: 7249-59.

3. Tomita, Y., Sachs, D.H., Khan, A., Sykes, M. *Additional mAb injections can replace thymic irradiation to allow induction of mixed chimerism and tolerance in mice receiving bone marrow transplantation after conditioning with anti-T cell mAbs and 3Gy whole body irradiation.* Transplantation 1996, 61: 469.

4. Charlton, B., Auchincloss, H. Jr., Fathman, C.G. *Mechanisms of transplantation tolerance.* Annu Rev Immunol 1994, 12: 707-34.

5. Waldmann, T. *Immune receptors: Targets for therapy of leukemia/lymphoma, autoimmune diseases and for the prevention of allograft rejection.* Annu Rev Immunol 1992, 10: 675-704.

6. Roitt, I.M. *OKT3: Immunology, production and pharmacokinetics.* Clin Transplant 1993, 7: 367.

7. Neylan, J. *New developments in immunosuppression: Ongoing clinical trials in solid organ transplantation.* Graft 1998, 1: 11-6.

8. Kreis, H. *Adverse effects associated with OKT3 immunosuppression in the prevention or treatment of allograft rejection.* Clin Transplant 1993, 7: 431.

9. Leo, O., Foo, M., Samelson, L.E., Bluestone, J.A. *Identification of a monoclonal antibody specific for a murine T3 polypeptide.* Proc Natl Acad Sci USA 1987, 84: 1374.

10. Kung, P.C., Goldstein, G., Reinherz, E.L., Schlossman, S.F. *Monoclonal antibodies define distinctive human T cell surface antigens.* Science 1979, 206: 347.

11. Nicolls, M.R., Aversa, G.G., Pearce, N.W. et al. *Induction of long-term specific tolerance to allografts in rats by therapy with an anti-CD3-like monoclonal antibody.* Transplantation 1993, 55: 459-68.

12. MacLean, J.A., Su, Z., Colvin, R.B., Wong, J.T. *Anti-CD3:anti-IL-2 receptor-bispecific mAb-mediated immunomodulation. Low systemic toxicity, differential effect on lymphoid tissue, and inhibition of cell-mediated hypersensitivity.* J Immunol 1995, 155: 3674-82.

13. Chavin, K.D., Qin, L., Lin, J., Kaplan, A.J., Bromberg, J.S. *Anti-CD2 and anti-CD3 monoclonal antibodies synergize to prolong allograft survival with decreased side effects.* Transplantation 1993, 55: 901-8.

14. Tomonari, K. *A rat antibody against a structure functionally related to the mouse T-cell receptor/T3 complex.* Immunogenetics 1988, 28: 455.

15. Mandel, T.E., Koulmanda, M. *Effect of immunosuppression with anti-T-cell monoclonal antibodies on the survival of organ-cultured fetal pig pancreas xenografts in nonobese diabetic mice.* Transplant Proc 1993, 25: 2926-7.

16. Hirsch, R., Bluestone, J., DeNenno, L., Gress, R. *Anti-CD3 $F(ab)_2$ fragments are immunosuppressive in vivo without evoking either the strong humoral response or morbidity associated with whole mAb.* Transplantation 1990, 49: 1117-23.

17. Blazar, B., Jenkins, M., Taylor, P. et al. *Anti-CD3e$F(ab')_2$ fragments inhibit T-cell expansion in vivo during graft-versus-host disease or primary immune response to nominal antigen.* J Immunol 1997, 159: 5821-33.

18. Alegre, M.L., Peterson, L.J., Xu, D. et al. *A non-activating "humanized" anti-CD3 monoclonal antibody retains immunosuppressive properties in vivo.* Transplantation 1994, 57: 1537-43.

19. Alegre, M.L., Tso, J., Sattar, H. et al. *An anti-murine CD3 monoclonal antibody with a low affinity for Fc-γ receptors suppresses transplantation responses while minimizing acute toxicity and immunogenicity.* J Immunol 1995, 155: 1544-55.

20. Lenchow, D., Bluestone, J. *T cell co-stimulation and in vivo tolerance.* Curr Opin Immunol 1993, 5: 747.

21. Vallera, D., Panoskaltsis-Mortari, A., Jost, C. et al. *Anti-graft-versus-host disease effect of DT390-anti-CD3sFv, a single-chain Fv fusion immunotoxin specifically targeting the CD3-ε moiety of the T-cell receptor.* Blood 1996, 88: 2342-53.

22. Ma, S., Hu, H., Thompson, J., Stavrou, S., Scharff, J., Neville, D.J. *Genetic construction and characterization of an anti-monkey CD3 single chain immunotoxin with a truncated diphtheria toxin.* Bioconjug Chem 1997, 8: 695-710.

23. Thomas, J., Neville, D., Contreras, J. et al. *Preclinical studies of allograft tolerance in rhesus monkeys.* Transplantation 1997, 64: 124-35.

24. Contreras, J., Wang, P., Eckhoff, D. et al. *Peritransplant tolerance induction with anti-CD3-immunotoxin: A matter of proinflammatory cytokine control.* Transplantation 1998, 65: 1159-69.

25. Ehrlich, P. *The relationship existing between chemical constitution, distribution, and pharmacological action.* In: The Collected Papers of Paul Ehrlich, Vol. 1. Himmelweite, I.F., Marquardt, M., Dale, H. (Eds.). Pergamon Press: London 1956, 596-618.

26. Vitetta, E.S., Thorpe, P.E., Uhr, J.W. *Immunotoxins: Magic bullets or misguided missiles?* Immunol Today 1993, 14: 252.

27. Vallera, D.A. *Immunotoxins: Will their clinical promise be fulfilled?* Blood 1994, 83: 309.

28. Thrush, G., Lark, L., Clinchy, B., Vitetta, E. *Immunotoxins: An update.* Annu Rev Immunol 1996, 14: 49-71.

29. Pietersz, G.A., Smyth, M.J., McKenzie, I.F.C. *The use of anthracycline antibody complexes for specific anti-tumor therapy.* In: Targeted Diagnosis and Therapy, Vol. 1. Rodwell, J.D. (Ed.). Marcel Dekker: New York 1988, 25-53.

30. Pietersz, G.A. *The use of drug-antibody conjugates for the treatment of cancer.* Cancer Forum 1993, 17: 116.

31. Young, R., Ozals, R., Myers, C. *The anthracycline antineoplastic drugs.* New Engl J Med 1981, 305: 139-53.

32. Crooke, S.T., Duverney, V.H., Mong, S. *Molecular pharmacology of anthracyclines.* In: Molecular Actions and Targets for Cancer Chemotherapeutic Agents. Sartorelli, C.A., Lazo, J.S., Bertino, J.R. (Eds.). Academic Press: New York 1981, 137.

33. Dorr, R., Alberts, D. *Pharmacology of doxorubicin.* In: Current Concepts in the Use of Doxorubicin Chemotherapy. Jones, S. (Ed.). Farmitalia Carlo Erba: Milano 1982, 1-20.

34. Neidle, S. *The molecular basis for the action of some DNA binding drugs.* Prog Med Chem 1979, 16: 139-53.

35. Israel, M., Modest, E., Frei, E. *N-Trifluoracetyl adriamycin-14-valerate, an analogue with greater experimental antitumor activity and less toxicity than adriamycin.* Cancer Res 1975, 35: 1365-8.

36. Hurwitz, E., Levy, R., Maron, R., Wilchek, M., Arnon, R., Sela, M. *The covalent binding of daunomycin and idarubicin to antibodies, with retention of both drug and antibody activities.* Cancer Res 1975, 35: 1175-81.

37. Latif, Z., Lozzio, B., Wust, C., Krauss, S., Aggio, M. *Evaluation of drug-antibody conjugates in the treatment of human myelosarcomas transplanted in nude mice.* Cancer 1980, 45: 1326-33.

38. Shen, W., Ryder, H. *Cis-aconityl spacer between daunomycin and macromolecular carriers: A model of pH-sensitive linkage releasing drug from a lipomotrophic conjugate.* Biochem Biophys Res Commun 1981, 102: 1048-54.

39. Garnett, M., Embleton, M., Jacobs, E., Baldwin, R. *Preparation and properties of a drug-carrier-antibody conjugate showing selective antibody-directed cytotoxicity in vitro.* Int J Cancer 1983, 31: 661-70.

40. Trouet, A., Masquelier, M., Baurain, R., Deprez de Campenée, D. *A covalent linkage between daunorubicin and proteins that is stable in serum and reversible by lysosomal hydrolases, as required for lysosomotropic drug-carrier conjugate: In vitro and in vivo studies.* Proc Natl Acad Sci USA 1982, 79: 626-9.

41. Braslawsky, G., Edson, M., Pearce, W., Kaneko, T., Greenfield, R. *Antitumor activity of adriamycin (hydrazone-linked) immunoconjugates compared with free adriamycin and specificity of tumor cell killing.* Cancer Res 1990, 50: 6608-14.

42. Zunino, F., Savi, G., Guiliani, F. et al. *Comparison of antitumor effects of daunorubicin covalently linked to poly-L-amino acid carriers.* Eur J Cancer Clin Oncol 1981, 20: 421-5.

43. Pietersz, G.A., Smyth, M.J., McKenzie, I.F.C. *Immunochemotherapy of a murine thymoma with the use of idarubicin-monoclonal antibody conjugates.* Cancer Res 1988, 48: 926.

44. Kanellos, J., Pietersz, G., Cunningham, Z., McKenzie, I. *Antitumor activity of aminopterin-monoclonal antibody conjugates: In vitro and in vivo comparison with methotrexate-monoclonal antibody conjugates.* Immunol Cell Biol 1987, 65: 483-93.

45. Hogarth, P., Edwards, J., McKenzie, I. *Monoclonal antibodies to murine Ly2.1 cell surface antigen.* Immunology 1982, 46: 135.

46. Potter, M. *Immunoglobulin-producing tumors and myeloma proteins of mice.* Physiol Rev 1972, 52: 631.

47. Smyth, M., McKenzie, I., Pietersz, G. *The effect of idarubicin monoclonal antibody treatment on first-set rejection of murine skin allografts.* Transplantation 1989, 48: 77-9.

48. Mottram, P., Wheelahan, J., McKenzie, I., Clunie, G. *Murine cardiac allograft survival following treatment of recipients with monoclonal anti-L3T4 or Ly-2 antibodies.* Transplant Proc 1987, 19: 2898-901.

49. Mottram, P.L., Pietersz, G.A., Purcell, L.J., Krauer, K., McKenzie, I.F. *Immunosuppression by aminopterin or idarubicin conjugated to anti-CD8 in the mouse heart allograft model.* Transplant Proc 1992, 24: 2301.

50. Mottram, P.L., Pietersz, G.A., Smyth, M.J., Purcell, L.J., Clunie, G.J., McKenzie, I.F. *Evidence that an anthracycline-anti-CD8 immunoconjugate, idarubicin-anti-Ly-2.1, prolongs heart allograft survival in mice.* Transplantation 1993, 55: 484-90.

51. Mottram, P.L., Pietersz, G.A., Purcell, L.J., Krauer, K., Clunie, G.J., McKenzie, I.F. *Deletion of graft reactive cells by idarubicin-anti-CD8 (Ly-2.1) immunoconjugate: Studies in the mouse heart graft model.* Transplant Proc 1991, 23: 499-500.

52. Alegre, M., Vandenabeele, P., Flamand, V. et al. *Hypothermia induced by anti-CD3 monoclonal antibody in mice: Role of tumor necrosis factor.* Eur J Immunol 1990, 20: 707-10.

53. Mottram, P., Han, W., Murray-Segal, L., Mandel, T., Pietersz, G., McKenzie, I. *Idarubicin anti-CD3: A new immunoconjugate that induces alloantigen-specific tolerance in mice.* Transplantation 1997, 64: 684-90.

54. Mackie, J.D., Pankewycz, O.G., Bastos, M.G., Kelley, V.E., Strom, T.B. *Dose-related mechanisms of immunosuppression mediated by murine anti-CD3 monoclonal antibody in pancreatic islet cell transplantation and delayed-type hypersensitivity.* Transplantation 1990, 49: 1150-4.

55. Cobbold, S.P., Qin, S., Leong, L.Y.W., Martin, G., Waldmann, H. *Reprogramming the immune system for peripheral tolerance with CD4 and CD8 monoclonal antibodies.* Immunol Rev 1992, 129: 164-210.

56. Chen, Z., Cobbold, S.P., Metcalfe, S., Waldmann, H. *Tolerance in the mouse to major histocompatibility complex-mismatched heart allografts and to rat xenografts, using monoclonal antibodies to CD4 and CD8.* Eur J Immunol 1992, 22: 805-10.

57. Mottram, P.L., Wheelahan, J., Mirisklavos, A., Clunie, G.J., McKenzie, I.F. *Prolongation of murine cardiac allografts following treatment of graft recipients with monoclonal anti-L3T4 and Ly-2 antibodies.* Transplant Proc 1987, 19: 582-5.

58. Pearson, T.C., Darby, C.R., Bushell, A.R., West, L.J., Morris, P.J., Wood, K.J. *The assessment of transplantation tolerance induced by anti-CD4 monoclonal antibody in the murine model.* Transplantation 1993, 55: 361.

59. Braun, W.E., Popowniak, K.L., Nakamoto, S., Gifford, R.W., Stafford, R.A. *The fate of renal allografts functioning for a minimum of 20 years (level 5A) - Indefinite survival or the beginning of the end?* Transplantation 1995, 60: 784-90.

60. Starzl, T.E., Demitris, A.J., Trucco, M. et al. *Chimerism and donor-specific nonreactivity 27 to 29 years after kidney allotransplantation.* Transplantation 1993, 55: 1272.