ELSEVIER

Transplant Immunology 10 (2002) 63–72

Transplant Immunology

www.elsevier.com/locate/trim

Remission and pancreas isograft survival in recent onset diabetic NOD mice after treatment with low-dose anti-CD3 monoclonal antibodies

Patricia L. Mottram[a,*], Lisa J. Murray-Segal[b], Wenruo Han[c], Julie Maguire[d], Alicia N. Stein-Oakley[d]

[a]*The Austin Research Institute, Studley Road, Heidelberg, Victoria 3084, Australia*
[b]*Immunology Research Center, St. Vincent's Hospital, Melbourne 3065, Australia*
[c]*Department of Surgery, University of Melbourne, Royal Melbourne Hospital, Victoria 3050, Australia*
[d]*Department of Medicine, Monash Medical School, Prahran 3181, Australia*

Received 1 August 2001; accepted 3 September 2001

Abstract

Diabetes in NOD mice is an autoimmune disease similar to Type I diabetes in humans. Prior to hypoglycemia, changes in the islet infiltrate led to autoreactive T cell activation and destruction of the insulin-producing β cells. If T cell activation can be inhibited before β cell destruction is complete, islet cell rescue and regeneration can occur. Female NOD mice >100 days old with blood glucose levels >20 mM/l for less than 7 days were selected as 'recent onset' mice. Untreated, all of these animals would die of diabetes in <40 days. Mice treated with anti-CD4 (GK1.5) achieved 14.3% permanent remission, while those treated with anti-CD8 (53.6.7) showed 33.3% permanent remission. Mice treated with anti-CD3 (145-2C11) also achieved 33.3% permanent remission, but 14% of these died of first dose syndrome. In mice treated with a low dose of anti-CD3 (10 μg KT3), which did not induce first dose syndrome, 50% remained in remission for >100 days. This dose of mAb reduced insulitis but did not deplete splenic CD3 cells. When mice in remission were challenged with a vascularized pancreas isograft at 50 days, 9/22 remained normal and 13/22 had recurrent disease in both transplanted and native pancreas. Of the long-surviving isografts 7/9 were in KT3 treated recipients. Histology showed activated T cell infiltration in the native and transplanted pancreases of mice with transient remission. Benign insulitis with macrophages, B cells, CD4>CD8 T cells and low levels of IL-2R, IL-2, IFN-γ and IL-4 was seen in islets from the native pancreas and in long surviving pancreas isografts in mice that remained in remission. Thus, using low dose KT3, it was possible to halt the development of diabetes in 50% of animals treated soon after diagnosis, despite significant islet cell destruction at this stage. Of the KT3 treated mice in permanent remission, 70% had re-established tolerance to autoantigen and did not destroy vascularized pancreas isografts.

Keywords: Recent-onset diabetes; Pancreas transplants; NOD; Anti-CD3

1. Introduction

Although research is in progress on the early detection and treatment of insulin dependent diabetes mellitus (IDDM), most patients are not diagnosed until significant autoimmune destruction of pancreatic islet cells has occurred, with up to 80% of insulin production lost [1,2]. Treatment with immunosuppressive agents can halt the progress of disease in some cases and promising results have been obtained from recent experimental work with anti-CD3 mAb [3]. Clinical trails of a humanized anti-CD3 are currently in progress [4]. These agents specifically block the development of the autoimmune response and have the potential to induce permanent remission, allowing rescue and regeneration of remaining islet tissue and preventing disease recurrence in transplanted tissue. The mechanism of action of these agents requires further investigation.

Studies of the non-obese diabetic mice (NOD) have been very useful in exploring disease mechanisms [5]. As with human IDDM, not all of the genetically susceptible mice develop diabetes, but all develop autoimmune insulitis, seen as an accumulation of T cells, B cells and macrophages around pancreatic islets. In those animals that develop disease (30–70% of females, depending on

*Corresponding author. Tel.: +61-3-92870666; fax: +61-3-92870601.
E-mail address: p.mottram@ari.unimelb.edu.au (P.L. Mottram).

0966-3274/02/$ - see front matter
PII: S0966-3274(02)00050-3

environmental conditions and the NOD strain used), insulitis can change rapidly from benign to aggressive, with the appearance of Th1 cytokines, activated T cells and consequent β cell destruction [6]. Although many treatments can prevent or delay the progress of disease in neonatal or pre-diabetic NOD mice [3], treatment of older mice and those with established diabetes is rarely effective [7,8]. Thus, once insulitis has switched from benign to aggressive, rescue of the remaining β cells and protection of transplanted islet tissue has proved difficult.

Treatment of recent onset diabetes will depend on understanding the critical steps in the switch from benign to aggressive insulitis. Recent findings in the NOD mouse have shown that β cell destruction correlates with changes in the infiltrate. These include the migration of dendritic cells, increased Th1 inflammatory cytokines [9], and the presence of activated CD4 [10] and CD8 T cells [11]. The role of NK T cells and IL-4 in protecting islet cells and limiting T cell mediated destruction has also been demonstrated [12]. Thus antibody treatment that prevents T cell activation and the secretion of Th1 cytokines can prevent progression of recent onset disease, as shown in earlier studies with ALS, anti-CD4 plus CD8 and anti-CD3 [13,14]. More recently, Chatenoud et al. showed that anti-CD3 treatment of NOD mice with T cell depleting antibody 145-2C11 or non-depleting F(ab′2) fragments of this antibody could induce remission in some mice [8]. This treatment was ineffective if used before the recent onset stage of the disease, suggesting that the antibody inhibits T cell activation during the switch from benign to aggressive autoimmune disease [8].

In this study we show that low doses of an alternative anti-CD3 are effective in changing the character of the insulitic lesion and inducing long-term remission. Others have used islet cells and skin grafts for in vivo challenge to test specific autoantigenic tolerance after anti-CD3 treatment of recent onset diabetes in NOD mice [15], but this is the first study to use primarily vascularized pancreas transplants for challenge, providing a large dose of unmanipulated autoantigen, readily accessible to the recipient's circulating immune cells. This is a more rigorous challenge than neovascularized islet transplants, and provides a model for pancreas organ transplantation as a supplementary source of islet tissue after rescue therapy. Such treatment may be required where anti-CD3 therapy cannot rescue sufficient islet tissue to maintain normal blood glucose levels.

2. Objectives

Since it is known that anti-CD3 treatment of NOD mice can induce remission in some mice [8] the aim of this study was to: (1) increase the rate of remission by using alternate anti-CD3 treatments; (2) study the immune response to islets during remission; and (3) to see if mice in remission had developed robust autoantigenic tolerance by challenge with a pancreas isograft.

3. Materials and methods

3.1. Mice

Inbred NOD/Lt ($K^dI\text{-}A^{g7}D^b$) and nude mice for ascites production were purchased from the Animal Resources Center, Perth, Australia or obtained from The Walter and Eliza Hall Institute for Medical Research (Melbourne, Australia) and were maintained according to the ethical guidelines of the National Health and Medical Research Council of Australia. The incidence of spontaneous diabetes in the NOD colony was 60% in females over the age of 80 days. Female mice were urine tested weekly using Labstix (Bayer Australia Ltd.). Those registering above 10 mM/l glucose in their urine were then blood tested using a Beckman Glucose Analyzer 2 (Beckman Instruments, Australia Pty Ltd.), to confirm their diabetic state. Those mice testing within the normal range 1 week (6–10 mM/l) and diabetic the next (>18 mM/l) were termed 'recent onset mice'; that is, mice that had been diabetic for less than 7 days. The day the blood test results revealed the diabetic state became day 1 of the study. Donor mice for transplantation were pre-insulitic NOD/Lt females <30 days of age.

3.2. Monoclonal antibodies and treatment protocols

145-2C11 (hamster-anti-mouse) anti-CD3 IgG2b [16], KT3 (rat-anti-mouse) anti-CD3 IgG2a [17], GK1.5 (rat-anti-mouse) anti-CD4 IgG2b [18] and 53.6.7 (rat-anti-mouse) anti-CD8 IgG2a [19] were produced and purified in the Department of Surgery, RMH. The 145-2C11 and 53.6.7 antibodies were harvested from tissue culture cell supernatants, while KT3 and GK1.5 were isolated from ascites fluid collected from pristane-primed nude rats. Separation of the antibodies was via a protein-G sepharose column (AMRAD Pharmacia-Biotech Ltd., Australia), dialysis against PBS and measurement of protein at OD 280 on a Du-65 spectrophotometer (Beckman Instruments Australia Pty Ltd.). All hybridoma cell lines were originally provided by Dr Tom Mandel, from the Walter and Eliza Hall Institute for Medical Research (Melbourne, Australia).

Due to its toxic nature, mice received relatively low doses of 145-2C11 (5 or 10 μg) for either 5 or 21 days intravenously (i.v.) following confirmation of the 'recent onset' status (day 1=first day of treatment). KT3, GK1.5 and 53.6.7 were independently administered at either 10, 50 μg or higher doses for 5 or 21 days.

3.3. FACScan studies

For analysis of T cell subsets, mice were killed and single-cell suspensions of spleens were prepared and examined by a FACScan Flow Cytometer (Becton Dickinson, Mountain View, CA) to determine the proportions of CD3, CD4 and CD8 cells. FITC-conjugated antibodies were purchased from Boehringer-Mannheim (Castle Hill, NSW, Australia) and used as recommended by the supplier. The antibodies used were 53.6.7 (CD8), H129.19 (CD4) and 145-2C11 (CD3). The cells were incubated in saturating concentrations of mAb-FITC (50 μl) for 30 min at 4 °C, washed and fixed for analysis: three or more mice were sampled in each test group. Histograms were compared after gating to concentrate analysis on small lymphocytes. The percentage of cells in the fluorescent peak of a normal mouse spleen was compared to the percentage of cells found in the same gated peaks of spleens from treated mice. A mean percentage of cells in a gated peak ±standard deviation was produced from the analysis of multiple samples within a treatment group.

3.4. Pancreas isografts

To test for stable remission of disease, vascularized segmental pancreas isografts were performed. Donor mice were 4-week-old pre-insulitic NOD/Lt females. The vascularized segment of pancreas attached to the donor splenic vessels was transplanted as described previously [20]. The spleen and pancreatic lymph nodes were not transplanted with the pancreas, which was attached to the abdominal vena cava and aorta of the recipient mouse using microsurgical techniques. Function was monitored twice weekly by testing glycouria using Labstix. Readings >10 mM/l were confirmed with a blood test. Grafts were deemed defunct when mice tested >18 mM/l on the glucose analyzer. Long surviving grafts were assessed by histology at >100 days.

3.5. Histology and immunohistology

Pancreas samples were removed and placed in Bouin's fixative for 2 h before transfer to 70% ethanol then embedded in paraffin. Glucagon and insulin were detected by a three-layer immunoperoxidase technique using rabbit anti-human polyclonal antibodies that cross-react with mouse insulin or glucagon (Dako, Carpinteria, CA 93013, USA). Islets were assessed by a semiquantitative score of 0–4 with 0 representing an islet with no insulin positive β cells. Scores of 2 or 3 were for islets with increasing numbers of insulin positive β cells and 4 represented a well-preserved islet with the majority of cells insulin positive. Infiltrates are also scored as 0 (no infiltrate), 1 (focal peri-islet or peri-ductal infiltrate), 2 (peri-islet infiltrate with 25–75% of the islet affected), 3 (100% peri-islet infiltrate and some intra-islet invasion and disruption of β cells), or 4 (extensive infiltrate throughout the islet). All individual islet scores for each treatment group were averaged and presented as mean ± S.D. Scores were compared using ANOVA and Student's *t*-test [21].

For other immunohistology, tissue was embedded in OCT medium and frozen in liquid nitrogen. Cryostat sections (4–6 μm) fixed in 4% paraformaldehyde were stained using the four-layer immunoperoxidase techniques described previously [22]. The antibodies used were GK1.5 (CD4), and 53.6.7 (CD8), affinity purified from ascites from nude rats in the Department of Surgery, RMH, Australia. Other antibodies used included XMG1-2 (IFN-γ), obtained from Dr Charmaine Simeonovic (Australian National University, Canberra, Australia), F4/80 (macrophages) and B220 (B cells) from Dr Thomas Mandel (the Walter and Eliza Hall Institute, Melbourne, Australia) and 11B11(IL-4), obtained from Dr Christina Cheers (University of Melbourne, Australia). Anti-IL-2 and IL-2R were purchased from Genzyme (www.genzyme.com). Labeled cells in areas around and within islets were described and photographed.

4. Results

4.1. Remission in recent onset diabetic mice treated with anti-T cell mAb (*Table 1*)

All of the anti-T cell mAb used for treatment could induce permanent remission in some of the treated mice, but the percent remission varied with the mAb used. We did not observe spontaneous remission in diabetic mice with blood glucose levels >20 mM/l kept for 21 days without insulin treatment ($n=10$). Similarly no remission >2 days was seen in mice treated with an inactive antibody for 5 days, then observed until day 21 ($n=8$). In most of the groups treated with mAb, some mice had transient remission, with blood glucose levels <10 mM/l for a short time. Mean transient remission time was 4.6 ± 2 days S.D., and did not depend on the mAb dose. Permanent remission was defined as maintaining normal blood glucose levels after treatment for the duration of the experiment (50 or 100 days).

Although 145-2C11 could induce up to 33% permanent remission, even at the low doses used here, first dose syndrome, due to cytokine release during T cell activation, precluded the use of higher doses of mAb. All of the mice treated with 145-2C11 became sick, with significant weight loss and 14% mortality, as described previously [23]. The less toxic KT3 antibody could, however, be used at much higher doses without cytokine release syndrome being a problem [23]. KT3 was a highly effective treatment, with 50% permanent remission seen with the 10-μg dose given over 5 days

Table 1
Anti-T cell mAb treatment of recent onset diabetic NOD mice

Monoclonal antibody	i.v. Treatment (number)	% Remained diabetic	% Complete remission
None	None ($n=10$)	100	0
Inactive mAb	50 μg/5days ($n=8$)	100	0
145-2C11 (anti-CD3)	5 μg/5 days ($n=14$)	85.7	14.3
	10 μg/ 5days ($n=22$)	19.1	9.5
	5 μg/21 days ($n=9$)	66.6	33.3
KT3 (anti-CD3)	10 μg/5days ($n=12$)	25	50
	50 μg/5days ($n=21$)	19	38
	50 μg/21 days ($n=9$)	44.5	44.5
	250 μg/4 days, then every 7 days (5)	80	0
GK1.5 (anti-CD4)	10 μg/5 days ($n=8$)	87.5	12.5
	50 μg/5 days ($n=6$)	100	0
	50 μg/21 days ($n=7$)	85.7	14.3
53.6.7 (anti-CD8)	50 μg/5 days ($n=12$)	66.6	33.3
GK1.5+53.6.7 (CD4+8)	50 μg each/5 days ($n=13$)	77	15

and slightly lower rates of remission (38 and 45%) seen with the 50-μg doses. Interestingly, when the KT3 dose was increased to 250 μg, causing CD3 T cell depletion in the spleen and lymph nodes [23], the effectiveness of the treatment decreased, with no permanent remission seen. This implied that maintenance of an immunoregulatory T cell population was required and that rigorous T cell depletion would not allow the establishment of a benign infiltrate.

Treatment with GK1.5 was less effective (12–14% complete remission) than the anti-CD3 agents, while anti-CD8 (33% complete remission) was as effective as 145-2C11, but less effective then KT3. Combining anti-CD4 with CD8 gave remission rates similar to those seen with GK1.5 alone. Other combinations and higher doses of antibody were tried, including 145-2C11 and KT3, GK1.5 and KT3, 53.6.7 and KT3 (data not shown). In all cases the combinations resulted in the same or less permanent remission than single antibody treatments. Combination with GK1.5 always reduced the rate of remission to that seen with GK1.5 alone. Thus it was detrimental to block CD4 T cell activity and it seemed the CD4 cells were required to induce remission.

4.2. Survival of pancreas isografts in mice with long-term remission

Isografts were performed to test for a response to fresh islet antigen in mice that remained in remission at 50 days following anti-T cell treatment. Survival times are shown in Table 2. In isografts from age-matched untreated diabetic NOD mice, disease recurrence was seen at 9–14 days (median 10.5 days). Anti-CD4 and/or CD8 treated recipients responded strongly to the isograft with 6/11 mice becoming diabetic by 16 days and only one mouse maintained normal blood glucose levels for >70 days. Survival times for these groups were not significantly different to untreated controls ($P>0.1$). Two GK1.5 treated mice in remission, which were not isograft challenged, maintained normal blood glucose levels to >100 days (data not shown). Thus, disease recurrence following challenge could destroy both native and transplanted islet function in these mice.

In the anti-CD3 treated groups, isografts in the 145-2C11 treated mice showed variation in survival times (range, 9 to >70 days) as seen in the anti-CD4 and 8 groups, and were not significantly different to controls ($P>0.1$). Only 2/10 survived >31 days. In contrast,

Table 2
Pancreas isograft challenges at day 50 in mice exhibiting remission, compared with untreated isograft controls

Original treatment[a]	Graft survival (days)	Median
None	9, 9, 10, 11, 12, 14	10.5
GK1.5	14, 21, 33	21
53.6.7	12, 12, 13, 64	12.5
GK1.5+53.6.7	12, 16, 34, >70[b]	25
145-2C11	9, 13, 13, 19, 20, 20, 30, 31, 92, >70[b]	20
KT3	28, 37, 42, >52[b], >65[b], >100[b], >100[b], >100[b], >100[b], >100[b]	100[c]

[a] Seven mice in permanent remission, without isograft challenge, remained normoglycemic for >100 days.
[b] These mice were normoglycemic when culled for histology.
[c] Significantly different to untreated controls ($P<0.001$) and to 145-2C11 treated mice ($P=0.001$).

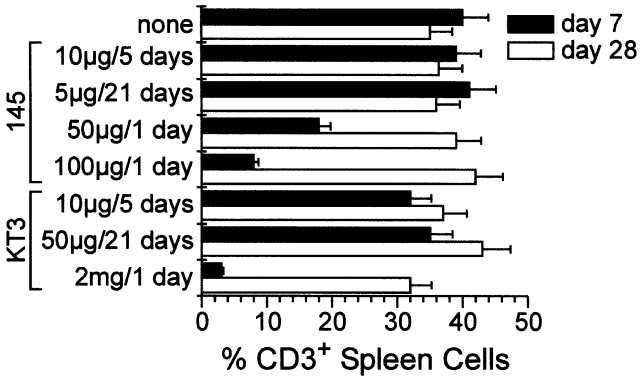

Fig. 1. FACS analysis of spleen cells from NOD mice treated with anti-CD3. KT3 was given i.v. at doses of 10 μg to 2 mg for between 1 and 21 days. 145-2C11 was given i.v. at doses of 5–100 μg for 1–21 days. Groups were sampled at 7 and 28 days (2–5 mice/group) after treatment commenced. Freshly isolated spleen cells were incubated with FITC-coupled anti-CD3, CD4 or CD8 mAb. Results for CD3 cells are shown as mean percent positive cells ± S.D. in the gated small lymphocyte populations.

KT3 treated mice maintained normal blood glucose levels for longer after isograft challenge (range 28 to >100 days) and 7/10 remained normal during the experiment (50–100 days). Median survival (>100 days) was significantly different to untreated controls ($P<0.0001$) and to the 145-2C11 group ($P=0.001$). All unchallenged mAb-treated mice remained in remission for >100 days ($n=5$, data not shown). Thus, KT3 treated mice showed more stable remission than the long-term normoglycemic mice from the other mAb treatment groups.

4.3. Low dose anti-T cell mAb treatment does not deplete peripheral T cells

Treatment of NOD mice with a single 2-mg dose of KT3 or 100-μg dose of 145-2C11 could reduce splenic CD3 cells to <10% of normal, 7 days after treatment (Fig. 1). This is similar to the target cell depletion previously reported by us for 1-mg doses of anti-CD4, CD8 and CD4 plus CD8 in NOD mice [22]. This level of spleen cell depletion was not seen with doses of antibody <50 μg. For example, 50 μg of KT3 given i.v. for 21 days or 10 μg given for 5 days was ineffective in reducing spleen CD3 T cell numbers. A 50-μg dose of 145-2C11 reduced splenic CD3 T cells to 18%, but T cell numbers rapidly returned to normal, with percent CD3 levels not significantly different to untreated controls 28 days after cessation of either 5 or 21 days treatment. The 5-μg dose of 145-2C11 for 21 days did not change splenic CD3 T cell numbers. The low doses of GK1.5 (anti-CD4) and/or 53.6.7 (anti-CD8) used here were also non-depleting for splenic T cells (data not shown). Thus, the effects of anti-T cell treatment were not due to systemic T cell depletion, but to specific effects on insulitis (see below).

4.4. T Cell depletion and long-term islet function in native and transplanted pancreases of KT3 treated mice

In each treatment group, individual islets were scored according to the number and condition of the β cells (islet score) and the extent of islet infiltrate (insulitis score). Since the KT3 treatment groups showed the full spectrum of response to treatment, a detailed analysis of islet function for these groups is shown in Table 3. In pancreases from untreated diabetic animals, harvested soon after onset (7–14 days) the low islet function and high insulitis scores were typical of aggressive disease, as reported previously [22]. In contrast, non-diabetic older mice (>120 days) with many insulin producing β cells had benign insulitis and mean islets scores >2. All of the KT3 treated recent onset diabetic groups had significantly lower insulitis scores ($P<0.001$) than untreated recent onset mice, indicating local depletion of T cells by the anti-CD3 mAb.

Typical responses seen after mAb treatment were:

i. Where mAb treatment was given too late after disease onset and there was no remission, or only transient remission (average of 4 days), treatment had reduced the infiltrate, but there were not enough

Table 3
Islet function and insulitis in KT3 treated recipients compared with untreated controls

Treatment group[a] (no. islets scored)	Pancreas sampled	Sample time point after onset (days)	Islet score mean ± S.D.	Insulitis score mean ± S.D.
Untreated diabetic (31)	Native	7–14	0.2 ± 0.4	3.9 ± 1.1[b]
Untreated non-diabetic (11)	Native	>100	2.4 ± 0.8	1.2 ± 0.6
KT3: remained diabetic (24)	Native	20–30	0.3 ± 1.0	1.9 ± 2.0
KT3: permanent remission (26)	Native	>100	2.0 ± 1.9	1.9 ± 1.7
KT3: isograft challenge (15)	Native	>160	3.3 ± 1.0	1.3 ± 1.5
KT3: isograft challenge (25)	Tx	>60	1.8 ± 0.9	2.4 ± 0.7

[a] KT3, 10–50 ug/day for 5 or 21 days i.v.

[b] Significantly greater than all other scores, $P<0.0001$ Scores in KT3 treated groups were not significantly different from each other ($P>0.05$).

insulin producing β cells remaining in the islets (low islet and insulitis scores) to maintain blood glucose levels.

ii. Treatment blocked the destructive phase of the disease and enough islets were rescued to allow regeneration of function. The animals remained in the benign phase of disease, with low insulitis scores indicating low number of T cells surrounding the islets. Challenge with NOD isografts did not disturb this state in most KT3 treated mice.

iii. Islets in long surviving isografts showed lower islet scores and higher levels of infiltration than the surviving native pancreas, indicating a persistent low level response to the isograft, but insulitis seemed benign (see below)

4.5. Immunohistology of islets in diabetic and non-diabetic mice

Fig. 2 shows representative paraffin sections of pancreas stained for insulin from: (a) a non-diabetic older mouse with intact islet function (score 4) and peri-islet infiltrate less than 50% (insulitis score 2); (b) a mouse in long-term remission with good islet function (islet score 3) and 75% peri-islet infiltrate (insulitis score 3); (c) a mouse with end-stage disease, no intact β cells (islet score 0) and extensive remaining infiltrate (insulitis score 4); (d) a transplanted islet with long-term function (islet score 4) and insulitis (75%, score 3) in a mouse with permanent remission. The lack of acinar tissue in this last sample is due to duct occlusion during transplantation [22]. This shows the presence of functioning β cells and persistent insulitis before and after antibody treatment, and in transplanted tissue. Staining for lymphocyte cell surface markers and cytokines revealed the benign nature of the infiltrate in long-surviving native and transplanted islets.

Although good frozen sections were more difficult to prepare than paraffin sections, and sections containing islets were not available for all the groups, immunohistology confirmed the findings summarized in Table 3. In untreated diabetics both peri-islet and intra-islet infiltrate was seen, dominated by T cells (CD4 = CD8, with many IL-2R positive cells), scattered macrophages, IFN-γ, IL-2 and IL-4 expression and a few B cells (not shown here: see published work [22,24]). In contrast, non-diabetic older mice and those with permanent remission had peri-islet insulitis with T cells (CD4 > CD8, few IL-2R cells), many B cells, and some macrophages at the edges of the infiltrate. The levels of cytokine expression were uniformly low (IFN-γ, IL-4 and IL-2). The distinct patterns of infiltration typical of this benign insulitis are shown in Fig. 3. These are representative serial sections of a single native islet from a KT3 treated mouse in permanent remission at >100 days. The dense peri-islet infiltrate included T cells, with CD4 cells (a) predominating over CD8 (b) and many B cells (c). Macrophages (d) lined the margins of the peri-islet region. There were very few scattered IL-2R positive cells (e) and low levels of the cytokines IFN-γ (f), IL-4 (g) and IL-2 (h) in the peri-islet region.

In summary, of the 38 mice from all treatment groups in remission at 50 days, 31 mice were challenged with isografts. Of these, 22/31 (71%) developed recurrent destructive disease in both the native and transplanted pancreas. In the KT3 treated groups 7/10 (70%) isograft challenged mice remained normal, with a benign peri-islet infiltrate. Although both 145-2C11 and KT3 produced high rates of remission, low-dose KT3 was the most effective treatment for both inducing remission and re-establishing tolerance to the autoantigen.

5. Discussion

The use of low-dose anti-CD3 in the treatment of recent onset autoimmune diabetes is effective in the immediate reduction of peri-islet insulitis, without causing deletion of systemic CD3 positive cells. The work described here provides further evidence that this treatment selectively deletes activated CD3 cells from the insulitis lesion, promoting long-term, autoantigen-specific tolerance that is sufficiently robust to allow transplantation of a vascularized pancreas to replace islet cell capacity. Anti-CD3 therapy provides the opportunity to use a known immunosuppressive agent in a new role and to explore the immune mechanisms in well established animal models. The pioneering work of Chatenoud et al. [8,15] has led directly to the recent establishment of clinical trials of a humanized anti-CD3 for the treatment of recent onset diabetes [4], so further exploration of anti-CD3 therapy and its impact on reactivity to islet autoantigens will have direct clinical implications.

Our data show that blood glucose levels and islet histology after anti-CD3 therapy falls into three patterns of response: (i) mice in which rescue came too late. These had no remission or only transient remission (average of 4 days) and histology showed very little infiltrate and no insulin producing β cells remaining in the islets. Although the infiltrate had been cleared, there was insufficient remaining β cell function to maintain normal blood glucose levels. (ii) In the second group of animals, treatment cleared insulitis, halting the destructive phase of the disease at a stage when enough β cells survived to maintain or allow regeneration of function. Some of these mice went into remission until challenged with a NOD isograft, when aggressive disease destroyed both transplanted and native pancreas. It seemed here that the transplant triggered recurrent disease, since none of the unchallenged group of mice in remission ($n = 7$) developed diabetes. A change in antigen load or presentation may have been the trigger for

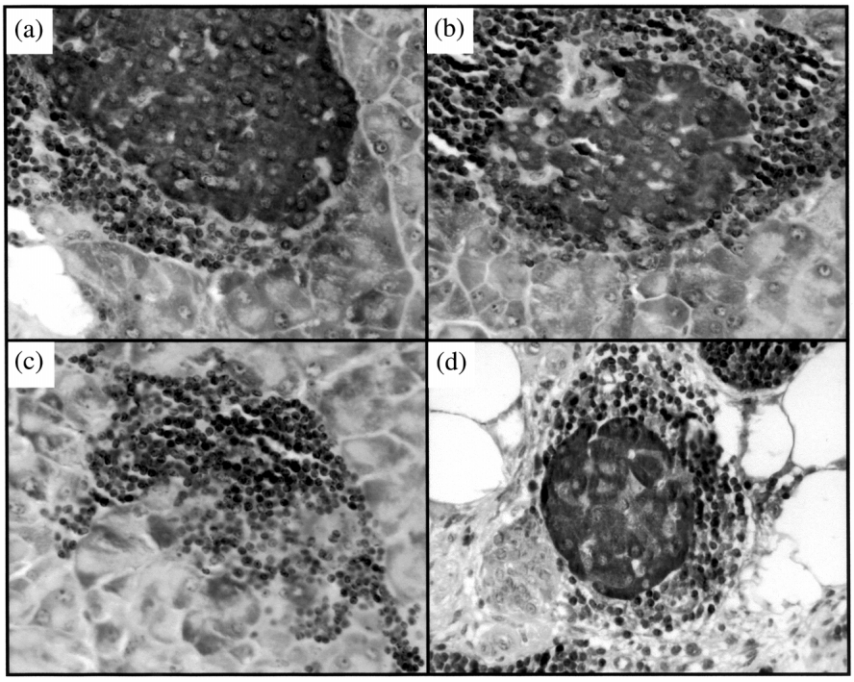

Fig. 2. Parafin sections of pancreas tissue were immunoperoxidase stained for insulin (original magnification 200×). Samples were from (a) a non-diabetic older mouse; (b) a mouse in long-term remission; (c) a mouse with recurrent disease in the native pancreas after isograft challenge; and (d) a transplanted pancreas with long-term function in a mouse with permanent remission.

disease recurrence here [25]. (iii) In the third group of animals, treatment blocked the destructive phase of the disease and enough islets were rescued to allow regeneration of function. Challenge with NOD isografts did not disturb this state and insulitis in both the native and transplanted pancreas remained benign, implying restoration of a tolerance to islet autoantigens.

The question of when and how the initial breakdown of tolerance occurs in NOD mice has been investigated extensively. The earliest changes occur between weaning and approximately 3 weeks when low levels of β cell apoptosis initiate dendritic cell (DC) migration into the islets [26] [27,28] followed by T cells and B cells, and culminating in a stable state of benign infiltration which persists for >70 days [6]. Investigators have reported predominantly Th2 (IL-4, IL-10) cytokine expression at this stage [29] and demonstrated the presence of immunoregulatory T cells (CD4+, CD62L+, CD25+) in the infiltrate [30]. Autoimmune reactions have been demonstrated in both the B and T cell fractions, but this does not progress to extensive β cell destruction, and cannot predict the development of diabetes in either male of female mice [6,31]. B cells are consistently seen in benign infiltrates and may play a role, as antigen presenting cells, in the development of the immunoregulatory CD4 T cells [32,33].

In 10–20% of male mice and up to 80% of female NOD mice there is a rapid switch from benign to destructive insulitis at 70–100 days. Invasive T, B and dendritic cell infiltration and Th1 cytokine expression, results in extensive β cell apoptosis and consequent diabetes occurs 14–21 days after the switch in infiltrate character [6,26]. Changes prior to diabetes onset may be triggered by an interaction of resident T cells with an altered peptide ligand, causing a cytokine switch from Th2 to Th1 in some islets. Cytokines within the islet and possibly from neighboring islets then cause CD3 cell activation, Th1 cytokine release and induction of Fas on β cells, which interact with Fas-L expressing CD4 and CD8 cells [11]. Th1 cytokine release is not a systemic response, since the degree of destructive insulitis in diabetic mice varies widely from islet to islet, suggesting that the changes occur as local events in individual islets. Islets differ in their degree of destruction within the same animals, so that it is only when populations of islets and mice are examined that a pattern of destructive or benign insulitis can be detected. Our histology and islet scores clearly showed this variable response, with intact islets existing beside others that had been subject to autoimmune destruction. The heterogeneous nature of insulitis makes detection of changes erratic, so early treatment to reverse destructive autoimmunity will be difficult, and rescue of variable numbers of islets, as seem in our results, will occur.

Chatenoud et al. showed that anti-T cell mAbs were effective in rescuing recent onset mice, yet not effective in pre-diabetic mice, or in treating established disease [8]. In some of the mice in remission, the autoimmune

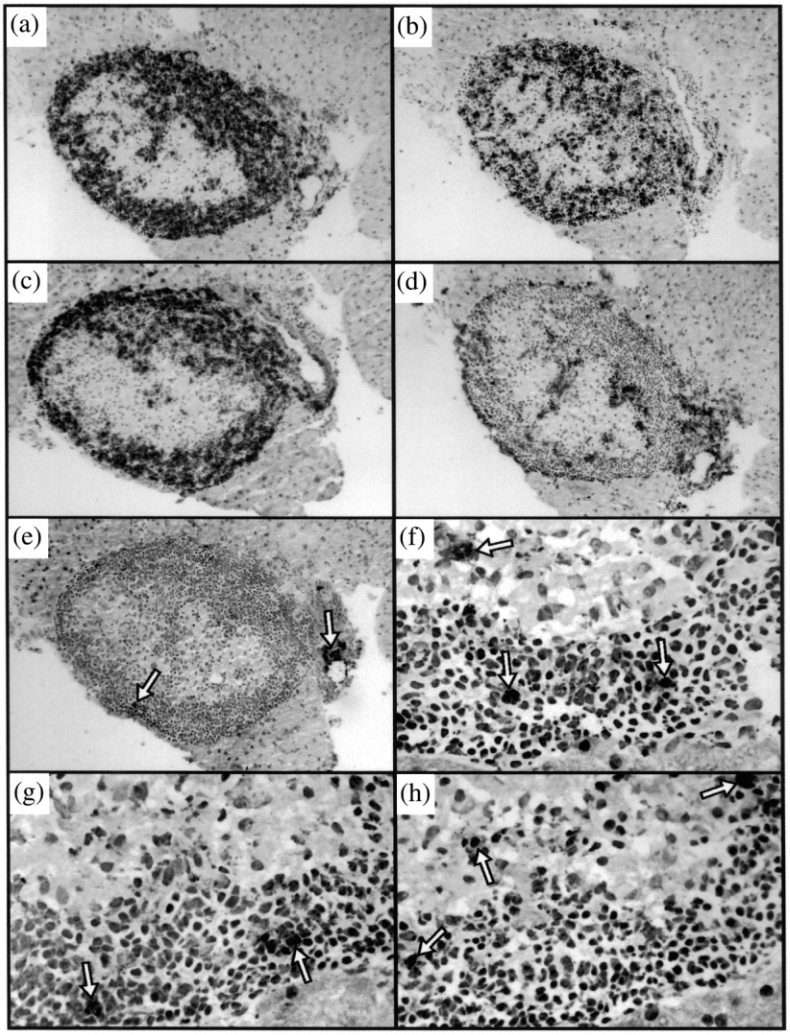

Fig. 3. Serial sections of a single islet (frozen tissue) from a representative KT3-treated mouse in long-term remission (>100 days) are shown with staining for CD4 cells (a), CD8 cells (b), B cells (c), macrophages (d) and IL-2R positive cells (e) (a–e, original magnification 200×). Higher power (original magnification 400×) pictures show IFN-γ (f), IL-4 (g) and IL-2 (h) scattered positive cells (arrows).

response remained destructive when presented with new antigen in the form of an isograft. Recurrent disease was then a secondary response to autoantigens, mediated by CD4 and CD8 T cells, and could only be controlled by high-dose immunosuppressive mAb [22]. Recurrent disease after isograft challenge in mice in apparent remission implied that a sufficient number of memory T cells resistant to anti-CD3 treatment [34] had remained and could mount a response to the new dose of autoantigen, and that this lead to destruction of the remaining native β cells. It seems that T cells must be targeted during a window of activation, at the time of or shortly after the switch from benign to destructive infiltrate, for anti-CD3 therapy to be effective.

Anti-CD3 treatment during T cell activation has been shown to preferentially delete Th1 cells, sparing Th2 cells [35]. Although abnormally low responses to anti-CD3 treatment have been documented in diabetic patients, possibly due to a signal transduction defect [36], response in newly diagnosed IDDM patients were significantly better than in long-term diabetics. Thus the window of opportunity for treatment with anti-CD3 corresponds with the recent onset phase of IDDM, when the numbers of activated Th1 cells peak. It also seems that low-dose anti-CD3 is more effective than high doses of mAb, and that systemic CD3 T cell depletion may actually inhibit the establishment of benign autoimmunity. Total loss of CD3 cells may remove the regulatory CD4/Th2 cells required to maintain the benign peri-islet infiltrate [37,38]. Thus the persistence of benign insulitis in long surviving native and transplanted islets does not signal the return of active disease. Our data, showing less remission with anti-CD4 treatment, and the dominance of CD4 over CD8 cells in benign insulitis, also suggests that intact CD4 cell function is required to maintain remission.

In summary, our findings show that good rates of remission can be induced with low-dose anti-CD3 and that anti-CD8 and anti-CD4 mAb were less effective. These treatments produced a common pattern of clearance of insulitis, but not peripheral T cells, after treatment. Benign insulitis was seen in long-term remission and in long surviving isograft tissue transplanted into mice with established remission. Histology showed the clearance of insulitis in all KT3 treated mice, activated T cell infiltration of the native and transplanted pancreases of those mice with transient remission or recurrent disease and benign insulitis in mice that remained in remission. Of the mice in permanent remission following KT3, anti-CD3 treatment, 70% had re-established tolerance to autoantigen and did not destroy transplanted vascularized isografts placed >50 days after treatment. With greater knowledge of the natural history of IDDM and an ability to identify the recent onset phase more accurately, we will be able to gain insight into and modify the anti-CD3 treatment of recent onset IDDM in humans [4].

Acknowledgments

This work was supported by the National Health and Medical Research Council and the National Heart Foundation of Australia. We thank Ms Janna Stickland for graphic design of the figures and Dr Tom Mandel for helpful comments on the manuscript.

References

[1] Todd JA. Genetic control of autoimmunity in type 1 diabetes. Immunol Today 1990;11:122–129.
[2] Atkinson M, Maclaren N. What causes diabetes? Scientific American 1990 (July):42–49.
[3] Bach J-F, Chatenoud L. Tolerance to islet autoantigens in type 1 diabetes. Annu Rev Immunol 2001;19:131–161.
[4] Herald K. Phase I/II trial of hOKT3gamma1(Ala-Ala) for treatment of type 1 diabetes mellitus. 2001, JDRF Task Force.
[5] Leiter EH, Serreze DV, Prochazka M. The genetics and epidemiology of diabetes in NOD mice. Immunol Today 1990;11:147–149.
[6] Gazda LS, Charlton B, Lafferty KJ. Diabetes results from a late change in the autoimmune response of NOD mice. J Autoimmunol 1997;10:261–270.
[7] Shizuru JA, Alters SE, Fathman CG. Anti-CD4 monoclonal antibodies in therapy: creation of nonclassical tolerance in the adult. Immunol Rev 1992;129:105–130.
[8] Chatenoud L, Primo J, Bach J-F. CD3 antibody-induced dominant self tolerance in overtly diabetic NOD mice. J Immunol 1997;158:2947–2954.
[9] Pakala S, et al. In autoimmune diabetes the transition from benign to pernicious insulitis requires an islet cell response to tumor necrosis factor alpha. J Exp Med 1999;189(7):1053–1062.
[10] Mora C, et al. Pancreatic infiltration but not diabetes occurs in the relative absence of MHC class II-restricted CD4 T cells: studies using NOD/CIITA-deficient mice. J Immunol 1999;162(8):4576–4588.
[11] Sainio-Pollanen S, et al. The role of CD8+ cells, cell degeneration, and Fas ligand in insulitis after intraperitoneal transfer of NOD splenocytes. Pancreas 1999;18(3):282–293.
[12] Lehuen A, et al. Over expression of natural killer T cells protects Valpha14-Jalpha281 transgenic nonobese diabetic mice against diabetes. J Exp Med 1998;188(10):1831–1839.
[13] Chatenoud L, et al. Remission of established disease in diabetic NOD mice induced by anti-CD3 monoclonal antibody. C.R. Acad. Sci III 1992;315(6):225–228.
[14] Maki T, et al. Long-term abrogation of autoimmune diabetes in nonobese diabetic mice by immunotherapy with anti-lymphocyte serum. Proc Natl Acad Sci 1992;89:3434–3438.
[15] Chatenoud L, et al. Anti-CD3 antibody induces long-term remission of overt autoimmunity in nonobese diabetic mice. Proc Natl Acad Sci USA 1994;91(1):123–127.
[16] Leo O, et al. Identification of a monoclonal antibody specific for a murine T3 polypeptide. Proc Natl Acad Sci 1987;84:1374–1378.
[17] Tomonari K. A rat antibody against a structure functionally related to the mouse T-cell receptor/T3 complex. Immunogenetics 1988;28:455–458.
[18] Dialynas DP, et al. Characterisation of the murine T cell surface molecule, designated L3T4, identified by the monoclonal antibody GK1.5: similarity of L3T4 to the human Leu-3/T4 molecule. J Immunol 1983;131:2445–2450.
[19] Ledbetter J, Herzenberg LA. Xenogeneic monoclonal antibodies to mouse lymphoid differentiation antigens. Immunol Rev 1979;47:63–90.
[20] Mottram PL, Purcell LJ, Kalina SL. Microsurgical techniques for transplantation of vascularised organs in mice. In: Green MK, Mandel TE, editors. Experimental transplantation models in small animals. Chur, Switzerland: Harwood Academic Publishers; 1995. p. 175–195.
[21] Brown M, et al. BMDP Statistical Software: printing with additions. Berkeley: University of California Press, Ltd, 1983.
[22] Mottram P, et al. Long-term survival of segmental pancreas isografts in NOD/Lt mice treated with anti-CD4 and anti-CD8 monoclonal antibodies. Diabetes 1998;47:1399–1405.
[23] Mottram P, et al. Idarubicin anti-CD3: a new immunoconjugate that induces alloantigen-specific tolerance in mice. Transplantation 1997;64(5):684–690.
[24] Mandel TE, Kovarik J, Koulmanda M. A comparison of organ cultured fetal pancreas allo-, iso-, and xenografts (pig) in non-immunosuppressed non-obese diabetic mice. Am J Pathol 1995;147(3):834–844.
[25] Healy JI, Goodnow CC. Positive versus negative signaling by lymphocyte antigen receptors. Annu Rev Immunol 1998;16:645–670.
[26] O'Brien BA, et al. Beta cell apoptosis is responsible for the development of IDDM in the multiple low-dose streptozotocin model. J Pathol 1996;178:176–181.
[27] Jansen H, et al. Histochemical characterisation of monocytes-macrophages and dendritic calls involved in the initiation of insulitis and beta cell destruction in NOD mice. Diabetes 1994;43:667–675.
[28] Dilts SM, Lafferty KJ. Autoimmune diabetes: involvement of benign and malignant autoimmunity. J Autoimmunol 1999;12:229–232.
[29] Green E, Flavell R. Tumor necrosis factor-alpha and the progression of diabetes in non-obese diabetic mice. Immunol Rev 1999;169:11–22.
[30] Lepault F, Gagnerault MC. Characterization of peripheral regulatory CD4+ T cells that prevent diabetes onset in nonobese diabetic mice. J Immunol 2000;164(1):240–247.

[31] Fox CS, Danska JS. IL-4 expression at the onset of islet inflammation predicts nondestructive insulitis in nonobese diabetic mice. J Immunol 1997;158:2414–2424.
[32] Noorchashm H, et al. B lymphocytes influence the shape of the mature preimmune CD4+ TCR repertoire. Transplant Proc 1999;31(1-2):832–833.
[33] Noorchashm H, et al. Impaired CD4 T cell activation due to reliance upon B cell-mediated costimulation in nonobese diabetic (NOD) mice. J Immunol 2000;165(8):4685–4696.
[34] Zhang X, et al. Unequal death in T helper cell (Th)1 and Th2 effectors: Th1, but not Th2, effectors undergo rapid Fas/FasL-mediated apoptosis. J Exp Med 1997;185(10):1837–1849.
[35] Varadhachary AS, et al. Differential ability of T cell subsets to undergo activation-induced cell death. Proc Natl Acad Sci USA 1997;94(11):5778–5783.
[36] Nervi S, et al. Constitutive impaired TCR/CD3-mediated activation of T cells in IDDM patients co-exist with normal co-stimulation pathways. J Autoimmunol 1999;13(2):247–255.
[37] Phillips JM, et al. Nondepleting anti-CD4 has an immediate action on diabetogenic effector cells, halting their destruction of pancreatic beta cells. J Immunol 2000;165(4):1949–1955.
[38] McLaren N, Lafferty K. Protective insulitis. Diabetes 1993;42:1099.