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Leiden
The Netherlands

Immunotherapy and beta-cell replacement in type I diabetes mellitus

Linde, P. van de

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Chapter 2

Mechanisms of antibody immunotherapy on clonal islet reactive T-cells

P. van de Linde, O.M.H Tysma, J.P. Medema, G. Hale, H.
Waldmann , D.L. Roelen, B.O. Roep.

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ABSTRACT

Clinical intervention trials evaluating the efficacy of antibody immunotherapy in type 1 diabetes are in progress. We tested effects on prediabetic islet-antigen specific autoreactive T-cells of anti-thymocyte globulin (ATG) and humanized monoclonal antibodies against CD3 (ChAglyCD3) or CD25 (daclizumab) with regard to down-modulation of the target protein, proliferation, cytokine production, complement-dependent cytotoxicity (CDC), antibody dependent cell cytotoxicity (ADCC) and survival. ATG leads to depletion of auto-reactive CD4+T-cells by ADCC, CDC and apoptosis, whereas anti-CD3 and anti-CD25 inhibited T-cell autoreactivity in a nondepleting fashion. ATG treatment led to a cytokine burst of Th1- and Th2- associated cytokines. Modulation of cytokine release through humanized monoclonal antibodies was moderate and selective: anti-CD25 led to increased release of IL-2 and reduced production of TNF α , whereas anti-CD3 decreased release of interferon- γ and IL-5, and increased secretion of IL-10. ATG and the humanized monoclonal antibodies displayed contrasting mechanisms of action.

INTRODUCTION

Type 1 diabetes is generally believed to result from a T-cell-mediated autoimmune destruction of the insulin-producing beta-cells in the pancreatic islets of Langerhans (1-4). Therefore T-cell modulation by selective and mild immune modulatory drugs is an attractive intervention therapy. Clinical trials in type 1 diabetes demonstrated that the use of immunomodulatory drugs such as cyclosporine, azathioprine and prednisone causes transient improvement in clinical measures and enhances the rate of remissions when initiated soon after diagnosis (5,6). Unfortunately, the toxic effects of such drugs, the associated risk of the immune suppression, and the need for continuous treatment in otherwise healthy, usually young subjects limit the use of these agents (7). Polyclonal antibody therapy with anti-thymocyte globulin (ATG) was demonstrated to be very effective in protecting beta-cell function in autoimmune diabetes (8) and after islet transplantation in type 1 diabetes patients, preventing recurrent islet autoreactivity and islet allograft rejection (9-11). Although very effective, ATG targets many functional T-cell molecules, and is broadly lytic for T-cells, and elicits a number of unwanted side effects. Preclinical studies suggested that a monoclonal antibody against CD3 could reverse hyperglycemia at presentation and induce tolerance to recurrent disease (12). Recent developments have allowed the clinical use of so-called humanized monoclonal antibodies produced with a mutated Fc region from human origin, which lack side effects seen with non-humanized monoclonal antibody therapy such as OKT3. Both anti-CD3 and anti-CD25 monoclonal antibodies are of particular interest. Treatment with the humanized anti-CD3 monoclonal hOKT3 γ 1(Ala-Ala) delayed the deterioration in insulin production and improved metabolic control during the first year of type 1 diabetes mellitus in a pilot clinical trial (7,13). Very recently, a phase 2 placebo-controlled trial with a humanized antibody, an aglycosylated human IgG1 antibody directed against CD3 (ChAglyCD3), involving 80 newly diagnosed patients was conducted (14). Patients receiving ChAglyCD3 continued to produce their own insulin and needed less supplemental insulin to maintain normal blood glucose levels compared with patients who received a placebo. This benefit was apparent up through 18 months after the treatment, suggesting the protective effect is lasting, although for how long is not yet known. Moreover, side effects were minor and short-lived, including flu-like symptoms. The humanized monoclonal antibody daclizumab directed against CD25, the α subunit of the interleukin-2 receptor, has also become available. CD25 is primarily expressed on activated T-cells and on regulatory T-cells. This characteristic provides the opportunity to target activated auto-reactive T-cells in recent onset type 1 diabetes albeit with the caveat that Treg function may also be compromised. Humanized monoclonal antibodies such as anti-CD3 and anti-CD25 are considered more selective and less aggressive than polyclonal antibody therapies.

ATG Fresenius (rabbit), ATG Merieux (rabbit), Daclizumab (humanized anti-CD25), and ChAglyCD3 (humanized anti-CD3) are used in several clinical trials to study the effect in recent onset type 1 diabetes patients and pancreas/islet transplant recipients. Studies on the mechanistic action have been performed on each of these compounds but never in a comparative fashion and in the context of human autoimmune disease.

We were interested in the effects of these compounds on pre-existent islet autoreactive T-cells. To this end, we tested the mechanism of action of these rabbit derived polyclonal and humanized monoclonal antibody therapies specifically focused on a unique islet antigen-specific autoreactive T-cell clone. This clone was of particular interest because it was isolated from a prediabetic individual and subsequently demonstrated to selectively home to islets and pancreas-draining lymph nodes in an *in vivo* model of humanized mice, and therefore may be of pathogenic relevance (15). Using polyclonal cell populations or in *in vivo* studies, different mechanisms that may obscure additional pathways, for instance by the presence of complement, NK cells, naïve T-cells, or regulatory T-cell progenitors, may operate. We wished to exclude the contribution of the antibody therapeutics on other immune components. We separated all possible effector pathways of the antibody therapeutics by focusing on a monoclonal T-cell population that was tested for induction of apoptosis, depletion by antibody-dependent cell cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC), inhibition of antigen-induced T-cell proliferation, and downmodulation in a type 1 diabetes related *ex vivo* setting.

MATERIALS AND METHODS

T-cells

The human GAD65-reactive T-cell clone PM1 11 was isolated from a patient with Stiff Man syndrome who subsequently developed type 1 diabetes. This T-cell reagent has been described in great detail elsewhere (16,17). In short, PM1 11 recognizes the GAD65339-352 epitope presented by HLA-DR3. The cytokine profile is Th0-like. Our T-cell clone mainly produced IFN γ , IL-10 and IL13 (18). All pilot mechanistic studies were performed on PBMC, but we present the results on the islet autoreactive T-cells to focus on possible relevance to type 1 diabetes.

APC's

Mature dendritic cells were generated from monocytes isolated from peripheral blood mononuclear cells from HLA-DR3 buffy coats obtained from healthy blood donors after informed consent, as described in detail before (18).

Antibody therapeutics

Two different polyclonal antibodies (ATG's) were tested. Anti-T-lymphocyte Globulin (ATG Fresenius; Fresenius Germany) (ATGf) was obtained from sera of rabbits immunized with the Jurkat T-cell lymphoma cell line. Anti-Thymocyte Globulin (Thymoglobulin; Sang-Stat, Lyon, France) (ATGm) was obtained from sera of rabbits immunized with human thymocytes. The aglycosylated anti-CD3 monoclonal antibody ChAglyCD3 is a modified humanised IgG1-lambda monoclonal antibody which is very similar to the antibody previously described (19,20) and used for the treatment of kidney transplant rejection (21). It was manufactured at the Therapeutic Antibody Centre, Oxford, UK. Daclizumab (Roche, UK) is a humanized monoclonal antibody directed against CD25.

Lymphocyte proliferation test

T-cells were stimulated as described (16). In short, 20.000 T-cells were cultured in tissue-coated, round-bottomed 96-well plates (Costar, Cambridge, MA, USA) in Iscove's modified Dulbecco's medium with 2 mmol/L glutamine (Gibco, Paisley, Scotland) supplemented with 10% human-type pooled serum in the presence of 4000 HLA-DR3-matched dendritic cells pre-incubated with suboptimal concentrations of GAD peptide (0.04 and 0.2 mg/ml) for 1 hour at 37 °C before T-cells were added. GAD peptide with and without therapeutic antibody was used in 200 µl at 37°C, 5% CO₂. The antibodies were tested in a concentration range designed around the trough levels that have been reported for each type of antibody to have therapeutic efficacy of clinical relevance: ATGf and ATGm were tested 0.4, 2.0, 10, 50, 250, and 500 mg/ml. ChAglyCD3 was tested at 0.4, 2.0, and 10 mg/ml, and daclizumab was tested 0.4, 2.0, 10, 50, and 250 mg/ml. After 24 hours, 0.5 mCi [³H] thymidine was added per well, and incubation was continued for 16 h. Cultures were then harvested and [³H] thymidine incorporation was measured by liquid scintillation counting in a betaplate counter (Wallace, Perkin Elmer). The results are expressed as counts per minute (means of triplicates ± standard deviation).

Antibody Dependent Cell Cytotoxicity

The ADCC assay was performed as described previously (22). Freshly isolated PBMCs were used as effector cells and tested in 100:1 and 150:1 effector: target cell ratios against 2000 target cells labeled with carboxyfluorescein (C-F). Labeling of the GAD65-reactive T-cells (target cells) took place as follows: target cells were resuspended in 1 ml phosphate-buffered saline containing 50 µg carboxyfluorescein diacetate and incubated in the dark at room temperature for 30 minutes. After one wash, the cells were resuspended in RPMI 1640 (Dutch Modification; Gibco) supplemented with 0.5% bovine

serum albumin. Antibody concentrations tested were 20, 100, and 500 mg/ml for ATG, 0.4, 2.0, and 10 mg/ml for ChAglyCD3, and 10, 50, and 250 mg/ml for daclizumab. ATG is used as a reference in our analysis. Then, 10 ml effector cells, 10 ml target cells, and 10 ml antibody solution were mixed in Terasaki-type trays with a well volume of 40 ml. After spinning the trays for 5 minutes at 150g, the relative number of C-F-labeled cells was determined, as an arbitrary fluorescence value per well, using an automated inverted epi-illuminated fluorescence microscope equipped with filters for C-F. All values were expressed as per tray normalized percentages. Next, the trays were incubated at 37°C for 3 hours in a humidified 5% CO₂ incubator and centrifuged for 5 minutes at 400g. Subsequently, 10 ml ink-suspension (Leitz ink; 1:300 in isotonic 5% EDTA, pH 7) was added to each well. The ink quenches the released medium C-F but not the C-F in the unlysed sedimented target cells. After a 10-minute equilibration, the amount C-F remaining in the target was determined with the automated microscope. Percent lysis was calculated as follows: the latter assay values are corrected for well to well variability by dividing them by the preincubation values and are used in the following formula $(1-(A/B)) \times 100$, where A = median value of (target cells + antibodies + effector cells) and B = median value of (target cells + medium + effector cells).

Complement-Dependent Cytotoxicity

Complement-dependent cytotoxicity was tested as previously described (23,24). Briefly, microtest trays were filled with 1 µl antibody solution (antibody concentrations were used as described for ADCC) under oil. Then, 1-µl suspensions of carboxyfluorescein diacetate (Biofine, Leiden, the Netherlands)-loaded T-cells at 3×10^6 /ml were added and incubated for 30 minutes in the dark at room temperature. Complement (Bioscope, Leiden, the Netherlands) was added at 5 µl and allowed to react for 1 hour. Cell suspensions (1 µl) and complement (5 µl) were dispensed using a LambdaJet (One Lambda, Canoga Park, CA, USA). After addition of 5 µl of an EDTA-Propidium Iodide (Sigma)-ink (Leitz, Wetzlar, Germany) solution, percentages of dead lymphocytes were read on a Leitz Patimed (Leitz) and calculated using Patimed-Software (version 4.0) from raw two-colourfluorescence data. Monoclonal antibody W4/W6, recognizing a pan leukocyte marker, served as our standard positive control and fresh culture medium served as negative control (23).

FACS Analysis to Study Downmodulation

A total of 300.000 T-cells were incubated with ChAglyCD3 (10 mg/ml) or daclizumab (10 mg/ml) for three and eight hours at 4 and 37 °C. At 4 °C downmodulation is blocked, while the degree of downmodulation can be tested at 37 °C. Thereafter, treated T-cells

were incubated for 30 minutes with 1:25 FITC-labeled rabbit-antihuman F(ab')₂ IgG, antibody (200mg/l) (Dako A/S, Denmark) to determine the amount of ChAglyCD3 or daclizumab bound on the cell surface. Staining intensity of FITC-labeled rabbit-antihuman immunoglobulins was determined by FACS analysis.

FACS Analysis to Test Induction of Apoptosis

To test apoptosis induction fresh cultured PM1 11 T-cells with and without 5% T-cell growth factor (TCGF) were used. T-cells were stimulated with TCGF to circumvent interference with TCR signalling by ATG and ChAglyCD3. Caspatag Fluorescein Caspase (VAD) Activity Kit was used according to manufacturer's protocol (Intergen Co., Purchase, USA). Briefly, GAD65-specific T-cells were incubated with ATGf and ATGm (100 mg/ml), ChAglyCD3 (10 mg/ml), or daclizumab (10 mg/ml) for up to 48 hours. Cells were stained with caspatag for 1 hour at 37°C and washed twice with washbuffer. Caspatag and Propidium Iodide staining was measured by FACS.

Cytokine assay

The Cytometric Bead Assay human Th1/Th2 cytokine kit (Becton Dickinson Biosciences, San Diego, CA) was used according to the manufacturer's instructions. Supernatant (50µl) was harvested after 24hrs of incubation of autoreactive T-cells stimulated with islet epitope presented by dendritic cells and tested for the amount of IFN γ , TNF α , IL-2, IL-10, IL-5, and IL-4.

RESULTS

Inhibition of GAD65-peptide induced T-cell proliferation.

Both humanized monoclonal antibodies (ChAglyCD3 and daclizumab) inhibited the proliferative response of autoreactive T-cells against GAD65 peptide in a dose-dependent manner, where ChAglyCD3 showed complete inhibition of proliferation. The decreases of proliferation for both ChAglyCD3 and daclizumab were significant (p-values were 5.4×10^{-7} and 4.5×10^{-3} , respectively).

In contrast, neither polyclonal rabbit antibody therapies (ATGf and ATGm) inhibited proliferation at trough levels (25,26). Notably, a nonspecific stimulation was measured after treatment with high concentration ATGm, suggestive of a mitogenic effect (Figure 1).

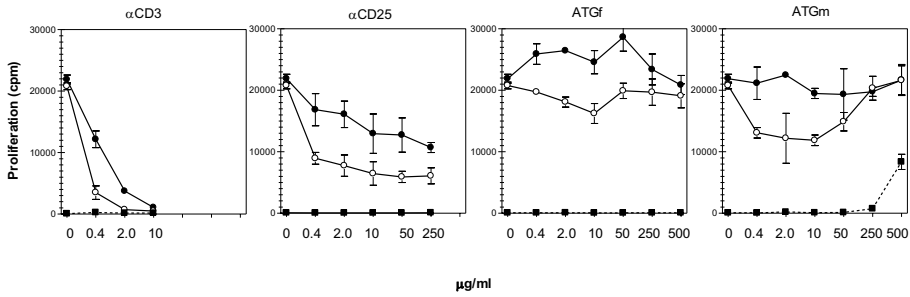


Figure 1. Proliferation assay: Dose-dependent effect of antibody therapy on antigen specific T-cell proliferation after 24 hours of stimulation without GAD65 (black squares), or with 0.04 µg/ml (open circles) and 0.2 µg/ml (filled circles) GAD65 peptide presented by dendritic cells. Antibody concentration is depicted on the x-axis. The mean ± standard deviation are shown. The experiments were repeated three times.

Antibody-Dependent Cell Cytotoxicity

The ADCC assay measures the ability of an antibody to recruit cellular CD16 (Fc receptor)-positive effector cells to perform killing of sensitized target cells. Antibody-dependent cell cytotoxicity was found only after treatment with the polyclonal antibodies (ATGf and ATGm) even at low effector/target ratio, while ChAglyCD3 and daclizumab did not induce ADCC in a standard 4-hour incubation period (Figure 2). The lack of ADCC in ChAglyCD3 is as expected from mutagenesis of the Fc region (19).

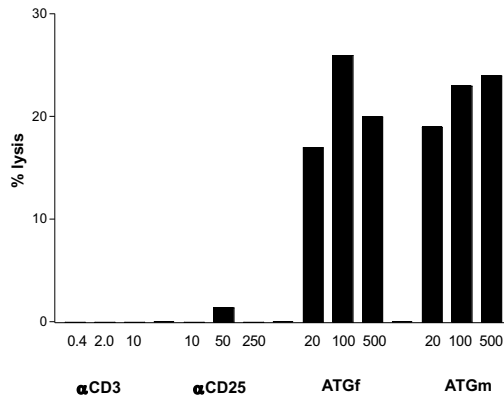


Figure 2. ADCC assay: Percentage of lysis due to antibody dependent cell cytotoxicity after antibody therapy. The mean are shown. The experiments were repeated twice.

Complement-Dependent Cell Lysis

In a complement-dependent cytotoxicity assay, polyclonal antibodies (ATGf and ATGm) elicited cell lysis of T-cells after 1 hour of incubation. In contrast, neither ChAglyCD3 nor daclizumab elicited complement-mediated cell lysis of the target cells (Figure 3). The lack of complement activation in daclizumab and ChAglyCD3 is as expected from the altered Fc region of both humanized monoclonal antibodies as described previously (19,27).

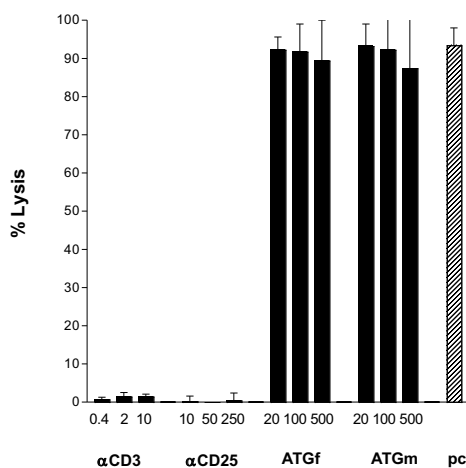


Figure 3. CDC assay: Percentage of lysis due to complement dependent cytotoxicity after antibody therapy. Monoclonal W4/W6 was used as a positive control (pc). The mean \pm standard deviation are shown. The experiments were repeated twice.

Modulation of Expression

Modulation was defined as internalisation and/or shedding of the antibody-receptor complex. The extent of monoclonal antibody binding to T-cells was measured by counterstaining with rabbit-antihuman F(ab')₂ IgG. The amount of ChAglyCD3 bound to the cell surface of GAD65-specific T-cells was decreased after 8 hours of incubation at 37°C compared to incubation at 4°C. For daclizumab-treated T-cells, decreased staining of daclizumab was found at 37°C in approximately half of the T-cells, which is indicative of antigenic modulation, but the staining intensity for the unmodulated cells was equal at 37 and 4°C (Figure 4). Both humanized monoclonal antibodies were demonstrated to have modulatory characteristics as defined above.

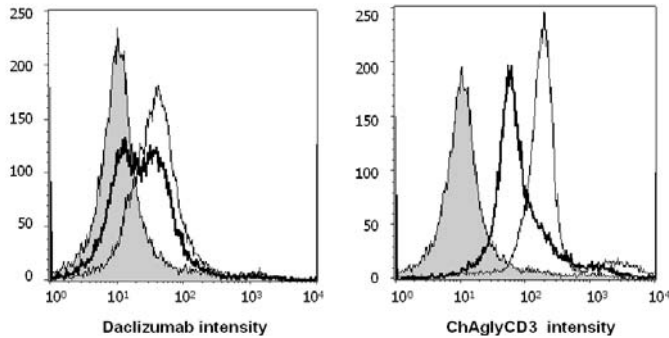


Figure 4. Modulation of receptor expression: The cell surface staining intensity of daclizumab and ChAglyCD3 on autoreactive T-cells after 8 hours of incubation at 37°C (thick lines) or 4°C (thin lines) compared to background (untreated cells; gray). This experiment was repeated once.

Apoptosis

Since proliferating T-cell blasts have been reported to stain Annexin V, we used caspatag to determine apoptosis in antibody-treated T-cells. Apoptosis induction was found after treatment with the polyclonal antibodies only, while ChAglyCD3 and daclizumab treatment did not induce apoptosis (Figure 5). ATG showed different patterns in the first 12 hours and between 12 and 48 hours. Induction of apoptosis was detected within 8 hours, whereas relatively more viable cells than untreated cells were present. Since the extent of apoptosis was normalized for cell death in untreated cells, it is likely that apoptosis continues in T-cells treated with polyclonal antibodies after 12 hours but that cell loss due to death by neglect in the untreated and unstimulated T-cells occurs to a relatively larger extent. In T-cells stimulated with T-cell growth factor death by neglect in the untreated T-cells was avoided. Even under activated conditions the monoclonal antibodies did not induce apoptosis, whereas ATG showed a sustained effect on stimulated T-cells compared to the early and temporary response in unstimulated cells (Figure 5).

Cytokine Assay

Both ATG preparations showed comparable response patterns. ATG treatment led to cytokine secretion of all cytokines tested. ChAglyCD3 reduced IFN γ and IL-5 secretion and elicited a slight but significant increase of IL-10 only ($p = 0.048$). ATGf and ATGm elicited a significant increase in IL-10 ($p = 0.0048$ and $p = 0.0097$, respectively). Daclizumab showed a trend for reduced TNF α and increased IL-2 secretion. All percentages were normalised to the cytokine secretion levels of T-cells triggered with their islet autoantigenic epitope presented by dendritic cells (Figure 6).

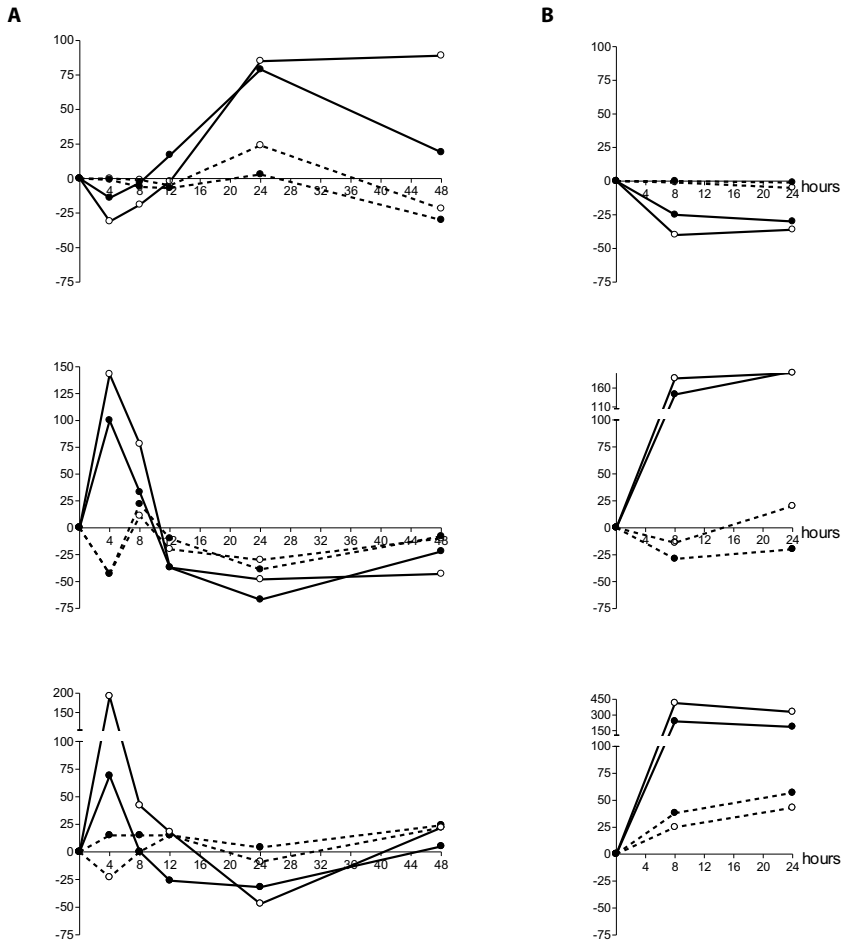


Figure 5. Apoptosis assay: Percentages of T-cell viability and death during treatment with ATGf (filled circles, straight line), ATGm (open circles, straight line), ChAglyCD3 (open circles, dotted line) and daclizumab (filled circles, dotted line) that were normalized for cell death in untreated cells (death by neglect), since the viability of untreated cells changes in time, as depicted. Percentages indicate the fractions of cell viability (or death) compared to untreated cells. The proportion of viability/death in untreated cells at each time point was set as baseline. Plotted values of treated cells represent the percentage difference from untreated cells. This experiment was repeated once. (A) Unstimulated autoreactive T-cells; (B) Autoreactive T-cells nonspecifically stimulated with T-cell growth factor.

DISCUSSION

We here demonstrate that the mechanism of action differs for these particular humanized monoclonal antibodies compared to the polyclonal antilymphocyte antibodies from rabbit origin. Aglycosylated humanized ChAglyCD3 and daclizumab modulated surface

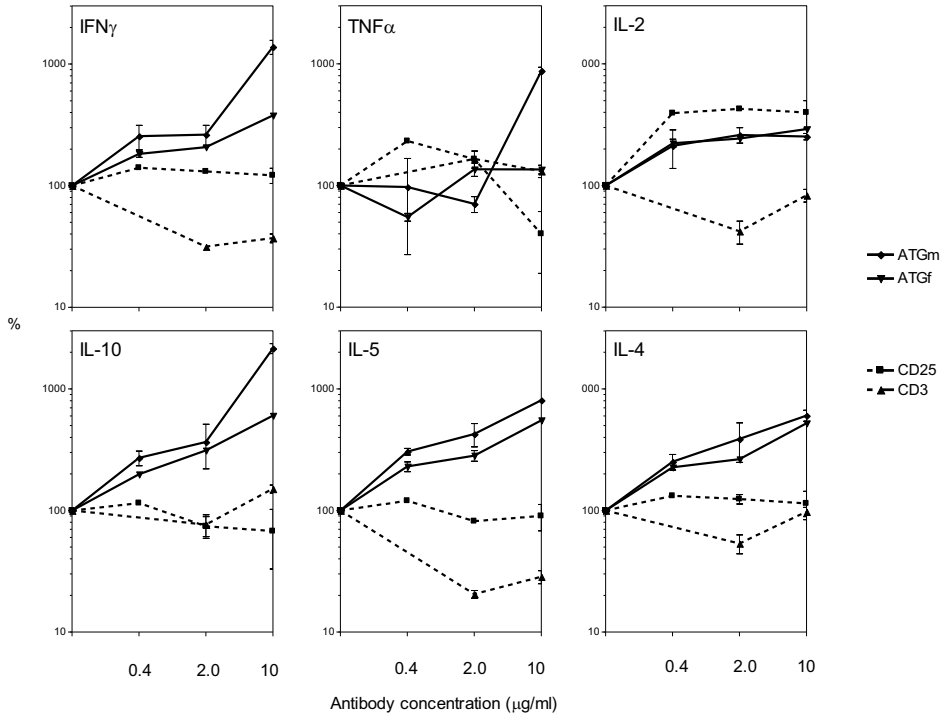


Figure 6. Cytokine production: cytokines levels were measured in supernatant of autoreactive T-cells stimulated with islet epitope presented by dendritic cells after 24hrs of incubation. To indicate changes in cytokine production related to treatment with antibodies, cytokine levels were normalized for the production of cytokines without antibody treatment. The mean levels (\pm SD) of cytokines by T-cells not treated with antibody were: IFN γ : 19,158 \pm 3,065 pg/ml, TNF α : 226 \pm 51 pg/ml, IL-2: 256 \pm 57 pg/ml, IL-10: 162 \pm 54 pg/ml, IL-5: 1,412 \pm 171 pg/ml, and IL-4: 571 \pm 115 pg/ml. Titration curves (0.4, 2.0, 10 μ g/ml antibody) are shown for all antibody therapeutics, experiments were repeated twice.

expression of target molecules and T-cell activity but did not deplete autoreactive T-cells, whereas, conversely, both ATGf and ATGm were shown to have several depleting characteristics. Furthermore, ATG treatment led to a burst of Th1 and Th2 cytokines, whereas ChAglyCD3 and Daclizumab led to a moderate and selective change in production of only a few cytokines that differed between these monoclonal antibodies. The consequences of these distinct modes of action for in vivo therapy are that ChAglyCD3 and daclizumab interfere with autoimmunity by modulation of T-cell activity, whereas ATG depletes T-cells regardless of their specificity and state of activity (Table 1).

The mechanism of action of antibody therapy in human type 1 diabetes has not been reported. Disease outcome after polyclonal and monoclonal antibody therapy was studied in type 1 diabetic pancreas transplant recipients and recent onset type 1 diabetes patients, respectively (7,9,11,14,26,28,29,30-32). Immunological effects were also studied in diabetes-related mouse models (13,33) and in in vitro transplantation settings (25-27,34,35).

Table 1.

Therapeutic	ADCC	CDC	Apoptosis	Inhibition of proliferation	Antigen modulation	Modulation of cytokine secretion	Projected in vivo outcome
ATGf	+	+	+	--	n.d.	Increases of all cytokines	Depletion
ATGm	+	+	+	--	n.d.	Increases of all cytokines	Depletion
aCD25	--	--	--	+	+	Increase of IL-2 Decrease of TNF α	Modulation
aCD3	--	--	--	+	+	Increase of IL-10 Decrease of IFN γ and IL-5	Modulation

This is the first study to compare the mechanism of various antibody therapies currently evaluated in clinical trials in type 1 diabetes using human islet autoreactive T-cells as target.

Polyclonal reagents like ATG have several drawbacks. As consequence of their production technique, polyclonal antibody preparations may vary in constituent antibodies. This unpredictability is associated with variable efficacy and adverse reactions that may differ between batches. There is an increased risk of infection and lymphoproliferative disease due to immunosuppression (36,37). The toxicity of any heterologous serum prepared against human tissue depends on its cross-reactivity with other tissue antigens and the induction of neutralizing antibodies. Although serum sickness is a potential complication of these preparations, this fortunately is rarely observed. Nonetheless, the presence of antibodies specific for non-T-lymphocyte antigens in polyclonal antibodies like ATG may cause thrombocytopenia, leucopenia, or anemia (36). This non-specific characteristic is also the reason that B-cells and regulatory T-cells are thought to be affected in addition to pathogenic T-cells. The depleting properties of ATG are documented in several clinical reports, but the mechanistic basis for this was not resolved (29,36). Our results in vitro are in agreement with those in vivo findings. In addition, we confirmed that ATG induces ADCC, CDC and apoptosis, comparable to in vitro alloreactive settings (25). This depleting nature combined with its ability to activate cytokine expression and its nonspecific polyclonal character might explain the immunological and clinical side effects seen with these antibodies. Two different ATGs were used to compare the effect of nonhumanized polyclonal antibody therapy with the different humanized monoclonal antibody therapeutics. ATG Fresenius is expected (according to the manufacturer) to be more selective for activated T-cells. We found similar results for both ATGs. This might be explained by the fact that our T-cell clone is activated and thus expresses the phenotype covered by both ATGs. At trough levels neither ATG showed inhibition of proliferation, but they elicited ADCC and CDC in a comparable fashion. In the apoptotic and cytokine assays a clear similarity was shown. This might reflect that

the nature of these antibodies, e.g., nonhumanized and polyclonal, is responsible for the comparable mechanism of action.

The apoptosis in T-cells stimulated with IL-2 by ATG treatment appears to be in discord with the marginal inhibition, and even enhancement, of antigen-induced T-cell proliferation following the same *in vitro* treatment. Nonspecific response in proliferation assays is a known phenomenon for high concentrations of polyclonal antibody therapeutics such as ATG. The main difference between stimuli in Figures 1 and 5 is the TCR triggering versus the IL-2R triggering, respectively. To test the direct apoptotic effect on autoreactive T-cells, the T-cells were tested with and without IL-2 addition. Antigen-presenting cells were not added, and their presence in the proliferation experiments of Figure 1 represents another variable. IL-2 alone has been shown to lead to activation-induced cell death. Apoptosis may be overruled in our proliferation assay by nonspecific stimulation of the T-cell in combination with adaptive immune responses.

Modulating characteristics are described for humanized antibody therapies. Pre-clinical studies in NOD mice on the effect of ChAglyCD3 indicated that regulatory T-cells were induced in antibody-treated mice. These 'protective' T-cells were found mostly in the CD4+CD25+ lymphocyte compartment. Transforming growth factor- β (TGF β) appears to have a central role in the long-term 'active-tolerance' phase (38). It remains to be determined whether TGF- β is essential as a mediator of regulation or whether it acts as a growth and/or differentiation factor for regulatory T-cells. Altogether, these data provide an explanation for the tolerogenic effects of CD3-specific antibodies in mice, rendering these antibodies a clinically applicable pharmacological agent that may stimulate immunoregulatory CD4+CD25+ T-cells. We here demonstrate that, in addition, these antibodies elicit a direct effect on pathogenic T-cells, which might also contribute to a long-term antigen-specific immune modulation. The precise signals that are transduced by CD3-specific antibodies that mediate their tolerogenic effect are not yet known (13). Whether these signals differ depending on the activation state or functionality of the target cell (pathogenic T-cells versus regulatory T-cells) also remains unresolved. Our data showing a modulatory mechanism of action of ChAglyCD3, without depleting characteristics, is in agreement with features previously described for aglycosylated antibodies (19,39). This nondepleting mechanism of action and changes in cytokine production towards an anti-inflammatory profile may in part explain their modulatory capacity, as shown previously (13). Inhibition of proliferation is clearly shown after ChAglyCD3 treatment. We speculate that the inhibition can be caused by the modulatory capacity of these antibodies (internalisation of antibody-receptor complex or shedding) or steric hindrance affecting the T-cell/APC interface.

The mechanism of action for daclizumab was previously studied in alloreactive *in vitro* settings only. Due to the differences in assays used in our study and other studies, our results cannot be compared easily. For instance, we used trough levels for the different

antibody therapeutics. Inhibition of proliferation by Daclizumab has been shown at the same concentration range as used in our study (27). In this study, CDC was inhibited in a comparable fashion, whereas ADCC was not affected (27). We speculate that differences in target cells contribute to this discrepancy (27,34). Our study is the first to demonstrate that daclizumab lacks depleting characteristics in an autoreactive setting. Since CD25 is expressed on activated lymphocytes, it might target pathogenic T-cells, in addition to activated B-cells and CD4+CD25+ regulatory T-cells. Although some of the properties described for ChAglyCD3 might also apply to daclizumab, the latter antibody displayed only partial inhibition of proliferation, and downmodulation of the antibody-receptor complex on a subpopulation of autoreactive T-cells. We speculate that the inhibition can be caused by shedding or internalization of antibody-receptor complex or by steric hindrance affecting the T-cell/APC interface.

The increased levels of IL-2 in the supernatant may have resulted from an impaired IL-2 consumption (under constant production) by inhibition of the IL-2R but could also explain the observed partial rather than complete inhibition in our proliferation assay. The ADCC results are in discord with a report claiming ADCC induction in daclizumab-treated leukemic cells in addition to inhibition of proliferation (27). We speculate that this apparent discrepancy is related to differences in target cells used in the ADCC assay. Leukemic cells that highly express CD25 may be more susceptible to ADCC than our GAD65 specific T-cells expressing moderate levels of CD25. Downregulation of the daclizumab-bound interleukin-2 receptor complex has been proposed as a mechanism of action. In kidney transplant recipients treated with daclizumab, a decline was found in the percentage of circulating lymphocytes expressing CD25 without an accompanying decrease in the absolute number of lymphocytes (31). This would be in concordance with our present findings *in vitro*.

Clinical use of antibody therapy is evolving to a more specific targeting of the pathogenic T-cells. ATG has been used for decades in transplantation settings and hemat oncology. The wide range of clinical and immunological side effects are acceptable in life-threatening diseases but hamper their use for diseases that are not immediately life threatening. In the current standard immunosuppressive treatment in islet transplantation polyclonal antibody induction treatment has been replaced by humanized monoclonal antibody therapy with daclizumab (30).

Due to increasing experience and success with humanized monoclonal antibody therapy in islet and pancreas transplantation settings, this therapeutic option gained interest as possible immunotherapy in recent onset diabetes. Currently, daclizumab is tested in recent onset type 1 diabetes in combination with mycophenolate mofetil (www.diabetestrialnet.org). The scientific basis for this approach is that CD25 is expressed by a proportion of the T-cells involved in T-cell-mediated autoimmune diseases (40,41). A European multicenter randomized placebo-controlled trial testing ChAglyCD3 at clinical onset of type 1 diabetes to preserve beta-cell function has recently been completed.

Evidence of clinical benefit of hOKT3gamma1 (Ala-Ala) (anti-CD3) and ChAglyCD3 monoclonal antibody therapy in recent onset type 1 diabetes has already been reported in phase I studies (7,14). Humanized monoclonal antibodies appear to be preferable for application in type 1 diabetes because of their precise and reproducible specificity and modulatory characteristics with milder side effects. Our data attest to this point. Consequently, humanized monoclonal immunotherapeutics may provide us with more selective and milder treatment opportunities to intervene in type 1 diabetes.

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