

Cellular and molecular changes accompanying the progression from insulinitis to diabetes

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Insulin-dependent diabetes mellitus (IDDM) is not a disease of unbridled destruction. The autoimmune attack on pancreatic beta cells has two distinct stages – insulinitis and diabetes – and progression of the former to the latter appears to be highly regulated. Identifying the factors controlling this transition has been difficult because it is a complex process that occurs non-universally and asynchronously. We have overcome these difficulties by coupling a simplified TCR transgenic (tg) model of IDDM and the immunosuppressive drug cyclophosphamide (CY). Young BDC2.5 TCR tg mice show insulinitis but not diabetes; CY treatment provoked diabetes in 100 % of animals with rapid, highly reproducible kinetics. This allowed a detailed temporal analysis of changes in cellular organization and cytokine gene expression within the lesion. The monokines IL-18, IL-12 and TNF- α were pivotal, their induction occurring almost immediately and their coordinate action being required for the onset of aggression. Other cytokines with direct toxicity for beta cells, including IL-1- β , IL-6 and IFN- γ , were subsequently induced; in contrast, there was no cellular or molecular evidence of cell contact-mediated mechanisms of beta cell death.

Key words: Autoimmunity / Diabetes / NOD mice / TCR transgenic mice / Cyclophosphamide

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1 Introduction

Insulin-dependent diabetes mellitus (IDDM) is an autoimmune disease characterized by specific destruction of the insulin-producing beta cells of the pancreatic islets of Langerhans [1]. In both human patients and the NOD mouse, the disease has two distinct stages [2]. An occult phase, termed insulinitis, begins with invasion of the islets by leukocytes and may persist quite innocuously for a long period; the overt phase, diabetes, develops once most of the beta cells have been destroyed. As the progression from insulinitis to diabetes is neither immediate nor automatic, it is thought to be regulated; however, the controlling factors have remained largely elusive, obscured by the complexity of the disease.

In order to generate a simpler, more manipulable, model of diabetes, several groups have established lines of TCR transgenic (tg) mice expressing a receptor that recognizes an antigen naturally or artificially synthesized by

beta cells [3–9]. Our group has focussed on the BDC2.5 line, which expresses the TCR from a CD4⁺ T cell clone derived from a diabetic NOD mouse [10]. This TCR recognizes an islet-specific antigen associated with beta cell granules [11], and T cells that express it are diabetogenic upon transfer into young NOD recipients [12, 13]. BDC2.5 TCR tg mice develop a florid insulinitis between 2 and 3 weeks of age; however, on the NOD genetic background, the infiltrate remains harmless for 3 to 6 months before terminal beta cell destruction and the onset of diabetes, and this occurs in only a minority of animals [14].

In BDC2.5 transgenics, as in standard NOD mice, the exact age of diabetes development in a given animal is impossible to predict because there is large scatter in individual onset times; in addition, by the time the animal is clinically diabetic, the destructive phase of the autoimmune process is essentially over. These complications hamper the analysis of events immediately associated with the progression from insulinitis to diabetes. One means to circumvent this problem is to employ cyclophosphamide (CY), an immunosuppressive drug known to accelerate diabetes in a proportion of standard NOD mice after multiple injections [15–20]. According to many criteria, the induced disease mimics the spontaneous

[1 18879]

Abbreviations: IDDM: Insulin-dependent diabetes mellitus
tg: Transgenic

one [15–18, 21–23]. We hoped that by coupling the TCR tg model and CY treatment we could develop a system with significantly enhanced synchrony and reproducibility.

2 Results

2.1 CY-induced acceleration of the BDC2.5/NOD disease

BDC2.5 mice were injected with a single dose of CY at 5 to 8 weeks of age. All animals became diabetic less than 10 days after injection (Fig. 1A). The kinetics of disease induction were rather stereotyped, the great majority of animals becoming hyperglycemic on days 7 or 8 (Fig. 1B). Diabetes was not observed in transgene-negative littermates because insulinitis is absent or very mild in such animals at this age, and it is known that the disease-inducing effect of CY requires an established infiltrate [15, 16].

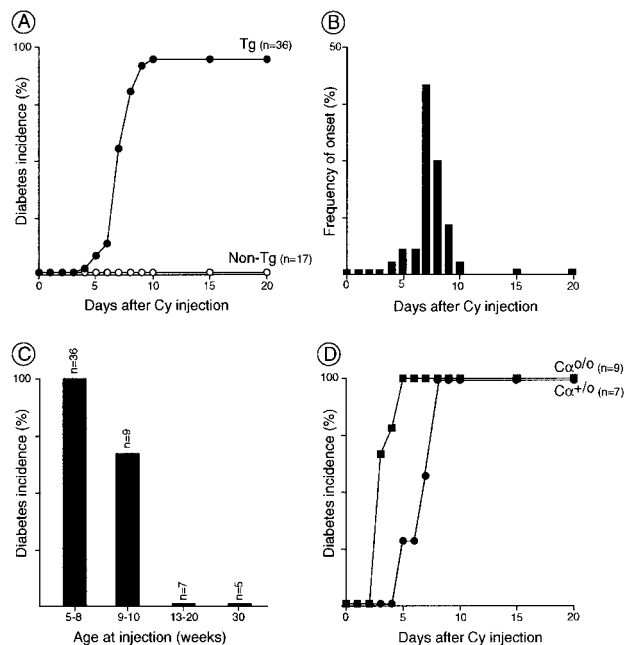


Figure 1. Effect of CY on the incidence of diabetes in BDC2.5/NOD mice. A. Five to eight week-old BDC2.5 tg and non-tg mice were injected intraperitoneally on day 0 with CY. Diabetes was monitored every other day for 20 days. B. The proportion of mice which became diabetic on each day after treatment. C. BDC2.5 mice of different ages were injected with CY and tested for diabetes over 1 month. The 30-week-old animals received two doses of CY two weeks apart and were followed for 3 months. D. Effect of CY on the development of diabetes in BDC2.5 mice lacking the endogenous TCR α chain ($C\alpha^{0/0}$), or their littermates ($C\alpha^{+/0}$).

Interestingly, older BDC2.5 mice were less susceptible to diabetes induction than were younger animals (Fig. 1C). Complete resistance was observed after 13 weeks, even though these animals exhibited marked insulinitis; disease could not be induced in 30-week-old animals even after two drug injections. Standard NOD mice also show reduced susceptibility to CY-induced diabetes [15].

It has been claimed that CY acts by preferentially killing suppressor T cells [16, 24]. Incomplete allelic exclusion of the TCR- α gene in BDC2.5 tg mice allows some T cells to rearrange endogenously encoded α chain genes and express a receptor other than BDC2.5 at the surface. To determine whether CY operates in the absence of such cells, we tested BDC2.5 mice carrying a TCR- α gene null mutation [25]. As shown in Fig. 1D, CY was even more efficient at provoking diabetes in the mutant animals, with an even earlier onset of diabetes. Therefore, CY does not require the recruitment of T cells displaying other $\alpha\beta$ TCR specificities to exert its effect.

Clearly, then, coupling of the BDC2.5 TCR tg model and CY treatment provides an attractive system for studying the progression of insulinitis to diabetes: disease onset is universal in young adults, with rapid and highly reproducible kinetics. We have exploited these characteristics for a detailed temporal analysis of the accompanying cellular and molecular events.

2.2 Cellular changes

Cellular alterations prompted by CY treatment of BDC2.5 transgenics were examined by both light microscopy and electron microscopy (EM). As detailed below, these studies revealed the progression from an organized, relatively innocuous insulinitis to one that, by day 5, was anarchic and destructive (Fig. 2).

On day 0, the light microscopic picture was typical of insulinitis in BDC2.5 mice carried on the NOD genetic background: an extensive infiltrate, but one that left a core mass of islet cells intact (panel A). The infiltrate had a uniform appearance, most cells exhibiting dense nuclei and little cytoplasm (panel B). Immunofluorescence of cryostat sections indicated that the infiltrating cells were mostly CD4⁺ T cells and B cells; the former were adjacent to the beta cells, while the latter resided at the periphery of the infiltrate; CD8⁺ T cells, macrophages and dendritic cells were scattered throughout (data not shown). EM showed the infiltrating cells to be organized in layers, with a thin connective membrane separating the inflammatory from the islet cells in many areas; when there was direct contact between the invading and resident cells, no particular signs of beta cell distress were

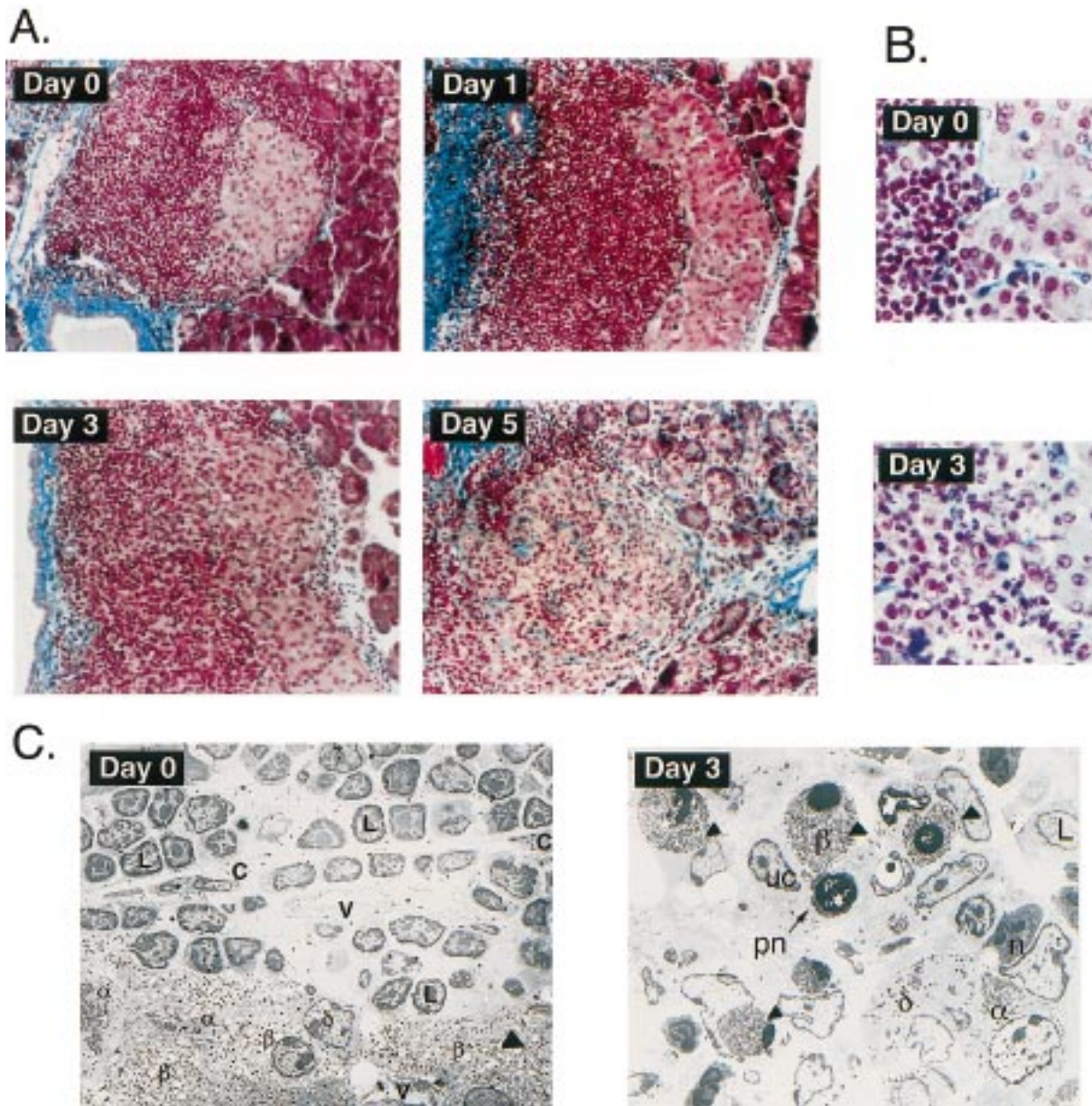


Figure 2. Histological examination of the pancreatic islets after CY administration. A. Islet sections from BDC2.5/NOD mice that were untreated (day 0) or treated with CY and analyzed 1, 3 and 5 days after injection (×125). B. Details (×500) of the zone of contact between beta and infiltrating cells in islets day 0 or 3 after injection. C. EM of islets at day 0 and 3 after injection (magnification ×3000). The day 0 micrograph shows a portion of an endocrine islet (at the bottom) containing numerous beta cells with intracytoplasmic secretory vesicles, characterized by a dense core and a peripheral lucent halo (caret), and numerous dilated rough endoplasmic reticulum cisternae. Note at the edge of the islet a delta cell with electron-dense secretory granules and, to the left, two alpha cells. At the periphery of the islet under the thin conjunctival membrane (C) surrounding the entire structure, numerous small lymphocytes (L) can be seen. At the top, the extralobular space is also invaded by lymphoid-like and fibrous tissue. V = blood vessel. On day 3, the beta cells involute massively; the necrotic beta cells are phagocytosed by neighbouring cells, perhaps undifferentiated beta cells. Necrotic remnants of the nucleus and beta secretory granules are still recognizable (caret). pn = picnotic nucleus; uc = undifferentiated cell. Several neutrophils (n) and lymphocytes (L) infiltrate the islet. At the bottom right, unaffected alpha and delta cells form an islet structure, well isolated from the damaged tissue.

evident, except for enlargement of the rough endoplasmic reticulum in places (panel C).

Little change was detectable on days 1 and 2 after CY treatment; histological preludes to diabetes onset were not evident until day 3. Light microscopy showed that the regular organization of the infiltrate had been lost, as had the demarcation between infiltrating and islet cells; also, most leukocytes had a more irregular shape (panels A, B). According to immunofluorescence analysis, the proportions of CD4⁺ T cells, other lymphocytes, and macrophages were not significantly altered (not shown). EM demonstrated distinct phenotypic changes in the infiltrating lymphocytes in the vicinity of the beta cells, e.g. a decreased nucleocytoplasmic ratio and the formation of pseudopodia (panel C). Neutrophils were found in some sections. Furthermore, the thin membrane separating regions of the inflammatory infiltrate from the beta cells appeared to have been breached, and the first indications of beta cell destruction were evident, cells in some islets showing clear signs of apoptosis, such as pycnotic nuclei and granulolysis (arrowhead in panel C). Examining multiple sections of multiple pancreata, we did not observe apoptotic or pre-apoptotic cells in intimate contact with T lymphocytes; rather, the degenerating beta cells were being engulfed by surrounding cells while alpha and delta cells remained intact. Marked signs of beta cell pathology like those described above occurred throughout the affected islets.

On day 5, most of the islets had lost their characteristic structure, being entirely taken over by inflammatory cells (Fig. 2, panel A). No recognizable beta cells remained, only a few alpha and delta cells, as confirmed by immunofluorescence with anti-glucagon and anti-insulin Ab (not shown). Beta cell loss was accompanied by edema in the connective spaces around the islets and blood vessels, separation of the surrounding acinar lobules, and some lesions in neighboring acinar cells. Slight fibrosis was first present at this time.

We also performed flow cytometric studies of T cells in CY-treated BDC2.5 mice (not shown). We did not detect any clear phenotypic changes in spleen, lymph node or intra-islet lymphocytes – no signs of widespread T cell activation (increase in CD69, CD25, CD44; loss of CD62L), nor of increased proliferation (save for a temporary drop in the number of proliferating cells at days 1 and 2, consistent with the known toxicity of the drug for dividing cells [26].) Thus, disease induction by CY is not associated with widespread systemic lymphocyte activation.

2.3 Molecular changes

To investigate the molecular alterations accompanying islet destruction, we quantitated cytokine mRNA levels in whole islet RNA by PCR analysis, prior to and at different times after CY administration. The results are summarized in Fig. 3A, where each point represents a titration value from an individual mouse. We initially concentrated on day 3 after injection, the time at which histological changes were first visible. It was impossible to go beyond this time-point because we could not recover enough islets from individual mice, no doubt reflecting the advanced state of islet destruction. Each treated mouse was directly compared with an untreated littermate. A single preparation of splenocyte RNA (whole spleen or Con A-activated) was used as a positive control for amplification and for comparison between experiments. Internal standards were thymidine kinase (TK), a “housekeeping gene”, and Thy-1, chosen to monitor gross changes in T cell representation; RNA levels for TK and Thy-1 were quite similar before and 3 days after drug treatment.

Levels of mRNA for seven cytokine genes – IL-18, IL-12p40, TNF- α , IL-1 β , IL-6, IL-2 and IFN- γ – were induced on day 3 after CY administration. This was true for all day 3/day 0 pairs, although there was substantial variation in the actual mRNA levels attained. The increase was highest for IL-6, IL-2 and IFN- γ , with average increases between 8- and 18-fold. IL-4, IL-10 and TGF- β gene expression was low prior to drug treatment and was minimally changed. Levels of inducible nitric oxide synthetase (iNOS) mRNA were already significant before treatment and did not change markedly.

The kinetics of induction were investigated for those genes whose transcript levels were elevated on day 3 after treatment (Fig. 3B). There appeared to be two patterns: some genes like IL-18, IL-12p40 and TNF- α showed increased expression already 1 day after drug injection, while expression of other genes such as IL-1 β , IL-6, IL-2 and IFN- γ was not augmented until 2 days later.

We also investigated the expression of Fas, recently suggested to play a role in the autoimmune destruction in NOD mice [27]. There was a slight increase in Fas mRNA levels on day 3 after CY injection (2.7-fold when averaging values from all mice), but an augmentation occurred in only half of the treated animals vis a vis their paired untreated littermates. In addition, Fas mRNA was visualized by *in situ* hybridization of pancreas sections at days 0, 1, 3, and 5 after CY treatment (data not shown). Fas transcripts were not detected in the beta cells, themselves, and there was only a low and constant expres-

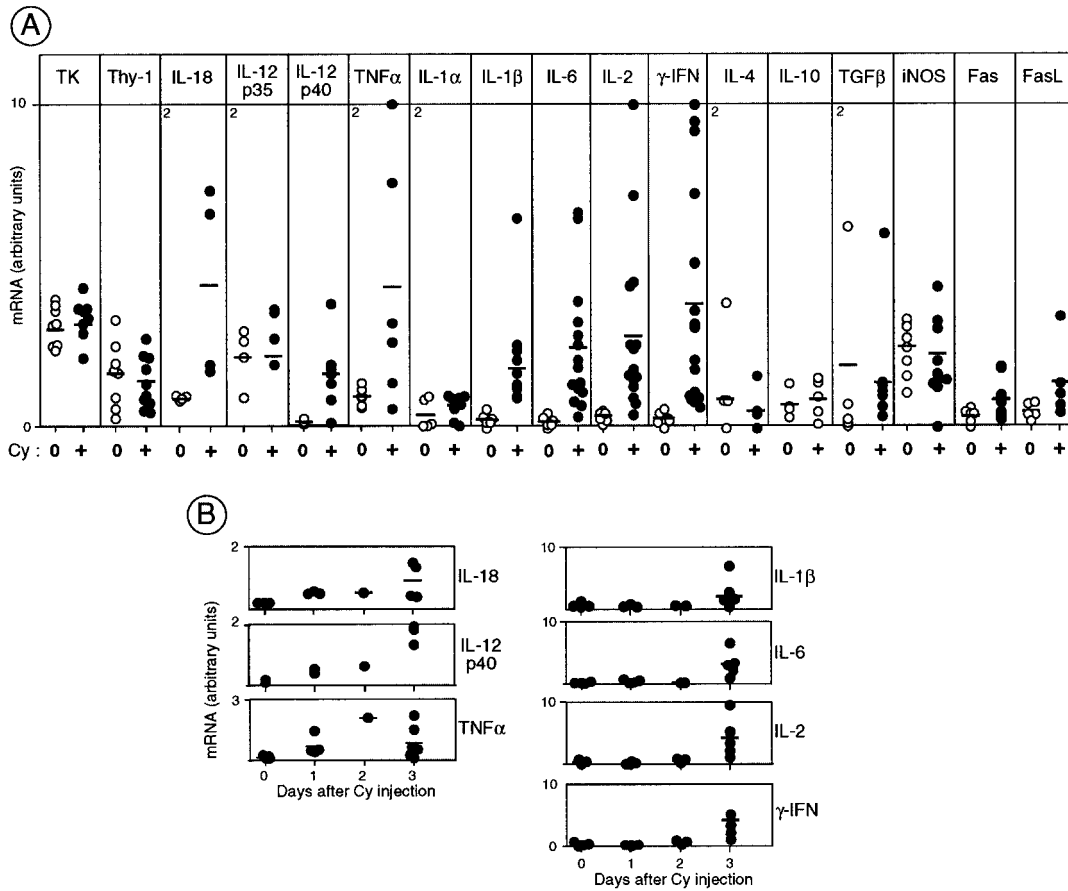


Figure 3. Cytokine mRNA expression in the pancreatic islets before and after administration of CY. **A.** Between 4 and 16 mice of each group (untreated (0) and 3 days after treatment (+)) were analyzed as littermate-pairs for the level (in arbitrary units) of different mRNA in the islets. Several dilutions of cDNA were amplified and each point was calculated from the quantity of PCR product obtained in two to three dilutions of cDNA in the pseudo-linear phase of the PCR where template quantity is limiting for the amplification. The value 2 indicated in certain cases represents a five-fold expansion of the scale. The bars represent the mean value for each cDNA species. **B.** Cytokine mRNA expression 0, 1, 2 and 3 days after CY injection, evaluated as for panel A.

sion in the infiltrating cells, arguing against the notion that a significant induction of Fas is required for beta cell destruction in the BDC2.5 model.

2.4 Cytokine blockade

To evaluate the significance of some of the molecular changes observed, we performed Ab blocking studies, injecting anti-cytokine reagents during the first 3 days after CY administration. Whereas all of the control mice (treated either with PBS or irrelevant Igs) became diabetic, induction of disease was substantially reduced in animals treated with anti-IL-12 and -TNF- α Ab, and partially reduced with anti-IL-1 β (Fig. 4). Anti-IFN- γ delayed the onset of diabetes after CY treatment but in most animals was unable to completely halt the disease process.

Anti-IL-6 had essentially no effect; neither did anti-IL-4 (not shown).

Histological analysis revealed that blocking IL-12, TNF- α or IL-1 β prevented the previously noted CY-induced disorganization of the islets (data not shown). We asked whether inhibiting these three cytokines influenced the increased cytokine mRNA levels (Fig. 5). Blocking IL-12 and TNF- α had clear effects, abrogating or dampening the increase of all transcripts except those for IL-18. In contrast, anti-IL-1 β treatment, although inhibiting diabetes induction, did not halt the increase of any of the cytokine transcripts.

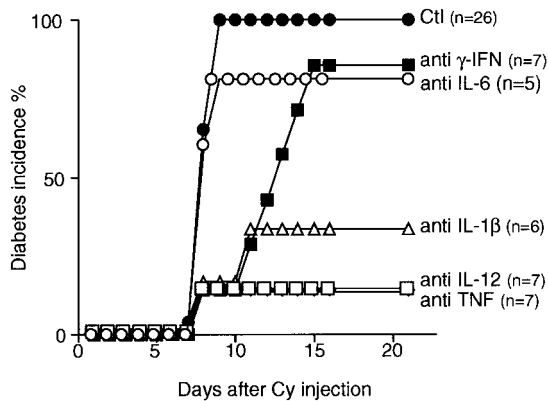


Figure 4. Effect of Ab blocking of cytokines on the incidence of CY-induced diabetes. BDC2.5 mice were injected at day 0 with CY and between day -1 and 2 with blocking Ab for IL-12, TNF- α , IL-1 β , IL-6, IFN- γ . The numbers of mice treated are indicated in brackets. Control mice (Ctl) were injected either with PBS ($n = 12$), rat IgG as ascites or purified antibody ($n = 10$), or rabbit IgG ($n = 4$).

3 Discussion

3.1 A good system?

CY treatment of BDC2.5/NOD transgenics provides a powerful system for studying the terminal events of IDDM. Progression from insulinitis to diabetes is provoked in 100 % of injected animals, initiates rapidly, and takes place essentially within a 3-day time window. This con-

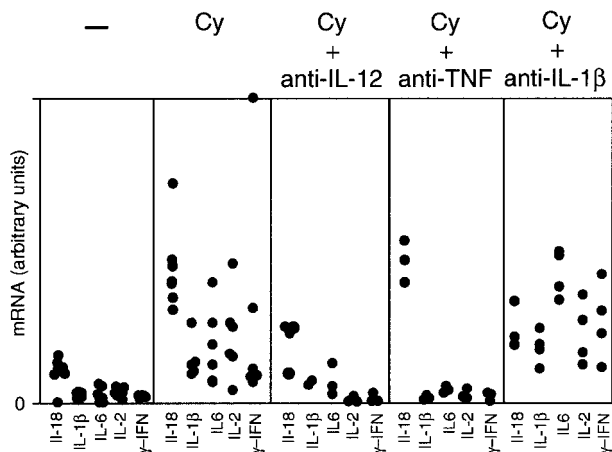


Figure 5. Effect of antibody blocking of cytokines on the induction of mRNA for other cytokines. Transcription was quantitated in seven untreated mice (-) and at day 3 after injection in 6 CY-treatment mice (CY), and 3 to 4 mice treated with CY and anti-IL-12 (CY + anti-IL-12), anti-TNF- α (CY + anti-TNF) or anti-IL-1 β (CY = anti-IL-1 β).

trasts with the months required for this transition in only a fraction of individuals in untreated NOD and BDC2.5 mice, and the weeks in CY-treated NOD animals.

Nonetheless, it is important to consider to what extent CY-induced diabetes mimics the spontaneous disease. This drug's preferential destruction of cycling cells does appear rather artificial. However, many previous reports have documented a close resemblance between the induced and spontaneous diseases, citing similar pathology, leukocyte cell-type requirements and transferability, as well as dependence on some of the same idd loci [15–18, 21–23]. Thus, it is likely that many conclusions concerning the induced disease will prove generalizable.

3.2 Cellular and molecular changes

At no time after treatment was there evidence of widespread lymphocyte activation in the peripheral lymphoid organs. This suggested that the heightened aggressivity of the BDC2.5 disease was probably not a systemic phenomenon, but rather that CY destabilizes the local regulatory balance in the pancreatic islets.

Within the islets, there was a striking evolution in the organization of the lesion. The infiltrate in untreated BDC2.5 mice was active, containing a substantial number of cycling cells and numerous activated CD4⁺ T cells [4, 28], but appeared quite innocuous. Starting 3 days after CY treatment, the infiltrate began to look much more aggressive, signalled by greater intermingling of invading and resident cells, the appearance of apoptotic bodies, and eventually edema and fibrosis. A similar dichotomy has been noted before with the BDC2.5 model – in BDC2.5 mice untreated vs. treated with an anti-CTLA-4 antibody [29], and in BDC2.5/NOD vs. BDC2.5/B6⁹⁷ animals [14]. It is tempting to draw an analogy between the innocuous insulinitic lesion, with its organized structure and lymphocyte partitioning and standard peripheral lymphoid organs, and to wonder whether the internal organization of the lesion, like that of the lymphoid organs, will prove to depend on specific cytokines [30] and chemokines [31].

Accompanying the evolution in the behavior of the inflammatory infiltrate were changes in the expression of certain cytokine genes. A clear induction in mRNA transcript levels was observed for several inflammatory cytokines, occurring in two waves. mRNA for “first-wave” cytokines (IL-18, IL-12p40, TNF- α) were detectably increased already 1 day after CY injection, and Ab blockade of these cytokines (when tested) inhibited essentially the whole process – the histological alterations, the up-

regulation of most other cytokines, and the onset of diabetes. In contrast, transcripts for the “second-wave” cytokines (IL-1 β , IL-6, IL-2, IFN- γ) were not detectably augmented until 3 days after drug administration, and Ab blockade generally had no or only moderate effects on any aspect of the process.

Three points concerning the waves of cytokine gene induction merit special comment. First, the first-wave cytokines are produced primarily by APC, *i.e.* are rather monokines than lymphokines. IL-18, IL-12 and TNF- α are known to act synergistically to up-regulate IL-1 β and IFN- γ synthesis [32–34], and thus probably set off the destructive process. An initiating role for monokines is consistent with reports that ablation of macrophages abrogates CY-induced diabetes in standard NOD mice [17, 35], but we are so far ignorant of how CY might act to augment monokine synthesis. Second, the first- and second-wave cytokines form a complex network of interacting mediators. They often have overlapping functions, and influence each other's and their own receptors' synthesis [36]. It is rather paradoxical, then, that blocking individual players can have such a drastic effect. The explanation may be that a very delicate balance of the individual cytokines is required, or that the functions of different cytokines never completely overlap, as is becoming increasingly apparent for IL-12 and IL-18, for example [32, 33, 37]. Third, several of the CY-induced cytokines are known to have direct cytotoxic effects on beta cells, at least *in vitro* [38–40]. This is particularly true of those induced at day 3 (IFN- γ , IL-6, IL-1 β) so it might be that this set of second-wave cytokines is directly involved in the final effector mechanism(s).

Expression of other cytokine genes was little changed after CY treatment. The absence of substantial decreases in IL-4, IL-10 or TGF- β mRNA levels does not plead in favor of the popular notion that the controlled behavior of early insulinitis can be attributed to these cytokines' activity. However, it is consistent with the recent finding of little or no change in IL-4 mRNA levels in CY-treated NOD mice [20].

How do the changes in cytokine gene expression found to accompany diabetes onset in CY-treated BDC2.5/NOD mice compare with those observed in other diabetes models? Induction of inflammatory cytokines in CY-treated standard NOD mice has also been reported, and the significance of the increases tested functionally in a few cases [20, 41–48]. One difference between the two sets of results is that blocking of second-wave cytokines, in particular IL-6 and IFN- γ , appeared to have a more profound effect in NOD than in BDC2.5 mice. This might reflect divergence in the terminal events, but it is also possible that the greatly elevated number of autore-

active T cells in BDC2.5 mice makes it more difficult to achieve complete inhibition. A recent study on another TCR tg model of diabetes (CD4⁺ T cells recognizing on influenza hemagglutinin Ag artificially expressed on beta cells [49]) reported findings very similar to those on BDC2.5: diabetes was accompanied by a large increase in IFN- γ gene expression and only minimal changes in IL-4, IL-10 and Fas-L gene expression. However, in this model, there was a decrease, rather than increase, in TNF- α transcripts in whole islets. This may reflect the different nature of diabetes in the two cases – spontaneous vs. CY-induced, short vs. extended time of survival after disease onset.

3.3 Beta cell destruction

Precisely how beta cells are killed during the development of diabetes remains controversial [50]. EM revealed apoptotic beta cells in islets from CY-treated BDC2.5 mice, consistent with previous reports of apoptosis in islets from BDC.SCID [51] and regular NOD [52] animals. Numerous electron microscopic images showed no evidence of direct interactions between the invading leukocytes and the dying beta cells. While fleeting contacts could well have been missed, these observations, together with the demonstration that cytokines directly toxic to beta cells were strongly induced, while Fas expression on beta cells was only slightly augmented (if at all), suggest that beta cell destruction may not be mediated via direct cell-cell contact in the BDC2.5 model. This appears to be at variance with recent claims of an important role for cell contact-mediated killing via Fas/FasL or perforin in other models, based on reduced diabetes incidence after introduction of the relevant null mutation [27, 53, 54]. However, the reliance on Fas/FasL may have been at the level of insulinitis rather than diabetes [55], and the dependence on perforin was only partial [54]. It is also possible that the prevailing mechanism of beta cell death varies in the different models, Fas/FasL- and perforin-mediated killing being more a feature of CD8⁺ effector cells. In BDC2.5 mice, diabetes depends entirely on CD4⁺ T cells and so killing is less likely to depend on perforin, while the lack of implication of Fas/FasL is similar to what was found in another TCR tg diabetes model dependent on CD4⁺ cells [49].

4 Materials and methods

4.1 Mice

The BDC2.5 TCR tg line has been described [4], and has been backcrossed onto the NOD genetic background for 16 generations. Except when indicated, all animals were 5 to 8 weeks old.

4.2 CY-induced diabetes

CY (Sigma) dissolved in PBS was injected i.p. at 200 mg/kg. Diabetes was evaluated by measuring glucose levels in the urine, with confirmation by blood measurement. A mouse was considered diabetic when its level was above 300 mg/dl.

4.3 Histochemistry and electron microscopy

Insulinitis was evaluated on paraffin sections as previously described [14]. Anti-glucagon and -insulin Ab were kindly provided by Dr. B. Ziegler, Institute of Diabetes, Karlsburg, Germany [56]. For EM analysis, animals were terminally anesthetized, perfused with 4 % paraformaldehyde in PBS, and the whole pancreas was rapidly removed, immersed overnight in fixative (3 % glutaraldehyde in 0.1 M phosphate buffer, pH 7.4), cut into small fragments, postfixed in 1 % osmium tetroxide, dehydrated in graded ethanol and embedded in Araldite-Epon. Islets were detected with the light microscope on semi-thin sections. Ultrathin sections were prepared from selected zones, contrasted with uranyl acetate and lead citrate.

4.4 RT-PCR on isolated islets

Islets were hand-picked after limited digestion of the pancreas with collagenase P (Sigma) and were mixed with HeLa cells to provide carrier RNA (50 000 HeLa cells per islet). Twenty islets were disrupted in 1 ml of 4 M guanidine thiocyanate, 5 mM sodium citrate, 0.1 M 2-ME, 0.5 % N-lauryl sarcosine with the help of a Dounce homogenizer. After addition of sodium acetate to 0.2 M, RNA was purified by phenol/chloroform extraction and isopropanol/ethanol precipitations.

After reverse transcription with AMV reverse transcriptase (Pharmacia), PCR was performed on 10 µl of fivefold serial dilutions of the cDNA – amounting roughly to 5000, 1000, 200, 40 and 8 cell equivalents (assuming 10 000 infiltrating cells per islet), and correct amplified product was quantitated by radioimaging (Fuji) after Southern blot hybridization. The oligonucleotides used for amplification and detection can be found on the Website: <http://www-igbmc.u-strasbg.fr/cbdrm>. None of the PCR primers amplified the corresponding band from the human RNA used as carrier. For each determination, a mean value was interpolated from two to three values within the linear part of the amplification. To eliminate variations between experiments, mostly due to differences in the time of exposure, the results were homogenized by comparing the positive controls and the values obtained in untreated mice.

4.5 Ab blocking

Ab were injected i.p. 1 day before and 2 days after CY injection, unless otherwise indicated. The following reagents were used: 1 to 2 mg of anti-IFN- γ (R4-6A2, Pharmingen), 1.5 mg of anti-IL-6 (MP5-20F3, Pharmingen), 500 µg of polyclonal rabbit-anti-IL-1 β ([46], a gift from C. Carnaud), 150 µl of anti-IL-12 ascites (a gift from G. Trinchieri), 400 to 600 µg of rat-anti-TNF- α (MP6-XT3, Pharmingen). Species-matched irrelevant sera or purified Ab were used as controls.

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