Acceleration of Autoimmune Diabetes by Cyclophosphamide is Associated with an Enhanced IFN-\(\gamma\) Secretion Pathway

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Introduction

Cyclophosphamide (CY), an alkylation cytostatic drug, is known for its ability to accelerate a number of experimental autoimmune diseases including spontaneous diabetes in NOD mice. The mechanism(s) by which CY renders autoreactive lymphocytes more pathogenic is largely unknown, but it has been postulated that the drug preferentially depletes regulatory (suppressor) T cells. It has been suggested that in cell-mediated autoimmune diseases, Th2-like lymphocytes secreting IL-4 and/or IL-10 provide protection, while Th1-like cells secreting IFN-\(\gamma\) are pathogenic. In this study, we analysed the effects of CY on autoimmune diabetes and cytokines in two mouse models: the spontaneous diabetes of NOD mice and the diabetes induced in C57BL/KsJ mice by multiple injections of low dose streptozotocin (LD-STZ). In both models, CY induced severe lymphopenia and accelerated the progression to hyperglycemia. This was associated with changes in splenic cytokine patterns indicating a shift towards the IFN-\(\gamma\)-secreting phenotype. We provide here evidence that IFN-\(\gamma\) producers are relatively resistant to depletion by CY and that Th0 clones can be shifted towards Th1. However, direct exposure of T lymphocytes to CY may not be a necessary condition for exacerbation of diabetes; NOD.scid mice treated with CY before adoptive transfer of NOD splenocytes developed diabetes at a higher rate than did controls. Thus, the acceleration of diabetes by CY seems to be a complex event, which includes the relatively high resistance of IFN-\(\gamma\) producers to the drug, their rapid reconstitution, and a Th1 shift of surviving T cell clones.

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diabetes of NOD mice. The data presented below show that CY augments the severity of the disease in both models. Analysis of cytokine secretion demonstrates that IFN-$\gamma$ producers are relatively resistant to the cytostatic action of CY and that a possible mechanism for this resistance may be a shift of individual Th0 clones towards the Th1 pathway. On the other hand, augmentation of the diabetogenic properties of the lymphocytes may not require a direct exposure of T cells to CY, suggesting a role for accessory cells in the acceleration of IDDM.

Materials and Methods

Animals

C57BL/KsJ (KsJ) mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). NOD and NOD.scid mice were bred at the Weizmann Institute Animal Breeding Center from the breeders obtained from The Jackson Laboratory, and were housed in a specific pathogen-free environment. The incidence of IDDM in our NOD colony is about 80% in females and about 30% in males by 28 weeks of age. All the mice used in the experiments were males, 6–8 weeks old, except diabetic NOD females, 24 weeks old, which were used as donors for adoptive transfer of the disease.

Reagents

Tissue culture media RPMI-1640 and PBS were obtained from the Weizmann Institute Biological Services. HEPES and fetal calf serum (FCS) were purchased from Biological Industries (Beit Haemek, Israel). Cyclophosphamide was bought from Sigma (St. Louis, MO, USA) and Streptozotocin was obtained from Boehringer Mannheim (Mannheim, Germany).

Matched pairs of monoclonal rat anti-mouse cytokine antibodies obtained from Pharmingen (San Diego, CA, USA) were JES6–1A12 (pure) and JES6–5H4 (biotinylated) anti-IL-2; BVD4–1D11 (pure) and BVD6–24G2 (biotinylated) anti-IL-4; JES5–2A5 (pure) and SXC-1 (biotinylated) anti-IL-10; C15.6 (pure) and C17.8 (biotinylated) anti-IL-12; R4–6A2 (pure) and XMG1.2 (biotinylated) anti-IFN-$\gamma$. For TNF-$\alpha$ determination, goat anti-mouse mouse TNF-$\alpha$ (R&D Systems, Minneapolis, MN, USA) was paired with biotinylated rabbit anti-mouse TNF-$\alpha$ (Pharmingen). Anti-mouse CD3$\varepsilon$ antibody for in vitro activation and in vivo depletion of T cells was monoclonal hamster 145–2C11 (ATCC, Rockville, MD, USA). The antibody was purified from tissue culture supernatants by ammonium sulfate and dialysis, and was filter-sterilized. For FACScan analysis, FITC-labelled 145–2C11 and G235–2356 (hamster anti-TNP as a negative control) were purchased from Pharmingen.

Induction of IDDM by STZ

To minimize the direct toxicity of STZ on pancreatic $\beta$-cells, an ultra low-dose protocol was used [16]. Mice were injected ip with STZ dissolved in 0.5 M citrate buffer (pH 4.4) at a dose 30 mg/kg b.w. daily for five consecutive days (days −4–0). To confirm the immune-mediated nature of the disease, a group of 10 mice was treated with anti-CD3$\varepsilon$ antibody twice a week at a dose 150 $\mu$g/mouse ip during the first 2 weeks after the last injection of STZ. Control mice were treated with citrate buffer.

Acceleration of IDDM by CY

CY was administered at a dose 200 mg/kg body weight twice at an interval of 1 week, starting from day 0. To avoid a possible interaction of CY with STZ, the first CY injection was given 8 h after the last administration of STZ. Diabetes was monitored by bleeding the mice from the tip of the tail and measuring glucose using the 'Companion-2' blood glucose analyzer (MediSense, Waltham, MA, USA). Mice were considered diabetic if their blood glucose exceeded 250 mg/dl.

Adoptive transfer experiments

Spleen cells from diabetic female NOD mice (3×10$^7$ / mouse) were injected into male NOD.scid recipients pretreated either with CY or PBS, and the development of diabetes was monitored. In a separate experiment, NOD.scid mice were inoculated ip with a NOD-derived Th0 T cell clone C9–2 (8×10$^6$ cells/mouse). The C9–2 clone was a spontaneous variant of the original C9 clone that was diabetogenic when first isolated [17] and expressed a Th1 phenotype (in preparation). This CD4$^+$ clone proliferated and secreted IFN-$\gamma$ and IL-4 in response to human hsp60 and was not diabetogenic when injected in NOD.scid mice. Two weeks after the last CY injection clone cells obtained from the recipient spleens were tested for cytokine production.

Estimation of T cell depletion

Spleen cells were obtained from the mice before (day 0) and 3, 6, 9 and 12 days after administration of CY. Erythrocytes were lysed and the total number of cells per spleen was counted. The percentage of T lymphocytes was estimated by flow cytometry using FACSscan (Becton Dickinson, San Jose, CA, USA) by counting CD3$^+$ cells in a total of 10$^4$ cells. The software used was Lysis II.

Cytokine determination

ELISA assays for IL-4, IL-10 and IFN-$\gamma$ were performed according to a standard sandwich protocol.
Five million total spleen cells were cultured in 1 ml RPMI-1640 medium in 24-well plates pre-coated with the anti-CD3 antibody for activation. After 48 h of incubation (37°C, 5% CO2 in air), the supernatants were collected and tested for their cytokine content which was estimated from calibration curves using recombinant mouse IL-4, IL-10 or IFN-γ (all from PharMingen). The lower detection limits of the assay were 37 pg/ml for IL-4 and IFN-γ, and 75 pg/ml for IL-10. The linear part of the curve was within 75 pg/ml–0.62 ng/ml for IL-4, 0.31–10 ng/ml for IL-10, and 75 pg/ml–2.5 ng/ml for IFN-γ. The data are expressed as the amount of the cytokine secreted per 10⁶ T cells.

The content of the cytokines in the supernatants of non-activated spleen cells was below the detection limits of the ELISA. To detect spontaneous cytokine secretion, we used a gel substrate ELISPOT assay [19] as described previously [13]. The numbers of cytokine producers (spot forming cells; SFC) per spleen were calculated and their changes after CY administration were expressed as per cent of the respective SFC numbers in PBS-treated animals.

Statistical analysis for comparison of mean blood glucose values was done using the Kruskal–Wallis non-parametric ANOVA test. The incidence of IDDM was compared by Fisher’s exact test. The difference between the groups was considered significant if the P value was <0.05.

Results

CY accelerates spontaneous and LD-STZ-induced diabetes

In NOD males aged 8 weeks, two injections of CY rendered 50% of the animals diabetic within 2 weeks, while PBS-treated littermate controls remained normoglycemic (Figure 1A). In the absence of treatment with insulin, the disease accelerated by CY was associated with a high mortality.

In KsJ mice, IDDM induced by ultra-low dose STZ manifested a slow onset (Figure 1B): the majority of mice (78%) developed blood glucose levels above 250 mg/dl only by day 45 (mean 278.4 ±23.6). Values above 400 mg/dl were seldom seen and there was no mortality during the whole observation period (100 days). CY treatment increased the progression of the disease: 70% of the animals were sick by day 7 with a mean blood glucose of 265.5 ±19.2 mg/dl. The peak of the disease was on day 14 (80% sick, blood glucose 320.8 ±25.2), but individual glucose values rarely exceeded 400 mg/dl and all the mice survived during the observation period. Treatment with anti-CD3 antibody attenuated the development of diabetes, indicating the pathogenic role of T cells in this model (data not shown).

It was conceivable that exacerbation of hyperglycemia by CY in the LD-STZ model might be due to either increased autoimmunity against the islets, or to some added direct cytotoxicity of STZ on the β-cells. Any direct toxicity to β-cells would be expected to be detectable in the absence of T and B lymphocytes. Therefore, we treated NOD.scid mice, which lack T and B cells, with both STZ and CY using the same protocol as for the KsJ mice.

CY does not enhance the toxicity of STZ on islet β-cells

NOD.scid mice lack mature T and B lymphocytes due to the scid mutation and are insulinitis-free, so they never develop IDDM spontaneously, or following CY administration. Nevertheless, these mice are sensitive
to STZ at ultra-low doses [20]. When we treated the NOD.scid mice with LD-STZ followed by CY, the disease was not accelerated (Figure 2), suggesting that there is no synergistic effect of CY on â-cell toxicity when the combination of both drugs is used. On the contrary, the mice treated with STZ and CY appeared to be protected from the development of diabetes. We can conclude, therefore, that the acceleration of IDDM by CY in the LD-STZ model is probably immune-mediated. This is compatible with the hypothesis of preferential removal of suppressors by CY, a mechanism suggested in the NOD mouse model. Since suppressors in IDDM are believed to be Th2-like cells, we studied the changes in the splenic T cell content and cytokine profiles of NOD and KsJ mice following CY administration.

**CY induces spleen cell depletion followed by reconstitution**

CY induced a transient depletion of splenocytes detected by a decreased total MNC content of the spleen, most marked at day 3 (Figure 3). NOD mice rapidly restored the total cell content and CD3+ cells in the spleen by day 6. Similar to the NOD mice, CY-treated KsJ mice developed a loss of MNC and T cells by day 3. In contrast to the NOD mice, the KsJ mice did not restore completely the total number of splenic MNC, and the absolute numbers of T cells per spleen remained low even at day 12 after CY treatment. Note, however, that in both strains the depletion of total MNC (Figure 3, the upper panels) coincided with an increased proportion of CD3+ cells (the middle panels), suggesting that non-T cells are relatively more sensitive to CY than are T cells. During the reconstitution phase, the percentage of CD3+ cells decreased, indicating that non-T cells predominated in repopulation of the spleen.

**CY upregulates activated cytokine secretion**

Spleen cells of naive NOD mice secreted detectable amounts of IFN-γ and IL-10 upon activation via CD3 cross-linking, while IL-4 was below the detection limit of the assay (Figure 4, left panels). Subsequent to CY treatment, these cytokines were downregulated on day 3, at the peak of MNC depletion (Figure 3). Starting from the day 6, the amount of the cytokines secreted per 10^4 T cells significantly increased: IL-4 could be detected, and the amounts of IL-10 and IFN-γ exceeded the initial values many-fold.

Spleen cells of KsJ mice that had not been treated with CY secreted detectable amounts of IL-4, IFN-γ and IL-10 when activated (Figure 4, right panels). After CY treatment, all the cytokines were down-regulated on day 3. On day 6, the amount of IL-4 and IL-10 secreted per 10^4 T cells started to rise and, beginning from day 9, IFN-γ was upregulated. Thus, all the cytokines tested were downregulated shortly after CY administration and increased many-fold during the reconstitution phase.

The ability of T cells to secrete cytokines in response to activation in vitro reflects their potential; the real in vivo balance between the different cytokines is not measured under these conditions. We were interested, therefore, to learn whether CY differentially affects cytokine secretion by cells activated spontaneously in vivo.

**Spontaneous IFN-γ producers are relatively resistant to CY**

Since spontaneous secretion of the cytokines tested was below the detection limits of our cytokine ELISA, we used a more sensitive ELISPOT assay. As shown on Figure 5, spleen cells secreting IL-2, IL-4, IL-12 and TNF-α were downregulated shortly after CY treatment in both KsJ and NOD mice, coinciding with the depletion of MNC. IL-2 and IL-4 SFC were the most sensitive in both mouse strains, while cells secreting IFN-γ appeared to be the most resistant. During the reconstitution phase, IL-2 producers were the most rapidly regenerating cells in the NOD mice while, in KsJ mice, splenocytes secreting IL-4 and IFN-γ repopulated the spleen at the highest rate. The IFN-γ/IL-4 ratio on the days 0, 4 and 11 was 0.21, 6.5 and 0.77 in the NOD mice and 0.8, 8.3 and 12.7 in the KsJ mice, respectively. Thus, CY shifts the cytokine balance towards IFN-γ shortly after the administration of the drug, reflecting a relative sensitivity of IL-4 producers to CY-induced lymphodepletion.

![Figure 2. Effects of CY on IDDM induction by LD-STZ in NOD.scid mice. Groups of 10 mice were treated by STZ (30 mg/kg×5) at days −4–0, and then received either CY or PBS at days 0 and 7 (†). Blood glucose was monitored weekly. Data are means ±SE; *P<0.005. Two experiments gave identical results. Treatment: STZ (——); STZ+CY (—–).](image-url)
The resistance of IFN-γ producers to CY may be an intrinsic property of these cells, but it is possible that CY might shift preexisting Th0 clones towards a Th1 phenotype. To test this, we transferred a Th0 NOD-derived T-cell clone into NOD.scid mice and treated the recipients either with CY or with PBS. Figure 6 shows the changes in the amounts of IFN-γ and IL-4 secreted by the clone after the in vivo passage and re-stimulation in vitro. In contrast to the control PBS-treated group, the T cells from CY-treated NOD.scid mice displayed a complete disappearance of IL-4 and an increased IFN-γ secretion per 10^4 T cells. Thus, the greater pathogenicity of T cells from CY-treated NOD mice may result from a shift of ‘neutral’ Th0 clones towards the Th1 phenotype.
Acceleration of IDDM does not require a direct exposure of T cells to CY

In both mouse strains, CY administration results in spleen cell depletion followed by an influx of numerous non-T mononuclear cells (Figure 3) that may act as APC and provide signals favorable for IFN-γ secretion. To test the possibility that accessory cells might be directly involved in CY-accelerated diabetes, we treated male NOD.scid mice with CY first, and on day 2 reconstituted them with $3 \times 10^7$ spleen cells from diabetic NOD females. The development of IDDM was monitored. As shown in Figure 7, NOD SCID recipients pretreated with CY developed diabetes faster than PBS-treated control mice, suggesting that CY may increase the pathogenicity of autoreactive T cells indirectly. The recipients treated with CY after the transfer developed IDDM later than did the controls, reflecting depletion of the transferred spleen cells, and no ability to generate mature T cells of their own.

![Figure 4](image_url)  

**Figure 4.** Effect of CY on activated cytokine secretion by NOD spleen cells. At the indicated days after CY treatment, spleen cells were activated by anti-CD3 cross-linking, and cytokines were measured by ELISA after 48 h incubation. The values were calculated per $10^6$ T cells based on the percentage of CD3+ cells determined by FACS. Data are the means of duplicate wells from a representative of two separate experiments.

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Acceleration of IDDM in NOD mice by CY has been widely used as a tool to study effector mechanisms in \( \beta \)-cell destruction because CY provides a convenient synchronization of the disease process. The mechanisms of CY action on the autoimmune process, however, remain largely unknown. It has been clearly demonstrated that the effect of the drug is not due to a direct toxicity on the islet \( \beta \)-cells [8], and it was postulated that suppressor cells are more sensitive to CY; that is, the suppressors are either preferentially eliminated or regenerate at a lower rate [7, 8, 21]. The role of suppressors in preventing the clinical manifestation of IDDM has been suggested [21–23], but further phenotypic characterization of the regulatory subset has been difficult. It seems that CD4\(^+\) T cells are responsible for both pathogenicity and protection [9, 22–24], while CD8\(^+\) T cells appear to be necessary for the initiation of insulitis [25] and for acceleration of the ongoing destruction of the islets [26]. Hence, no direct evidence has been provided to support the hypothesis of CY-sensitive regulatory cells.

Another approach to discriminate between the pathogenic and the suppressor cells has been based on the dichotomy of the T-helper maturation towards the Th1 (secreting IFN-\( \gamma \)) or Th2 (secreting IL-4 and IL-10) phenotypes [27]. In IDDM, the pathogenic role of IFN-\( \gamma \) and the protective role of IL-4/IL-10 have been generally confirmed [10]. Pathogenic T cell clones capable of transferring IDDM are IFN-\( \gamma \) producers [28], the amount of IFN-\( \gamma \) in the lesion correlates with pathogenicity [13, 29], and treatment with anti-IFN-\( \gamma \) antibody provides protection [30]. In contrast, IL-4 and IL-10 are up-regulated in the non-destructive insulitis characteristic for mice protected from IDDM [29, 31], and systemic administration of Th2 cytokines [32] or their transgenic expression in the islet \( \beta \)-cells [33] or lymphocytes [34] can prevent diabetes. Indeed, the diabetogenic process in NOD mice could be arrested by the induction of a burst of specific Th2 reactivity using a peptide vaccine [35].

The aim of the present study was to test the preferential sensitivity of regulatory cells to CY by studying the cytokine profiles of spleen cells in two different conditions.
models of autoimmune diabetes. We could demonstrate that CY augments the severity of the disease in both the spontaneously diabetic NOD mice and KsJ mice rendered diabetic by multiple LD-STZ (Figure 1).

To confirm the autoimmune mechanism of LD-STZ diabetes, we ameliorated the disease by T cell depleting anti-CD3 mAb treatment. The problem, however, is that induction of diabetes by LD-STZ is associated with an initial toxic damage to islet cells [36, 37]. Subsequent administration of CY, another alkylating agent, could provide additional damage to islet cells and (or) to their precursors, resulting in a more severe hyperglycemia in KsJ mice. If this was the case, administration of both drugs would also be synergistic in immunodeficient mice, like the NOD.scid that carry the STZ-sensitive NOD background [20] and the scid mutation depriving them of mature T and B lymphocytes. Our present results show that a direct augmentation of STZ cytotoxicity by CY is unlikely, since CY did not accelerate the LD-STZ-induced IDDM in NOD.scid mice (Figure 2). On the contrary, CY appeared to provide resistance to IDDM—a new finding that cannot be easily explained.

The sensitivity of NOD/scid mice to LD-STZ-induced diabetes has been reported previously as an argument against a pivotal role of autoimmunity in β-cell destruction [20]. Indeed, the NOD.scid mouse may be extremely sensitive to the toxic effect of STZ because the scid mutation is associated with a defect in DNA repair [38], and it seems unlikely that CY can compensate for it. However, we could speculate that CY may inhibit male sex hormones, and this may render β-cells more resistant to STZ direct toxicity. Early studies of the LD-STZ IDDM model clearly showed that males are more prone to the disease [36, 39]. Interestingly, in the NOD mouse model, male islets also appear to be more susceptible to autoimmune attack than are female islets [14]. Additional studies are needed to test our suggestion, but the important conclusion at this point is that in KsJ mice acceleration of LD-STZ IDDM by CY is immune-mediated rather than toxic.

CY induces depletion of splenic MNC followed by reconstitution (Figure 3). The peak of depletion coincides with a relative increase in the proportion of CD3+ cells in the spleen, indicating that T cells are more resistant to the cytotoxic action of the drug than are non-T cells. During the reconstitution phase, the proportion of CD3+ cells decreases, indicating that the spleen is repopulated mainly by non-T cells. Which of these two phases, the depletion or the reconstitution, is responsible for acceleration of diabetes is uncertain. On the one hand, the IFN-γ/IL-4 ratio is the highest at the peak of the depletion reflecting a relative resistance of IFN-γ SFC to CY, and this short period may be sufficient for destruction of a critical β-cell mass resulting in accelerated hyperglycemia. On the other hand, depletion of the effector cells seems not to be necessary, since adoptively transferred IDDM is accelerated in NOD.scid recipients exposed to CY two days before the injection of spleen cells (Figure 7). The inability of NOD.scid mice to generate their own T and B lymphocytes strongly suggests the contribution of non-lymphoid bone marrow emigrants (macrophages and dendritic cells) in the acceleration of the disease.

Dendritic cells (DC) are known to be the most potent antigen-presenting cells [40], and their influx into the spleen may explain the increased cytokine secretion by polyclonally activated spleen cells on a per T cell basis (Figure 4), and the higher numbers of spontaneous cytokine producers per spleen (Figure 5). Myeloid but not lymphoid DC has been suggested recently to be responsible for the presentation of self-antigens in a pathogenic context [41]. Future studies will show whether myeloid DC have an advantage over other APC in repopulating the body after CY-induced depletion.

Acceleration of IDDM was associated with an increased IFN-γ/IL-4 ratio of spontaneous cytokine producers estimated as SFC in both models. This ratio was the highest at day 4 post CY, reflecting a higher resistance of IFN-γ producers to the depletion. The ratio remained higher than in non-treated mice by day 11, in spite of a rapid reconstitution of IL-4-secreting cells. These data fit the hypothesis of a relative resistance of pathogenic cells to CY in IDDM. Another possible mechanism might be a shift of non-depleted Th0 clones to Th1, as we showed using the adoptive transfer of an established Th0 clone to the NOD.scid mouse (Figure 6). It would be interesting to elucidate whether established Th2 clones, too, could be shifted into Th1 clones.

A decision for selecting the Th1 or Th2 pathway is made upon antigen presentation; several factors provided by APC were shown to be important, like B7 costimulatory molecules and IL-12 [42]. In this study, IL-12 producers detected in the spleen were sensitive.
to depletion by CY treatment (Figure 5), suggesting that newly generated IL-12-secreting cells (DC?) may be responsible for the shift towards IFN-γ production. A visible disagreement of our data with previously reported upregulation of IL-12 mRNA in the pancreas and the spleen 3 days after CY [43] could be due to differences in experimental design (one CY injection vs. two injections in this study) or readout (mRNA vs. protein product), or reflect a different sensitivity to CY in different NOD colonies (NOD/Bom vs. NOD/LtJ).

The spontaneous diabetes of the NOD mouse and the disease induced by LD-STZ are quite different autoimmune models (see [44] for the most recent comparative review). The major contrast between them is that only NOD diabetes can be adoptively transferred by lymphocytes into a MHC-matched immuno-compromised recipient. We were not able to transfer LD-STZ-induced IDDM from KsJ (H-2d) mice into C.B-17 scid (H-2q7) mice, or from NOR (H-2q7) into NOD.scid mice (H-2q7), even when the disease was accelerated in the donor by CY. For successful adoptive transfer, the recipient’s β-cells have to be modified by STZ treatment [45]. Therefore, in contrast to the spontaneous autoimmune diabetes of the NOD, the LD-STZ disease results from immunity to a ‘changed self’. Which of the models better reflects type 1 diabetes of the human is uncertain. A few cases of autoimmune diabetes of the human is uncertain. A few cases of non-obese diabetes-prone mice by cyclophosphamide. Diabetes 37: 1108–1112

In conclusion, our findings show that LD-STZ-induced IDDM in KsJ mice, like the spontaneous disease in NOD mice, is accelerated by CY and that this acceleration is associated with a systemic cytokine shift towards IFN-γ secretion pathway. A possible clinical relevance of CY-accelerated diabetes has been recently demonstrated in a case report of type 1 diabetes resulting from CY treatment of a patient with a lymphoma [48].

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