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Functional status of the immune system after chronic administration of 2'-deoxycoformycinin the BB rat

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Summary. Insulin-dependent diabetes mellitus (IDDM) is caused by autoimmune destruction of pancreatic beta cells with the primary mechanism being cell mediated. The BB rat develops insulitis and IDDM with many features analogous to the disease in man. In previous studies we reported that weekly administration of 2'-deoxycoformycin (dCF) for four months reduces significantly the incidence of IDDM in the BB rat by 70%, and that the animals remain free of diabetes for a minimum of two months after drug withdrawal. Since the diabetes-prone BB rat is lymphopenic, with a reduction of both CD4 and CD8 cells, the continuous failure of dCF treated animals to develop diabetes may have been due to generalized immunosuppression. To test this possibility, the ability of dCF treated diabetes-free BB rats to mount an immune response after challenge with Ovalbumin was examined five months after drug withdrawal. The results showed that the post-immunization levels of total IgG and specific IgG in these animals did not differ from those observed in non-dCF treated controls nor those of control diabetes-resistant non-lymphopenic BB rats. Moreover, FACS analysis indicated no change in the percentages or total numbers of CD4+ or CD8+ cells between the two groups of animals. Histological assessment of the pancreata of the post-dCF treated animals showed varying degrees of mononuclear cell infiltrates in the islets. These data demonstrate that treatment by dCF is not permanent, and may require intermittent or continuous administration to prevent development of diabetes. Further studies are needed to determine the mechanism of action of dCF in this model of IDDM.

Key words: Diabetes mellitus, Deoxycoformycin, Immune system

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Introduction

Development of insulin-dependent diabetes mellitus (IDDM) results from the autoimmune destruction of pancreatic beta cells, with macrophages and T lymphocytes playing an essential role (Lee et al., 1988; Boitard et al., 1991). Clinical studies with immuno-suppressive agents, such as cyclosporine, prednisone, azathioprine and anti-lyrnphocyte globulin, which have been used to prevent the progression of diabetes have produced significant adverse effects, most notable nephrotoxicity (Wilson and Eisenbarth, 1990; Martin et al., 1991). Moreover, clinical symptoms of IDDM redevelops in all patients within six months after discontinuation of drug therapy. Thus, at present there are no safe and efficacious drugs available to treat IDDM and prevent insulin dependency.

The purine nucleoside analogs fludarabine, 2-chlorodeoxyadenosine, and 2'-deoxycoformycin exhibit impressive activity in lymphoproliferative malignancies of adults and children (Slomiany et al., 1997). Their mechanism of action is not clear. Studies have suggested that their use is associated with significant myelo-suppression, immunosuppression, and in some circumstances, increased infection with viral and opportunistic pathogens (Johnston et al., 1988; Catovsky, 1996; Slomiany et al., 1997). We recently examined the immunosuppressive effects of 2'-deoxycoformycin (dCF) by assessing its ability to prevent induction of diabetes in the BB Wistar rat, a strain that spontaneously develops insulitis and IDDM, exhibiting many features similar to the human disease (Marliss et al., 1982; Castano and Eisenbarth, 1990). In that study, a linear dose effect was observed with protection against IDDM, and 80% of the animals remained free of diabetes at the highest dose tested, i.e, 10 mg/kg/wk two months following cessation of dCF treatment (Thliveris et al., 1997).

Strikingly, subsequent monitoring demonstrated that the protective effect persisted for at least five months following discontinuation of this drug. We speculated that the protective effect of dCF against IDDM is

mediated through induction of a profound, long lasting depletion of T cells. This would result in an animal which is severely immunocompromised and consequently unable to mount adequate immune responses, a major hindrance to its potential clinical application. Thus, the purpose of the present study was to address the hypothesis that the striking protective effect obtained upon dCF treatment is the result of global immunosuppression.

Materials and ethods

Following our previous study (Thliveris et al., 1997), diabetes-free animals (n=12) were monitored for an additional 5 months, after discontinuation of dCF treatment, with weekly assessment of blood glucose levels. All of these animals remained normoglycemic (5.93±1.67mmol/l) for the duration of this time period. For the present study, a group of these animals (n=6) were sensitized on day zero with a 0.5 ml i.p. injection of 200 μg Ovalbumin in alum to assess immune response capability. On day 15, blood was collected via tail vein bleed for specific and total IgG determinations by ELISA. The animals then received a boost of Ovalbumin on day 25 at the same concentration as used on day zero. On day 35, blood was collected via cardiac puncture for specific and total IgG determinations by ELISA, and for assessment of CD4+ and CD8+ lymphocytes by flow cytometry.

An additional group of age-matched diabetes resistant (DR) control animals (n=8), as well as a group (n=8) of diabetes-prone (DP) pre-diabetic control animals were included. Neither of these groups received dCF. Moreover, the latter group of animals was not age-matched due to the fact that diabetes occurs between the ages of 60-120 days.

The remaining group of diabetes-free animals (n=6) from our previous study, and additional DP pre-diabetic (n=3) and DR (n=3) rats were used to assess percentages and numbers of CD4+ and CD8+ cells in the thymus by FACS analysis.

Total and OVA-specific IgG levels were determined in capture ELISAs utilizing goat anti-rat IgG and OVA (Sigma) respectively followed by biotinylated goat anti-rat IgG (Fc specific) in a Streptavidin-alkaline phosphatase system set up similarly to that previously reported (Wang et al., 1998).

For flow cytometry, blood was collected into sterile glass tubes containing EDTA. Aliquots (100μl) were placed into 12x75 mm tubes and the recommended amounts of anti-CD4-PE (10μl) and anti-CD8-FITC (10μl) were added (mouse anti-rat CD4-PE and CD8-FITC antibodies were obtained from Serotec, Ltd., Mississauga, Ontario, Canada). Following incubation at room temperature for 30 minutes, the samples were processed with the Q-Prep lysing and fixing system of Beckman Coulter Inc. and analyzed with an EPICS-XL flow cytometer. The standard optical filter combinations were employed and the data collected in listmode format. The data files were analyzed using the Coulter Elite workstation software ver.4.01. Forward versus side light scatter histograms were collected in order to identify and set gates for single intact lymphocytes; all fluorescence histograms were based on 5,000 gated events.

After blood collection for the aforementioned studies, the animals were killed by a lethal injection of sodium pentobarbitol administered subcutaneously, and the thymuses collected for flow cytometry, as noted above, and for histology. Pancreata were also harvested for light microscopy. Haematoxylin and eosin stained sections were collected for assessment of the pancreatic islets and the thymus from all animals.

Statistical analyses of the data was performed using ANOVA and Tukey's procedure. Values less than 0.05 were considered statistically significant.

Results

Diabetes prone BB rats typically exhibit spontaneous development of IDDM by 60-120 days (Butler et al., 1983). We recently reported that administration of dCF markedly reduces the incidence of IDDM in this model, ie., when rats were treated with 2.5, 4 or 10 mg/kg/wk, the percentage of diabetes-free animals was 40, 60, and 80% respectively, compared to 10% for controls (Thliveris et al., 1997). Most importantly, this protection persisted for up to 270 days of age, the term of the experiment, notwithstanding the fact that dCF treatment was withdrawn at 120 days of age.

Given that dCF is a potent immunosuppressive agent, used clinically to inhibit both B and T cell function (Slomiany et al., 1997), we addressed the hypothesis that the failure of IDDM to spontaneously develop following cessation of dCF treatment reflected a persistant immunosuppression in these rats. As one indicator of immunocompetence, we examined the total serum IgG antibody levels in diabetic prone rats vs diabetic prone/dCF treated rats. The data in Table 1 indicate that dCF treated rats examined 150 days following cessation of treatment exhibit total serum Ig levels at or above those seen in non-drug treated, healthy, diabetes-prone pre-diabetic BB controls at each time point examined. The observation that total serum Ig levels in dCF treated rats were consistently above those

Table 1. dCF treated diabetic prone BB rats are not globally immunodeficient.

GROUP	TOTAL IgG (μg/ml)		
	d.0	d.15	d.35
Diabetic prone	562±89	547±97	953±219
Diabetic prone/dCF treated	1,866±358	1,889±222	2,236±306

Total serum IgG levels (mean ± SEM, n=8 and 6 rats respectively) were determined by ELISA as described at Materials and Methods. DP vs DP/dCF $p<0.005$ in all cases.

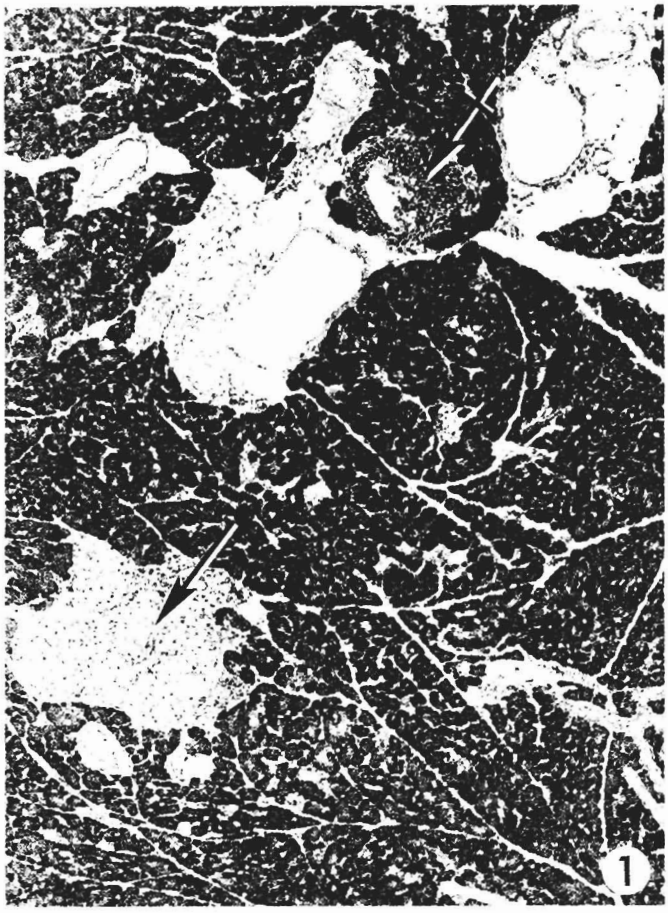
of DP controls likely reflects the fact that the dCF treated rats are significantly older at the initiation of the study (mean age of 270 days vs 40 days in pre-diabetic controls). Indeed, total IgG levels in dCF treated/DP rats are also at or above those in age matched DR controls (data not shown), arguing against a generalized inability of these rats to mount serum antibody responses to environmental antigens.

As a second approach to evaluating immunocompetence, the capacity of rats which had completed a course of dCF treatment and remained diabetes-free for five months to mount specific IgG responses upon immunization with ovalbumin was determined. OVA-specific serum IgG levels were detemined by ELISA

Table 2. dCF treated diabetic prone BB rats mount Ag-specific antibody responses equivalent to non-drug treated controls.

GROUP	OVA-SPECIFIC IgG (ELISA TITER)			
	d.0	d.15	d.35	d.42
Diabetic prone	<250	6,965±2,971	401,286±214,899	433,800±211,249
Diabetic prone/dCF	<250	7,750±2,670	480,769±125,363	425,000±119,434
Diabetes resistant	<250	22,625±2,846	909,500±300,456	656,250±178,825

Rats were immunized at d.0 and boosted at d.28 with 100 μg OVA (alum). Serum OVA-specific IgG levels for individual rats, independently analyzed, were determined in ELISA as described at Materials and Methods. Data expressed as mean±SEM reciprocal ELISA titer (n=8, 6, 8 for the three groups examined). DP vs DP/dCF $p>0.05$ in all cases. DP or DP/dCF vs DR $p<0.005$ at d.15, $p>0.05$ at all other time points.



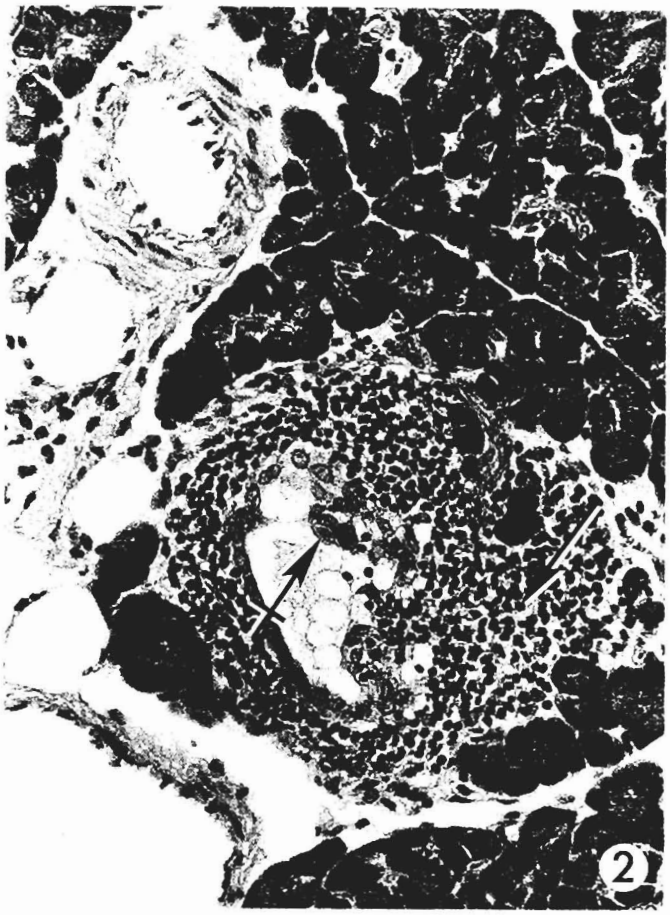


Fig. 1. Low power micrograph of pancreas from a post-dCF treated BB rat seven months after drug withdrawal. Note normal islet (arrow) and an islet with mononuclear cells (crossed arrow). x 25

Fig. 2. High power micrograph of islet with mononuclear infiltrate in Fig. 1. The mononuclear cells (arrow) have destroyed most of the islet, with only a few endocrine cells remaining in the centre (crossed arrow). x 50

immediately prior to OVA (alum) immunization (d.0), at the time of the peak primary antibody response (d.15), and at the peak secondary response (d.35), ten days after OVA (alum) booster. This response was selected as an indicator of immunocompetence as it requires effective collaboration between antigen presenting (APC), T and B cell arms of the immune response.

As evident from the data in Table 2, none of the rats exhibited detectable OVA-specific serum IgG prior to immunization, ruling out crossreactivity with environmental/food antigens or the generation of substantial OVA specific antibody responses prior to dCF treatment. Both DP and DP/dCF treated rats generated strong, and most importantly, equivalent OVA specific primary (d.15) and secondary (d.35) IgG responses to OVA ($p>0.05$). DR rats generated somewhat more intense OVA specific IgG responses (Table 2, $p<0.005$) that developed slightly more rapidly in that they were readily detectable at d.12 (mean ELISA titer 10,500), in contrast to those in DP rats with or without dCF treatment (mean <250, data not shown). However, secondary OVA-specific IgG responses did not differ between any of the three groups. Taken together, the data argue against persistent defects in the capacity of dCF treated rats to mount adaptive immune responses.

In subsequent studies we examined the CD4:CD8 balance among peripheral blood mononuclear cells of dCF treated vs control diabetes-prone BB rats. As indicated in Table 3, neither the percentage nor total numbers of CD4+ or CD8+ T cells in DP BB rats differed significantly as a consequence of dCF treatment. The DR rats exhibited similar numbers of CD8+ cells but somewhat greater numbers of CD4+ cells in the circulation than did either of the DP groups. Analyses of thymic lymphocytes showed a greater percentage of CD4+ cells in the DP/dCF than in the DP/ non-dCF treated animals. Moreover, dCF-treated animals had greater numbers of CD4+ cells compared to the DP/non-dCF treated and DR animals (Table 3).

Histological assessment of the pancreatic islets from post-dCF treated animals showed varying degrees of mononuclear infiltration (Figs. 1-4). The infiltrate was seen in the islets of all animals, regardless of whether they were challenged or non-challenged with Ovalbumin. There were no detectable morphological changes in the thymuses from any of the animals in the study, nor were there changes in the islets from the DP pre-diabetic or DR animals.

Table 3. Post immunization flow cytometry of CD4+ and CD8+ cells in whole blood and thymus.

GROUP	CD4%	CD8%	CD4 Cells	CD8 Cells
Blood				
A (n=3)	23±3	13±2	1006±120	552±85
B (n=6)	30±3	14±1	1252±118	746±50
C (n=3)	53±1[a]	20±1	2466±16[b]	912±42
Thymus				
A (n=3)	10±1	9±1	405±59	356±58
B (n=6)	15±1[c]	7±1	773±42[d]	376±30
C (n=3)	12±1	11±1	534±58	439±57

Data expressed as mean±SEM. A: diabetes prone; B: diabetes prone + dCF: C: diabetes resistent. [a]: C>A,B; p 0.01; [b]: C>A,B; p 0.01; [c]: B>A; p 0.05; [d]: B>A,C; p 0.05

Discussion

The BB rat model of human insulin dependent diabetes (IDDM) is characterized by autoimmune mediated lymphocytic infiltration of the islets of Langerhans, insulin deficiency and the presence of islet cytotoxic antibodies (Dyberg et al., 1982; Seemayer et al., 1982; Eisenbarth, 1986). While the mechanism of action of dCF mediated protection remains ill-defined, it has been suggested that dCF treatment may alter immunologic activity by inhibiting interferon gamma (IFNγ) mediated class II major histocompatibility complex (MHC) expression of macrophages (Inaba et al., 1986), resulting in reduced antigen presentation to CD4+ cells. More recent studies in normal rats treated with dCF have demonstrated marked lymphopenia and a

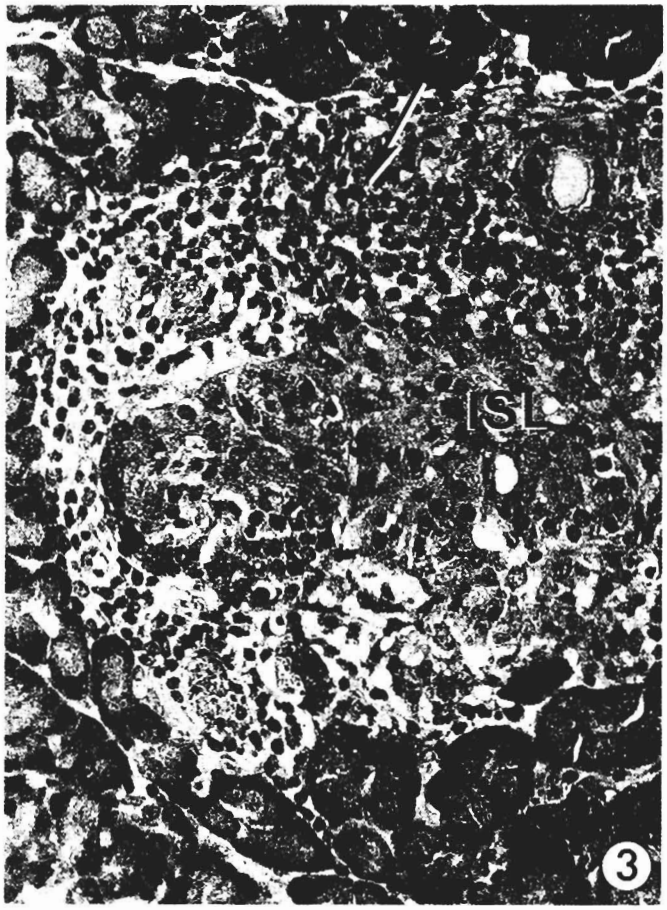


Fig. 3. Micrograph of islet (ISL) with marked mononuclear infiltrate (arrow). x 100

decrease in lymphocyte proliferation to Con A (Arrajab et al., 1995) whether it was administered as daily injections or as a continuous infusion. Similar results have been shown in normal mice (ie. non-diabetic prone) receiving daily injections of 0.4 mg/kg b.w. dCF (Sordillo et al., 1981). Continuous infusion of dCF at a lower dose of 0.3 mg/kg b.w. resulted in a potent depression of immune function and greater lymphotoxicity, with a decrease in the leucocyte count from 5,200 mm^2 (normal) to 2,300 mm^2 after continuous infusion (Sordillo et al., 1981). Adenosine deaminase was inhibited by 84-94% in lymphoid tissues, and histological examination showed depletion of T cells, atrophy of lymph nodes, spleen and thymus. There was also significant depression in blastogenesis induced by the pan mitogen phytohemagglutinin (PHA) and the T-cell specific mitogen Con A. Others have reported that multiple daily single injections of 1.0 mg/kg b.w. dCF into mice had no effect on the proliferation response of lymphocytes to Con A or of splenocytes in a mixed lymphocyte culture in spite of a high degree of ADA inhibition (Burridge et al., 1977). Thus, dCF administrartion can clearly lead to a concentration dependent inhibition of lymphocyte function in vivo. For that reason, it was important to directly determine if the striking protective effects that we previously identified for dCF (Thliveris et al., 1997), and that we now demonstrate to persist for a minimum of five months after cesation of dCF treatment, were attributable to a global lack of immunocompetence. We report here that the immune status of dCF protected DP BB rats is not impaired by phenotypic or functional criteria and that the capacity of these rats to remain healthy is not a result of persistent immunosuppression.

The choice of the BB rat with its constitutive lymphopenia is a recognized limitation of this animal as a model for IDDM. Although these rats mount vigorous autoimmune responses, previous studies have also reported impairment of components of T cell dependent immune responses in both DP and DR BB rats (Poussier et al., 1982; Elder and Maclaren, 1983; Prudhomme et al., 1984). DR BB rats on the other hand have normal lymphocyte numbers and phenotype and do not become diabetic spontaneously. The percentages of T cell subsets and histology of DP thymuses are comparable to DR rats (Elder and Maclaren, 1983; Jackson et al., 1983). The observations in our current study were similar to those in the aforementioned reports with respect to CD4+ and CD8+ levels in DR and DP animals, as well as the histological appearance and T cell subsets in the thymus.

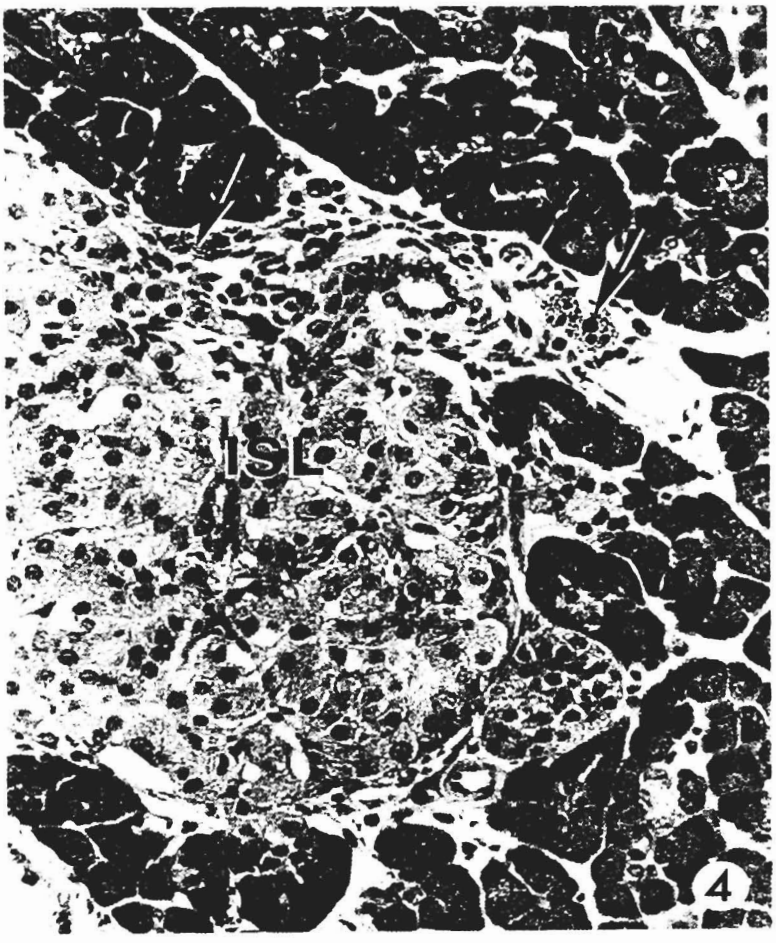


Fig. 4. Micrograph of islet (ISL) with sparse mononuclear cells at the periphery of the islet (arrows). x 100

Strikingly, the analyses of DP and DP/dCF treated rats indicated comparable findings in the DP animals regardless of whether or not they were treated with dCF. Moreover, notwithstanding the fact that BB rats are lymphopenic, they are indeed able, as we have shown, to mount substantial total and antigen specific immune responses requiring T:B cell collaboration. Again, the ability of dCF-protected DP rats to mount exogenous Ag specific responses did not differ from the non-dCF treated animals. Taken together, the data argue that the extended protection from IDDM that is conferred by dCF treatment is not a consequence of global immunosuppression.

Of further interest was the histological appearance of the pancreatic islets. Varying degrees of mononuclear infiltrates were observed in the islets of the post-dCF treated animals, with evidence for the beginnings of islet destruction in some sections. The question arises as to whether the immunization regimen used resulted in this activtion and the subsequent infiltration of the islets by immunocompetent cells. We consider this unlikely since a similar degree of infiltration was seen in all post-dCF treated animals, regardless of whether or not they were challenged with OVA. We speculate that a more likely cause is the gradual loss of the protective effects of dCF over the period between two and seven months following dCF treatment. These mononuclear infiltrates are in marked contrast to our previous study (Thliveris et al., 1997) which showed normal islet histology as well as normoglycemia in animals two months after cessation of drug treatment. At the same time, the fact that all post-dCF treated animals in the present study remained normoglycemic suggests that there was sufficient reserve of beta cell function. Thus taken together, it would appear that the clinically protective effect of dCF is not compromised between for at least seven months after drug withdrawal.

An alternative explanation for the infiltrates we observed is that they may play an active protective role in preventing islet destruction. It is well recognized that stimulation of antigen-specific CD4+ cells leads to secretion of IL-2, which is required for clonal expansion and further differentiation of these cells. Studies have shown that over-expression of IL-2 in mice resulted in an inflammatory response directed at beta cells, with the cellular infiltrate composed of B cells, CD4+ and CD8+ cells (Allison et al., 1994). Expression of IL-2 in islets not only leads to a marked insulitis but to the destruction of islet cells, which results in diabetes. Considerable evidence supports the hypothesis that by skewing the response toward CD4+ Th2 cells and TGF-beta producing cells, the induction of diabetes is delayed or prevented (Liblau et al., 1995; Cameron et al., 1997; Avanzini et al., 1998; King et al., 1998; Maron et al., 1998; Mathieu et al., 1998).

At the same time, a causative relationship between Th2-like activation and protection from clinical disease remains to be demonstrated. Several recent studies examining the relationship between an altered balance of type 1 vs type 2 immunity and different strategies that are capable of preventing the development of IDDM concluded that the protective effects were unlikely to be due to induction of protective Th2 immune reactivity but rather to general downregulation of immune activation in the pancreas, and hence also of Th1 autoimmunity, a finding that may be applicable to our own data as well (Balasa et al., 1997; Heurtier and Boitard, 1997; Kolb et al., 1997; Mueller et al., 1997). At present, the mechanism of action of dCF on the various lymphocyte populations is not known. Further studies will be required to determine optimal duration and frequency of dCF therapy and to ascertain the mechanism of action of dCF on the immune system in this model of IDDM and whether dCF or similar acting agents have a role in the prevention and treatment of the disease in humans.

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