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EVALUATION OF THERAPEUTIC ROLE OF FILARIAL ANTIGENS ON THE DEVELOPMENT OF TYPE 1 DIABETES

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ABSTRACT

Increasing evidence is available in support of an inverse relationship between worm infection and T helper type 1/17 (Th1/17) - based inflammatory disorder such as Type 1 diabetes suggesting the therapeutic potential of helminth molecules in this condition. The objective of the study was to evaluate the therapeutic potential of filarial immune modulators (Bm mf's/ Bmmf'ES'/ rBmCys) on the development of type 1 diabetes. A study was designed to validate therapeutic efficacy of filarial proteins (Bm mf'S/ Bmmf'ES'/ rBmCys) in streptozotocin (STZ) induced diabetic mice. Following experimental induction of diabetes the mice were either treated with or without the said proteins using alum as adjuvant (25 µg) for 2 months. After treatment the blood glucose level and pancreatic histopathological changes were measured. Therapeutic treatment of diabetic mice with filarial proteins reduced the severity of disease by decreasing the blood glucose levels. Mice treated with rBmCys in alum adjuvant showed significantly lower blood glucose level as compared to the diabetic mice treated with only Alum($p<0.001$). Also there was significant reduction in the glucose level in diabetic group of mice treated separately with filarial native proteins (Bm mf's and Bmmf'ES) compared to diabetic mice treated with only Alum($p<0.002$ and$p<0.001$). Almost 70% of the mice treated with Bmmf'ES showed recovery from diabetes at the end of the experiment period. Although all mice had evidence of ongoing pancreatic islet cell inflammation by histology, mice treated with filarial proteins had greater numbers of total intact islets and non-infiltrated islets than untreated group of mice. These findings suggest that filarial derived proteins play pivotal role in the amelioration of disease condition in mice and act as novel therapeutic candidates in the treatment of type 1 diabetes.

Introduction

Increased incidence of allergic or autoimmune inflammatory diseases in the West has been attributed to the popular 'hygiene hypothesis' that proposes direct link between the absence of appropriate priming of the immune response by infectious agents during childhood and apparent increases in autoimmune disease and allergy in areas of the world with improved health care and sanitation.[1,2]Epidemiological data from the World Health Organization (WHO) largely supports this hypothesis, indicating that westernized countries are facing alarming increase in childhood allergic conditions like rhinitis, atopic dermatitis and asthma[3] ,inflammatory bowel diseases (IBD) and autoimmune disorders like type 1 diabetes, multiple sclerosis and rheumatoid arthritis[4]. In contrast, several autoimmune disorders have reduced incidence and severity in geographical regions with high parasite load[5] .

The parasitic infections might attenuate the host immune system to be more tolerant and avoid exacerbated inflammatory response. The helminth parasites thus could be a rich source of immunomodulators with potential therapeutic value for these diseases. In a bidirectional relationship parasites develop strategies, including mechanisms to escape detection and active manipulation of hosts' immune cells, to circumvent or dampen the host response(s). This ability to interfere with the host's immune responses affords parasites the opportunity to establish, develop, reproduce and complete their life cycles.[6]

There is consequently intense interest in understanding the molecular basis of parasite driven immunomodulation with a therapeutic application for autoimmune diseases. The nematodes *Heligmosomoides polygyrus* and *Trichinella spiralis* have been shown to play vital role to suppress the development of the experimental autoimmune encephalitis[7], and experimental colitis[8,9].

T1D is a chronic autoimmune disorder characterized by the progressive loss and selective destruction of insulin-producing pancreatic beta cells[10]. Changes in autoimmune T1D includes the inflammatory cells infiltration into the islets, insulitis followed by destruction of beta cells. It accounts for 5–10% of the total cases of diabetes worldwide. The geographical distribution of allergic and autoimmune diseases is a mirror image of the geographical distribution of various infectious diseases, including gastrointestinal infections and parasitic or helminthic infections. One environmental change that may be responsible for the recent increase in autoimmune diseases like T1D as reasoned earlier is the loss of chronic parasitic infections in developed countries. Also persons infected with chronic parasitic worm infections are found to have lower rates of autoimmune diseases than others living in the same environment[11].

Experimentally, a number of helminth parasites, including infection with a tissue-invasive filarial nematode, *Litomosoides sigmodontis* have prevented the onset of type 1 diabetes in NOD mice by altering the levels of IFN-γ and IL-10[12]. Multiple studies conducted in India have found that individuals infected with chronic filarial parasitic worm infections have lower rates of type 1 diabetes than others living in the same environment[13,14].

Filarial nematode *Acanthocheilonema viteae*, derived excretory-secretory (ES) phosphorylcholine-containing glycoprotein, ES-62 of adult stage has been shown to inhibit the development of allergic responses associated with collagen-specific pro-inflammatory/Th1 cytokines (TNF-γ, IL-6, and IFN-β) release and finally suppression of the LPS- induced rheumatoid arthritis[15]. Filarial cystatin, a cysteine protease inhibitor has also been shown to account for a major proportion of the immunosuppressive activity of secreted filarial proteins and therefore is suggested to be considered as a major pathogenicity factor of filariae[16].

In view of the above considerations the present study was planned to assess the therapeutic potential of filarial proteins viz. *Brugia malayi* Cystatin (rBmCys) , microfilarial excretory-secretory (Bm mf ES) and microfilarial soluble (Bm mf S) antigens in type 1 diabetes using mouse model.

Material and methods:

i)Experimental Animals and *B. malayi* Parasites

After getting clearance from the Institutional Animal Ethics Committee, BALB/c mice (of 8-10 weeks of age and weighing 25-30 g) bred and maintained in the animal house of the institute were used in this study as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals, Government of India (CPCSEA). The animals were maintained under standard laboratory conditions with free access to animal

chow and drinking water ad libitum and all the surgical procedures were performed under the strict aseptic conditions.

Micro-filariea were obtained by lavage of the peritoneal cavities of jirds with intraperitoneal filarial infection of 3 months or more duration. Brugia malayi infective stage (L3) larvae used in this study were obtained using Baermann's technique.[17]

ii)Filarial native antigens (Bm mf ES & Bm mf S):
Bm mf ES antigen was prepared as described by Chenthamarakshan et al.,[18]. Bm mf S antigen was prepared as described by Kaliraj et al.[19] with few modifications.

iii)Recombinant Cystatin antigen (rBmCys):
rBmCys antigen was expressed and purified. The recombinant gene constructs pRSETA-BmCys was maintained in Top 10F' *E.coli* host. *E.coli* BL21(DE3) pLysS was transformed with the desired gene construct in pRSET-A. A single colony of fresh transformant was inoculated into 1.5 ml LB and grown overnight. 1M IPTG was added for the expression of recombinant protein. Collected supernatant was passed through immobilized cobalt metal affinity column chromatography (Clontech, Mountain View, CA) to purify the His tagged recombinant proteins. The expression pattern and purity were analysed by 15% SDS-PAGE. Presence of Histidine-tag in the purified protein was detected using penta His-HRP monoclonal antibodies (Qiagen Valencia, CA). Concentration of protein was estimated by micro BCA method (Thermo Fisher).

iv)Induction and assessment of Multiple Low-Dose Streptozotocin-induced Diabetes (MLDS):
Type I diabetes was induced in BALB/c mice using low dose Streptozotocin (STZ) protocol (40 mg STZ / Kg/ day, injected intraperitoneally for five consecutive days) as described by Santos-Junior et al[20].

v)Therapeutic effect of filarial proteins:
5 groups of mice (n=6-8 in each group) were treated with intraperitoneal (i.p) injection of different agents as detailed below and followed up for 7 weeks

1. *STZ-rBmCys*: STZ induction followed by rBmCys.
2. *STZ-Bm mf ES*: STZ induction followed by Bm mf ES antigen.
3. *STZ-Bm mf S*: STZ induction followed by Bm mf S antigen.
4. *STZ-Alum*: STZ induction followed by only alum adjuvant.
5. *STZ*: STZ Induction of diabetes (control group).

vi)Analysis of therapeutic potential of filarial antigens on mice with type I diabetes:

a) *In vitro* proliferation assay of spleen cells by modified MTT cell viability method: After 72 hr incubation, the extent of cell proliferation was measured by modified MTT cell viability assay i.e. MTS method using Cell Titer 96 R aqueous non-radioactive cell proliferation assay (Promega, USA).

b) Analysis of cytokines by ELISA: Supernatants collected from the spleen cell cultures were used for measuring IL-10, IL-4, IL-5, IFN-γ and TNF- α by two site sandwich Enzyme Immuno assay (Invitrogen, USA) as per the protocol supplied by the manufacturer.

c) Monitering of diabetes status in mice: The mice were monitored for fasting glucose levels in blood using standard glucometer (Gluco Chek, Major Biochemical Corporation, Taiwan). Mice with blood glucose level of ≥ 200 mg/dl in samples for two consecutive weeks were considered as diabetic.

d) Anti-insulin antibodies in mice sera: Serum from all the above experimental and control groups of mice were collected to evaluate the anti-insulin antibodies (IgG, IgG1, IgG2a and IgM Abs) using standard enzyme-linked immunosorbent assays (ELISA).

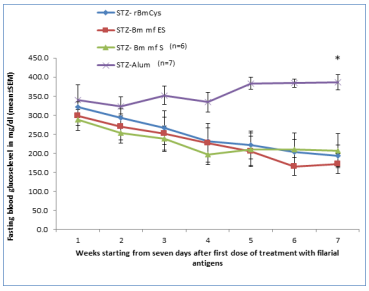
e) Histological assessment of pancreas inflammation: Total islets were counted and the changes looked for in each group were islets morphology number of islets, inflammation, lymphocyte infiltration. Numbers of islets of four longitudinal sections of each pancreas were assessed. The severity of insulitis was scored as non-infiltrated , peri-insulitis or intra-insulitis.

Statistical analysis:
Results for various parameters are presented as mean ± standard error of the means (SEM). The tested for significance applied were One way ANOVA followed by Dunn's post-hoc test for multiple comparisons, student *t* test. P-values < 0.05 were considered significant. Spearman's correlation study was performed to find any association between damage score and the alteration of blood glucose level and also with cytokines response. The statistical analysis was carried out in SPSS 20.0 version.

Result:

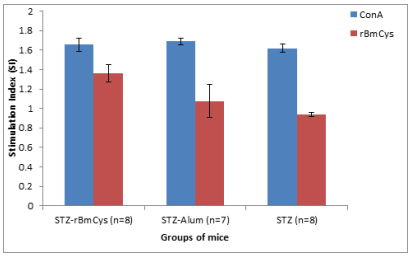
1. **Diabetes status in mice :**There was significant reduction ($p<0.0001$) in fasting blood glucose levels in diabetic mice treated with rBmCys or with Bm mf ES antigen compared to the levels in diabetic mice treated with only alum . Similarly, the group of diabetic mice treated with Bm mf S showed significantly lower fasting blood glucose levels compared to the levels alum treated diabetic mice. (Figure 1)

Figure 1: Fasting blood glucose levels and diabetes status in mice followed by treatment with filarial proteins:



2. **Effect on cellular proliferation :**Proliferative response of spleen cells from diabetic group of animals treated with rBmCys (STZ-rBmCys) showed significantly pronounced splenocyte proliferation response ($p<0.001$) upon stimulation with rBmCys compared to the spleen cells from mice treated with alum alone (STZ-Alum).(Figure 2)

Figure 2: Proliferation of splenocytes of STZ-induced diabetic mice treated i.p. with rBmCys



3. **Analysis of cytokines in the supernatants of splenocyte cultures:** Splenocytes from STZ induced diabetic mice treated with rBmCys, Bm mf ES or Bm mf S showed significantly higher secretion of anti-inflammatory cytokine IL-10, IL-4 and reduced level of IL-5 cytokine compared to other groups. Similarly, the level of TNF-α and interferon-γ (IFN-γ) was found to be significantly lower in the splenocyte culture supernatant of rBmCys group, Bm mf ES or Bm mf S compared to other groups.(Figure3-7)

Figure 3: Cytokine levels to stimulation with rBmCys or Bm mf ES or Bm mf S in IL-10

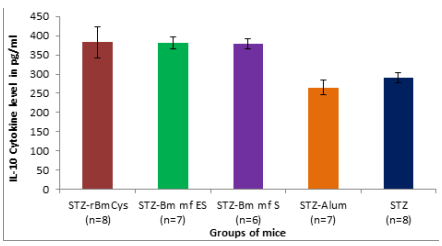


Figure 4: Cytokine levels to stimulation with rBmCys or Bm mf ES or Bm mf S in IL-4

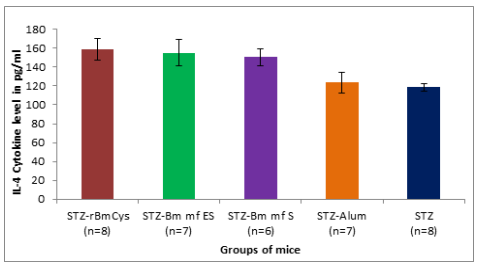


Figure 5: Cytokine levels to stimulation with rBmCys or Bm mf ES or Bm mf S in IL-5

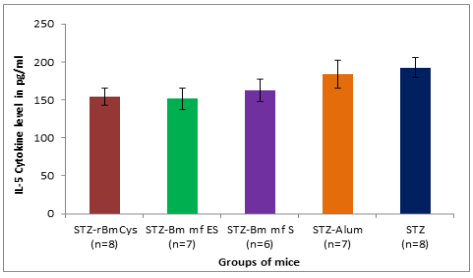


Figure 6: Cytokine levels to stimulation with rBmCys or Bm mf ES or Bm mf S in TNF-α

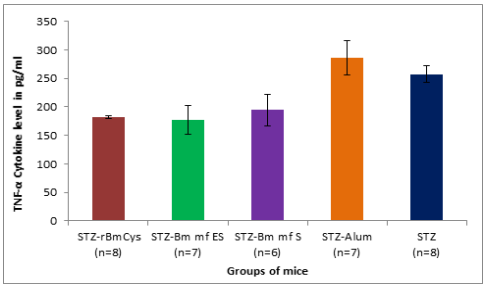
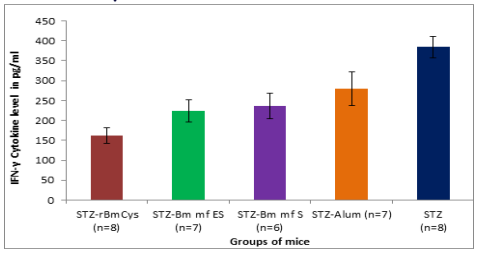


Figure 7: Cytokine levels to stimulation with rBmCys or Bm mf ES or Bm mf S in IFN-γ



4. Levels of anti-insulin antibodies: Significantly higher levels of insulin specific IgG1, IgM antibodies were found in diabetic group of mice treated with Bm mf ES compared to the levels in STZ-Alum ($p<0.002$) or STZ (0.005) group of diabetic mice. In contrast, significantly lower level of anti-insulin IgG2a antibodies were found in rBmCys or Bm mf ES or Bm mf S group of mice compared to the untreated STZ group of mice.(Figure 8-10)

Figure 8: Anti-insulin IgG1 auto-antibodies in STZ-induced diabetic mice

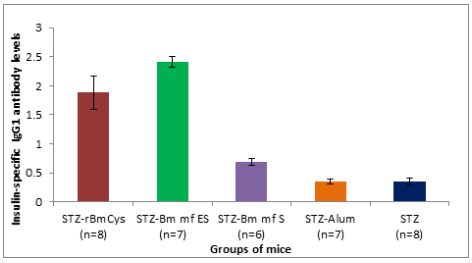


Figure 9: Anti-insulin IgM auto-antibodies in STZ-induced diabetic mice

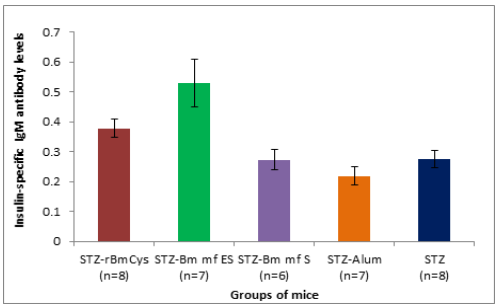
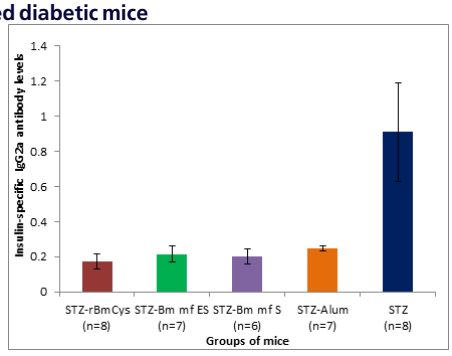
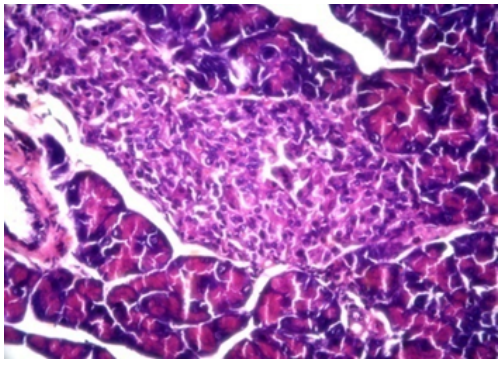


Figure 10: Anti-insulin IgG2a auto-antibodies in STZ-induced diabetic mice



5. **Histopathological assessment of pancreas:** The groups of diabetic mice treated with rBmCys showed significant number of healthy pancreatic islets cells, whereas pancreas from the diabetic groups of mice treated with alum showed, as expected, either severe insulitis or complete destruction of islets.(Figure 11)

Figure 11: Figure shows islets as severe insulitis in alum treated mice



Discussion:

Gillespie and colleagues[21] have shown a strong inverse association between age at diagnosis and prevalence of HLA alleles conferring susceptibility to T1D. Asia-Pacific region is being considered as an important contributor to the epidemiology of diabetes. According to the 'hygiene hypothesis', the decreasing incidence of infections in western countries and more recently in developing countries is at the origin of the increasing incidence of autoimmune diseases. The balance of Th1/ Th2 responses has been recognized as a critical factor in the development of T1D and it is well established that diabetes is associated with the development of a pathogenic Th1 response.

Although the parasitic nematodes as such have been successfully explored in the treatment of autoimmune diseases, it is logical to identify and use their defined molecules for therapeutic purpose. Thus, helminth -derived products not only have therapeutic potential but can also be used as unique tools for defining key molecular events in the induction of an anti-inflammatory response and therefore, for defining new therapeutic targets[22].

In the present study, we have investigated post-disease therapeutic treatment with recombinant *Brugia malayi* cystatin (rBmCys), Excretory secretory (Bm mf ES) and microfilarial soluble (Bm mf ES) filarial proteins could induce protection from the development of STZ (Streptozotocin) induced T1D.

When treated with rBmCys or Bm mf ES or Bm mf S showed significant downfall in the glucose level after 3rd week of treatment compared to the control groups of mice, which showed continued elevation of glucose level till the end of experimental period. In the experiment where mice with established T1D were treated with filarial antigens, though there was lymphocytic infiltration and extensive destruction in the islets cells by STZ, some part of the islets remained healthy showing recovery areas in the Bm mf ES and rBmCys treated diabetic groups.

The cytokine IL-10 is known to suppress immune responses in general and immuoregulate the Th-cell response. The higher levels of IL-10 in this situation also indicate the presence of regulatory T cells and possible involvement of such cells in prevention of T1D[23]. There are likely several factors which contribute to the protection by helminths infection by inducing Th2 environment that might counteract the proinflammatory responses (TNF- , IFN-) that are necessary to generate diabetic condition.[24,25]

IL-4 has been shown to play an important role in the protection from spontaneous development of diabetes due to Th1-mediated destruction of pancreatic -cells in female NOD mice when they were exposed to *S. mansoni* [23,26]. In agreement with this, in our study elevated levels of cytokine IL-4 were found in the diabetic group of mice treated with filarial proteins compared to the diabetic group of mice treated only with alum though the difference was not statistically significant.

Th1 induce production of IgG2a antibodies suggesting that helminth infection also induces a Th2 shift with respect to the auto antigens involved in diabetes[12,27]. Consistence with this finding, our results have shown significantly elevated levels of insulin specific IgG1 in the mice treated with Bm mf ES.

The findings of the present study are in consistence with this concept as the filarial proteins rBmCys, Bm mf ES and Bm mf S seems to be mediating through anti-inflammatory cytokine IL-10 to shift the immune response towards Th2 type to suppress T1D. Hubner et al.,[12]have shown that the inhibition of T1D in filarial infected diabetic mice is associated with Th2 response and induction of FoxP3+ regulatory T cells.

Conclusion:

From the findings of this study it can be envisaged that the treatment of mice with filarial antigens (rBmCys, Bm mf ES and Bm mf S) after the onset of T1D had protective effect against this autoimmune disease. This protection was found to be associated with changes in humoral, cellular and cytokine responses that reflected a shift from Th1 to Th2 type of immune response. Further studies on the dose dependent responses and long term follow up of treated animals for T1D and other autoimmune diseases.

Conflict of interest: All the authors have declared that they have no conflict of interest.

Ethical approval: This study does not contain any studies with human participants performed by any of the authors. This study was approved by Institutional Animal Ethics Committee and national guidelines as per Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) for the care and use of animals were followed.

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