

Molecules of Parasites as Immunomodulatory Drugs

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Abstract: Parasite molecules offer unique advantages for the treatment of immunological disorders, and several candidate molecules have been shown to be effective. In our studies, it was shown that a factor inducing immunoglobulin E from filarial nematode parasites was suppressive in animal models of immunological disorders such as allergy and insulin dependent diabetes mellitus (IDDM). The Th1/Th2 paradigm of CD4+ T helper cell subsets can provide the basis for the development of new types of drugs and of novel strategies for the treatment of allergic and autoimmune disorders by parasite molecules. In our experimental system, parasite molecules from a filarial nematode parasite led to the down-regulation of the allergic reaction in animal models. In the majority of hosts, infection with helminths is associated with markedly reduced cellular immune reactions and polarization of T cell responses to Th2 and Th3 types. Some studies have suggested that the stimulation of host immunoregulatory networks with parasite molecules leading to the synthesis of anti-inflammatory cytokines (interleukin10, transforming growth factor-beta (TGF- β) and others) can provide new therapy for immunological disorders. It is known that parasites produce some types of molecule that mimic host molecules such as CD40 ligand, TGF- β and macrophage migration inhibitory factor. These molecules are also candidates for medicinal agents. This review describes many of the latest possibilities in this field and shows how they can be best put to use for the development of medicinal agents, molecular target identification, and for prioritization.

INTRODUCTION

The concept of the parasite as a source of medicinal agents is perhaps not a familiar one to medicinal chemists, judging by the lack of references to these agents in standard textbooks. Most parasitologists investigate substances derived from parasites with respect to mechanisms of parasite evasion of host immunity or physiologic research. Such substances from parasites are seen as possible immunomodulatory agents, or more particularly as possible sources of new pharmaceuticals.

In the last few years, the prevalence of atopy, allergic diseases and autoimmune diseases have been steadily increasing in the Western world, in contrast to Africa and Asia [1]. Even within Europe several studies conducted in the 90's have shown significant East-West differences [2], hardly attributable to genetic differences but that can probably be explained by differences in levels of helminth infection.

This paper proposes that a new source of pharmaceuticals for immunological disease is parasite-derived molecules. Parasite infection can impact on the course of autoimmune disease, both in disease-inducing and disease-protecting capacities [3]. Parasitic helminth infection has been shown to induce immunomodulation of host immunity characterized by mast cell hyperplasia, eosinophilia [4] and markedly elevated levels of circulating antigen non-specific

immunoglobulin E (IgE). These responses were related to host protection [5,6]. Despite the fact that simplistic views are not recommended, it seems that polyclonal IgE production, bias of Th2 immunity, mimicry of host molecules and mainly the stimulation of host immunoregulatory networks leading to synthesis of anti-inflammatory cytokines (IL-10, TGF-beta and others) can provide new insights into mechanisms by which parasites have developed throughout their evolution.

Those mechanisms that are useful for parasite evasion and persistence might also be used in humans in order to provide new approaches to immunological disorder prevention. Specifically, the parasite proteins may be useful in further attempts to use the mechanisms involved in parasite immune defense to prevent and treat certain autoimmune conditions.

POSSIBILITY OF TH1 IMMUNE-DISEASE THERAPY USING THE TH2 BIAS INDUCED BY PARASITIC HELMINTH MOLECULES.

In the past few years, considerable evidence has accumulated to suggest the existence of functionally polarized responses by the CD4+ T helper (Th) and the CD8+ T cytotoxic (Tc) cell subsets that depend on the cytokines they produce. The Th1 and Th2 cellular immune responses provide a useful model for explaining not only the different types of protection, but also the pathogenic mechanisms of several immunopathological disorders. Both environmental and genetic factors act in concert to determine the Th1 or Th2 polarization. The factors responsible for the polarization of specific immune responses into a predominant Th1 or Th2 profile have been extensively investigated in mice and humans. Evidence has accumulated

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from animal models [7] to suggest that Th1-type lymphokines are involved in the genesis of organ-specific autoimmune diseases, such as experimental autoimmune uveitis, experimental allergic encephalomyelitis, and insulin-dependent diabetes mellitus. Accordingly, data so far available in human diseases favor a prevalent Th1 lymphokine profile in target organs of patients with organ-specific autoimmunity. Th1-dominated responses are involved in the pathogenesis of organ-specific autoimmune disorders [8], Crohn's disease, sarcoidosis, acute kidney allograft rejection, multiple sclerosis and some unexplained recurrent abortions. By contrast, Th2-cell predominance was found in the skin of patients with chronic graft-versus host disease, progressive systemic sclerosis, systemic lupus erythematosus, and allergic diseases. Further, Th2-dominated responses play a pathogenic role in both progressive systemic sclerosis and cryptogenic fibrosing alveolitis, and favor a more rapid evolution of HIV infection towards the full-blown disease [9]. The Th1/Th2 concept suggests that modulation of the relative contribution of Th1- or Th2-type cytokines regulate the balance between protection and immunopathology, as well as the development and/or the severity of some immunologic disorders. The Th1/Th2 paradigm can provide the basis for the development of new types of drug and of novel strategies for the therapy of allergic and autoimmune disorders by parasite-derived molecules. In this section, we discuss the paradigm of Th1 and Th2 cytokines in relation to autoimmunity and parasite molecules.

The type of helper T cell response that develops following an infection by parasites is critical to the outcome of the infection [10]. The early events that mediate polarization of the helper T cell subsets towards either Th1 or Th2 during parasite infection are not well characterized, but it is likely that multiple factors influence the induction of a Th1 or Th2 type response, just as multiple effectors mechanisms are involved in parasite expulsion. Although there is intense ongoing investigation and debate on this issue, the review of experimental [11], clinical [12] and epidemiological [13] data apparently shows that helminth infections can have beneficial aspects in regard to the pathogenesis of atopy and allergic diseases. It is known that in murine models of *Schistosoma mansoni* infection, egg production is associated with a switch from a Th1 to Th2 response [14]. This subsequent dominance of a Th2 response in *S.mansoni* infected mice has been shown to influence the response to other infectious agents or antigens. Cooke *A et al.* showed that infection with this helminth significantly reduced the spontaneous incidence of IDDM [15] (see also Chapter 3). IDDM was also prevented by injecting parasite eggs alone. The simplest interpretation of the protective effect of *S. mansoni* is that it alters the cytokine profile such that a Th2 response predominates. Interestingly, a recent experiment has shown that oral administration of insulin to nonobese diabetic (NOD) mice induced T cells of a Th2-like phenotype, and its effect was enhanced in a synergistic manner by the oral administration of *S. mansoni* soluble egg antigen (SEA) [16]. Previous studies by the same group have shown that induction of oral tolerance to insulin delays the onset of IDDM in NOD mice [16]. Because until relatively recently humans might expect to succumb to a variety of

infectious agents, the current freedom from infection might permit the expression of a genetic predisposition to autoimmune pathology and be responsible for the increased incidence of IDDM. The presence of parasitic helminth infections may play an important role in determining the outcome of any concurrent immune responses whether to an infectious agent or to the expression of an autoimmune pathology. IDDM is a disease, which is increasing dramatically in incidence in certain countries [17]. This increase in disease incidence could be interpreted as reflecting an increased exposure to a diabetogenic agent. However, because this disease was lethal until relatively recently and primarily affected individuals prior to reproduction, the large increase in disease incidence might rather be a reflection of the removal of some environmental factor which suppresses the expression of the disease phenotype. The decreased worm burden in the population and the lowered incidence of diseases such as tuberculosis may permit the increased abundance of IDDM [18] and thus lead to the rising incidence of IDDM seen in many developed countries. These experimental results suggest that parasitic helminths could produce molecules that suppress insulin dependent diabetes mellitus.

We previously purified an IgE inducing factor in the excretory and secretory antigens (ES) derived from *Dirofilaria immitis*. This factor has a molecular weight of 15 kDa and behaves as a positively charged protein [19]. It is also remarkably stable to various proteases, to periodate digestion, and to physicochemical treatment. We found that the total ES extract enhanced CD23 expression and potentiated non-specific IgE synthesis through increased generation of Th2-related cytokines. To further understand the inflammatory reactions of the host-parasite relationship at a molecular level, we purified the ES protein and determined its amino acid sequence. The purified ES protein was named the DiAg (*Dirofilaria immitis*-derived antigen) [20]. In our experiments, a DNA sequence analysis showed that DiAg is 387 bp in length and is homologous with with neutrophil chemotactic factor (DiNCF), polyprotein antigen (DiPA), and cuticular antigen (Di5) from the *D. immitis* adult worm. The DiAg is composed of 129 amino acid residues consist with the molecular weight of about 15 kDa referred to earlier. We produced recombinant DiAg (rDiAg) in *E.coli* and found that it induced antigen non-specific IgE production (Fig. 1), proliferation of B cells, and enhancement of CD23 expression *in vitro*, as well as in mice [20]. Parasitic helminth infections induce immune responses in infected hosts. We demonstrated that DiAg is an essential factor in inducing antigen non-specific IgE during helminth infection. It is also known that parasitic helminth infection generates impressive Th2 responses. Immune responses to parasitic helminth are usually characterized by two quite mysterious phenomena: dominance of Th2-like immunity [21] and antigen-non-specific IgE secretion [22]. DiAg might cause these mysterious phenomena in the case of filarial nematode infection. This hypothesis can be conveniently tested using the NOD mouse, which provides an excellent animal model of the human autoimmune disease, IDDM. The NOD mouse spontaneously develops Th1 cell-dependent autoimmune diabetes. We investigated the effects of rDiAg on the initiation and progression of this

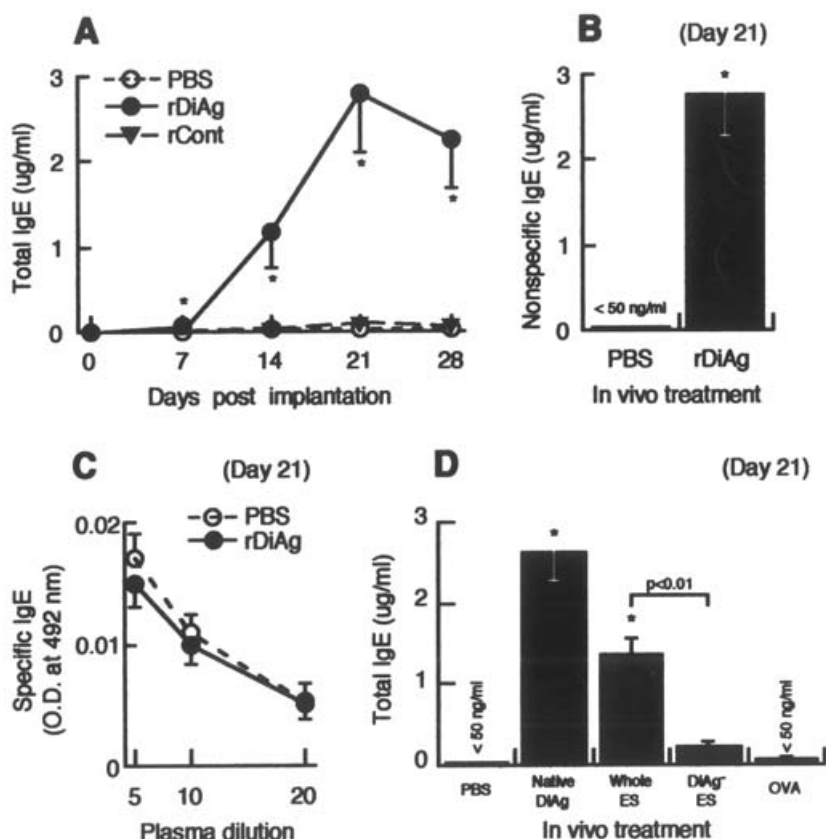


Fig. (1). Kinetics of plasma IgE levels in mice administered rDiAg. BALB/c mice received s.c. implants of a micro-osmotic pump filled with rDiAg, rCont (ABA-1; amino acid residues 1 to 67), native DiAg, whole ES, DiAg-depleted ES (DiAg-ES), or PBS (each at 100 μ g/mouse), and plasma was collected weekly. Total (A and D), nonspecific (plasma of affinity absorbed with DiAg) (B), and specific (C) IgE levels in plasma samples were measured by ELISA. The data represent the mean values and standard deviations (error bars) from five independent experiments. *, $P < 0.01$, when compared with mice administered PBS or rCont.

disease [23]. To determine the effect of rDiAg on the development of spontaneous autoimmune diabetes, NOD mice were treated, starting at 6 wk of age, with 10 μ g/mouse ($n=7$) or 100 μ g/mouse ($n=7$) of rDiAg or phosphate buffer saline (PBS) alone ($n=8$). The mice were killed at 27 wk of age, the time points when the cumulative incidence of diabetes reaches between 80% and 90% for NOD female mice in our specific pathogen-free NOD colony. The results are summarized in the table (Table 1). Insulin autoantibodies spontaneously develop in NOD mice and prediabetic humans. The immunoglobulin G (IgG) autoantibody profile for NOD mice at various ages is shown (Fig. 2). Insulin autoantibody levels in sera from control NOD mice peaked at 15 wk. In contrast, insulin autoantibody levels in rDiAg-infused mice remained low. rDiAg prevented appearance of the anti-insulin antibody that is normally seen in most NOD mice as they approach overt diabetes. This failure to develop an IgG response suggests a failure of the T cell activation required for class switching rather than the development of a Th2 response. Thus, rDiAg impaired the islet antigen-specific Th1 cell response *in vivo*.

The prevention of diabetes by rDiAg was associated with switching of the response from a Th1 to a Th2 profile. Since rDiAg clearly prevented the insulinitis by inhibiting the development and further accumulation of pathogenic Th1 cells to islets of Langerhans, we conclude that DiAg is a native Th2 inducer during filarial nematode infection and that Th1 responses are required for early events in the development of spontaneous autoimmune diabetes. The presence of parasitic helminth infections may thus play an important role as an immunomodulator in some autoimmune diseases or allergies.

Relapsing experimental autoimmune encephalomyelitis (R-EAE) is a Th1-mediated central nervous system (CNS) autoimmune disease with a pathology similar to that of relapsing-remitting multiple sclerosis [24]. In our preliminary experiment, rDiAg significantly reduced disease in the spontaneous disease relapse model (data not shown). Thus, DiAg has a Th2 polarization bias for helper T cell subsets in the host animal, and prevents some immunological disorders. In fact, rDiAg favors increases in Th2-type cytokine levels in serum [20]. It is well known that IL-4, a

Table 1. NOD Mice Given rDiAg Prevents Development of Their Characteristic Autoimmune Diabetes. The Female NOD Mice of Indicated Ages were Treated with rDiAg or PBS as Described in MATERIAL and Methods. The Blood Glucose Levels were Measured Once Every 2 wks. Mice were Considered Diabetic when the Blood Glucose Levels were > 300 mg/dl. The Paraffin-embedded Section were Stained by H & E. Approximately 10 to 20 islets were Scored for Each Pancreas. Note that rDiAg Treated Mice had Islets with no Insulitis or Few Islets with Peri-insulitis and a Higher Proportion of Normal Islets. Percentage of Islets was Calculated by the Following Formula: (Number of Islets Normal, with Peri-insulitis or Insulitis or Completely Damaged/ Total Number of Islet). * Denotes Values Significantly Different from those of Controls ($p < 0.005$). p Values were Calculated Using the Fisher's Exact Test.

Group	mice (n)	No. of mice with diabetes/ total No. of mice	Histologic Analysis of Pancreatic islets				
			Total	Normal	Peri-insulitis	Insulitis	Islet remnants
Control	n=8	7/8 (87.5%)	127	2 (1.6%)	12 (9.4%)	73 (57.5%)	40 (31.5%)
rDiAg (10 µg/head)	n=7	1/7 (14.3%) p=0.0101	124	62 (50%)	57 (46%)	5 (4%)	0 (0%)
rDiAg (100 µg/head)	n=7	0/7 (0%) p=0.0014	108	91 (84.3%)	17 (15.7%)	0 (0%)	0 (0%)

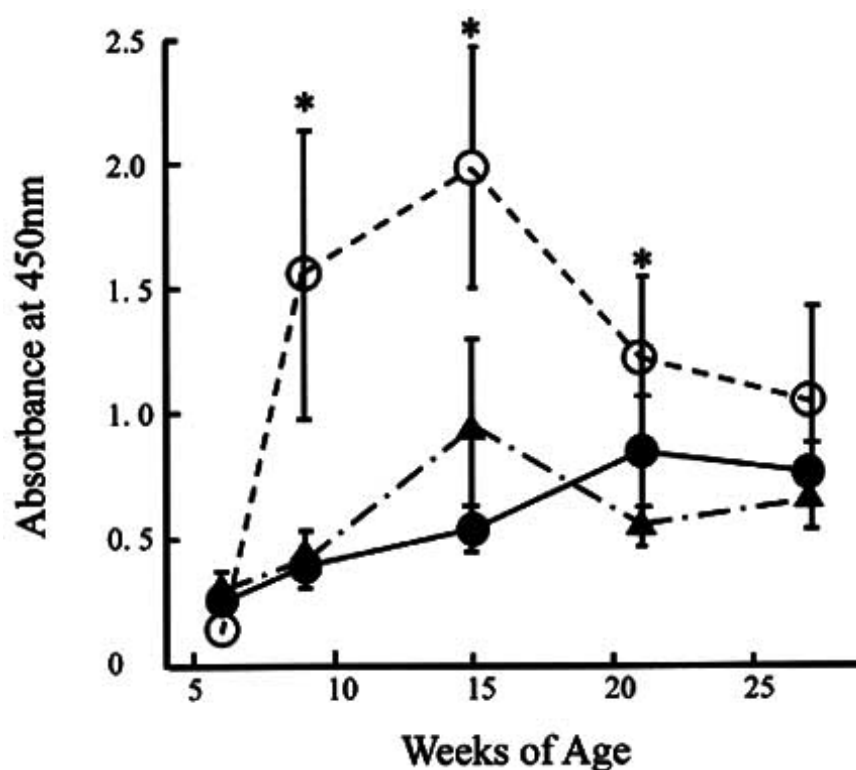


Fig. (2). Anti-insulin autoantibody levels of female NOD mice. Female NOD mice were subcutaneously implanted with osmotic pumps filled with rDiAg (closed circle: 100 µg/mouse and closed triangle: 10 µg/mouse) or PBS (open circle) and serum was collected. Serum anti-insulin autoantibody levels were measured by ELISA. Data are shown as the mean $\pm$ SE of seven or eight animals. * $p < 0.05$ by Student T test as compared with anti-insulin autoantibody levels in 100 µg/mouse of rDiAg-infused mice.

Th2-type cytokine, is an inducer of IgE production, while interferon gamma (IFN- γ), a Th1-type cytokine, has an inhibitory effect [25]. IL-10, often considered a Th2-type cytokine, has been shown to enhance IL-4-induced non-specific IgE production and to inhibit IFN- γ production [26-29]. To determine cytokine responses induced by rDiAg stimulation, we monitored levels of these three cytokines in serum from BALB/c mice administered rDiAg. IL-4 concentration increased and peaked around 7 to 14 days after implantation (Fig. 3A). IL-10 first became detectable at 7 days after rDiAg administration and reached a peak at 21 days (Fig. 3B). In contrast to Th2-type cytokines, IFN- γ concentration increased transiently at 7 days and then decreased to the baseline level (Fig. 3C). In PBS-administered mice, the production of these cytokines remained at background levels observed on day 0. These results suggest that rDiAg-induced non-specific IgE production is dependent not only on up-regulation of IL-4 concomitant with down-regulation of IFN- γ but also on up-regulation of IL-10.

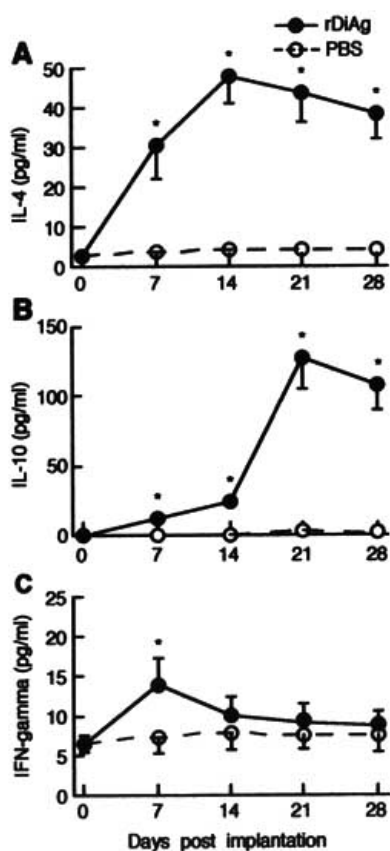


Fig. (3). Kinetics of cytokine levels in sera from mice administered rDiAg. BALB/c mice received s.c. implants of micro-osmotic pumps filled with rDiAg (100 μ g/mouse) or PBS, and serum was collected weekly. IL-4 (A), IL-10 (B), and IFN- γ (C) levels in serum were measured by ELISA. *, $P < 0.01$, when compared with mice administered PBS.

Elliott D.E. *et al.* [30] show that helminths can inhibit ongoing colitis that results from dysregulated Th1 responses, and that helminthic parasites suppress colitis even in the absence of the critical immunoregulatory cytokine IL-10. Crohn's disease is common in developed countries but rare in lesser-developed tropical countries. Helminthic parasitosis has an opposite global distribution. It is also known that Crohn's disease is a Th1-mediated inflammatory bowel disease [31]. Crohn's disease results from excessive intestinal inflammation associated with dysregulated Th1 (IFN- γ , IL-12, tumor necrosis factor- α (TNF- α)) cytokine production. IL-10-deficient C57BL/6 mice develop colitis that resembles Crohn's disease [32]. The colitis requires intestinal bacteria, can be transferred between mice and is progressive and fatal. Mice lacking IL-10 spontaneously develop colitis after 2-3 months of age. To produce a more uniform colitis, IL-10 $-/-$ mice given low-dose piroxicam develop severe colitis that persists after the NSAID is discontinued [33]. Wild type C57BL/6 mice do not develop colitis from piroxicam. Five-week-old IL-10 $-/-$ mice were given piroxicam for two weeks. After this treatment all mice developed colitis and piroxicam was then discontinued. Mice then next exposed to *Schistosoma mansoni* eggs and schistosome egg antigen by weekly intraperitoneal injection. Control mice received injections of PBS. At 10 weeks of age, the mice were sacrificed and colons removed, fixed, and sectioned for histologic examination. IL-10 $-/-$ mice treated with piroxicam and control injections of PBS uniformly had severe colitis (average score 3.7 ± 0.24 SE). However, animals exposed to *S. mansoni* had significantly less intestinal inflammation (1.2 ± 0.32 , $p < 0.001$ by Students' *t* test). Exposure to *S. mansoni* reduces established non-steroidal anti-inflammatory drug (NSAID)-synchronized colitis in IL-10 $-/-$ mice. Infection of *S. mansoni* can inhibit ongoing colitis that results from dysregulated Th1 responses. Helminthic parasites suppress colitis even in the absence of the critical immunoregulatory cytokine IL-10. Schistosome egg exposure attenuated trinitrobenzene sulphonic acid (TNBS) colitis, and protected mice from lethal inflammation [34]. Also, schistosome egg exposure diminished IFN- γ and enhanced IL-4 production from alpha CD3-stimulated spleen and mesenteric lymph node cells of TNBS-treated mice. Finally, schistosome egg exposure decreased colonic IFN- γ but increased IL-10 mRNA expression in TNBS-treated mice. Intact Stat6 was required for attenuation of colitis. Exposure to helminths can decrease murine colonic inflammation. According to this experimental evidence, Th2-dominant responses by parasite-derived molecules lead to the down-regulation of the Th1-type cytokines associated with organ-specific autoimmune disorders.

POSSIBILITY OF ANTI-ALLERGIC DISEASE

Only in the last 100 years or so have people living in areas of high development with sophisticated water and sewage systems been able to escape helminth infection [35]. Allergies are as conspicuously present in these human populations as they are absent in populations that are still regularly exposed to helminths. Furthermore, in populations with endemic helminthoses there is little overt expression of allergic pathology that could be connected to the acquisition

or elimination of helminth parasites [36]. Allergic reactivity may be rarely present in helminth-infected individuals because allergic reactions do not function to regulate helminth infections. There are many discussions when examining the link between helminth and atopy and at least three hypotheses have to be considered: helminth infections contribute to the atopic state; helminth infections prevent the development of the atopic state; there is no relationship between helminth infections and atopy. Recently we experimentally showed that helminth infections might prevent the development of the atopic state. In this section, we discuss the possibility that parasite molecules affect on atopic disease based on our experimental data.

It is known that functionally polarized responses by the CD4⁺ T helper (Th) cell toward Th2 contribute to the development of the atopic state [37]. When parasitic helminth infections are established, there are accompanying host responses that are dominated by a Th2 profile and the emergence of parasite-specific immunosuppression [38-41]. The increased production of IgE is especially characteristic of host animals infected with parasites. These infections result not only in the production of parasite-specific IgE but also in the production of a large amount of parasite-non-specific IgE. However, there is little available information about the nature of the factors that positively modulate the non-specific IgE response in helminth infections. Generally, the increase in IgE is thought to exacerbate allergic reactions. However, high levels of non-specific IgE may alter some features of anaphylactic reactions.

There are at least two possibilities to consider. First, high levels of non-specific IgE may increase allergic disease by actively stimulating the production of Th2-related cytokines and the high-affinity IgE receptor Fc ϵ RI. Second, high levels of non-specific IgE may protect against unrelated allergies by means of competition for mast cell receptors. We investigated the effect of rDiAg on PCA (passive cutaneous anaphylaxis) reactions in rats, and sought to identify the mechanism of suppression in PCA reactions [42]. In the rDiAg-infused group, the PCA reaction activated by anti 2,4-dinitrophenol (DNP) IgE was significantly inhibited compared with the control group (Fig. 4). However, the number and viability of peritoneal mast cells were not different between the two groups. The histamine contents in the culture supernatants in the presence and absence of rDiAg were not significantly different. Thus, the rDiAg does not directly inhibit histamine release from peritoneal mast cells. The plasma and ascitic IL-10 concentrations were analyzed on day 21 after pump implantation. In rDiAg-infused rats, IL-10 was significantly increased in serum and in the ascitic fluid. In addition, IL-10 production was marked in the supernatant of splenic cells primed with rDiAg. These data suggest that rDiAg induces IL-10 production. Fc ϵ RI contains four polypeptide subunits: an α -subunit that binds to the Fc portion of the IgE molecule, a β -subunit, and two γ -subunits [43]. In rats, transfection studies have shown that the β -subunit of Fc ϵ RI is necessary for expression of the α -subunit and γ -subunits on the cell membrane [44]. Here, we examined mRNA levels of the β -subunit to investigate the density of Fc ϵ RI on the peritoneal mast cells derived from

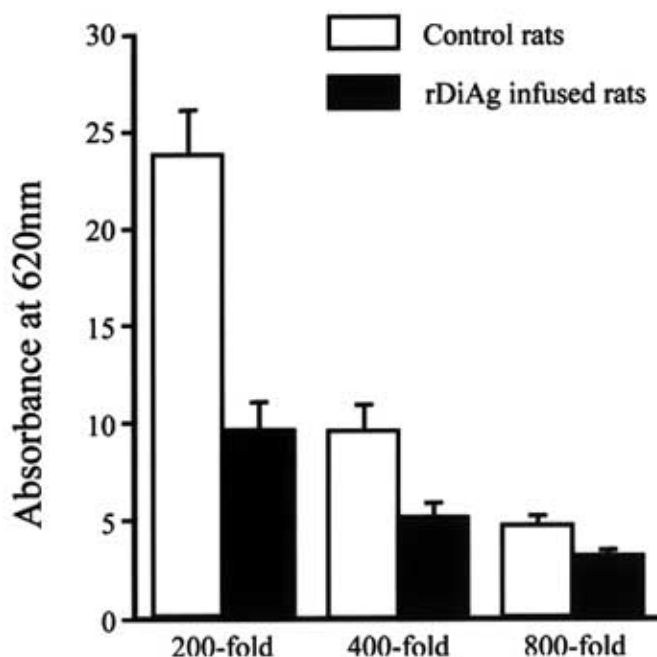


Fig. (4). Effect of rDiAg on PCA reactions. The amount of Evans blue dye on PCA reaction sites was measured. Reactions were examined 21 days after osmotic pump implantation. The anti-DNP-As IgE was diluted in saline (200-, 400- and 800-fold). The rats were injected intradermally with 100 μ l of anti DNP-As IgE into each skin site. The rats received an injection of 1 mg of DNP-As after 48 h. The dorsal skin was removed at 30 min after challenge. Values are means $\pm$ SE of 8 rats. $p < 0.05$, vs. control.

control or rDiAg-infused rats on day 21 after osmotic pump implantation. The expression of the β -subunit of Fc ϵ RI mRNA was up-regulated in peritoneal mast cells from rDiAg-infused rats compared with control rats. To clearly demonstrate that Fc ϵ RI expressed in peritoneal mast cells from rDiAg-infused rats is saturated with non-specific IgE, ^{125} I-IgE binding assays were undertaken. ^{125} I-IgE bound to peritoneal mast cells from control rats. The level of ^{125} I-IgE binding to the mast cells increased with the concentration of ^{125} I-IgE. In contrast, ^{125} I-IgE did not bind to peritoneal mast cells from rDiAg-infused rats. The ^{125} I-IgE binding to peritoneal mast cells from control rats suggests the presence of a very small number of high-affinity receptors (Kd=3086.5 pM; 1500 sites/cell), according to a scratchard plot analysis. The Kd of IgE binding to the mast cells is similar to that of other mammalian Fc ϵ RI [45].

Non-specific IgE production was induced in rDiAg-infused rats. The presented data support the hypothesis that non-specific IgE might protect against unrelated allergies by means of competition for mast cell receptors. rDiAg, which can induce non-specific IgE and IL-10, is an essential factor in the tolerance of immune responses against invasion by helminth parasites. IgE and antigen-dependent activation of mast cell mediator secretion is thought to be an important factor in the pathogenesis of asthma and other allergic diseases [46]. Some reports have shown a strong positive correlation between serum IgE levels and numbers of Fc ϵ RI expressed on mast cells [44,47,48]. These data suggest that the increase in IgE may exacerbate allergic reactions. However, high levels of antigen-non-specific IgE might alter the features of anaphylactic reactions.

While there is a hypothesis [11] that allergic reactivity is rarely present in helminth-infected individuals because allergic reactions do not function to regulate helminth infections, a functional role for allergic reactions in protecting against helminths has been confirmed by our experimental work. Regarding therapy by parasitic molecules for allergic disorders, there is a misgiving that the Th2 bias of Th polarization may induce exacerbation of the symptoms [49]. However, the possible immunoregulatory effects of helminths in allergies seem not to be due to a less intense Th2 response, since high IgE levels are a common feature of these infestations. As described in the next section, the suppression of the allergic response by rDiAg might be the effect of inducing immunoregulatory cytokines, because rDiAg induces the production of IL-10 *in vitro* and *in vivo* [20].

POSSIBILITY OF IMMUNOREGULATORY CYTOKINE INDUCERS

The majority of hosts infected with helminths demonstrate markedly reduced cellular immune reactions and polarization of T cell responses to Th2 and Th3 types. It is believed that these alterations are a key element in the persistence of the worms within their host. The down-regulation of inflammatory host immune reactions has been suggested to be induced by secreted products of the helminths. An aspect which has been recently focused on is the ability of the parasite to produce or to induce the production by host cells of immunoregulatory cytokines,

such as IL-10 or TGF- β [50]. This increased cytokine production could compensate for the reduced levels that are seen in patients with immunological disorders such as allergy and Crohn's disease [51]. Some studies described a 3 to 10 fold lower IL-10 concentration in bronchoalveolar lavage fluid of allergic patients than in normal controls [52]. The dimensions of wheals in skin prick tests to house dust mite extracts have been inversely correlated with IL-10 production by mononuclear cells of allergic patients when incubated with house dust mite extracts [53]. On the other hand, some studies on patients chronically infested with filarial nematodes or schistosomes have revealed an increase in IL-10 and TGF- β production by mononuclear cells [54,55]. Lymphocyte proliferative responses both to parasite and non-parasite antigens were reduced in these patients [56]. The inhibitory effects of IL-10 and TGF- β were confirmed, because pretreatment with anti-IL-10 or anti-TGF- β monoclonal antibodies restored lymphocyte proliferative responses.

Our experimental results showed that a factor (DiAg) that induces IgE derived from a filarial nematode parasite induces production of IL-10 *in vitro* and *in vivo* [20]. IL-10 induction may act in protection against helminths. IL-10 is a cytokine that shows immunosuppressive effects due to its ability to suppress the production of IL-12 and IFN- γ [57], to down-regulate MHC class II expression [58], and to reduce the expression of the adhesion molecules CD54 (ICAM-1) [59] as well as the costimulatory molecules CD80 and CD86 on antigen presenting cell (APC) [60]. IL-10 is a potential human therapeutic agent for immunological disorder such as IDDM [61] rheumatoid arthritis [62] and inflammatory bowel diseases [63]. We found that IL-10 production induced with rDiAg alone is higher in peritoneal B cells as compared with splenic B cells (Fig. 5). These results are supported by report that peritoneal B-1 cells are a main source of B cell-derived IL-10. IL-10 also acts as an autocrine growth factor and plays an important role in the terminal differentiation of B-1 cells in BALB/c mice, as continuous anti-IL-10 antibody treatment prevents the development of B-1 cells. Furthermore, IL-10 enhances proliferation of human B cells activated with anti-CD40 mAbs and the *in vitro* viability of mouse splenic B cells. Thus, B cell proliferation by rDiAg may be further enhanced in an autocrine or paracrine manner by IL-10 resulting from each B cell subset stimulated with this recombinant protein. We found that rDiAg-induced IgE production by splenic B cells is enhanced by coculture with peritoneal B cells and also confirmed that IgE production by rDiAg was less in CBA/N xid mice as compared with CBA/J wild-type mice. In fact, peritoneal B cells from CBA/N xid mice do not express detectable levels of IL-10, as these mice lack B-1 cells in their peritoneal cavity. These results suggest that IL-10 producing B-1 cells play an important role in polyclonal IgE production during filarial nematode infections. IL-10 induces up-regulation of Th2 responses concomitant with down-regulation of the Th1 response. IL-10 also enhances IL-4 plus anti-CD40 mAb-induced polyclonal IgE production by human peripheral blood mononuclear cells (PBMC). Thus, rDiAg may enhance Th2 responses, which can induce preferentially IgE production through a large amount of IL-10 production by peritoneal B cells *in vivo*. In

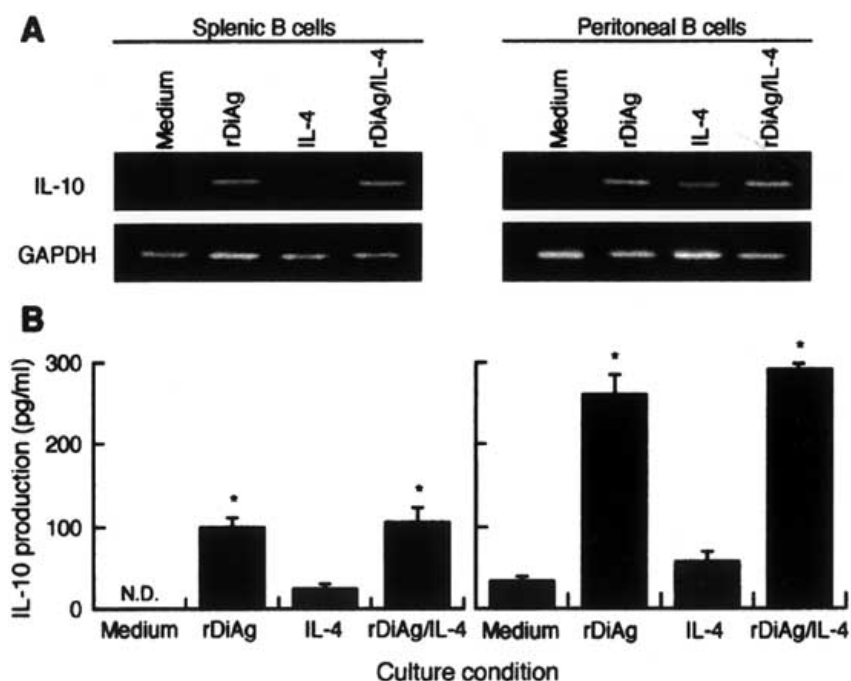


Fig. (5). rDiAg induces IL-10 production by B cells. Splenic or peritoneal B cells (5×10^6 /ml) from naive BALB/c mice were stimulated with rDiAg (10 μ g/ml) and/or IL-4 (10 U/ml) for 24 h (for RT-PCR) or 48 h (for ELISA). (A) Total RNA from B cells was analyzed for IL-10 mRNA expression by RT-PCR. The housekeeping gene GAPDH was amplified as an internal control. Data represent one typical experiment out of five. (B) IL-10 production in culture supernatants was measured by ELISA. The data represent the mean values and standard deviations (error bars) from five independent experiments. N.D., not detected; *, $p < 0.05$, when compared with medium alone.

addition, the production of IL-10 by peritoneal B cells induced with rDiAg was additively enhanced in the presence of IL-4. On the other hand, no enhancement of IL-10 production by splenic B cells was observed in the presence of IL-4, suggesting that IL-4 does not effectively act on rDiAg-induced IL-10 production by B cells.

Recombinant onchocystatin (rOv17) from the filarial nematode *Onchocerca volvulus*, a biologically active cysteine protease inhibitor that inhibited among others human cysteine proteases cathepsins L and S, suppressed the polyclonally stimulated and the antigen-driven proliferation of human PBMC [64]. Stimulated as well as unstimulated PBMC in the presence of onchostatin produced significantly more IL-10, which was paralleled in some situations by a decrease of IL-12 p40 and preceded by an increased production of tumor necrosis factor- α (TNF- α). IL-10 deficient mice show a much slower cutaneous migration of schistosomes and a much more exuberant cutaneous cellular infiltrate than normal mice.

Infection with *Trypanosoma brucei brucei* (Tbb), the protozoan causative agent of African Sleeping Sickness, could ameliorate the course of collagen induced arthritis (CIA) in the Dark Agouti rat, an experimental model which shares many features with human rheumatoid arthritis [65]. Infection of animals with living, but not inoculation with dead Tbb resulted in complete or significant reduction of clinical arthritic symptoms. Infection prior to collagen

immunization was more effective than a later treatment, and this effect was related to the level of parasitaemia. TGF- β could be detected in the lymph nodes in four out of six animals that had received Tbb. In the joints, immunohistochemistry revealed reduced production of TNF- α in Tbb-treated animals relative to controls. The most striking difference between Tbb-infected and control groups was the down-regulation of anti-collagen II IgG antibody responses in parasite-infected animals. Live parasites can exert an immunomodulatory and protective effect in CIA in which several mechanisms may work in parallel, although the almost complete down-regulation of the anti-collagen antibody response may alone explain the protective effect in CIA. The protective effects in CIA by Tbb infection might be related to the immunoregulatory responses by TGF- β such as down-regulation of proinflammatory cytokines. These experimental data suggested that immunoregulatory cytokine accumulation induced by helminth molecules could lead to the development of new therapies for immunological disorder.

MIMICRY OF IMMUNOREGULATORY MOLECULES BETWEEN THE HOST AND PARASITE

It is known that parasites make forms of mimicry molecules to allow successful interaction between the host and parasite. The mimicry between the host and parasite molecules may enable parasites to protect themselves from the immune system and induce a state of immuno-

modulation. Although classic autoimmune diseases appear less common amongst patients with chronic parasitic infections than in the general population, it is clear that autoimmune activity does occur in some patients with chronic parasite infections. Exposure to parasite infection represents one environmental modifier, and although it has been suggested that parasites might activate autoimmune disease through antigenic mimicry, it is also equally likely as has been shown that infection might inhibit the development of autoimmune disease.

In our experiments, we concluded that a factor (DiAg) that induces IgE and that is derived from a filarial nematode parasite is an agonist of human CD40 [66]. Dendritic cells (DC) are professional antigen-presenting cells (APC) that are specialized in the initiation of T cell-dependent immune responses. It is known that human DC secrete high levels of TNF- α and IL-12 upon either activation by lipopolysaccharide (LPS) or CD40 engagement [67]. The addition of IL-10 during DC stimulation profoundly inhibited the production of the cytokines by LPS-activated DC. In contrast, when DC were activated by co-culture with CD40L transfectants, TNF- α was not significantly affected by the addition of IL-10, but IL-12 levels were markedly decreased [68]. It is also known that antigen-presenting THP-1 cell lines produce proinflammatory cytokines such as IL-12 and TNF- α by CD40L stimulation [69]. We found that rDiAg directly stimulated a human immune cell line, THP-1 cell, and induced proinflammatory cytokine production. When THP-1 cells were stimulated with rDiAg or recombinant

human soluble CD40L (rsCD40L), IL-12 and TNF- α were induced (Fig. 6). These results demonstrate that human APC shows similar responses to CD40L and rDiAg. Also, the production of IL-12 was inhibited by addition of recombinant human soluble CD40 (rsCD40) (Fig. 7). In addition, rsCD40L competitively inhibited rDiAg binding to the membrane of these cells. ^{125}I -rDiAg or ^{125}I -rsCD40L bound to the cell membrane fraction and this binding was competitively inhibited by addition of rsCD40L or rDiAg respectively. Moreover, rDiAg bound directly rsCD40 (Fig. 8). rDiAg was not effective in CD40-deficient mice. The experimental evidence obtained using the CD40-deficient mice showed that the *in vivo* T cell-dependent, but not the T cell independent, antibody response is affected [70]. Our experimental results showed that polyclonal IgE induction by DiAg is affected in CD40-deficient mice. This means that DiAg induces polyclonal IgE by a mechanism that is not related to the T cell-independent antibody response. DiAg might induce the IgE class switching via the CD40 molecule with the help of IL-4. From these experiments, we concluded that DiAg is a native analog for soluble CD40 ligand. Soluble CD40L is known to be a potent immunoregulatory molecule and a candidate for a therapeutic agent for diseases such as cancer or chronic lymphocytic leukemia [71].

TGF- β is a stable, multifunctional extracellular growth factor with an extremely wide range of biological activities in metazoan animals. TGF- β is a particularly important modulator of the growth, differentiation, and activities of cells of the immune system, and multiple members of the

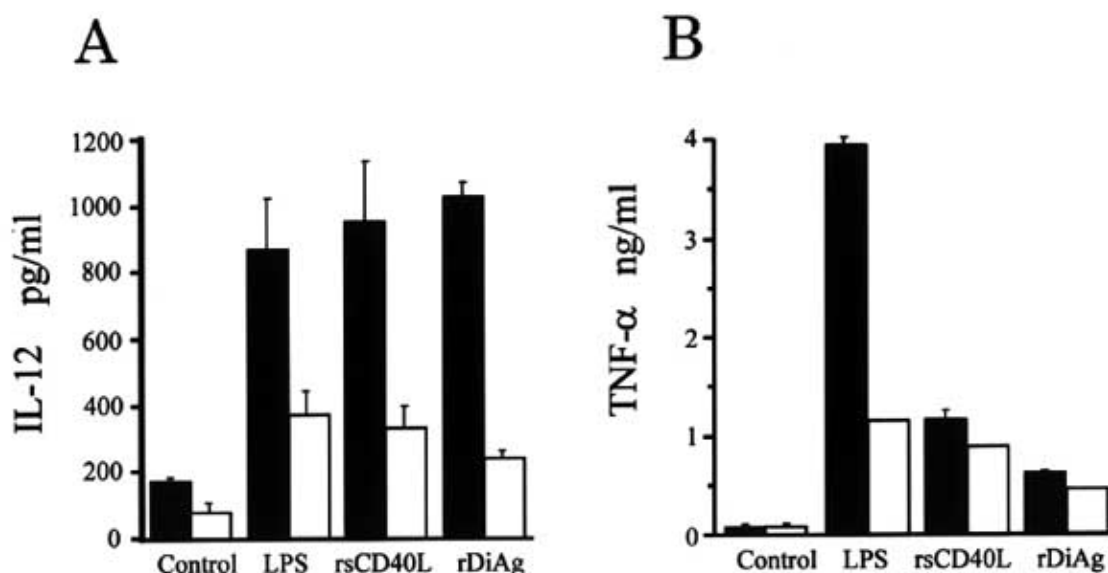


Fig. (6). Differential effects of IL-10 on the production of IL-12 and TNF- α by THP-1 cells stimulated with rDiAg, rsCD40L or LPS. **A)** THP-1 cells (2×10^6) were cultured in 24-well plates in 1 ml culture medium (RPMI 1640 supplemented with 10% fetal calf serum) with rDiAg (10 $\mu\text{g/ml}$), rsCD40L (10 $\mu\text{g/ml}$) or LPS (10 $\mu\text{g/ml}$), in the absence (open column) or presence (closed column) of IL-10 (20 U/ml). After incubation for 24 hr the culture supernatants were measured for IL-12 by means of ELISA (ENDOGEN). **B)** THP-1 cells (2×10^6) were cultured in 24-well plates with 10 $\mu\text{g/ml}$ of rDiAg, rsCD40L and LPS in 1 ml culture medium with 2 mM hydroxyurea and 50 $\mu\text{g/ml}$ silica powder and with or without 20 U/ml IL-10 at 37 $^\circ\text{C}$ for 24 hr in a humidified atmosphere of 5% CO_2 . Levels of TNF- α produced in the absence (open column) or presence (closed column) of IL-10 (100 U/ml) were determined by ELISA (R&D SYSTEMS) after 16 hr of culture. Data are shown as the mean $\pm$ SE of triplicate assays. * $p < 0.01$ by Student T test as compared with cytokine levels in absence of IL-10. Similar results were obtained in three separate experiments.

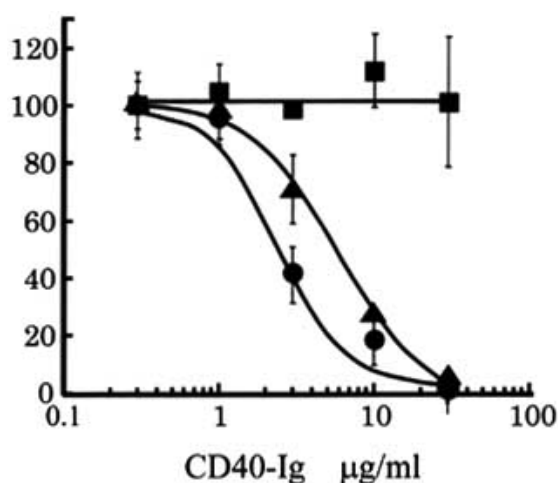


Fig. (7). Inhibitory effects of rsCD40-Ig on the production of IL-12 by THP-1 cells stimulated with rDiAg, rsCD40L or LPS. Levels of IL-12 produced in the presence of 1_ g/ml of rDiAg (triangles), rsCD40L (circles) and LPS (squares) with various concentration (0.3-30 µg/ml) of rsCD40-Ig were determined by ELISA after 16 hr of culture. Data are shown as the mean $\pm$ SE of triplicate assays. * $p < 0.01$ by Student T test as compared with cytokine levels in absence of IL-10. Similar results were obtained in three separate experiments.

superfamily are now associated with immune inhibition [72]. In parasitic infection, TGF- β has emerged as one of the key cytokines [73], together with IL-4 and IL-10, which down-regulate cellular responses and compromise immunity to a spectrum of intracellular infections, including those caused by *Leishmania* species, *Mycobacterium tuberculosis*, *Plasmodium chabaudi*, *Toxoplasma gondi*, and *Trypanosoma cruzi*.

A novel member of the TGF- β family has been identified in the filarial nematode parasite *Brugia malayi* [74] by searching the recently developed Expressed Sequence Tag (EST) database produced by the Filarial Genome Project [75]. Designated tgh-2, this new gene shows most similarity to a key product regulating dauer larva formation in *Caenorhabditis elegans* (DAF-7) [76,77] and to the human down-modulatory cytokine TGF- β . Similarity to human TGF- β is restricted to the C-terminal domain, over which there is a 32% identity between TGH-2 and TGF- β 1, and includes every cysteine residue. Expression of tgh-2 mRNA has been measured over the filarial nematode life cycle. It is maximal in the microfilarial stage, with lower levels of activity around the time of molting within the mammal, but continues to be expressed by mature adult male and female parasites. Expression in both the microfilaria, which is in a state of arrested development, and the adult, which is terminally differentiated, indicates that tgh-2 may play a role other than purely developmental. This is consistent with observation that TGH-2 is secreted by adult worms *in vitro*. Recombinant TGH-2 expressed in baculovirus shows a low level of binding to TGF- β -receptor bearing mink lung epithelial cells (MELCs), which is partially inhibited (16 to 39%) with human TGF- β , and activates plasminogen activator inhibitor-1 transcription in MELCs, a marker for

TGF- β -mediated transduction. Further tests will be required to establish whether the major role of *B. malayi* TGH-2 (Bm-TGH-2) is to modulate the host immune response via the TGF- β pathway.

Filarial nematode parasites establish long-term chronic infections in the context of an antiparasite immunity that is strongly biased toward a Th2 response. The mechanisms that lead to this Th2 bias toward filarial antigens are not clear, but one possibility is that the parasites produce molecules that have the capacity to proactively modify their immunological environment. Filarial parasites of humans secrete a homologue of the human proinflammatory cytokine macrophage migration inhibitory factor (MIF) that has the capability of modifying the activity of human monocytes/macrophages [78]. A cDNA clone isolated from a *B. malayi* infective-stage larva expression library encoded a 12.5-kDa protein product (Bm-MIF) with 42% identity to human and murine MIF. MIF homologues were also found to be expressed in the related filarial species *Wuchereria bancrofti* and *O. volvulus*. Bm-mif was transcribed by adult and larval parasites, and the protein product was found in somatic extracts and in the parasite's excretory-secretory products. Immunohistochemistry revealed that Bm-MIF was localized to cells of the hypodermis/lateral chord, the uterine wall, and larvae developing in utero. Unexpectedly, the activities of recombinant Bm-MIF and human MIF on human monocytes/macrophages were found to be similar. When placed with monocytes/macrophages in a cell migration assay, Bm-MIF inhibited random migration. When placed away from cells, Bm-MIF induced an increase in monocyte/macrophage migration that was specifically inhibited by neutralizing anti-Bm-MIF antibodies. Bm-MIF is the first demonstration that helminth parasites produce cytokine homologues that have the potential to modify host immune responses to promote parasite survival. Two homologues of the human cytokine MIF have been isolated from *B. malayi*. BmMIF show parallel functions to human MIF [79]. They are chemotactic for human monocytes and activate them to produce IL-8, TNF- α , and endogenous MIF [80]. Both biological activity and crystal structure reveal remarkable conservation between a human cytokine and its parasite counterpart despite the considerable phylogenetic divide among these organisms. The strength of the similarity implies that MIF-mediated pathways play an important role in nematode immune evasion strategies. These molecules have played the role of immunomodulators in the host for parasite protection.

DISCUSSION

In this review, we propose that parasite proteins have played an important part in inducing immunomodulation in the host and provide a new source of medicinal agents for immunological disorders. It is suggested that parasite proteins have medicinal potential in immunological disorders such as allergies, autoimmune disease, and rheumatoid arthritis. Many studies of immunomodulation by parasite infection or proteins suggest that parasite proteins would be good candidates for medicinal purposes. However, there are at least two problems in the development of drugs from parasite proteins. Parasite proteins present as much of a risk

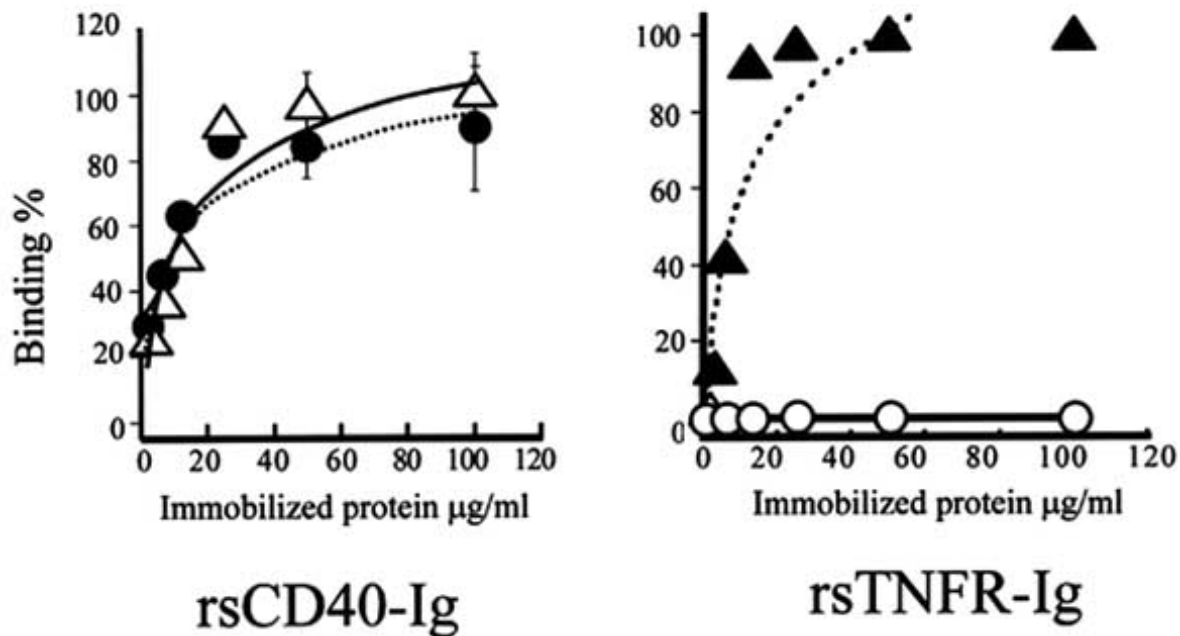


Fig. (8). Binding activity of rDiAg and rsCD40L or TNF- α to rsCD40-Ig or rsTNFR-Ig. Serial dilutions of purified rDiAg, rsCD40L and TNF- α were coated onto a 96-well plate (Maxisorb, Nunc) at 4 °C for 16 hr. The wells were then washed with PBS and blocked with milk casein (BlockA, Yukijirushi) for 1 hr at 37 °C. After washing with PBS, 100 μl of rsCD40-Ig (2 $\mu\text{g/ml}$ diluted in 0.1% bovine serum albumin in PBS) was added and was incubated for 1 hr at 4 °C. After washing with PBS, bound antibody was detected by peroxidase-conjugated anti-IgG Fc antibody (Jackson). **A**, This figure shows the binding of rsCD40L (open triangles) and rDiAg (closed circles) to rsCD40-Ig as described under "Methods". Curves represent the percentage of maximum binding of rsCD40L to rsCD40-Ig. Data are shown as the mean $\pm$ SE of triplicate assays. Similar results were obtained in two separate experiments. **B**, This figure shows the binding of rTNF- α (closed triangles) and rDiAg (open circles) to rsTNFR-Ig. Curves represent the percentage of maximum binding of rTNF- α to rsTNFR-Ig. Similar results were obtained in two separate experiments.

for antigenicity as recombinant protein drugs from non-human origins. They might have to be administered with a release rate as slow as the parasite's secretion of the protein. It has been suggested that *Trypanosoma cruzi* infection induces the up-regulation of both Fas and Fas ligand (FasL) molecules on B cells and renders them susceptible to B cell-B cell killing mediated via Fas/FasL [81]. More strikingly, the death pathway selectively affects IgG (+) B cells reactive to parasite but not self-antigens. The blockade of Fas/FasL interaction in the B cell compartment of *T. cruzi*-infected mice enhanced the anti-parasitic humoral immune response without affecting host tolerance. This data shows that some parasite-derived molecules can promote the down-regulation of antigenicity of themselves. In fact, the administration of rDiAg to a mouse did not induce production of DiAg specific antibody during the period of administration or later [20]. These findings might suggest that the antigenicity of rDiAg is not highly marked. The antigenicity of parasite proteins may be related to the choice of host species. More preferentially, the molecules of parasites that are able to infect humans should be selected for clinical use.

Because healing is a multistep process, parasite molecules may need to be applied at different times to achieve a satisfactory response. In our experiments, rDiAg was administrated by an osmotic pump that is capable of

releasing molecules for 2 weeks (0.25 $\mu\text{l/h}$) into animals to achieve maximum effect.

Although we show in this paper that parasite proteins have the potential to be effective treatments for immunological disorders, it might be unlikely that the parasite proteins themselves will be employed as drugs. It is much more likely that parasite proteins present an opportunity to develop a molecular target in order to try to produce a molecule that has both the essential biological and desirable chemical properties to become a drug development candidate. The path of a new drug from idea to product may be divided into two phases, namely drug discovery and drug development. In the realm of drug discovery a dramatic change is taking place. In drug discovery, it becomes more important to search and validate the target molecules that control diseases. Due to remarkable progress in understanding and explaining the underlying cause of many diseases by identifying and sequencing the genes encoded within DNA, it has become possible with new methods like molecular biology or gene technology to develop simple assays by which a large number of compounds may be tested in regard to their biological efficacy. For the screening of immunological disorder agents, the identification of molecular targets in animals has been performed, with much effort and funding, by traditional approaches from genomics,

proteomics and bioengineering. However, these methods are not necessarily efficient for cost performance. Over several hundred million years of evolution, the parasites have acquired ways to escape the host immunity without killing the host. Studies of immunomodulation by parasite molecules might be more effective in discovering molecular targets in immunological disorders than the latest novel gene technologies. Continued research in this area of innovative medicinal agents involving parasite molecules is expected.

ABBREVIATIONS

Ig	=	Immunoglobulin
IL	=	Interleukin-
TGF- β	=	Transforming growth factor-beta
Th	=	T helper
Tc	=	T cytotoxic (Tc)
IDDM	=	Insulin dependent diabetes mellitus
CNS	=	Central nervous system
SEA	=	Soluble egg antigen
NOD	=	Nonobese diabetic
ES	=	Excretory and secretory antigen
PBS	=	Phosphate buffer saline
R-EAE	=	Relapsing experimental autoimmune encephalomyelitis
IFN- γ	=	INTERFERON gamma
TNBS	=	Trinitrobenzene sulphonic acid
PCA	=	Passive cutaneous anaphylaxis
DNP	=	2,4-Dinitrophenol
ICAM	=	Intercellular adhesion molecule
APC	=	Antigen presenting cell
PBMC	=	Peripheral blood mononuclear cell
Tbb	=	Trypanosoma brucei brucei
CIA	=	Collagen induced arthritis
TNF- α	=	Tumor necrosis factor-alpha
LPS	=	Lipopolysaccharide
DC	=	Dendritic cells
rsCD40	=	Recombinant human soluble CD40
rsCD40L	=	Recombinant human soluble CD40L
EST	=	Expressed Sequence Tag
MELCs	=	Mink lung epithelial cells
MIF	=	Migration inhibitory factor

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