

Lewis^X-Containing Oligosaccharide Attenuates Schistosome Egg Antigen-Induced Immune Depression in Human Schistosomiasis

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ABSTRACT: The proliferative and interleukin (IL)-10 responses to Lacto-n-fucopentaose III (LNFPIII) that contains Lewis^X(Le^X)-trisaccharide was assessed in PBMC from humans infected with *Schistosoma mansoni*. All patient groups with low, medium, and high egg counts in their feces responded to polyvalent LNFPIII-HSA (where HSA = human serum albumin) conjugate. PBMC of all subjects showed a significant proliferative response to this sugar conjugate. However, the levels of interleukin (IL)-10 induced by LNFPIII-HSA were higher in groups with low and medium egg counts than those with high egg. Soluble egg antigens (SEA) also induced IL-10 production by PBMC from infected patients. Interestingly, the SEA-induced IL-10 production was remarkably inhibited by pretreatment of PBMC with free ligands of LNFPIII (monovalent form). These LNFPIII-pretreated

PBMC displayed appreciable increase in the level of proliferation to SEA stimulation. We propose that the observed bystander immune potentiation rendered by free LNFPIII is due to the reduced IL-10 level which, presumably, up-regulate expression of co-stimulatory molecules on APC. The ensemble of results indicates that the Le^X-containing LNFPIII is a potent immunoreactive epitope in SEA that negatively influences PBMC response against this parasite antigens via IL-10. *Human Immunology* 61, 225–232 (2000). © American Society for Histocompatibility and Immunogenetics, 2000. Published by Elsevier Science Inc.

KEYWORDS: Helminth antigens; Lewis^X-trisaccharide; T-helper function; immune regulation; immunotherapy

ABBREVIATIONS

SEA soluble egg antigens
LNFPIII lacto-n-fucopentaose III
LNT lacto-n-tetraose

Le^X Lewis^X-trisaccharide
HSA Human serum albumin
IL-10 interleukin-10

INTRODUCTION

Schistosomiasis is a chronic blood-vascular disease caused by the blood fluke, *Schistosoma mansoni*. A striking feature of the disease is generation of a granulomatous inflammatory reaction around the liver tissue-trapped parasite

eggs, which is accompanied by induction of a variety of host immune reactions [1]. Importantly, SEA induces a predominant Th-2 type immune responses in murine as well as human schistosomal infection [2–5]. SEA are highly glycosylated [6, 7], and the glycoprotein fractions of SEA appear to be immunogenic [6–10]. In murine model of this disease, the native form of SEA, but not its deglycosylated form, induced *in vitro* polyclonal B-cell activation [10]. In schistosome-infected humans, SEA stimulated the PBMC to proliferate and secrete IL-10 [4, 5]. These studies have primarily utilized SEA from whole egg homogenate, and in some of these reports a possible role for carbohydrate moieties of SEA in lymphocyte activation has been indicated [6, 7, 10].

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Previously, we have identified an immunoreactive oligosaccharide from egg antigens: LNFPIII that contains the Le^x-trisaccharide [8]. Le^x is the basic structure that constitutes the various biological ligands of leukocyte adhesion molecules especially P-, E-, and L-selectins [11]. In addition, LNFPIII is also known as stage-specific embryonic antigen-1 that is expressed mostly in adult brain tissue [8]. It is important to mention here that the schistosomes are unique among helminthes in expressing the LNFPIII pentasaccharide [12]. LNFPIII is abundant and ubiquitously expressed in many membrane-bound glycoproteins of the adult schistosomes [8, 12], glycoproteins/lipids released from the eggs [8, 9, 12], and in circulating parasite-derived antigens of the infected host [13, 14].

Aside from these biological and biochemical understandings, recent appreciation of immunological significance of LNFPIII/Le^x oligosaccharide is evident from several studies. Previously, we have reported that LNFPIII induced the splenic B220⁺ B-cells from infected animals to proliferate, and these cells secreted IL-10 and PGE₂ [15]. Recently, Jacobs et al. [16] have demonstrated that mice sensitized with LNFPIII/Le^x mounted an increased cellular response towards SEA-coupled beads implanted in the liver, resulting in the formation of large periparticular granulomas. These two studies clearly indicate that LNFPIII/Le^x actively participates in the host's cellular immune responses to schistosomal infection. Further, this oligosaccharide also participates in humoral arm of the immune responses: the serum of infected rodents [17], monkeys [18], and human patients [12, 14, 18, 19] contained Le^x-reactive antibodies. These antibodies from infected humans and monkeys were shown to mediate specific complement-dependent cytolysis of human myelocytic leukemic cells, thus implying an autoimmune phenomenon. To this end, we have earlier demonstrated that mice injected with LNFPIII-coated sepharose beads showed an increase in the percentage of autoreactive B-1 cells (CD5⁺ B-cells) in the peritoneal cavity [20]. These pioneering reports converge to offer a promising clue that LNFPIII/Le^x plays a major role in the host immune responses generated against this parasitic infection. However, the precise role of LNFPIII in the regulation of cellular responses of schistosome-infected humans has yet to be investigated. Understanding the basis for oligosaccharide-mediated regulation of immune responses in human would support formulating a sugar-based therapy in several infectious diseases. Here we present data to show that the LNFPIII-HSA activates PBMC of schistosome-infected patients, and the free form of LNFPIII attenuates the ability of SEA to negatively influence the PBMC response.

TABLE 1 Characteristics of samples used in this study^a

Sample groups	Egg counts ^b (per gm feces)	Number of samples		Age (years)
		Male	Female	
Health controls	0	5	6	18–42
Low egg	<100	7	4	14–22
Medium egg	100–400	3	5	16–24
High egg	>400	3	1	18–32

^a Groups have A, B, AB, and O blood groups distributed equally among samples, and contain varied HLA haplotypes. None of the subjects had any sign of ectopic illness at the time of sampling. Patients were classified into three groups based on the egg counts per gm stool weight.

^b Three stool samples were collected from each patient on different days. The egg counts represent the arithmetic mean of number of eggs per gm feces weight.

MATERIALS AND METHODS

Studied Subjects

Studies on subjects living in an endemic area (Itaquara, Bahia, Brazil) were carried out on 23 individuals. Control group includes students and institute employees (Centro de Pesquisas Gonzalo Moniz, Bahia, Brazil) who were free of parasitic infection (Table 1). Samples were collected with written consent of the subjects, or that of their parents or guardians. All donors were informed of the nature of this study before sampling. Patients were classified to three groups based on the quantitative parasitological stool examinations and observations of *S. mansoni* eggs [21].

Reagents

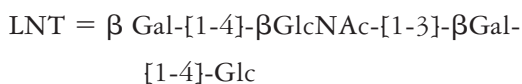
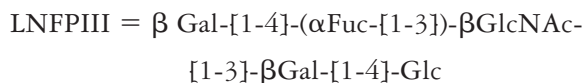
RPMI-1640, L-glutamine, penicillin, streptomycin, sodium pyruvate, sodium bicarbonate, HBSS containing Ca²⁺Mg²⁺, and FCS were purchased from Gibco BRL (Rockville, MD, USA). Lyophilized normal human AB serum, extravidin peroxidase, and HSA were from Sigma Chemical Co. (St. Louis, MO, USA). Ficoll-paque was obtained from Pharmacia Biotechnology (Piscataway, NJ, USA). Flat-bottom 96-well tissue culture clusters and microtiter plates for ELISA were from Corning Costar Corp. (Acton, MA, USA) and Nunc (Roskilde, Denmark), respectively. ³H-thymidine was purchased from Amersham (Arlington, IL, USA). Monoclonal Ab to hIL-10 and purified recombinant hIL-10 were from PharMingen (San Diego, CA, USA).

Antigens and Mitogens

SEA was prepared as described elsewhere [22]. Briefly, *S. mansoni* eggs were recovered from the livers of CD-1 mice 7 to 8 week post-infection. The saline soluble fractions were isolated, sterile filtered, and stored at -70°C until use. SEA was used at a final concentration of 2 µg/well. We have purchased LNFPIII in two different physical

forms: one conjugated to HSA (polyvalent) and the other in a free unconjugated form (monovalent). Hence forward, these two forms will be denoted as LNFPIII-HSA and free LNFPIII ligands, respectively.

The molecular structures of the oligosaccharides were as follows:



LNFPIII- and LNT-HSA were used at 10 $\mu\text{g/well}$ final concentration. Free LNFPIII and LNT were added in inhibition experiments at a final concentration of 7 $\mu\text{g/well}$. All oligosaccharides were obtained from Accurate Chemical and Scientific Corp. (Westbury, NY, USA). PHA (1 $\mu\text{g/well}$) and LPS (5 $\mu\text{g/well}$) were from Sigma.

PBMC Separation and Cell Culture

PBMC were isolated from heparinized blood by centrifugation on Ficoll-paque ($400 \times g$, 30 min, 15°C). PBMC were washed in RPMI medium containing L-glutamine (4 mM), sodium pyruvate (2 mM), sodium bicarbonate (0.2% w/v), penicillin (100 units/ml), and streptomycin (100 $\mu\text{g/ml}$). The cells were finally resuspended in the same medium supplemented with 1% (v/v) normal human serum (male, AB-blood group).

Lymphocyte Blastogenesis Assay

PBMC were distributed at 2×10^5 cells per well in a 96-well flat-bottom micro-titer plates. After the addition of antigen or mitogen, cells were incubated at 37°C in a 5% CO_2 atmosphere in a humidified incubator for 4 days. Cultures were pulsed with ^3H -thymidine (1 $\mu\text{Ci/well}$) for the final 16 h, and then harvested. The ^3H -thymidine uptake was measured using a LKB beta scintillation counter.

For IL-10 assay, the supernatants were collected from these cultures 36 h post-incubation and quantitated on the same day.

Pretreatment of PBMC with Free LNFPIII/anti-hIL-10

The PBMC from patients were treated with either free LNFPIII [15] or anti-hIL-10 [5] as described in these reports. In experiments using the monovalent forms of oligosaccharides, PBMC were pre-incubated with free LNFPIII (7 $\mu\text{g/well}$) or LNT (7 $\mu\text{g/well}$) for 30 min at 4°C , and then subsequently stimulated with either SEA (2 $\mu\text{g/well}$) or LPS (2 $\mu\text{g/well}$) as described previously. In some cases, the cultures contained either anti-hIL-10

mAb (clone JES3-9D7; 20 $\mu\text{g/well}$) or a rat IgG1 isotype control Ab.

IL-10 ELISA

The levels of IL-10 were measured in the 36-h culture supernatants using a two-site capture ELISA [15]. In brief, polystyrene micro-titer plates were coated with hIL-10 specific mAb (JES3-9D7), blocked, and then washed.

Culture supernatants were added to wells followed by addition of biotinylated anti-hIL-10 mAb (JES3 12G8). The bound Abs were detected using avidin-peroxidase system, and the colored product was measured at 450 nm in a Uvmax automated plate reader. Serial dilutions of recombinant IL-10 were included to construct a standard curve, and to extrapolate relative IL-10 concentrations in the culture supernatants.

Statistical Analysis

Data were expressed as mean \pm S.E. Comparisons between groups were made by Student's *t*-test. A value of $p < 0.05$ was considered significant.

RESULTS

Proliferation and IL-10 Production of PBMC *In Vitro* to LNFPIII-HSA Conjugate

The present study was initiated to address the immunological significance of Le^x-containing LNFPIII pentasaccharide in human schistosomiasis. The age, sex, and egg counts of patients with schistosome infection living in an endemic area are described in Table 1. None of the patients were under treatment for schistosomiasis at the time of sampling.

To evaluate the lymphostimulatory role of LNFPIII-HSA, the PBMC were stimulated either with LNFPIII-HSA conjugate or with its nonfucosylated homologue, Lacto-n-tetraose (LNT)-HSA as control. PBMC from healthy controls did not proliferate in response to LNFPIII-HSA stimulation as compared to the stimulation with HSA alone (Table 2). However, PBMC from all three patient groups responded to LNFPIII-HSA stimulation (cpm ranging from 1200 to 1900 over the HSA control). However, there was a trend toward higher side in the level of proliferation of PBMC from low egg patients (cpm = 1900) as compared to that of medium and high egg groups (cpm = 1200). All infected subjects showed only a marginal level of proliferation to LNT-HSA. A positive response to PHA was demonstrated in all samples (Table 2). These data indicate that LNFPIII in a polyvalent form is antigenic in stimulating the PBMC from schistosome-infected patients.

In our earlier study, we have demonstrated that in murine schistosomiasis the LNFPIII-HSA stimulates

TABLE 2 Proliferative response of PBMC to Lewis-related oligosaccharide conjugate

Sample groups	^3H -Thymidine uptake (cpm)				
	Medium ^a	PHA	LNFPIII-HSA	LNT-HSA	HSA
Healthy	1063 ± 165 ^b	4804 ± 509	1432 ± 170	1507 ± 136	1433 ± 185
Low egg	1016 ± 95	5170 ± 561	3575 ± 343 ^c	2400 ± 386	1690 ± 292
Medium egg	1610 ± 175	3916 ± 693	2722 ± 618	2019 ± 269	1528 ± 231
High egg	1562 ± 356	7286 ± 2574	3338 ± 306	2199 ± 345	2032 ± 149

^a PBMC (2×10^5) were stimulated in duplicate with either medium alone, or in the presence of oligosaccharide conjugates (10 µg/well). PHA (1 µg/well) and HSA were positive and negative controls respectively. The cpm was measured at 96-h cultures.

^b Arithmetic mean ± S.E. of 4 to 11 samples in each group.

^c $p < 0.05$ versus medium control or healthy subject.

splenic B-cells to secrete IL-10 *in vitro* [15]. Therefore, in the present study we wanted to see whether the PBMC of infected subjects secrete IL-10 in response to LNFPIII-HSA stimulation. The levels of IL-10 in the culture supernatants are presented in Table 3. Culture supernatants of PBMC from healthy controls had a minimal level of IL-10 (14 pg/ml) on stimulation with LNFPIII-HSA. The PBMC from low and medium egg groups secreted significant levels of IL-10 in response to LNFPIII-HSA. However, the level of IL-10 was higher in cultures with PBMC of low egg group (94 pg/ml over the HSA stimulation) as compared to cultures with PBMC of medium egg group (79 pg/ml). By contrast, PBMC of high egg group did not respond to this sugar stimulation in terms of IL-10 secretion. The control sugar, LNT-HSA, did not induce any significant levels of IL-10 in all of these four groups. All groups showed a positive IL-10 response to LPS stimulation. Together, these data indicate that LNFPIII is a potent carbohydrate antigen that induces proliferation as well as IL-10 secretion of PBMC from schistosome-infected patients especially those with low and medium egg counts.

LNFPIII Regulates Cellular Immune Response *In Vitro* Via IL-10

Several studies have reported that SEA induces PBMC proliferation and IL-10 secretion [4, 5, 23]. In experi-

ments with murine schistosomiasis, SEA-induced immune responses were largely attributed to LNFPIII present in complex milieu of SEA [7, 8, 12–15, 24]. Therefore, we next asked whether LNFPIII is a candidate antigenic motif in SEA-induced proliferation and IL-10 secretion of PBMC from infected patients. To answer this question, we had established an *in vitro* inhibitory bioassay in which PBMC of infected subjects (low and medium egg groups) were preincubated with free LNFPIII ligands thereby obstructing (LNFPIII)-SEA binding to lymphocyte cell surface receptors. We then stimulated this LNFPIII-treated PBMC with SEA and measured the levels of IL-10 secretion (Figure 1) and lymphocyte proliferation (Figure 2).

Figure 1 illustrates that SEA induced IL-10 secretion by PBMC of infected subjects (level ranging from 107 to 326 pg/ml with mean value of 239 pg/ml). Interestingly, this SEA-induced IL-10 secretion was dramatically reduced when the PBMC were pre-incubated with free LNFPIII, but not LNT, ligands (0–97 pg/ml with mean of 26). However, preincubation of PBMC with LNFPIII did not influence the level of IL-10 secretion by PBMC stimulated with LPS. This observation reveals that, indeed, the LNFPIII is the dominant motif present in SEA that is responsible for the SEA-induced IL-10 secretion by PBMC. We have additionally analyzed the effect of LNFPIII pretreatment on the proliferative response of

TABLE 3 Secretion of IL-10 by PBMC stimulated with oligosaccharide conjugates

Sample groups	Level of IL-10 (pg/ml) ^b				
	Medium ^a	LPS	LNFPIII-HSA	LNT-HSA	HSA
Healthy	0	544 ± 82 ^c	14 ± 10	0	0
Low egg	0	527 ± 69	96 ± 7 ^d	2 ± 1	2 ± 2
Medium egg	0	959 ± 174	104 ± 15	30 ± 15	25 ± 10
High egg	0	696 ± 205	12 ± 4	1 ± 1	0

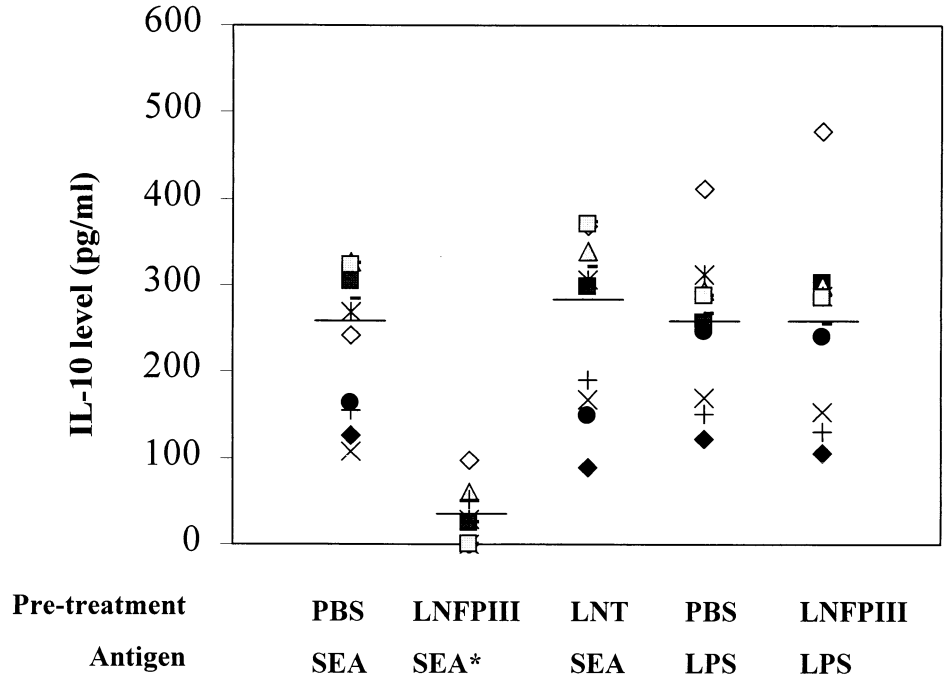
^a PBMC (2×10^5 cells/well) were treated as in Table 2. LPS (5 µg/well) was used as positive control.

^b The levels of IL-10 in the culture supernatants were measured by double-sandwich ELISA at 36-h post-culture.

^c Arithmetic mean ± S.E. of 4 to 11 samples in each group.

^d $p < 0.01$ versus healthy or high egg group.

FIGURE 1 Inhibition of SEA-induced IL-10 secretion by free LNFPIII ligands. PBMC (1×10^5 /well) of low and medium egg groups were pretreated with free ligands of LNFPIII (7 μ g/well) or LNT in PBS. PBMC treated with PBS alone were used as controls. Cells were then subsequently stimulated with either SEA (2 μ g/well) or LPS (2 μ g/well). The levels of IL-10 in 36-h culture supernatants were measured by double-sandwich ELISA. Cultures stimulated with medium alone had no detectable level of IL-10. Bars, arithmetic means. *Statistically different from PBS control.



PBMC to SEA (Figure 2). Surprisingly, we observed that pretreatment of PBMC with free LNFPIII, but not LNT, significantly enhanced the lymphoproliferative response to SEA (SEA alone, cpm ranging from 2100 to 6200 with mean of 3834; SEA+LNFPIII treatment, cpm ranging from 3900 to 10500 with mean of 5681; SEA+LNT treatment, cpm ranging from 2700 to 6400 with mean of 4111). As seen with IL-10 secretion, pre-

incubation of PBMC with free LNFPIII did not have any effect on the LPS-induced blastogenesis (Figure 2).

Based on this preceding experiment, a role for IL-10 in the immune response of PBMC to SEA was directly documented in the following experiment. PBMC from schistosome-infected patients (low and medium egg groups) were stimulated with SEA in presence of medium alone, anti-hIL-10 mAb, or rat IgG1 isotype con-

FIGURE 2 Effect of free LNFPIII on proliferative response of PBMC to SEA. PBMC were treated as described in legend for Figure 1. The thymidine incorporation was measured at 96-h cultures. Cultures stimulated with medium alone had cpm less than 1100. Bars, arithmetic means. *Statistically different from PBS control.

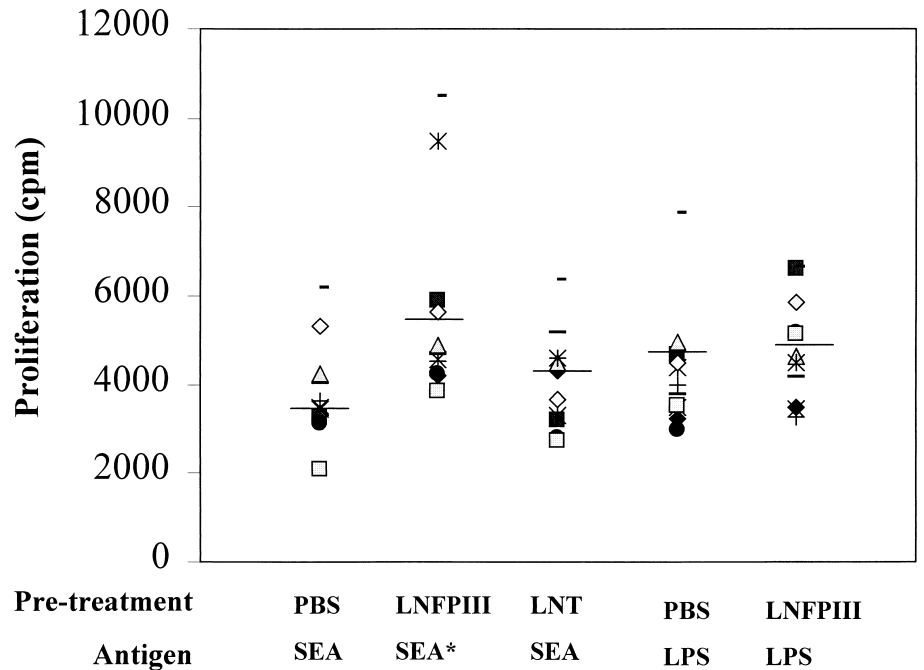
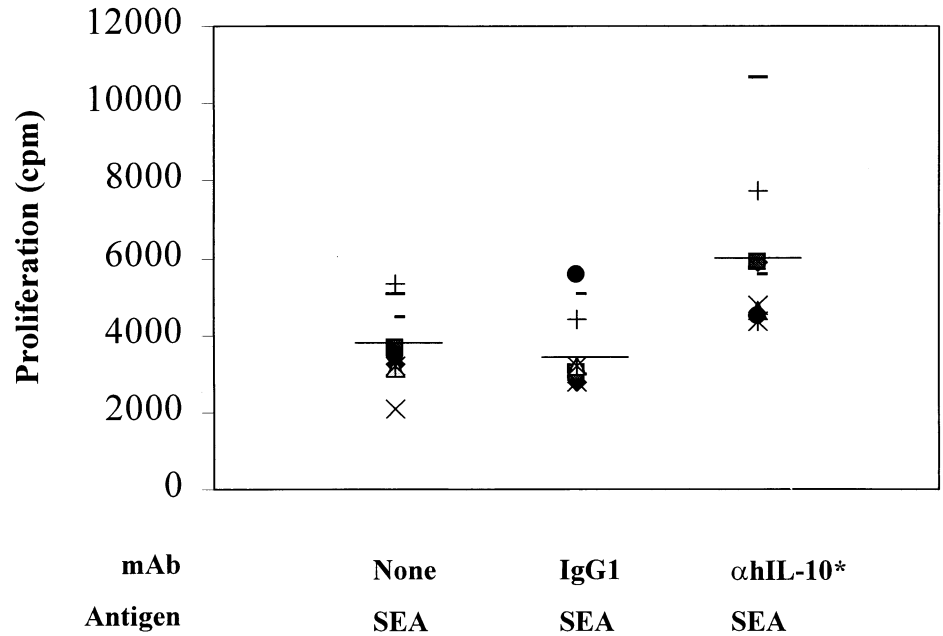


FIGURE 3 Treatment with anti-hIL-10 mAb up-regulates SEA-induced PBMC proliferation. PMBC (2×10^5 /well) of low and medium egg groups were stimulated in duplicate with SEA alone, or SEA plus anti-hIL-10 mAb (20 μ g/well). Isotype-matched rat Ig was used as control. The thymidine incorporation was measured in 96-h cultures. The cultures stimulated with medium or anti-hIL-10 alone had cpm less than 1100 and 1060, respectively. Bars, arithmetic means. *Statistically different from SEA alone.



trol Ab. As noticed with pretreatment with free LNFPIII, anti-hIL-10 also significantly augmented the proliferation of PBMC in response to SEA (Figure 3). The increase in cpm for cultures stimulated with SEA plus anti-hIL-10 mAb ranged from 4400 to 10600 with mean of 6000 as compared to the cpm for cultures stimulated with SEA alone that ranged from 2100 to 5300 with mean of 3700.

DISCUSSION

Parasite-derived oligosaccharide, LNFPIII/Le^x, is clearly a major focus of the immune responses generated in the schistosome-infected hosts. Our data show that LNFPIII-HSA induced proliferation of PBMC from schistosome-infected subjects. The level of proliferation induced by LNFPIII-HSA was comparable to that induced by SEA. This observation suggests that LNFPIII-HSA is as immunogenic as SEA, and that cellular responses initiated in infected subjects are predominantly specific for LNFPIII present in glycolipids/glycoproteins of SEA. It is worth mentioning here that LNFPIII exists in SEA as N-linked glycans [13], and in the present study the LNFPIII used was chemically coupled to HSA as to contain multiple LNFPIII terminals (~15 residues/HSA molecule). Therefore, this molecular orientation of LNFPIII in the carrier molecules allows exposure of multiple epitopes in a close proximity thus making LNFPIII-HSA to be a potent immunogen. The present results using PBMC of infected subjects are in consensus with earlier reports that have used infected animals. In murine schistosomiasis, LNFPIII-HSA induced splenic B-cells to pro-

liferate and secrete IL-10 and PGE₂ [15], the two soluble mediators that are known to have negative influence on Th-1 T-cell functions [25, 26]. Similarly, in the present study we show that PBMC from infected subjects elaborated IL-10 in the culture supernatants in response to LNFPIII-HSA.

Comparing the IL-10 responses of PBMC from various groups, the level was higher in the low and medium egg groups than that seen with the high egg group. The results imply that carbohydrate-mediated IL-10 induction could be an early event in the course of disease progress, and this reaction could set a stage to favor the generation of Th-2 type responses observed during the chronic phase of the infection. Several cell types can elaborate IL-10, including macrophages and B-cells. B-1 cells are the main source of B-cell derived IL-10 production [27]. Malignantly transformed human B-cell lines, consisting predominantly of B-1 cells, produced IL-10 [28]. The IL-10 produced endogenously by surface Ig-activated B-cells acts as an autocrine growth and differentiation factor [29]. We have shown in our earlier study that splenic B-cells from infected mice secreted IL-10 in response to LNFPIII stimulation [15]. In addition, SEA-primed peritoneal B-1 cells also secreted IL-10 upon stimulation with LNFPIII [20]. On this line, Martin-Filho et al. [30] have analyzed the lymphocyte profile of PBMC from schistosome-infected individuals and showed a higher percentage of circulating B-cells with an increase in the B-1 cell population. From these previous reports, it is conceivable to presume in the present study that B-1/B-2 cells could be a potential source for the observed IL-10 production in response to LNFPIII.

Our experiments thus far indicated that LNFPIII is a potent immunoreactive moiety that stimulates PBMC of infected subjects. We then proceeded to investigate whether LNFPIII is responsible for SEA-mediated lymphocyte reactivity. Results of the inhibitory assay reveal that the influence of free LNFPIII is remarkable in that the preincubation of PBMC with free LNFPIII significantly reduced the level of SEA, but not LPS, induced IL-10 production. These findings prompted us to propose that, in the IL-10 production, LNFPIII is the major reactive glycan of SEA. In addition, we suggest that LNFPIII binds to lymphocyte surface receptors other than that seen by LPS. Here, it is likely that LNFPIII containing Le^x trisaccharide could recognize an as-yet-unidentified molecule(s) present on the cell surface that has a C-type lectin sequence.

It is clear from the aforementioned results that free LNFPIII attenuates the ability of SEA to induce IL-10 production by PBMC. Consequently, startling results obtained from blastogenesis assay showed that pretreatment of PBMC with free LNFPIII considerably increased the SEA, but not LPS, induced proliferative response. One valid explanation for this LNFPIII-effected up-regulation in lymphoproliferative response to SEA is the blocking of SEA-induced IL-10 production by free LNFPIII. IL-10 has been shown to hinder antigen-specific Th-1 cell activation by preventing the antigen-presenting capacity of accessory cells through down-regulation of MHC class II and B7 co-stimulatory molecules on APC [31, 32]. Thus, it is plausible to conclude that free LNFPIII, by diminishing SEA-induced IL-10 production, indirectly supports the expression of these co-stimulatory molecules on the APC surface. If these molecules are up-regulated, then the efficiency of lymphocyte recognition of parasite antigens will be enhanced significantly, leading to our observed increase in lymphocyte proliferation. In support of this view, Araujo et al. [5] have demonstrated that neutralization of IL-10 by anti-IL-10 mAb resulted in enhanced T-cell proliferation to the parasite antigens in PBMC cultures. In the present study, we also have formally shown that the treatment of cultures with anti-hIL-10 mAb resulted in enhanced proliferative response to SEA.

In human schistosomiasis, the parasite exploits glycoproteins/glycolipids containing the Lewis-related oligosaccharides (LNFPIII/Le^x) to down-regulate the host's protective immune responses largely via induction of IL-10. Interestingly, free LNFPIII attenuates this effect thereby alleviating the immune depression caused by the parasite antigens. Collectively, these results indicate that treatment with free oligosaccharides could have a potential therapeutic value in several human disease states wherein the protective immune responses are severely compromised.

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