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Research Article

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Immunoregulation in Onchocerciasis

Functional and Phenotypic Abnormalities of Lymphocyte Subsets and Changes with Therapy

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Abstract

To help define the immunoregulatory defects in patients with onchocerciasis, flow cytometric analysis of circulating lymphocyte subpopulations was performed in parallel with functional assays. No significant differences in CD4/CD8 ratios were seen when microfilariae-positive individuals from Guatemala were compared with Guatemalan controls. However, the infected individuals had significantly increased numbers of circulating CD4⁺CD45RA⁺ lymphocytes (mean 38.3%) when compared with controls (mean 16.0%). Coexpression of the activation marker HLA-DR was significantly increased on CD4⁺ cells from infected individuals. In contrast, no up-regulation of HLA-DR was seen on CD8⁺ or CD19⁺ cells. At 1 year after initiation of treatment with semiannual doses of the microfilaricide ivermectin, there were significant increases ($P < 0.05$) in the percentage of CD4⁺CD45RA⁻ cells, the percentage of CD4⁺HLA-DR⁺ cells, and mitogen-induced lymphokine production (IL-2, IL-4). Despite these changes, parasite-specific IL-2 and IL-4 production which had been undetectable before treatment did not manifest itself even by the 2-yr follow-up. Defects in the T-cell activation pathway in *Onchocerca volvulus*-infected individuals may thus exist at several independent points; a state of parasite antigen-specific tolerance appears to remain even after the relative reversal of other generalized immunoregulatory defects. (*J. Clin. Invest.* 1991. 88:231-238.)
Key words: CD45RA • flow cytometry • interleukins • ivermectin

Introduction

Onchocerca volvulus infects approximately 18 million people in Africa and Latin America (1). As a result of the combined effects of the visual impairment, the blindness, and the skin disease that are sequelae of infection, onchocerciasis is believed to be second only to poliomyelitis as a cause of long-term disability in the developing world (2).

Though the pathologic manifestations of disease are secondary to the host immune response to the parasite (3), it is the modulation of potentially deleterious parasite specific immune responses that is thought to be the reason why the severe patho-

logic complications are seen only in a minority of infected individuals. Both in vitro lymphocyte responses (blastogenesis, IL-2, and IFN- γ production; 4-6) and in vivo cutaneous delayed-type hypersensitivity (DTH)¹ reactivity (7, 8) to *O. volvulus* antigens are impaired or absent in individuals with the generalized form of onchocerciasis. Additionally, there is evidence for a generalized depression of cellular immune responses to non-parasite antigens in *O. volvulus*-infected individuals. This non-specific hyporesponsiveness is manifested clinically by increased prevalence rates for lepromatous leprosy (9) and malaria parasitemia (10) in individuals with onchocerciasis. Additionally, impaired skin test reactivity to tetanus (11) and purified protein derivative (PPD) (12) in onchocerca-infected individuals recently vaccinated with tetanus toxoid and Calmette-Guérin bacillus (BCG) has been reported. When in vitro assessment of immune function has been carried out, depressed lymphocyte blastogenesis in response to soluble streptococcal antigen (13) and decreased IFN- γ responses to PPD (4) have been demonstrated in *O. volvulus*-infected individuals.

The mechanisms responsible for the observed immunoregulatory abnormalities are unclear; in particular there has been no detailed analysis of the functional or phenotypic status of the specific lymphocyte subsets that may be mediating this immunologic defect. Thus, the aim of the present study was to identify the functional subpopulations of CD4⁺, CD8⁺, and CD19⁺ lymphocytes that correlate with the immunoregulatory abnormalities seen in diseased individuals. To this end, the expression of the "activation" markers HLA-DR and the IL-2 receptor (14, 15) on circulating cell populations from *Onchocerca*-infected individuals from Guatemala (INF) were examined by flow cytometric analysis and compared to those of Guatemalan controls (CTRL). Of special interest were any perturbations in numbers of CD45RA-"memory" T cells (16) as have been described in other immunologically mediated diseases such as leprosy (17), multiple sclerosis (18), rheumatoid arthritis (19), or visceral leishmaniasis (20).

Further, because enhanced posttreatment immunologic responsiveness to filarial (21, 22) and (in one study) to nonfilarial antigens (6) have been described after antifilarial chemotherapy, a second goal of the study was to assess phenotypic and functional immunoregulatory changes seen in INF individuals as a result of semiannual therapy with the microfilaricide ivermectin over a 2-yr period.

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1. Abbreviations used in this paper: CI, confidence interval; CTRL, normal Guatemalan control; DTH, delayed-type hypersensitivity; INF, *O. volvulus*-infected individual; OvAg, crude *O. volvulus* adult antigen; PE, phycoerythrin; PPD, purified protein derivative; TNF, tumor necrosis factor.

Methods

Study population. 14 previously untreated adult male volunteers (age range 15–56 yr) with *Onchocerca volvulus* infection (the INF), who were residents of a finca near Patalul, Guatemala (an area holoendemic for onchocerciasis), were selected for study. Utilizing a corneal scleral biopsy punch and following a previously described protocol (4), all patients had skin snips obtained for microfilarial counts. As part of a community-wide treatment campaign all 14 INF individuals had initial treatment with 150–200 µg/kg of the microfilaricide ivermectin immediately after the blood samples were drawn for baseline studies; all were treated again at semiannual intervals with the same dose of ivermectin. Five Guatemalan field assistants (age range 24–59 yr) of low socioeconomic status who were employed by the onchocerciasis control program but with no history of residence in areas endemic for onchocerciasis, served as controls (CTRL) for the flow cytometric analysis; these individuals did not receive ivermectin. For assessment of functional changes in cytokine production as a result of ivermectin treatment, the pretreatment levels of IL-2 and IL-4 for each INF served as the appropriate control value for the follow-up values. PBMC from most ($n = 12$) of the INF individuals were available for functional immunologic studies at yearly intervals after the initial evaluation. On each occasion blood was drawn prior to ivermectin administration. The logistical difficulties of performing flow cytometric analysis on cells obtained in rural Guatemala resulted in our obtaining pretreatment parameters on all 14 INF individuals for most cell surface markers studied, but follow-up data was available on 8 of 14 for HLA-DR and 6 of 14 for CD45RA. Lymphocyte staining and supernatant generation were performed in Guatemala using freshly drawn PBMC.

Antibodies. The following monoclonal antibodies were used for flow cytometric analysis: Leu 3a (anti-CD4), Leu 2a (anti-CD8), Leu 12 (anti-CD19, B-cell associated antigen), Leu 18 (anti-CD45R, high molecular weight isoform of T200), L_{243} (anti-HLA-DR), 2A3 (anti-CD25, IL-2 receptor)—all from Becton-Dickinson Immunocytometry Systems, Mountain View, CA. MABs utilized for two-color flow cytometry analysis were directly conjugated either to phycoerythrin (PE) CD4, CD8, CD19) or to fluorescein isothiocyanate (FITC) HLA-DR, IL-2R, CD45RA) and used in combinations previously shown to be compatible (23, 24).

Isolation of lymphoid populations. PBMC were collected from heparinized patient blood by sedimentation on a Ficoll-diatroazate gradient Lymphocyte Separation Medium [LSM], Organon Technika, Durham, NC) at 400 *g* for 40 min at 20°C. Cells at the interface were collected and washed three times in RPMI-1640 (Biofluids, Inc., Rockville, MD), and viable cells were counted by trypan blue exclusion.

Two-color flow cytometric analysis. Two-color flow cytometric analysis was performed on fixed PBMCs stained with previously evaluated combinations of PE and FITC-conjugated MABs according to a protocol shown to preserve fluorescence intensity on cells stored in the dark at 4°C for up to 2 wk (25, 26). Briefly, 5×10^5 cells per tube were stained for 30 min at 4°C by simultaneous addition of PE- and FITC-conjugated MAB in various combinations. Each individual MAB was diluted according to the manufacturer's specification. After staining, cells were washed two times with FACS buffer (HBSS, 0.1% BSA, 0.1% sodium azide) and a third wash was done using PBS. Fixation of the cells was then carried out by addition of 0.4 ml of freshly made 1% paraformaldehyde for 15 min at 4°C. After two more washes in FACS buffer, cells were resuspended in 0.5 ml of HBSS with 1% BSA for transport at 4°C to the United States. Flow cytometric analysis was performed within 8 d in all cases.

Analysis of 10,000 cells per sample was performed on an Epics 773 (Coulter Electronics, Inc., Hialeah Park, FL) flow cytometer linked to a PDP11 computer. With PE fluorescence represented on the *x*-axis, FITC fluorescence on the *y*-axis and number of events on the *z*-axis, two-color contour maps were plotted. The percent of CD4⁺, CD8⁺, or CD19⁺ (PE staining) cells expressing each of the "secondary" markers (HLA-DR, IL-2R, or CD45RA; FITC staining cells) was calculated by taking the percentage of doubly stained cells and dividing by the sum of

the percentage of singly PE-stained cells plus the percentage of doubly stained cells. The mean number of CD4⁺ or CD8⁺ cells obtained from three independent combinations of each of these two PE-conjugated MABs with three different FITC-conjugated antibodies were used for calculation of the CD4/CD8 ratio in each patient.

Cytokine production by patient PBMC. 1×10^6 patient PBMC/ml of RPMI 1640 containing 10% FCS, gentamicin 80 µg/ml and 25 mM HEPES were incubated in 48-well tissue cluster plates for 24, 48, and 120 h at 37°C in a 5% CO₂ incubator in the presence of medium alone, phytohemagglutinin (PHA) 10 µg/ml (Wellcome Diagnostics, Research Triangle Park, NC), crude *O. volvulus* adult antigen (OvAg) 5 µg/ml, or with appropriate nonfilarial control antigens (4). Supernatant fluid was harvested and frozen in liquid nitrogen for later analysis.

IL-2 was assayed using the CTLL assay as described (25). IL-4 was assayed by ELISA using a polyclonal antibody to IL-4 and the monoclonal antibody 11B4 (kindly provided by DNAX Research Institute, Palo Alto, CA) as described (26). In preliminary experiments, the working range of the IL-4 assay was found to be 195–5,000 pg/ml. Interassay coefficient of variation was < 10%. There was no cross reactivity with IL-1, tumor necrosis factors TNF α and TNF β , IL-2, IL-3, IL-5, IL-6, granulocyte/macrophage-colony-stimulating factor (GM-CSF), or IFN- γ . All samples were assayed in triplicate.

Parasite-specific antibody. *O. volvulus*-specific IgG antibodies in serum were measured using a crude adult OvAg-based ELISA as previously described (4). Results are expressed in arbitrary units defined by a reference pool of sera from patients with onchocerciasis.

Statistical analysis. Geometric means were used throughout. Except where specified, samples were compared using the Mann-Whitney U test (unpaired samples) or the Wilcoxon signed rank test (paired samples).

Results

Study population. Table I summarizes the relevant parasitologic and serologic data for the individuals who met the criteria for inclusion in the study. Clinically, all 14 of the INF had the generalized form of onchocerciasis; the geometric mean skin microfilaria level was 7.21 microfilariae/mg of skin. None had received previous drug therapy for onchocerciasis. As expected, when *O. volvulus*-specific IgG levels were examined in the two groups, the INF had significantly elevated levels (mean 1,357 U/ml) when compared to the uninfected Guatemalan normals (mean 29 U/ml). However, when the mean CD4/CD8 ratio was examined in the INF group (1.29 [95% CI 1.11–1.58]) and compared to the CTRL group (1.77 [95% CI 0.33–3.78]), no significant difference was found (Table I).

CD4⁺CD45RA⁺ T lymphocytes. In order to investigate in more detail the differences in the circulating CD4⁺ subset in patients infected with onchocerciasis, two-color flow cytometric analysis was used to quantitate the expression of CD45RA on CD4⁺ cells. As can be seen from the representative individuals shown in the contour plot in Fig. 1, there is a larger population of doubly staining CD4⁺CD45RA⁺ T lymphocytes (i.e., "naive" CD4⁺ cells) in the INF patient (Fig. 1, right panel) when compared to a CTRL individual (Fig. 1, left panel). When the study groups as a whole were examined (Fig. 2), there was a significantly higher ($P < 0.02$) mean percentage of CD4⁺ cells that were CD45RA⁺ in the infected group (mean 38.3%; 95% CI 29.1–50.3) when compared to the normal controls (mean 16.0%; 95% CI 2.9–35.4).

Distribution of activation markers on lymphocyte subsets. Possible differences in the circulating lymphocyte populations with respect to the degree of "activation" of the lymphocyte subsets defined by the CD4, CD8, and CD19 phenotypes was

Table I. Comparative Parasitological and Serological Findings in the Study Population

Patient number	Skin microfilariae	<i>O. volvulus</i> -specific IgG	CD4/CD8 ratio
	mf/mg	U/ml	
Infected individuals			
3	17	1,790	1.08
5	5	671	1.12
7	4	1,877	2.11
13	2	7,067	1.5
14	64	872	1.36
16	18	2,794	1.66
18	2	583	2.11
26	3	1,353	1.36
27	25	1,958	0.99
28	26	731	0.84
29	4	2,424	1.54
30	1	529	1.18
31	12	1,914	1.01
32	7	668	0.91
Geometric mean	7.21	1,357*	1.29
Guatemalan normal controls			
Controls			
33	0	32	1.27
34	0	144	1.54
35	0	1	0.95
36	0	86	4.42
37	0	40	2.1
Geometric mean	0	29	1.77

* $P < 0.001$.

investigated next (Table II). When HLA-DR expression was examined on CD4⁺ cells using two-color flow cytometric analysis, it was found that in the INF group 10.7% (95% CI 9.1–12.6) of the CD4⁺ cells were HLA-DR⁺, as compared to 6.2% (95% CI 3.9–9.0) that were HLA-DR⁺ in the CTRL ($P < 0.002$). In contrast, no significant difference was found between the two groups in the expression of this activation marker, HLA-DR,

on either CD8⁺ or CD19⁺ lymphocytes (Table II). No upregulation of another activation marker, the IL-2 receptor (CD25), was found in either study group (data not shown).

Phenotypic changes in the CD4⁺ population after treatment. The mean CD4/CD8 ratio in the INF group did not change significantly over time (1.29 pretreatment compared to 1.39 at 1-yr follow-up after ivermectin therapy).

To test the possibility that either microfilaricidal therapy itself with ivermectin or the decrease in parasite antigen load (mean percent reduction of microfilariae counts in six INF individuals = 93% at 1 yr; mean of 7.4 microfilariae/mg pretreatment compared to 0.55 microfilariae/mg at follow-up) associated with this treatment could affect the increased numbers of CD4⁺CD45RA⁺ T cells observed initially, PBMC from six INF individuals were reexamined 1 year later. As is shown in Fig. 3 A, there was a significant decrease from 38.3% to 19.9% (95% CI 15.5–25.6) ($P < 0.05$) in the percentage of CD4⁺CD45RA⁺ cells (i.e., an increase in CD4⁺CD45RA⁻ memory cells) with therapy. Furthermore, every individual patient showed a decrease in the percentage of CD4⁺CD45RA⁺ cells with time. In contrast, there was no significant change in CD4⁺CD45RA⁺ cells at 1-yr follow-up in the Guatemalan normal controls (mean percent change over time = -1.73%).

When expression of the activation marker HLA-DR on CD4⁺ cells from the INF group was examined at the one-year follow up (Fig. 3 B), there was a significant increase in its expression ($P < 0.05$) from the pretreatment mean of 10.7% to a mean of 17.9% (95% CI 12.4–25.8). Furthermore, except for one patient who did not change, every individual patient examined showed an increase in the percentage of CD4⁺HLA-DR⁺ cells over time. In contrast, the CD4⁺HLA-DR⁺ cells in the Guatemalan normal CTRL did not change significantly. The pretreatment mean was 6.2% compared to a mean of 7.6% at 1-yr follow-up.

Expression of HLA-DR on CD8⁺ T lymphocytes did not change over time (not shown).

Regulation of cytokine production before and after treatment. As both CD4⁺CD45RA⁻ cells and CD4⁺HLA-DR⁺ cells increased significantly and because these cells have been implicated in IL-4 (CD45RA⁻) and IL-2 (activated CD4 cells) production, PBMCs from available INF individuals were examined quantitatively pretreatment, at 1 yr, and again at 2 yr for antigen and mitogen induced cytokine (IL-2 and IL-4) production.

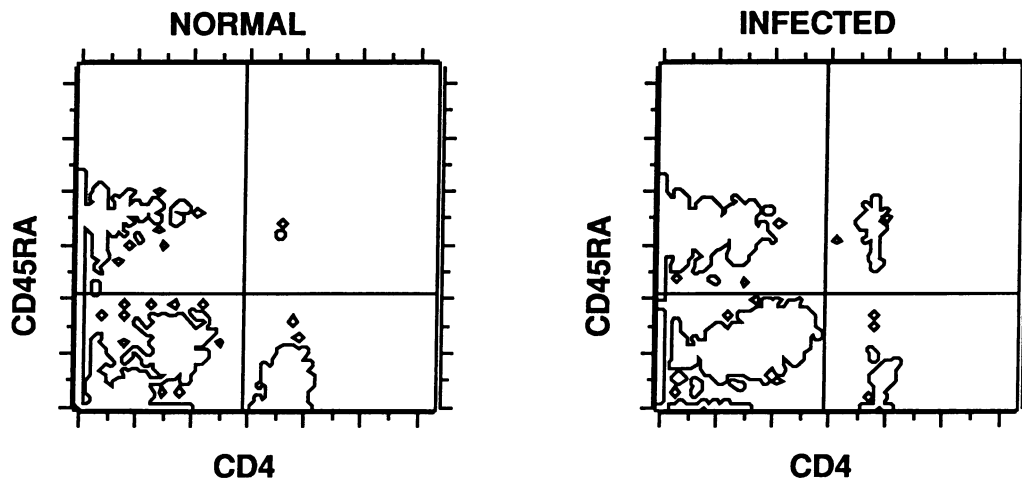


Figure 1. Two-color flow cytometric profile of peripheral blood lymphocytes co-expressing CD4 (PE staining; x-axis) and CD45RA (FITC staining; y-axis) at baseline before ivermectin treatment. Increased numbers of doubly stained CD4⁺CD45RA⁺ cells are seen in the representative INF patient (right) when compared to the representative CTRL patient (left).

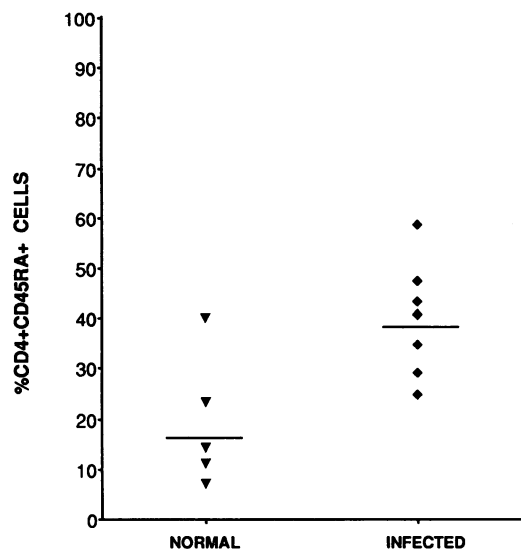


Figure 2. Proportion of doubly staining CD4⁺CD45RA⁺ (“naive,” unprimed) lymphocytes expressed as a percentage of total CD4⁺ cells in patients with onchocerciasis (infected) and in control individuals (normal) at baseline. The mean percentage doubly stained cells was significantly higher in the infected group (38.3%) when compared to the control group (16.0%), $P < 0.02$.

Pretreatment, there was found to be a significant inverse correlation between the percentage of CD4⁺CD45RA⁺ cells and PHA-induced IL-4 production (filled symbols in Fig. 3 C; $P < 0.05$; Spearman rank correlation). As is shown in Fig. 3, A and B, at 1-yr follow-up when the CD45RA⁻ population had significantly increased (i.e., decrease in CD45RA⁺ cells) in the INF individuals, mean mitogen-driven IL-4 production in these same individuals, had also increased significantly ($P < 0.05$) from 304 (95% CI 231–401) to 450 pg/ml (95% CI 351–576). To show that this correction of the pretreatment defect in IL-4 production by patient PBMC was sustained and showed no significant variability with time, PHA-induced IL-4 production by the same individuals was assessed at the 2-yr follow-up examination. The mean percent increase in IL-4 production in the INF over pretreatment values (which had been 200.1±SE 40.5% at the 1-yr follow-up) was 213.0±SE 46.2% at

Table II. Expression of HLA-DR on Lymphocyte Subsets at Baseline*

	Normal (n = 5)	Infected (n = 14)	
CD4 ⁺ DR ⁺	6.2 [‡] (3.9–9.0)	10.7 (9.1–12.6)	$P < 0.002$
CD8 ⁺ DR ⁺	15.3 (9.2–23.1)	16.8 (14.4–20.6)	NS [§]
CD19 ⁺ DR ⁺	68.6 (57.0–81.3)	42.6 (35.4–58.9)	NS

* Percentage of CD4⁺, CD8⁺, or CD19⁺ population expressing HLA-DR.

[‡] Values are geometric mean; (95% confidence interval).

[§] Not significant.

the 2-yr follow-up. The increases at these two time points do not differ significantly from each other. Flow cytometry was not, however, performed at 2-yr follow-up.

OvAg-driven IL-4 levels were also measured in PBMC supernatants that were generated and measured simultaneously and in parallel with the PHA-driven supernatants. At no time pretreatment or posttreatment (1- and 2-yr follow-up) was any antigen-driven IL-4 detected in any patient supernatant (assay sensitivity 195 pg/ml).

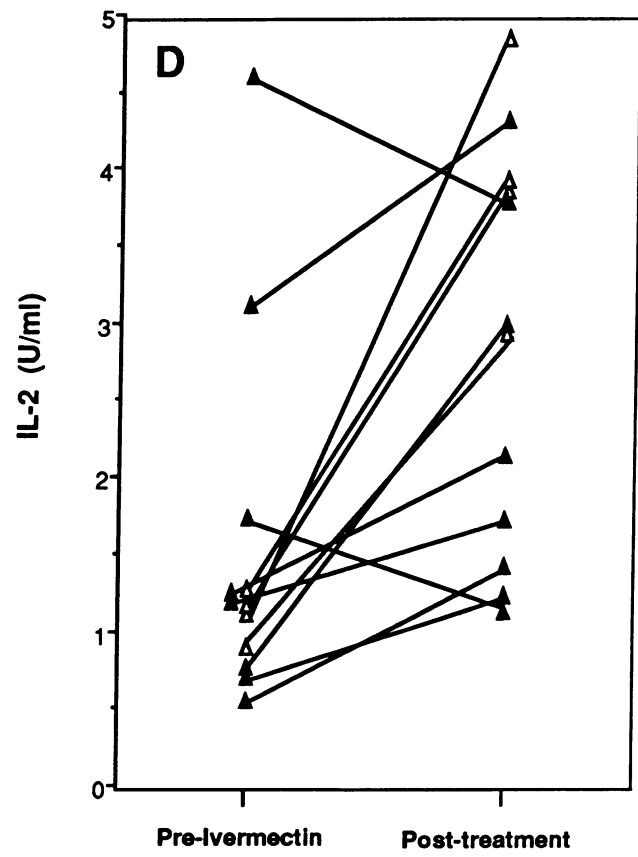
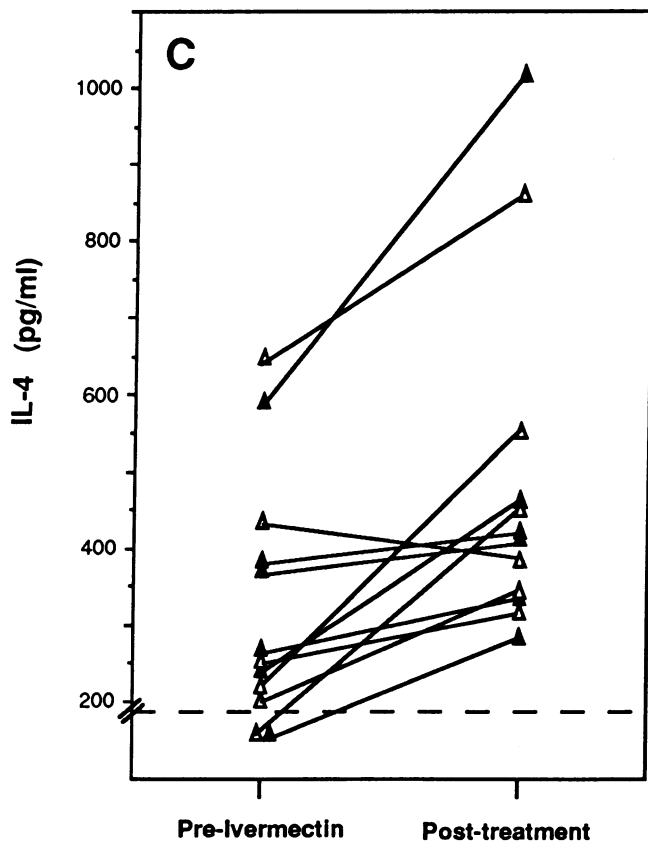
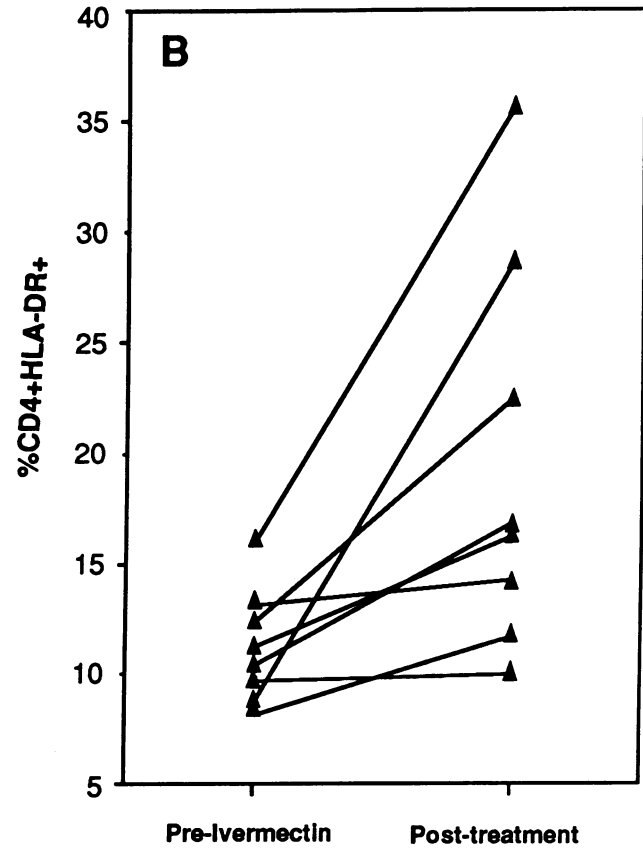
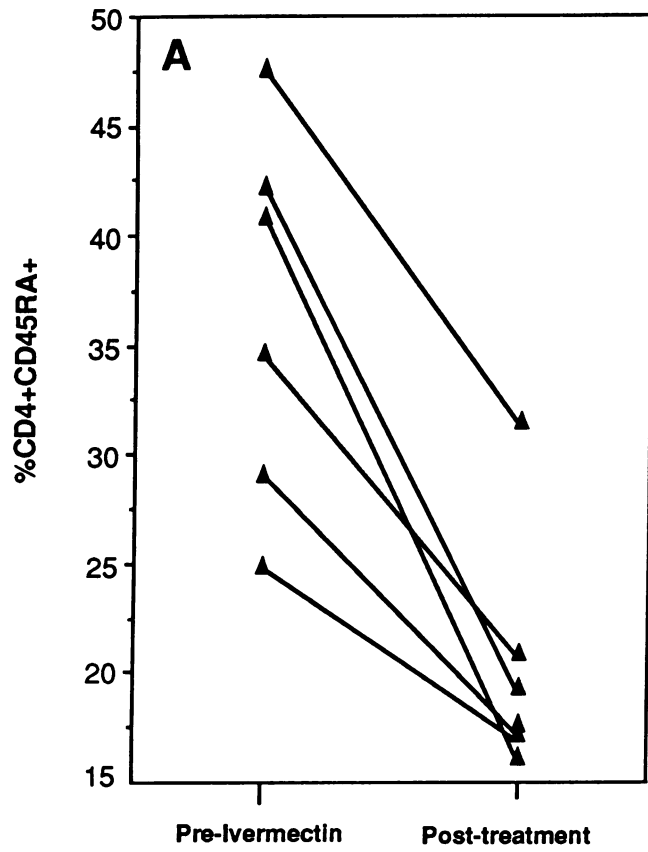
As is shown in Fig. 3 D, at 1 yr after the first ivermectin dose, when the CD4⁺HLA-DR⁺ population had significantly increased, mitogen-driven IL-2 production in the INF group had also increased significantly from 1.291 (95% CI 0.885–1.884) to 2.581 U/ml (95% CI 1.862–3.572). The mean percent increase over pretreatment values in PHA-driven IL-2 production which had been 232.2±SE 34.8% at 1 yr was 236.0±40.1% at 2 yr, thus demonstrating a sustained and reproducible increase.

OvAg-driven IL-2 levels were measured in PBMC supernatants that were generated and measured simultaneously and in parallel with the PHA-driven supernatants. At no time pretreatment or posttreatment (1- and 2-yr follow-up) was any antigen-driven IL-2 detected in any patient supernatant (assay sensitivity 0.03 U/ml). Consistent with these results, pretreatment in vitro proliferative responses (³H]thymidine uptake) in INF individuals in response to OvAg gave stimulation indices almost uniformly < 3.0. As would be expected from the IL-2 data above, proliferation capacity at 1 yr was not different from pretreatment values (data not shown).

Discussion

As has been demonstrated both by in vivo DTH reactions and by in vitro studies, individuals with generalized onchocerciasis exhibit severely impaired and frequently absent cell-mediated immune responses to specific parasite antigen (4–8, 26). Specifically, in studies of both African and Latin American onchocerciasis, PBMC from virtually every infected individual were incapable of generating detectable IL-2, IFN- γ , or lymphocyte blastogenic responses when stimulated with parasite antigen (4, 5). Furthermore, a more generalized suppression of the cellular immune response in *O. volvulus*-infected individuals has been often reported (4, 9, 11–13, 27). Although addition of exogenous IL-2 to cultured patient PBMC ameliorated the immunologic unresponsiveness to parasite antigen in one study (5), the cell lineages and underlying pathophysiologic mechanisms responsible for the defects in cellular activation in infected patients have been unclear.

To help understand these immunoregulatory abnormalities in chronic *O. volvulus* infection, the present study was designed to address two specific issues. First, we used two-color flow cytometry to examine phenotypic characteristics of circulating lymphocyte subsets in patients with generalized onchocerciasis when compared to uninfected local controls. Secondly, the effect of drug therapy on the characteristic antigen-specific and nonspecific immunologic unresponsiveness was assessed. Mitogen- and filarial-antigen induced cytokine production (IL-2 and IL-4) by patient PBMC at 1 and 2 yr after initiation of ivermectin therapy was compared to levels that were measured at baseline. In order to assess concurrent effects of ivermectin therapy on the phenotypic profile of patient lymphocytes, flow



cytometry was also performed at 1 yr after initiation of treatment.

In the first (pretreatment) phase of the study, no significant differences in the major T lymphocyte subsets (CD4, CD8) were found when Guatemalan individuals with generalized onchocerciasis (the INF) were compared with an uninfected, seronegative Guatemalan control group (the CTRL) never infected with onchocerciasis (Table I). Interestingly, a study in Liberia (28) utilizing the less sensitive technique of fluorescence microscopy for the comparison of major lymphocyte phenotypes among patient groups made a similar conclusion in an African population with generalized onchocerciasis.

To investigate in more detail possible differences in lymphocyte subsets between INF and CTRL individuals, the coexpression of CD45RA on CD4⁺ cells was assessed. CD45RA⁺ and CD45RA⁻ T-cells have been associated with different states of maturation of the T lymphocytes and are thought to represent "naive" and "memory" T cells, respectively (16). Formation of a memory cell, which occurs as a result of activation by specific antigen at some time after the exit of a naive T cell from the thymus, is uniformly associated with loss of the CD45RA marker (29). When expression of the CD45RA marker was examined in the two patient groups (Fig. 2), the INF individuals as a group were found to have a significantly higher percentage of CD4⁺CD45RA⁺ cells (38.3%) when compared to CTRL individuals (16.0%).

Quantitatively similar perturbations in the expression of CD45RA have been reported in a variety of immunologically mediated diseases, although its expression has not been investigated in any of the chronic helminthic infections. An ongoing immunologic response to antigen is thought to be responsible for the 1.5–2-fold decrease (compared to healthy controls) in CD4⁺CD45RA⁺ naive cells seen in the circulation in such immune mediated diseases as acute visceral leishmaniasis (20, 30), Reiter's syndrome (31), rheumatoid arthritis (19), and diabetes mellitus (32). Possible clinicopathologic relevance of this type of analysis was shown in multiple sclerosis, where individuals with the progressive form of the disease were found to have significantly fewer CD4⁺CD45RA⁺ cells (18.9%) when compared to those with stable disease (30.2%) or to healthy controls (41.0%) (18). Furthermore, elevated levels of CD4⁺CD45RA⁺ naive cells were seen in a group of patients with definite or classic rheumatoid arthritis who were also anergic to a panel of recall antigens (33), when they were compared to a clinically similar but nonanergic rheumatoid arthritis patients. In a similar fashion, the abnormally elevated population of unprimed, naive, CD4⁺CD45RA⁺ T cells seen in the INF individuals in the present study in the face of constant stimulation by *O. volvulus* antigen is in keeping with the characteristic state of functional immunological hyporesponsiveness seen in these patients (4–12).

The percentage of CD4⁺CD45RA⁺ cells (16.0%) in our all male control group (CTRL) was lower than has been generally

reported. Published values for percentages of CD4⁺CD45RA⁺ cells in normal individuals, initially reported to be ~ 40% (34), have varied from 20% to 55% in various studies. In one study where gender differences were examined, the male normals were found to have significantly lower percentages (19.3%) of CD4⁺CD45RA⁺ cells when compared to the normal females (32.9%) (35). The large number of bacterial, viral, and parasitic pathogens that residents of the developing world, like Guatemalan normal individuals constantly encounter, may account for the relatively low numbers of circulating CD4⁺CD45RA⁺ (naive) T cells seen in these controls when compared to published values for European and North American controls.

Expression of HLA-DR and the IL-2 receptor, which is seen only on low proportions of resting lymphocytes in normal individuals, may be increased up to severalfold in immune-mediated diseases, or after experimental challenge with soluble antigens like tetanus toxoid or PPD (14, 15). Abnormal elevations of these activation markers on T lymphocyte subpopulations in chronic helminthic infection have been shown to correspond with disease pathology. A group of individuals with inflammatory pathology secondary to Bancroftian filariasis was shown to have significantly elevated levels of CD8⁺DR⁺ T-cells (16.4% vs. 8.9%) when compared to asymptomatic infected individuals without such pathology; a role for this activated T cell population as effector cells in lymphatic obstructive disease was hypothesized (24). When African individuals with the pathologically and immunologically distinct localized form of onchocerciasis (called "sowda") were compared to individuals with generalized onchocerciasis, HLA-DR expression on the total circulating lymphocytes population was increased slightly less than twofold (28). When expression of HLA-DR on circulating lymphocyte subsets was assessed in the present study, the INF had a significantly higher percentage of CD4⁺DR⁺ cells (10.7%) when compared to CTRL (6.2%) individuals. In view of the repeated antigenic stimulus facing the infected host by virtue of the ongoing release of microfilariae into the tissues by the long-lived adult worms, the modest increase in numbers of activated CD4⁺DR⁺ cells over control levels is still suggestive of a relative immunoregulatory defect in the activation capabilities of CD4⁺ T cells in INF individuals. This hypothesis is supported by the further and significant increase in CD4⁺DR⁺ cells from 10.7% to 17.5% in the INF individuals 1 yr after reduction of their parasite burdens with ivermectin therapy (Fig. 3 B). No abnormal population of CD8⁺DR⁺ or CD19⁺DR⁺ cells was found in the INF individuals.

In order to assess mechanisms underlying the functional immunoregulatory defects that occur in INF individuals, a second goal of the study was to examine the effects of reducing the host's antigenic burden by reducing the total microfilarial load with the drug ivermectin. Ivermectin is microfilaricidal but does not kill the long-lived adult worms. Skin and ocular microfilariae, which are the stage of the parasite responsible for

Figure 3. (A) Decreased numbers of doubly staining CD4⁺CD45RA⁺ lymphocytes, expressed as a percentage of total CD4⁺ cells, in INF individuals after two courses of ivermectin therapy when compared to baseline levels 1 yr earlier. (B) Increased proportion of HLA-DR⁺ "activated" CD4⁺ cells in INF individuals at 1-yr follow-up after initiation of ivermectin treatment. (C) Increase in PHA-driven IL-4 production by PBMC from INF individuals 1 yr after initiation of ivermectin therapy. (▲) Same INF individuals for whom concurrent CD45RA staining is shown in A; (△) rest of the INF. Lower limit of IL-4 assay is 195 pg/ml. (D) Increase in PHA-driven IL-2 production when compared to baseline pretherapy levels. (▲) Same INF individuals for whom concurrent HLA-DR staining is shown in C; (△) rest of the INF.

inducing all of the immune mediated pathology associated with infection, are killed within 1–2 d of drug administration but reappear in tissue within 6 mo to a year. Because we were assessing the immunoregulatory changes produced as a result of semiannual ivermectin treatment in *Onchocerca*-infected individuals, the pretreatment levels of IL-2, IL-4, or lymphocyte phenotype were able to serve as appropriate control values for the follow-up values in each individual.

In 12 INF individuals followed sequentially after the initiation of ivermectin therapy, mitogen-induced IL-2 production by PBMC at the 1- and 2-yr time points had increased by an average of 232% and 236%, respectively, over pretreatment baseline control values in each individual. Follow-up supernatants were assayed at the same time as the baseline supernatant. The sustained and reproducible increase in IL-2 production once stable therapy was initiated demonstrates that there is little inherent variability over time in the production of this cytokine in these patients. As a clinical correlate of this finding, one previous study has shown increased levels of nonspecific skin test reactivity after diethylcarbamazine therapy of onchocerciasis (6). Because activated CD4⁺ cells might be expected to produce increased amounts of IL-2, the increase in mitogen induced IL-2 production over a 2-yr time period suggests a possible immunoregulatory significance for the increased numbers of activated CD4⁺HLA-DR⁺ cells (Fig. 3 B) seen.

In 12 INF individuals followed sequentially after the initiation of ivermectin therapy, mitogen induced IL-4 production by PBMC at the 1- and 2-yr time points had increased by an average of 200% and 212%, respectively, over pretreatment baseline control values in each individual. Further, 11 of 12 individuals showed increased IL-4 production by the 1-yr time point and sustained the increases with little variability subsequently. Several patients had seemingly minor rises in IL-4 production. Incremental yet consistent changes in IL-4 production must be viewed as potentially important in the context of the small number of IL-4-producing cells likely present in cultures of unfractionated PBMCs. In situ hybridization studies on normal donors have shown that only 3–4% of maximally mitogen stimulated CD4⁺ T cells were capable of producing IL-4 mRNA (36). IL-4 production has been shown to be restricted to CD45RA⁻ cells (36, 37). A dramatic decrease in CD4⁺CD45RA⁺ cells (an increase in CD4⁺CD45RA⁻ cells) (Fig. 3 A) was seen in six INF individuals for whom follow-up flow cytometry was available at 1 yr after the initiation of ivermectin therapy. A similar reversal of abnormally low numbers of CD45RA⁺ cells after antiparasitic chemotherapy in five patients with visceral leishmaniasis has been described (20). The concomitant increases in CD4⁺CD45RA⁻ memory T cells and mitogen driven IL-4 production suggests a possible functional significance for this increase in circulating CD4⁺CD45RA⁻ cells.

As discussed above, a sustained increase in mitogen-driven cytokine (both IL-2 and IL-4) production and increased numbers of circulating cells (CD4⁺DR⁺ and CD4⁺CD45RA⁻) capable of producing increased amounts of these cytokines were found in the INF individuals. Nevertheless, parasite antigen-specific production of IL-2 and IL-4, which had not been detectable pretreatment did not manifest in sensitive assays even after repeated ivermectin therapy over 2 yr. Thus, some yet to be elucidated defect in the ability of CD4⁺ T cells to respond to specific parasite antigen remains after ivermectin therapy.

Our results indicate that infection with *O. volvulus*, may lead to several independent defects in the T cell activation pathway. In the face of constant antigenic stimulation, such as is seen in microfilaria positive individuals before therapy, reversible defects in CD4⁺ cell activation were manifest by depressed expression of HLA-DR, depressed mitogen-driven lymphokine (IL-2 and IL-4) production and by decreased numbers of CD45RA⁻ memory cells when compared to posttreatment values. However, even after the relative reversal of these generalized immunoregulatory defects subsequent to therapy, the fundamental defect in parasite-specific antigen responsiveness apparently remains, suggesting that immunological tolerance (as opposed to active suppression) may account for the immunological hyporesponsiveness seen in generalized *O. volvulus* infection.

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