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FLOW CYTOMETRIC ANALYSIS OF THE TH1–TH2 BALANCE IN HEALTHY INDIVIDUALS AND PATIENTS INFECTED WITH THE HUMAN IMMUNODEFICIENCY VIRUS (HIV) RECEIVING A PLANT STEROL/STEROLIN MIXTURE

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The Th1–Th2 balance plays a pivotal role in determining the outcome of an immune response to an infectious organism. It is proposed that during HIV infection, disease progression is characterized by a loss of Th1 activity, a shift to a more 'allergic' Th2-type response and hence loss of cytotoxic cell activity against infected host cells. This study was undertaken to investigate this balance in three groups of individuals: HIV-negative volunteers (n=10), a group of HIV-infected patients on no therapy (n=10) as well as a group of patients managed with a mixture of plant sterols/sterolins (n=9). In parallel, their response to mitogens and the subsequent expression of the activation antigen CD69 was measured. This study was conducted by three-colour flow cytometry in order to obviate the less sensitive cytokine secretion assays that have yielded controversial results. The results indicate that HIV-infected patients on no therapy exhibit a pre-dominant Th2 response (IL-4 secretion), whereas those on the sterol/sterolin mixture exhibit a beneficial Th1 response (IFN-γ). Surprisingly, in both patient groups, the expression of CD69 was abnormally low when compared to the uninfected volunteers, implying that chronic activation is already present *in vivo*. It appears that the detrimental Th2 driven response might be swung to the more beneficial Th1 response with the immune modulatory sterols/sterolin mixture. Clinical use of this mixture in HIV infection has yielded results which corroborate the above observations in that patients using the plant sterol/sterolin mixture maintain their CD4 cell numbers over an extended period of time in the absence of any anti-retroviral therapy.

KEYWORDS: cytokines; Th1-cytokines; Th2-cytokines; cytoplasmic detection; HIV; plant sterols/sterolins; immunomodulation; flow cytometry.

INTRODUCTION

It has been known for many years that different microbes or antigens administered under different conditions elicit very different types of immune responses that are best able to eliminate these microbes or antigens. For instance, intracellular bacteria, such as most mycobacteria, stimulate macrophages, giving rise to delayed type hypersensitivity reactions, and include the production of opsonizing and complement fixing antibodies. In striking contrast, helminthic parasites induce eosinophilic inflammation and the production of IgE antibodies. These differences are likely to result from the production of functionally distinct cytokines produced by a number of immune and non-immune cells. T lymphocytes, especially CD4-positive cells, play a central role in the immune response to protein antigens. These functions are mediated by the secretion of cytokines in

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a supposedly polarized fashion—classified as either a Th1- or Th2-type cytokine.

The dichotomy between Th1 and Th2 has been identified in murine CD4+ T cells (Mosman and Coffman, 1989) and the analysis of T cell clones in humans have shown an analogous, although not identical, cytokine synthesis heterogeneity (Romagnani, 1994). Th1 and Th2 CD4+ T cells are characterized exclusively by differences in cytokine expression: Th1 cells produce interleukin (IL)-2 and interferon (IFN)-γ, whereas Th2 cells express IL-4, IL-5, IL-6, IL-10 and IL-13 (Romagnani, 1995). This cytokine heterogeneity is not restricted to CD4+ T cells, as other cell types also contribute to the secretion of regulatory cytokines. Thus, the terms Th1-type and Th2-type cytokine or cells are used to characterize the cytokine profile of different CD4 cell types.

Apart from the loss of CD4+ T cells, defects in T-cell immune function such as the reduced expression of Th1 cytokines can be detected in the peripheral blood and lymph nodes of HIV-infected individuals (Shearer and Clerici, 1991; Clerici, 1993; Meyaard *et al.*, 1993; Klein *et al.*, 1994, 1996). Moreover, the regular feature of a polyclonal B-cell activation and hypergammaglobulinaemia suggests that the Th2-type cytokines' expression may be increased. However, the observed changes in the pattern of Th1- and Th2-type cytokine expression during the course of HIV infection are controversial: certain authors reported a Th1 to Th2 cytokine shift (Meroni *et al.*, 1996; Barcellini *et al.*, 1994) while others proposed either a Th1 to Th0 shift (Rogmanani *et al.*, 1994a; Maggi *et al.*, 1994) and others still refute such a change of Th1/Th2 cytokine pattern (Graziosi *et al.*, 1994; Fakoya *et al.*, 1997). Many of these controversial reports may be due to the methodology employed to determine the Th profiles of the cells obtained from the patients: cytokine secretion into the culture supernatants was measured by means of ELISA. However, this is known to be problematic since many of the cytokines are utilized in autocrine fashion and hence the measurements may be low.

An understanding of the immunological mechanisms associated with protection from retrovirus infection and disease is likely to have an important impact on vaccine design and new therapeutic strategies. Natural acquired immunity against infection with the human immunodefficiency virus was studied in various cohorts of seronegative high-risk individuals. A specific group of prostitutes in Kenya was shown to remain HIV-1-negative despite repeated exposure and high-risk activity. Members of this group were found to have cellular immune responses to several regions of the virus (Kaul *et al.*, 1997). The presence of HIV-specific cytotoxic T lymphocytes (CTLs) has been demonstrated in this group. Uninfected partners of seropositive individuals have also been studied. They were found to have cellular immune responses indicative of primed Th1-type immune responses and/or CTL responses (Zerhouni and Touraine, 1997). In HIV-1-seropositive cohorts, approximately 5% of infected individuals remain healthy without evidence of disease progression. With respect to major histocompatibility complex (MHC) class II-related events, studies of the effects on Th cell activation, apoptosis and anergy as well as the production of Th1- and Th2-type cytokines and their relationship with disease progression were presented (Romagnani *et al.*, 1994b; Romagnani and Maggi, 1994).

Protective immunity in the presence of HIV-1 infection appears to depend on intact Th responses that are responsible for primary cellular as well as humoral responses. The plant sterols β-sitosterol (BSS) and its glucoside (BSSG), have been investigated as immune modulators of T cell activity (Bouic *et al.*, 1996). Both have been found to enhance the *in vitro* proliferation responses of T cells stimulated by sub-optimal concentrations of phytohaemaglutinin (PHA) several fold and the mixture of these plant sterols/sterolins preferentially enhance the activity of Th-1 CD4 cells (Bouic and Albrecht, 1993). Indeed, *in vitro* activated T cells expressed more activation antigens and they release more growth factors (IL-2 and IFN-γ) in their supernatants.

Based on these observations, several clinical studies have been undertaken. The clinical efficacy of the plant sterol/sterolin mixture was shown in patients treated for pulmonary tuberculosis (Donald *et al.*, 1997), in an open-labelled study in patients infected with HIV (Bouic, 1997) as well as in healthy individuals participating in an ultra-marathon (Bouic *et al.*, 1999). An animal model of HIV (FIV-infected domestic cats) has confirmed our clinical studies in HIV-positive patients (Bouic and Lamprecht, 1999).

In this paper we wish to report on the effect of the plant sterol/sterolin mixture on the cytokine secretion of cells from HIV+ patients' peripheral blood. Three groups of volunteers' cells were compared: healthy donors, HIV+ individuals not undergoing any anti-retroviral treatment, and HIV+ patients taking plant sterol/sterolin. Flow cytometry was used to characterize and distinguish CD4+ T cells as Th1 or Th2, solely defined by their

cytokine production. This method permits the detection on a single cell level without previous cell sorting and due to the fact that the activated cells are prevented from secreting the cytokines into their supernatants, the methodology may reflect the real nature of the cytokine responses. We detected the proposed dichotomy and demonstrate enhanced IFN-γ secretion by lymphocytes donated by HIV+ patients on plant sterol/sterolin treatment (Moducare™).

MATERIALS AND METHODS

Study populations

Peripheral blood was obtained after informed consent from ten healthy adult donors, ten HIV-infected patients on no treatment and nine HIV-infected donors receiving sterols/sterolins. Patients suffering from TB, malignancies, allergies, asthma were excluded from this study.

The healthy volunteers were HIV-negative laboratory personnel ($n=10$). The patient populations were recruited from the Infectious Diseases Clinic in the hospital. Those not on any antiretroviral treatment ($n=10$) were bled after informed consent. Those patients using the plant sterol/sterolin mixture supplement ($n=9$) were all on a clinical trial registered with our Ethics Committee. This trial is investigating the long term effects of Moducare™ on the immune parameters of HIV-infected patients. These patients were selected in order that the study populations exhibit similar CD4 cell counts and hence, similar disease stages. Blood sampling was part of the clinical follow up. These patients had been using the Moducare™ treatment for at least 6 months: this consisted of ingesting three capsules per day, each containing 20 mg sterol and 0.2 mg sterolin.

Cell preparation

Venous blood, collected in preservative-free lithium heparin tubes, was obtained from donors. The blood was diluted 50% with Tris-buffered RPMI. Peripheral blood mononuclear cells (PBMC) were isolated by Ficol-Histopaque (Sigma, Diesendofen, Germany) density gradient centrifugation and washed three times with the Tris by centrifugation. Cells were adjusted to a concentration of 2×10^6 cells/ml in RPMI 1640 medium (Gibco, Karlsruhe, Germany) containing 10% heat-inactivated fetal calf serum (Gibco), 100 U/ml penicillin and 100 μg/ml streptomycin.

Culture

PBMC were stimulated with 25 ng/ml PMA (phorbol-12-myristate-13-acetate) in combination with 1 μg/ml ionomycin (Sigma) in flat-bottom culture wells. Brefeldin-A (Sigma) was added in a concentration of 10 μg/ml to prevent cytokine release, by disrupting intracellular Golgi-mediated transport and allowing cytokines to acculmulate.

Cultures without exogenous stimuli were included to record the spontaneous activation and secretion of cytokines by CD4+ T cells. The optimal duration of incubation (at 37°C, 7% CO_2) for the induction of each cytokine had been determined previously in time kinetic experiments: the Th1-type cytokine IFN-γ was synthesized earlier than the type 2 cytokine, IL-4. The detection was therefore conducted at two cut-off points, namely, after 7 h and after 18 h incubation as described above.

Fixation, permeabilization and cytokine-staining

After incubation the cells were stained according to methods described by Becton Dickinson Immunocytometry Systems, BD Biosciences, Erembodegem, Belgium (BDIS) for flow cytometry. The cells were incubated in 2 ml Lysing Solution (BDIS) for 10 min at room temperature. Fluorescent Activated Cell Sorter (FACS) Lysing Solution (BDIS) helps to fix the surface epitopes and optimize the permeabilization process. The cells were pelleted. FACS Permeabilizing Solution (BDIS) overcomes the limitations of saponin-based permeabilizing reagent in ensuring consistent sensitivity and low background staining. Cells were incubated in 500 μl of FACS Permeabilizing Solution for 10 minutes at room temperature in the dark. Cells were washed with paraformaldehyde and pelleted. The following cytokine-specific monoclonal antibodies (mAbs were used: anti-IFN-γ-fluorescein isothiocyanate (FITC) (BDIS) and anti-IL-4-phycoerythrin (PE) in order to detect either Th-1 or Th-2 cytokines respectively.

Surface phenotyping was performed using a combination of CD3-Peridine chlorophyll a protein (PerCP)-labelled mAb and CD4-FITC-labelled mAb; and for activation detection, a monoclonal antibody against CD69-PE-labelled was used. All monoclonal antibodies were from Becton Dickinson. After three final washes, the cells were analysed in a flow cytometer. Routinely 20,000 events were analysed. The results were expressed as percentage of the cells positive for the antigen detected by the monoclonal antibodies.

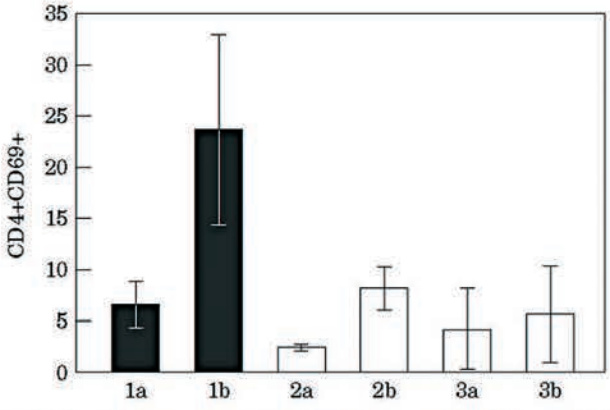

Fig. 1. CD69 expression (median ± SE percentage) on CD4 T cells following mitogenic activation for 4 h. Cells from HIV-negative volunteers (cultures 1a and 1b); HIV-positive patients on no treatment (cultures 2a and 2b) and HIV-positive patients on plant sterol/sterolin supplementation (cultures 3a and 3b) are shown. Cultures were incubated with medium alone (unstimulated: 1a, 2a and 3a) or were activated with PMA and Ionomycin (stimulated: 1b, 2b and 3b) as described in Materials and Methods section.

Statistical analysis was performed using non-parametric methods (Kruskal–Wallis and median tests).

RESULTS

Activation and CD69 expression

The difference in the percentage of cells expressing CD69 between those stimulated with PMA and those cultured in medium alone (unstimulated) is pronounced in the healthy group and is statistically significant ($P<0.05$). On the other hand, cells from the HIV+ group (either that having received sterols/sterolins treatment or those that were not treated) do not show such a significant difference in the percentage of activated cells (CD69-positive cells). The percentage of CD69-positive cells is only slightly higher in the PMA-stimulated cells than in the unstimulated cells (Fig. 1). It therefore appears that the activation process leading to the expression of CD69 is defective in HIV infection and that this possibly correlates to the stage of disease. Similar defects (lack of proliferation to stimuli, defective cytokine secretion by T cells, etc.) have been described in HIV disease.

Cytokine secretion

The Th1-type cytokine IFN-γ was induced only upon PMA addition to the cultures of the three groups. The markedly raised secretion of IFN-γ by the HIV+ patients taking the sterols/sterolins mixture was significantly higher ($P<0.04$) than the healthy individuals and the untreated HIV+ patients, in both the 7 h and 18 h cultures (Fig. 2). Indeed, significantly more cells staining for the presence of IFN-γ were detected in the cultures from HIV+ patients taking the sterols/sterolins when compared to those obtained from the healthy individuals and the non-treated HIV+ patients.

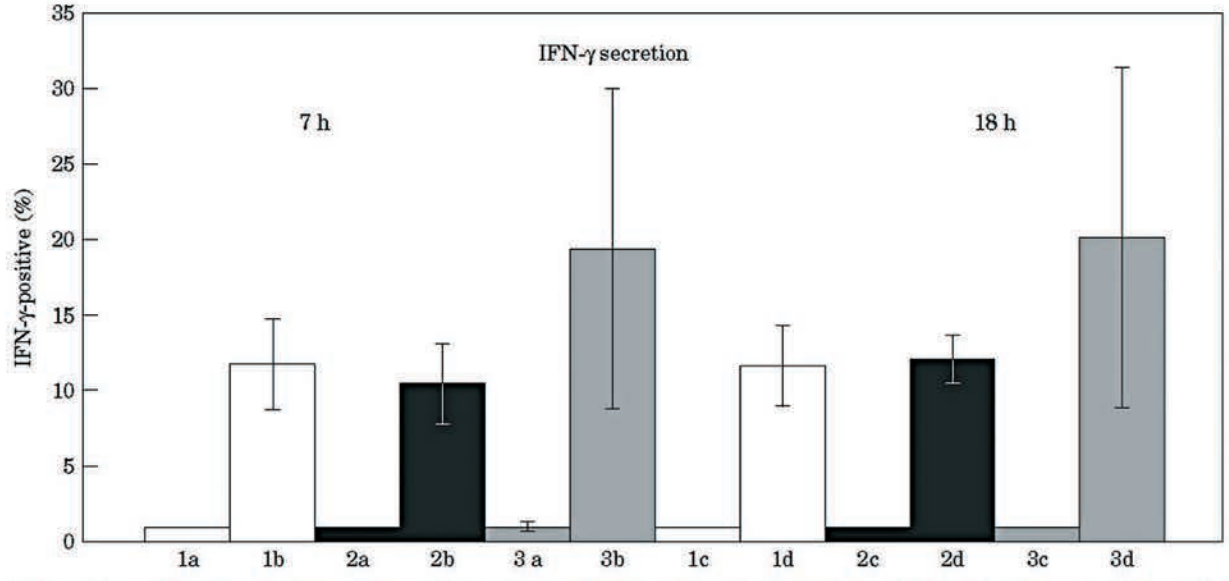

Fig. 2. Detection of IFN-γ-containing cells (median ± SE) following mitogenic activation of CD4 T cells for 7 or 18 h. Cells from HIV-negative volunteers (cultures 1a, 1b, 1c and 1d); HIV-positive patients on no treatment (cultures 2a, 2b, 2c and 2d) and HIV-positive patients on plant sterol/sterolin supplementation (cultures 3a, 3b, 3c and 3d) are shown. Cultures were incubated with medium alone (unstimulated: 1a, 1c, 2a, 2c, 3a and 3c) or were activated with PMA and Ionomycin (stimulated: 1b, 1d, 2b, 2d, 3b and 3d) as described in Materials and Methods section.

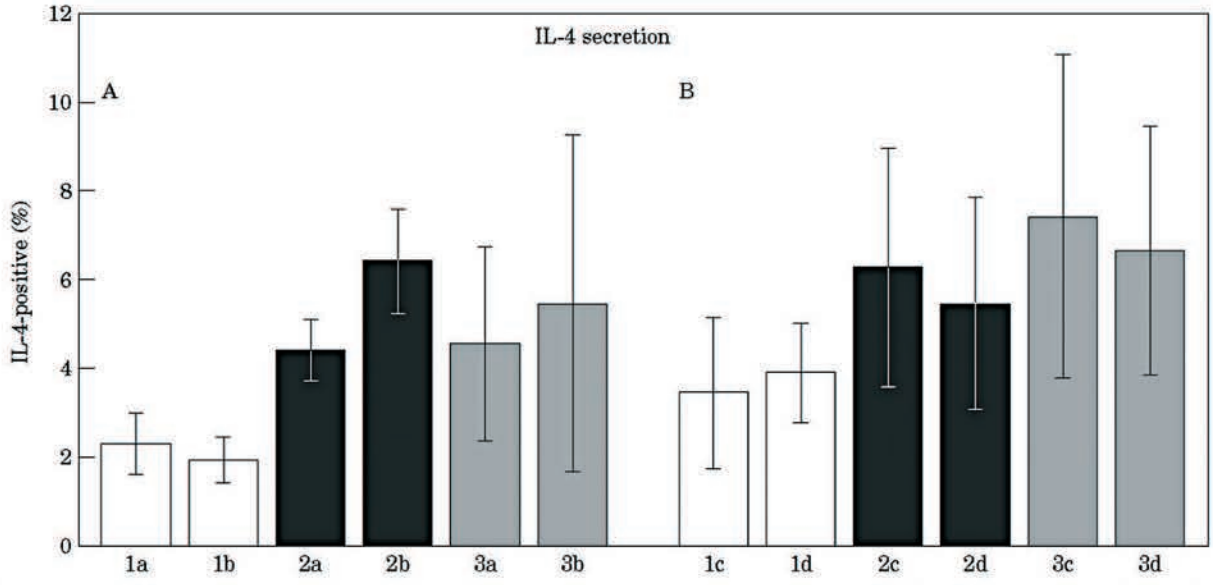

Fig. 3. Detection of IL-4-containing cells (median ± SE) following mitogenic activation of CD4 T cells from 7 or 18 h. Cells from HIV-negative volunteers (cultures 1a, 1b, 1c, 1d); HIV-positive patients on no treatment (cultures 2a, 2b, 2c and 2d) and HIV-positive patients on plant sterol/sterolin supplementation (cultures 3a, 3b, 3c and 3d) are shown. Cultures were incubated with medium alone (unstimulated: 1a, 1c, 2a, 2c, 3a and 3c) or were activated with PMA and Ionomycin (stimulated 1b, 1d, 2b, 2d, 3b and 3d) as described in Materials and Methods section. A: 7 h; B: 18 h.

The IL-4 secretion on the other hand showed a different trend. After 7 h culture, the spontaneous secretion (non-stimulated cultures) from both patient groups was higher when compared to that from the healthy individuals. The IL-4 secretion by the non-treated HIV+ patients was higher than that of the healthy individuals as well as the patients taking the sterols/sterolins (Fig. 3A). The secretion of IL-4 at 18 h showed the same trends: the median secretion, upon PMA stimulation, in the HIV+ untreated group was higher than in the other groups and the spontaneous secretion was higher in the patients groups. However, it failed to reach statistical significance (Fig. 3B).

When a ratio between IFN-γ and IL-4 was calculated for the individual groups using the median values of both the 7 h and 18 h cultures, it is interesting to note that both the healthy, uninfected group and the HIV+ group taking the sterols/sterolins had similar profiles (Table 1). This indicates that the sterols/sterolins mixture is able to maintain a predominant Th-1 secretion profile in the group of patients ingesting the sterols/sterolins mixture (this would explain partly the stable CD4 cell numbers seen in these patients on this clinical study). Second, it would indicate that the HIV+ patients on no treatment demonstrate a predominant Th-2 profile of cytokine secretion and that these patients are likely to progress with the disease as a result of loss of Th-1 activity and therefore loss of CTL activity against infected host cells.

DISCUSSION

During the past few years, a very simple theory that seeks to explain what causes the relentless and ultimately fatal decline in the CD4 cells of HIV/AIDS patients has received considerable attention. The theory holds that CD4 cells from HIV-infected patients switch from a Th1 to a Th2 state with a subsequent loss of CTL activity and finally the disease progresses. If correct, the theory could have important implications both in vaccine development for prevention of HIV infection and in treatment of the disease. As the Th1 state is mainly responsible for cell-mediated responses (whereas the Th2 state preferentially results in antibody responses), it was inferred that the goal of immunization to prevent or control HIV infection should be activation of the Th1-type cell-mediated, rather than the Th2-type antibody-mediated, arm of the immune system (Salk *et al.*, 1993).

Table 1.
Ratios of IFN-γ: IL-4 detected in the cultures from the study groups at 7 and 18 h, as determined by flow cytometry

	7 h	18 h
Healthy: uninfected	15.75	9.46
HIV-infected: no treatment	5.64	5.03
HIV-infected: sterols/sterolins treatment	30.72	24.64

The present study confirms previous reports of such a switch in HIV infected patients (Clerici and Shearer, 1993). In our small group of patients, we were able to show that infected but non-treated patients demonstrate a predominant Th-2 profile of cytokine secretion whereas patients ingesting plant sterol/sterolin capsules are able to mount a typical Th-1 cytokine pattern. *In vivo*, this would translate into stable CD4 cell numbers and slower or no disease progression. This was demonstrated in a clinical study reported in a preliminary format (Bouic, 1997) and confirmed in an animal model (Bouic and Lamprecht, 1999).

The strange and unexplained observation of abnormal CD69 expression by CD4-positive cells from HIV-infected patients cannot be ascribed to defective activation pathways since cytokine gene expression followed by protein synthesis was observed in the cultures. On the other hand, it is known that cellular activation markers seem to be restricted to the CD8 subset of T cells (CD3+ CD8+ CD38+ cells) and such activation marker expression decreases after the initiation of effective anti-retroviral therapy (Raffi *et al.*, 1997). It is postulated that these activated cells are cytotoxic in nature, are negatively correlated to the absolute CD4 cell numbers but correlate positively with viral burden. No such correlation has been attempted in the present study since viral burden was not determined in the non-treated patients. Other studies have also shown that following the introduction of active anti-retroviral therapy, CD4 cell function recovers with a concomitant decrease in the activation status of the CD8-positive cells (Sondergaard *et al.*, 1999).

Plant sterols have so far been used in man for their cholesterol-lowering effect (Salen *et al.*, 1970). Only recently have many biological activities been ascribed to this plant molecule. These activities include its potential as an anti-cancer agent in a rat model of colon carcinoma (Raicht *et al.*, 1980); it has been described as having anti-inflammatory activity (Yamamoto *et al.*, 1991) and anti-pyretic activity as well (Gupta *et al.*, 1980). Our group has demonstrated the immune-modulating properties of the mixture of plant sterol/sterolin in several clinical studies (Bouic *et al.*, 1996; Donald *et al.*, 1997; Bouic, 1997; Bouic *et al.*, 1999) and we feel that this mixture of naturally occurring plant molecules have important implications in the management of HIV-infected patients.

Here in South Africa, with one of the fastest growing HIV epidemics in the world, the patients who test sero-positive are not offered any forms of therapy due to financial constraints of the health departments and state hospitals' budgets. It has therefore become imperative to find alternatives for the management of the growing population faced with the ultimate outcome of this infection: such studies include the use of high doses of multi-vitamin supplementation, mineral and anti-oxidant usage. Our clinical work using Moducare™ has shown promising and exciting results in many chronic conditions. This safe and natural plant-derived immune modulator may represent a cheap alternative to those infected patients since it is able to maintain an effective Th1 response, a response that ultimately controls viral replication. It also does not preclude the simultaneous use of effective therapies specifically aimed against the virus itself. Such studies are currently underway.

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