ABSTRACT The effect of consumption of Immulina, a high-molecular-weight polysaccharide extract from the cyanobacterium *Arthrospira platensis*, on adaptive immune responses was investigated by evaluation of changes in leukocyte responsiveness to two foreign recall antigens, *Candida albicans* (CA) and tetanus toxoid (TT), *in vitro*. Consumption of Immulina by 11 healthy male volunteers caused an immediate, but temporary, increase of CA-induced CD4+ T-helper (Th) cell proliferation (*P* < .02). TT-induced Th cell proliferation was increased in individuals over 50 years of age (*P* < .05) and correlated with age (*P* < .02). Consumption for 8 days enhanced the CA-induced B cell proliferation (*P* < .02), but after 56 days a diminution was seen (*P* < .03). The CA-elicited production of the Th1 cytokines tumor necrosis factor (TNF)-α, interleukin (IL)-2, and interferon (IFN)-γ was increased after Immulina administration for 3 days (*P* < .001, < .03, and < .007, respectively), and increased IL-2 production persisted after 56 days (*P* < .004). The TNF-α, IFN-γ, and IL-6 responses to TT were enhanced after 8 and 14 days (*P* < .002–.05), while IL-5 responses increased significantly within 3 days (*P* < .04) and fell below baseline levels after 14 days (*P* < .008). Conversely, consumption for 3 days inhibited the IL-4 responses to both CA and TT (*P* < .008 and *P* < .03, respectively). No effects on IL-10 responses were observed. Upon addition to normal mononuclear cells *in vitro*, Immulina elicited strong TNF-α, IL-1β, and IL-6 responses, indicating that it acts by inducing a pro-inflammatory state. Taken together, the data suggest that Immulina causes an age-dependent, temporary enhancement of adaptive immune responses.

KEY WORDS: • adaptive immunity • cytokines • Immulina • immunomodulation • Spirulina

INTRODUCTION

Immulina is a high-molecular-weight polysaccharide extract from the cyanobacterium (blue-green algae) *Arthrospira platensis* (previously also called *Spirulina platensis*, and commonly known as *Spirulina*), designed for human consumption.1 Extracts of *Spirulina* have been utilized as a nutritional supplements for more than 15 years without any apparent undesirable effects,2 and several toxicological studies in animals have established their safety.3–7 In 2003, the U.S. Food and Drug Administration labeled the dry biomass of *Spirulina* as a substance generally recognized as safe (http://www.cfsan.fda.gov/~rdb/opa-g127.html).

A number of studies have addressed the effects of dietary *Spirulina* or *Spirulina* extracts, including Immulina, on the immune system. As regards the innate immune system, administration of *Spirulina* products has been shown to enhance significantly the production of interleukin (IL)-1 by peritoneal macrophages in mice8 and the phagocytic activity of macrophages in chickens.7 Administration of a hot water extract of *Spirulina* also augments innate immunity in humans by increasing the production of interferon (IFN)-γ by natural killer (NK) cells and enhancing, in two of four cases, the cytolytic activity of NK cells upon stimulation with IL-12 *in vitro*.9

The effects on the adaptive immune system have been more contradictory. On the one hand, a dietary hot water extract of *Spirulina* had no effect on IFN-γ production by CD4+ T cells (T-helper [Th] cells) and had only a marginal effect on the production by CD8+ T cells (cytotoxic T cells) in humans.9 In addition, Hayashi et al.8 found that addition of a hot water extract of *Spirulina* scarcely affected the proliferation of thymus cells. While T cell responses thus do not seem to be affected by *Spirulina* consumption, several studies point towards an effect on antibody production in
mice. Thus, the primary immune response to sheep red blood cells, as assessed by the number of antibody-producing cells in the spleen, and the mitogen-induced proliferation of splenic cells were enhanced in animals fed a diet containing the algae. In contrast, hardly any effect on the secondary immune response was observed. In another study, Spirulina extract augmented the rise in blood levels of immunoglobulin G1 antibody and the immunoglobulin A antibody levels in the intestine and supernatants of spleen cells and mesenteric lymph node cells, upon oral administration of antigen in mice.

As regards innate immunity, crude extracts of Spirulina and Immulina have pro-inflammatory effects by stimulating monocyte/macrophage functions, e.g., the production of tumour necrosis factor (TNF)-α and IL-1β, as well as the chemokines IL-8, macrophage chemotactic protein-1α, macrophage inflammatory protein-1β, and IFN-γ inducible protein-10 in vitro. The mechanism involved appears to be binding to CD14 and Toll-like receptor (TLR)-2 on human monocytes, which leads to activation of nuclear factor κB. On the other hand, Romay et al. found that phycocyanin, a pigment from Spirulina, administered to mice 1 hour before lipopolysaccharide (LPS) inhibited the LPS-induced induction of TNF-α levels in serum, indicating that Spirulina may have anti-inflammatory actions as well. However, in the interpretation of the apparently conflicting data above, one should distinguish between studies based on consumption of whole dry biomass versus crude or refined extracts of Spirulina. For example, dried biomass contains large amounts of phycocyanin, which Immulina does not (D. Pasco, personal communication).

To examine the effect of Immulina on adaptive immune responses in humans, we have here assessed the effect of Immulina consumption on the responses of mononuclear cells isolated from healthy donors, in terms of CD4+ T cells and B cell proliferation and cytokine production, when challenged with two common, secondary antigens, Candida albicans (CA) and tetanus toxoid (TT) in vitro.

MATERIALS AND METHODS

Study group

Eleven healthy male volunteers (26–69 years old, mean age 52 years) with no history of uncommon disease were included in the study after informed consent. We chose to include male subjects only in the analyses in order to avoid any influence of cyclic changes in the level of female hormones. Administration of Immulina and stimulation of peripheral blood mononuclear cells (PBMCs) with antigens were approved by the local ethics committees of Copenhagen and Frederiksberg and of the counties of Vejle and Funen, Denmark.

Study design

All subjects received Immulina in the form of Immolina® tablets (Nordic Phytopharma A/S, Havdrup, Denmark), 2 × 200 mg a day for 56 days. Blood samples were drawn from the 11 subjects 3 days before and immediately before start of Immulina ingestion and at days 3, 8 (n = 7, for technical reasons), 14, 36 (n = 6, for technical reasons), and 56 of intake. The average values of measurements carried out at the two time points before Immulina intake were used as the baseline with which measurements obtained later in the course were compared. The experiments were carried out by BioMonitor ApS (Copenhagen, Denmark).

Antigens

TT was a gift from Dr. Claus Koch, State Serum Institute, Copenhagen, and cultures of live CA were a kind gift from Else Svejgaard. Thyroglobulin (TG), purified from human thyroids, was purchased from Biogenesis (Poole, UK), and a preparation of purified Immulina was donated from Nordic Phytopharma. PBMCs from all 11 subjects included in the study were tested for in vitro responses to CA and TT, while PBMCs from five subjects were also tested for in vitro responses to Immulina and TG.

Stimulation of PBMCs with antigen

PBMCs were isolated by density centrifugation in Lymphoprep™ (Nycomed Pharma AS, Oslo, Norway) and suspended in RPMI (Gibco/Invitrogen, Taastrup, Denmark). 5-Carboxy-2',7'-dichlorofluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, Eugene, OR), kept as a 5 mM stock solution in dimethyl sulfoxide, was diluted to 180 μM in RPMI 1640 medium and added to the cell suspension to a final concentration of 2 μM. After incubation for 10 minutes at 37°C the cells were washed, resuspended in RPMI 1640 medium supplemented with glutamine and gentamicin, distributed in 96-well flat-bottomed Nunclon™ MicroWell™ microtiter plates (Gibco/Invitrogen), and incubated with or without antigen for 7 days at 37°C in 5% CO2 in humidified air. Each well contained a total volume of 100 μL: 50 μL of cell suspension (2.5 × 105 cells), 30 μL of autologous serum (30% vol/vol), and 20 μL of antigen suspension (10 μg/mL). Fifty microliters of culture supernatant was removed at days 1, 4, and 7 and replaced with 100 μL of RPMI 1640 medium supplemented with glutamine and gentamicin.

Analysis of fluorescence data

CFSE-labeled B cells and CD4+ T cells, labeled with PE-anti-CD19 and PerCP-anti-CD4, respectively, were analyzed for proliferation by measurement of their FL-1 intensities. Undivided cells were seen as cells within the peak of highest fluorescence intensity (prime peak), while divided cells were identified as cells with diminished CFSE content. Thus, cells belonging to generation n exhibited mean FL-1 intensities of 1/2n times that of the prime peak. The overall proliferation of B cells and CD4+ T cells was determined as the total number of daughter cells (in generations ≥1 for B cells and ≥2 for
CD4+ T cells) arising per 1,000 B cells or CD4+ T cells subjected to antigen challenge (determined from the sum of the nonproliferating cells and estimated precursor cells in the preparation).

**Measurement of cytokines in culture supernatant**

The content of TNF-α, IL-1β, IL-6, and IL-10 was quantified in culture supernatants at day 1 by means of the Cytometric Bead Array Inflammatory Cytokine kit (Becton-Dickinson, Copenhagen), and the content of TNF-α, IFN-γ, IL-2, IL-4, IL-5, and IL-10 was measured in day 7 supernatants by means of the Th1/Th2 cytokine kit (Becton-Dickinson) using a FACS caliburr™ flow cytometer (Becton-Dickinson). Data analyses were performed using the Cytometric Bead Array Software (Becton-Dickinson).

**Statistics**

When nothing else is stated, Wilcoxon matched pairs test was used to assess changes in the variables measured. The values at any time point during the course of Immulina consumption were compared to the average of two samples taken before administration for each test person. Values of $P < .05$ were considered significant. Spearman’s rank sum coefficient was used for calculation of correlations.

**FIG. 1.** Antigen-induced proliferation of CD4+ T cells and B cells. PBMCs were isolated at various time points after the beginning of ingestion of Immulina, labeled with CFSE, and cultivated for 7 days in the presence of CA (A and D), TT (B), or TG (C) before flow cytometric assessment. The resulting net proliferation of CD4+ T cells (A–C) and CD19+ B cells (D), after subtraction of the background proliferation of unstimulated cells (median <1% at day 0, increasing to 2% at day 56), is shown. The proliferation index corresponds to the percentage of cells having undergone more than one division. Assessments at day 0 were made in duplicates. Horizontal bars represent median values. $P$ values were calculated by the Wilcoxon matched pairs test.
RESULTS

Effect of Immulina ingestion on antigen-induced proliferation of CD4+ T cells and B cells

To test the effect of Immulina on antigen-elicited immune responses, PBMCs were isolated before and at several time points after ingestion of Immulina tablets (200 mg x 2 daily). The PBMCs were labeled with CFSE and stimulated for 7 days with the recall antigen TT or CA. The CA-elicited proliferation of CD4+ T cells contained in the PBMCs was significantly increased after ingestion of Immulina for 3 days (Fig. 1A), while tendencies to increased TT responses were observed after 3 and 8 days (Fig. 1B). The proliferative responses reverted to baseline or slightly lower than baseline after ingestion for 56 days.

While Immulina thus seemed to enhance CD4+ T cell proliferation in response to foreign, secondary antigens, we wished to examine whether this was also true for potentially detrimental responses to self-antigens and therefore incubated PBMCs from five donors with the self-antigen TG for 7 days (Fig. 1C). An increase in the response to TG was observed in four of five tested subjects after ingestion of Immulina for 14 days. When added directly to the culture wells, Immulina per se induced a moderate CD4+ T cell proliferation, which was dose-dependent and peaked at 10 μg/mL (data not shown).

Concomitantly with the assessment of CD4+ T cell proliferation, we examined the CD19+ B cell proliferation induced by the various antigens. CA induced a B cell proliferation, which was significantly increased by administration of Immulina for 8 days but was reduced after 56 days (Fig. 1D). In contrast, no significant B cell proliferation was seen in response to TT, TG, or Immulina (data not shown).

Age dependence of Immulina-mediated increase in T cell response

Since T cell function depends on maturation in the thymus and the thymus is known to degenerate with age, we chose to investigate whether the apparent increase in T cell function mediated by ingestion of Immulina depended on the age of the recipient. Indeed, we observed a significant correlation between age and the increase in TT-induced CD4+ T cell response mediated by 14 days of Immulina administration (Fig. 2), at which time point recipients over 50 years of age displayed a significant increase over baseline (P < .05). This increase was significantly higher in recipients over 50 years of age than the corresponding increase in younger recipients (P < .03). Significant correlations were not found with respect to CA responses. The difference in age dependency between the two antigens may be explained by the fact that subjects older than 50 years of age showed relatively high responses to CA even before consumption of Immulina, in contrast with their generally low baseline responses to TT.

To further characterize the response of T cells and other cell populations in the PBMC preparations, we quantified Th1 and Th2 cytokines in the culture supernatants after the various stimuli. Ingestion of Immulina for 3–8 days caused a significant increase in the TNF-α response to both CA and TT (Fig. 3A and B), and thereafter the effect of Immulina declined progressively. It remained borderline significant, however, with both antigens in five donors tested at day 36 (P = .06; data not shown). At day 56, the response to TT was lower than the baseline level (Fig. 3C).

Administration of Immulina for 3 days mediated a significant increase in the CA-elicited IL-2 production, as did administration for 56 days (Fig. 3C). No significant changes were found with respect to the TT-induced IL-2 production (Fig. 3D). Significantly increased production of a third Th1 cytokine, IFN-γ, to CA and TT was observed after ingestion of Immulina for 3 days and 14 days, respectively (Fig. 3E and F). At day 56, however, the IFN-γ responses to TT were lower than baseline (Fig. 3F). Neither of the antigens stimulated significant IL-12 responses (data not shown). Taken together, these data indicate that Immulina mediates a temporary enhancement of Th1 responses.

Effect of Immulina ingestion on production of Th2 cytokines

In concert with the enhancing effect of Immulina on B cell proliferation described above, the production of the B cell differentiation factor IL-614 in response to the foreign recall antigens CA and TT increased significantly after administration for 14 days and 8 days, respectively (Fig. 4A and B). The production of another Th2 cytokine, IL-5, was significantly increased after stimulation with TT when Immulina had been ingested for 3 days but was inhibited after 14 and 56 days (Fig. 4D). The antigen-elicited IL-4 responses decreased markedly after administration of Immulina for 3 days, and while the IL-4 response to CA reverted to normal towards the end of the observation period,
FIG. 3. Effect of Immulina on antigen-elicited Th1 cytokine responses. PBMCs were isolated at various time points after intake of Immulina and cultivated in the presence of stimulating foreign antigen, TT or CA. After 7 days of stimulation, the Th1 cytokines TNF-α (A and B), IL-2 (C and D), and IFN-γ (E and F) were quantified in the culture supernatants by means of cytometric bead array. The data shown represent median ± interquartile range of 11 subjects after subtraction of the background levels observed in the absence of antigen stimulation (the median values of which were <5 pg/mL for TNF-α and 0 pg/mL for IFN-γ and IL-2).
FIG. 4. Effect of Immulina on antigen-elicited Th2 cytokine responses. PMBCs, isolated at various time points after intake of Immulina, were cultivated in the presence of CA or TT for 7 days. The Th2 cytokine IL-6 (A and B) was quantified in culture supernatants at day 1, and IL-5 (C and D) and IL-4 (E and F) levels were measured at day 7. The data shown represent median ± interquartile range of 11 subjects after subtraction of background levels in the absence of antigen stimulation (medians <10 pg/mL for IL-10 and IL-5 and 0 pg/mL for IL-4, at all time points).
the IL-4 response to TT remained low. Throughout the observation period the IL-10 response remained unaffected by consumption of Immulina (data not shown).

Cytokine responses to Immulina in vitro

When added to PBMC cultures in vitro, Immulina per se was a more potent stimulus for the production of the pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 than CA or TT at equal concentration (Fig. 5A–C) and appeared to be as potent a stimulus for CD4+ T cell proliferation as TT (Fig. 5D). In particular, the effect of Immulina on IL-6 production was dramatic, which may explain the stimulating effect of Immulina on B cells.

DISCUSSION

Previous studies have demonstrated an effect of products derived from *A. platensis*, known as *Spirulina*, on adaptive immune responses in mice and on innate immune responses in mice, chickens, and humans. To our knowledge, this is the first study to assess the effect of *Spirulina*-derived products on antigen-specific immune responses in humans. We found that consumption of Immulina, a high-

**FIG. 5.** Responses of mononuclear cells to Immulina *in vitro*. PBMCs were isolated from five donors prior to administration of Immulina. To compare the pro-inflammatory effect of Immulina with those of conventional antigens, the cells were incubated with CA, TT, or a preparation of Immulina. The resulting production of the pro-inflammatory cytokines (A) TNF-α (B) IL-1β, and (C) IL-6 after 1 day and (D) the CD4+ T cell proliferation after 7 days are shown. Paired *t* tests were employed. Horizontal bars represent mean values.
molecular-weight polysaccharide Spirulina extract, enhanced the capacity of CD4+ Th cells and B cells to proliferate in vitro in response to stimulation with CA and, in men over 50 years of age, in response to stimulation with TT. The positive correlation between age and CD4+ T cell responses suggests that elderly persons with a relative T cell deficiency might benefit from the apparent immunostimulatory action of Immulina to a greater extent than younger persons. The enhancing effect of Immulina on the proliferation of lymphocytes seemed to be temporary, however, since it was neutralized within 14 days of intake. At day 56, the CA-induced B cell proliferation and CD4+ T cell proliferation were lower and borderline significantly lower, respectively, than before intake of Immulina. This may be a result either of the induction of a refractory state in the circulating lymphocytes after stimulation with Immulina over longer periods or of the disappearance of lymphocytes that have been primed by Immulina from the peripheral blood as a result of homing to lymphoid organs. The T cells likely to be affected are those harbored in large numbers, in Peyer’s patches and mesenteric lymph nodes, and these T cells are known to express various homing molecules upon activation, allowing them to migrate, e.g., to the small intestine.15 The proliferation in the absence of antigenic stimuli, as well as the spontaneous production of all cytokines measured, was negligible both before and after Immulina administration, indicating that a nonspecific mitogenic effect of Immulina was not pronounced, if existing at all.

Ingestion of Immulina for 3 days mediated a significant increase in the antigen-specific production of the Th1 cytokines TNF-α, IL-2, and IFN-γ upon antigen stimulation of PBMCs in vitro. While the positive effect on TNF-α and IFN-γ production apparently turned into an inhibitory effect at day 56 (for the same possible reasons as suggested above), the enhancing effect on IL-2 production by CA-stimulated cells was seen both at day 3 and at day 56. However, in view of the lack of effect on TT-stimulated cells, the IL-2 data should be interpreted with caution. Monocytes and monocyte-derived cells are major producers of TNF-α, and our data thus are in concordance with previous findings of enhanced TNF-α production by monocytes and macrophages in mice fed on Spirulina and with an effect of high-molecular-weight polysaccharide extracts from Spirulina on the production of TNF-α by human monocytes.1,8,11 The mechanism involved seems to be stimulation via CD14 and TLR-2.1 Our data suggest that Immulina is taken up from the intestine in a form that allows binding to these receptors in vivo, thereby lowering the threshold for subsequent activation in vitro. Thus, we confirmed that Immulina has pro-inflammatory effects. By contrast, Romay et al.13 reported that phycocyanin administered to mice 1 hour before LPS inhibited the LPS-induced induction of TNF-α levels in serum, indicating that Spirulina may have anti-inflammatory actions as well.

IL-2 is a T cell-specific cytokine, and IFN-γ is produced by T cells and NK cells. Our finding of enhanced antigen-elicited production of IFN-γ, and possibly IL-2, following consumption of Immulina indicates that Immulina affects T cells, and possibly NK cells, as well as monocytes. In accordance with our data, Hirahashi et al.9 demonstrated that administration of a hot water extract of Spirulina in humans mediated enhanced IFN-γ production by NK cells upon stimulation with IL-12 in vitro. They found no effect on the IFN-γ production by CD4+ T cells and only a little effect on the production by CD8+ T cells, however, and therefore suggested that Spirulina affects innate immunity, but not adaptive cellular immunity, in humans. Accordingly, Hayashi et al.8 found that addition of a hot water extract of Spirulina scarcely affected the proliferation of thymus cells. While these investigations employed nonspecific stimuli, our study indicates that administration of Spirulina-derived high-molecular-weight polysaccharides promotes antigen-specific Th1 cytokine responses.

With respect to the production of Th2 cytokines, consumption of Immulina enhanced IL-6 responses to both antigens and IL-5 responses to TT significantly, and with borderline significance to CA, within the first week. Since IL-6 promotes B cell growth,14 this is in accordance with an enhanced proliferative response of B cells to CA observed within the first week after administration. Notably, Th2 cytokine production was not promoted in general, since Immulina decreased the antigen-elicited IL-4 responses within 3 days and the TT-elicited IL-5 responses after 2 weeks.

When added to PBMC cultures, Immulina itself elicited a marked proliferation of CD4+ T cells, as well as a production of the pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 that, in five consecutive experiments, were higher than those seen after stimulation with any of the other antigens tested. These data are in keeping with previous findings of a stimulating effect of Immulina on the production of TNF-α and IL-1β by murine monocytes in vitro,1,11 and of an enhancement of IL-1β production by peritoneal macrophages in mice fed on a Spirulina diet.8 Pugh et al.1 found that Immulina stimulated monocytes primarily via TLR-2 in vitro, via a pathway involving nuclear factor κB, and on the basis of our results it seems likely that antigen-presenting cells, and subsequently T cells, are primed by Immulina in vivo via a similar mechanism. Immulina may thus mimic the setting of an acute bacterial infection, where antigen-presenting cells increase their expression of T cell costimulatory molecules, such as CD80, that lower the threshold for T cell activation.16

We observed no side effects during the present study. However, while Immulina seems to promote inflammatory responses against foreign microorganisms, it may cause undesirable side effects in subjects suffering from autoimmune diseases, e.g., rheumatoid arthritis. TNF-α and IL-6, the production of which is promoted by consumption of Immulina, are pro-inflammatory cytokines known to play major roles.
in the pathogenesis of autoimmune disease\textsuperscript{17–21} and lymphoproliferative disorders.\textsuperscript{22} Lee and Werth\textsuperscript{23} found a severe flare-up of dermatomyositis in a subject having ingested a supplement containing \textit{Spirulina} in combination with \textit{Aphanizomenon flos-aquae}, cayenne pepper, and organic sulfur. However, she had also taken various other substances within the preceding month, including BioChoice immune 26, which caused diarrhea, so the agent causing her symptoms could not be firmly established. Moreover, we observed that Immulina mediated increases in the CD4+ T cell proliferation elicited by TG in four of five donors in the present study. Thus, usage of \textit{Spirulina} extract as a therapeutic agent, as suggested by Tzianabos et al.,\textsuperscript{24} may not be appropriate—unless perhaps in the event of flare-up of tuber-

phoproliferative disorders.\textsuperscript{22} Lee and Werth\textsuperscript{23} found a significant increase in the CD4+ T cell proliferation elicited by TG in four of five donors in the present study. Thus, usage of \textit{Spirulina} extract as a therapeutic agent, as suggested by Tzianabos et al.,\textsuperscript{24} may not be appropriate—unless perhaps in the event of flare-up of tuberculosis or other serious opportunistic infections in association with TNF-\textalpha inhibitor administration.\textsuperscript{25} Again, it should be pointed out that significant differences may exist between the effects of whole dry biomass and different extracts of \textit{Spirulina}.

Given that the observed immune responses to CA and TT tended to decline after 56 days of ingestion, we cannot rule out that Immulina may have long-term mild immunosuppressive effects. To establish whether this is the case, and whether it applies to different preparations of \textit{Spirulina}- or other algae-derived products too, further investigation is needed.

In conclusion, this first study of the influence of \textit{Spirulina}-derived products on adaptive immune responses in humans shows an age-dependent and temporary priming effect on the responses of peripheral Th1, Th2, and B cells to antigenic stimuli, which is likely to be related to a strong pro-

inflammatory effect of Immulina \emph{per se} on normal human mononuclear cells.

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