the major responsibility for carrying out the activities of the immune system; their number is about 10^{12} in healthy person. The two major classes of lymphocytes are B cells, which grow to maturity in bone marrow, and T cells, which are processed in the thymus. Both B cells and T cells recognize specific antigen targets. B cells work chiefly by secreting soluble substances called antibodies into the body fluids, or humors (humoral immunity). Antibodies typically interact with circulating antigens such as bacteria and toxic molecules, but are unable to penetrate living cells. T cells, in contrast, interact directly with their targets, attacking body cells that have been commandeered by viruses or undergone malignant transformation (cellular immunity).[26,27,44,45] *Chlorella* powder actively inhibits B and T cell proliferation, and may be regarded as a possible immunosuppressant for autoimmune disease treatment.

Binding of phorbol esters to receptors initiates multiple changes in cell function. The typical phorbol ester represents the paradigm for a tumor promoter established in multistage carcinogenesis mediated by protein kinase C enzymes. However, novel phorbol ester receptors have recently been discovered with Rac-GTPase-activating protein activity, and identified as Ras guanyl-releasing proteins that enhance Ras signaling.[28,46] Inhibition of phorbol ester binding by *Chlorella* powder may have potential for blocking cell transformation.

CONCLUSION

In vitro screening of *Chlorella* powder revealed a moderate inhibition of a wide variety of cytokines, MMPs, B cell and T cell proliferation, and phorbol ester binding. *Chlorella* strongly inhibited PTPs such as CD45 and PTP1C and moderately inhibited PTP1B and TC-PTP. If the activities are confirmed in *in vivo* systems they could have important implications for inflammatory disease as well as diabetes, immune dysfunctions, cell proliferation disorders, and disease of the brain. These results identify important activities that should be more closely investigated in future studies.

REFERENCES

1. Hsiuan-Lin W, Ruey-Shyang H, Liang-Ping L: Identification of *Chlorella* spp. isolates using ribosomal DNA sequences. *Bot Bull Acad Sin* 2001;42:115–121.
2. Inoue K, Mukaiyama Y, Oka H, Misawa H: Clinical effects of *Chlorella* in hypertensive men. *J Jpn Soc Nutr Food Sci* 1995; 48:485–489.
3. Murakami T, Iiduka Y, Okamoto K, Oogaki M: Effect of *Chlorella* on blood pressure, cerebral stroke lesions, hypertensive vascular changes and life-span in spontaneously hypertensive rats. *J Jpn Soc Nutr Food Sci* 1987;40:351–359.
4. Shibata S, Orita K, Iwabuchi A, Misawa H: Serum cholesterol lowering action of hardly digestible fraction of *Chlorella*. *J Jpn Soc Nutr Food Sci* 1998;52:100.
5. Tanaka K, Konishi F, Himeno K, Taniguchi K, Nomoto K: Augmentation of antitumor resistance by a strain of unicellular green algae, *Chlorella vulgaris*. *Cancer Immunol Immunother* 1984;17: 90–94.
6. Konishi F, Tanaka K, Himeno K, Taniguchi K, Nomoto K: Antitumor effect induced by a hot water extract of *Chlorella vulgaris* (CE): resistance to Meth-A tumor growth mediated by CE-induced polymorphonuclear leukocytes. *Cancer Immunol Immunother* 1985;19:73–78.
7. Miyazawa Y, Murayama T, Ooya N, Wang LF, Tung YC, Yamaguchi N: Immunomodulation by a unicellular green algae (*Chlorella pyrenoidosa*) in tumor-bearing mice. *J Ethnopharmacol* 1988;24:135–146.
8. Tanaka K, Koga T, Konishi F, Nakamura M, Mitsuyama M, Himeno K, Nomoto K: Augmentation of host defense by a unicellular green alga, *Chlorella vulgaris*, to *Escherichia coli* infection. *Infect Immun* 1988;53:267–271.
9. Morita K, Matsueda T, Iida T: Effects of Chlorella, spirulina and chlorophyllin on fecal excretion of polychloronated dibenzo-p-dioxins in rats. *Jpn J Toxicol Environ Health* 1997;43:42–47.
10. Komiyama K, Hirokawa Y, Morota T, Umezawa I: Antitumor activity and immunological response. *Chemotherapy* 1986;32: 302–307.
11. Kanouchi H, Okabe M, Doi S, Yamada H, Tachibana H, Yamada K: Dietary effect of *Chlorella pyrenoidosa* powder on immunoglobulin productivity of Sprague-Dawley rats. *Nippon Shokuhin Kagaku Kaishi* 2001;48:634–636.
12. Johnson P, Ostergaard HL, Wasden C, Trowbridge IS: Mutational analysis of CD45: a leukocyte specific protein tyrosine phosphatase. *J Biol Chem* 1992;267:8035–8041.
13. Zhang ZY, Thieme-Sefler AM, Maclean D, McNamara DJ, Dobrusin EM, Sawyer TK, Dixon JE: Substrate specificity of the protein tyrosine phosphatases. *Proc Natl Acad Sci U S A* 1993;90:4446–4450.
14. Pei D, Lorenz U, Klingmuller U, Neel BG, Walsh CT: Intramolecular regulation of protein tyrosine phosphatase SH-PTP1: a new function of src homology domains. *Biochemistry* 1994;33:15483–15493.
15. D'Ambrosio D, Hippen KL, Minskoff SA, Mellman I, Pani G, Siminovitch KA, Cambier JC: Recruitment and activation of PTP1C in negative regulation of antigen receptor signalling by FrcγRIIB1. *Science* 1995;268:293–296.
16. Wiener JR, Kerns B-JM, Harvey EL, Conaway MR, Iglehart JD, Berchuck A, Bast RC Jr: Overexpression of the protein phosphatase PTP1B in human breast cancer: association with p185–c-erb-2 protein expression. *J Natl Cancer Inst* 1994;86:372–378.
17. Turner BC, Tonks NH: Interleukin 2 regulates Raf-1 kinase activity through a tyrosine phosphorylation-dependent mechanism in a T-cell line. *Proc Natl Acad Sci U S A* 1993;90:5544–5548.
18. Knight CG, Willenbrock F, Murphy G: A novel coumarin-labelled peptide for sensitive continuous assays of the matrix metalloproteinases. *FEBS Lett* 1992;296:263–266.
19. Johnson LL, Dyer R, Hupe DJ: Matrix metalloproteinases. *Curr Opin Chem Biol* 1998;2:466–471.
20. Olson MW, Gervasi DC, Mobashery S, Fridman R: Kinetic analysis of the binding of human matrix metalloproteinase-2 and -9 to tissue inhibitor of metalloproteinase (TIMP)-1 and TIMP-2. *J Biol Chem* 1997;272:29975–29983.
21. Thornberry NA, Bull HG, Calaycay JR, Chapman KT, Howard AD, Kostura MJ, Miller DK, Molineaux SM, Weidner JR, Aunins J, Elliston KO, Ayala JM, Casano FJ, Chin J, Ding GJ-F, Egger LA, Gaffney EP, Limjuco G, Palyha OC, Raju SM, Rolando AM, Salley JP, Yamin TT, Lee TD, Shively JE, MacCross M, Mumford RA, Schmidt JA, Tocci MJ: A novel heterodimeric cysteine protease is required for interleukin-1β processing in monocytes. *Nature* 1992;356:768–775.

22. Mittl PR, Di Marco S, Krebs JF, Karanewsky DS, Priestle JP, Tomaselli KJ, Grutter MG: Structure of recombinant human CPP32 in complex with the tetrapeptide Acetyl-Asp-Val-Ala-Asp fluoromethyl ketone. *J Biol Chem* 1997;272:6539–6547.
23. Talanian RV, Quinlan C, Trautz S, Hackett MC, Mankovich JA, Banach D, Ghayur T, Brady KD, Wong WW: Substrate specificities of caspase family proteases. *J Biol Chem* 1997;272:9677–9682.
24. Boldin MP, Goncharov TM, Goltsev YV, Wallach D: Involvement of MACH, a novel MORT1/FADD-interacting protease, in FAS/APO-1– and TNF receptor-induced cell death. *Cell* 1996; 85:803–815.
25. Welker P, Lippert W, Kruger-Krasagakes S, Möller A, Czarnetzki B: Glucocorticoid-induced modulation of cytokine secretion from normal and leukemic human myelomocytic cells. *Int Arch Allergy Immunol* 1996;109:110–115.
26. Dayton JS, Turka LA, Thompson CB, Mitchell BS: Comparison of the effects of myoribine with those of azothiaprine, 6 mercaptopurine and myophenolic acid on T-lymphocyte proliferation and purine ribonucleotide metabolism. *Mol Pharmacol* 1992;41: 671–676.
27. Mishell BB, Shiigi SM, eds.: Cell proliferation. In: *Selected Methods in Cellular Immunology*, W.H. Freeman Co., San Francisco, 1980, pp. 153–161.
28. Ashendel CL: The phorbol ester receptor: a phospholipid regulated protein kinase. *Biochim Biophys Acta* 1985;822:219–242.
29. Fischer EH, Charbonneau H, Tonks NK: Protein tyrosine phosphatases: a diverse family of intracellular and transmembrane enzymes. *Science* 1991;253:401–406.
30. Schieven GL, Wahl AF, Myrdal S, Grosmaire L, Ledbetter JA: Lineage-specific induction of B cell apoptosis and altered signal transduction by the phosphotyrosine phosphatase inhibitor bis(maltolato)oxovanadium(IV). *J Biol Chem* 1995;270:20824–20831.
31. Katagiri T, Ogimoto M, Hasegawa K, Mizuno K, Yakura H: Selective regulation of Lyn tyrosine kinase by CD45 in immature B cells. *J Biol Chem* 1995;270:27987–27990.
32. Tan X, Stover DR, Walsh KA: Demonstration of protein tyrosine phosphatase activity in the second of two homologous domains of CD45. *J Biol Chem* 1993;268:6835–6838.
33. Flint AJ, Gebbink MF, Franza BR Jr, Hill DE, Tonks NK: Multisite phosphorylation of the protein tyrosine phosphatase, PTP1B: identification of cell cycle regulated and phorbol ester stimulated sites of phosphorylation. *EMBO J* 1993;12:1937–1946.
34. Brown-Shimer S, Johnson KA, Hill DE, Bruskin AM: Effect of protein tyrosine phosphatase 1B expression on transformation by the human neu oncogene. *Cancer Res* 1992;52:478–482.
35. Zhai YF, Beittenmiller H, Wang B, Gould MN, Oakley C, Esselman WJ, Welsch CW: Increased expression of specific protein tyrosine phosphatases in human breast epithelial cells neoplastically transformed by the neu oncogene. *Cancer Res* 1993;53(10 Suppl):2272–2278.
36. Cool DE, Tonks NK, Charbonneau H, Fischer EH, Krebs EG: Expression of a human T-cell protein-tyrosine-phosphatase in baby hamster kidney cells. *Proc Natl Acad Sci U S A* 1990;87:280–7284.
37. Nagase H: Activation mechanisms of matrix metalloproteinases. *Biol Chem* 1997;378:151–160.
38. Nagase H, Woessner JF Jr: Matrix metalloproteinases. *J Biol Chem* 1999;274:21491–21494.
39. Thornberry NA, Lazebnik Y: Caspases: enemies within. *Science* 1998;281:1312–1316.
40. Green DR: Apoptotic pathways: the roads to ruin. *Cell* 1998; 94:695–698.
41. Cohen MC, Cohen S: Cytokine function: a study in biologic diversity. *Am J Clin Pathol* 1996;105:589–598.
42. Ishihara K, Hirano T: IL-6 in autoimmune disease and chronic inflammatory proliferative disease. *Cytokine Growth Factor Rev* 2002;13:357–368.
43. Henderson B, Blake S: Therapeutic potential of cytokine manipulation. *Trends Pharmacol Sci* 1992;13:145–152.
44. Han SB, Park SH, Jeon YJ, Kim YK, Kim HM, Yang KH: Prodigiosin blocks T cell activation by inhibiting interleukin-2R expression and delays progression of autoimmune diabetes and collagen-induced arthritis. *J Pharmacol Exp Ther* 2001;299:415–425.
45. Zenke G, Strittmatter U, Fuchs S, Quesniaux VFJ, Brinkmann V, Schuler W, Zurini M, Enz A, Billich A, Sanglier JJ, Fehr T: Sanglifehrin A, a novel cyclophilin-binding compound showing immunosuppressive activity with a new mechanism of action. *J Immunol* 2001;166:7165–7171.
46. Ron D, Kazanietz MG: New insights into the regulation of protein kinase C and novel phorbol ester receptors. *FASEB J* 1999;13:1658–1676.

TABLE 3. CHLORELLA POWDER INHIBITION OF HUMAN RECOMBINANT CASPASES 1, 3, 6, 7, AND 8

Peptidase caspase assayed	IC_{50} (μg/mL)
Caspase 1	~300
Caspase 3	203
Caspase 6	301
Caspase 7	291
Caspase 8	261

Chlorella powder was tested in duplicate at serial concentrations from 300 to 0.3 μg/mL in 1% DMSO for each enzyme assay. IC_{50} values were calculated by non-linear regression analysis.

TABLE 4. CHLORELLA POWDER INHIBITION OF CYTOKINE PRODUCTION IN HUMAN PBMNS

Mediator release assayed	IC_{50} (μg/mL)
IL-1β	44.9
IL-2	14.8
IL-4	49.2
IL-6	34.7
IFN-γ	31.6
TNF-α	11

Chlorella powder was tested in duplicate at serial concentrations from 300 to 0.03 μg/mL in 0.1% DMSO for each cytokine assay. IC_{50} values were calculated by non-linear regression analysis.

TABLE 5. CHLORELLA POWDER INHIBITION OF CELL PROLIFERATION IN MOUSE SPLENOCYTES AND THYMOCYTES

Cell proliferation assay	IC_{50} (μg/mL)
B cells + LPS	16.6
T cells + Con A	54.2

Chlorella powder was tested in duplicate at serial concentrations from 300 to 0.03 μg/mL in 0.4% DMSO for each cell proliferation assay. IC_{50} values were calculated by non-linear regression analysis.

TABLE 6. CHLORELLA POWDER INHIBITION OF PHORBOL ESTER RECEPTOR BINDING IN ICR MICE

Binding assayed	IC_{50} (μg/mL)
Phorbol ester	152

Chlorella powder was tested in duplicate at serial concentrations from 300 to 0.3 μg/mL in 1% DMSO for the binding assay. IC_{50} values were calculated by non-linear regression analysis.

DISCUSSION

We screened a *Chlorella* powder in a battery of 52 *in vitro* assay systems for inhibition of immune cell proliferation, cytokine production, and various enzyme activities.

Chlorella powder was found to very potently inhibit the CD45 and PTP1C PTPs. The CD45 PTP is a member of the receptor-like transmembrane protein phosphatase family. Studies have shown that CD45 is essential for the T-cell receptor to couple to second messenger pathways, IL-2 production, and the proliferative response of T cells to specific antigens. One model of CD45 action postulates that its role is to dephosphorylate lck on the regulatory tyrosine, making it possible for the kinases to respond to T-cell receptor occupancy.[12,13,29–32] Inhibition of CD45 tyrosine phosphatase activity would likely have immunomodulatory effects.

PTP1C, also known as HCP, SHP and SH-PTP1, is a non-transmembrane type protein tyrosine phosphatase that contains two src homology-2 (SH2) domains. The SH2 domain is believed to have dual roles: as a facilitator promoting interactions between cytoplasmic signaling molecules and specific phosphotyrosyl residues on activated (*i.e.*, autophosphorylated) growth factor receptors, and as a self-regulator of phosphatase activity. Its function has recently been linked to immune regulatory activity.[14,15,30] Inhibition of PTP1C activity by *Chlorella* has the potential for therapeutic benefits for autoimmune diseases. Other PTPs such as PTP1B and TC-PTP are also moderately inhibited by the *Chlorella* powder.

PTP1B is a major intracellular PTP first isolated from human placenta. It consists of a single catalytic domain and a hydrophobic carboxyl terminus responsible for localizing the enzyme to the endoplasmic reticulum. PTP1B is involved in regulating tyrosine kinases by controlling their phosphorylation status. Overexpression of this enzyme has been linked to several oncogenic diseases. Studies conducted on frozen sections from ovarian and breast cancer patients indicate increased expression of PTP1B along with other protein tyrosine kinases.[16,33–35] Inhibitory activity by *Chlorella* powder has the potential for the prevention and treatment of breast and ovarian cancers.

TC-PTP belongs to a diverse family of PTPs that, along with other kinases, regulate the critical level of phosphorylation necessary for intracellular signaling, cell growth, and differentiation. TC-PTP is a 48-kDa intracellular phosphatase consisting of a single catalytic domain and a non-catalytic C-terminal segment that plays an important role in the regulation and localization of phosphatase activity.[17,36] T-cell tyrosine phosphatase inhibition by *Chlorella* powder has potential as an inhibitor of T-cell proliferation.

Chlorella powder was found to inhibit the MMPs MMP-1, -3, -7, and -9. The inhibition of MMP-7 was about seven to 13 times stronger than that of the other three MMPs when comparing their IC_{50} values. The MMP family shares significant sequence homology and a common multidomain structure. On the basis of their preferred substrates, they can be divided into five main classes: collagenases, gelatinases, stromelysins, membrane-type MMPs, and others. These enzymes demonstrate very low activity in normal tissue but

are up-regulated and/or activated during inflammation and physiological remodeling processes in response to specific stimuli, including cytokines, growth factors, and extracellular matrix interactions.[18–20,37,38] Inhibition of MMPs by *Chlorella* extract has potential for intervention in cancer, rheumatoid arthritis, autoimmune diseases, periodontitis, tissue ulceration, atherosclerosis, aneurysm, and heart failure.

Chlorella powder had a weak inhibitory activity against caspases 1, 3, 6, 7, and 8. Caspases are the executioners of programmed cell death (apoptosis), a normal process used physiologically to eliminate specific cells or cell types without damaging surrounding cells or causing an inflammatory response. All caspases belong to a family of proteases characterized by a cysteine residue in the active site and display specificity for Asp residues. Caspases are present in all cells as a procaspase, which becomes activated through proteolytic cleavage by other proteases, including other caspases.[21–24,39,40] Caspase inhibition by *Chlorella* powder could have potnential therapeutic efficacy for such diseases as Alzheimer's disease, Parkinson's disease, and cerebral or myocardial ischemia, but would be contraindicated for other diseases such as cancer.

Chlorella powder moderately blocked IL-1β, IL-2, IL-4, IL-6, IFN-γ, and TNF-α release from human PBMNs. IL-1β activates adenylate cyclase to elevate cyclic AMP levels, resulting in activation of protein kinase A, and induces nuclear factors that serve as cellular gene transcription activators. IL-1β induces synthesis of enzymes that generate prostaglandins, resulting in fever. At low concentrations, the effects of IL-1β are primarily immunoregulatory. IL-1β acts with polyclonal activators to facilitate $CD4^+$ T lymphocyte proliferation as well as B lymphocyte growth and differentiation. IL-1β stimulates multiple cells to act as immune or inflammatory response effector cells, and also induces its own synthesis as well as that of IL-6 by mononuclear phagocytes and vascular endothelial cells. IL-1β resembles TNF in its pro-inflammatory properties.

IL-2 facilitates the formation of other cytokines produced by T lymphocytes, including IFN-γ and lymphotoxins. Inadequate IL-2 synthesis may lead to antigen-specific T lymphocyte anergy. IL-2 promotes natural killer (NK) cell growth and potentiates the cytolytic action of NK cells through generation of lymphokine-activated killer cells. IL-2 promotes the responsiveness of immature bone marrow cells to other cytokines. In the thymus, IL-2 may promote immature T cell growth.

IL-4 serves as a growth and differentiation factor for B cells, mast cells, and macrophages and is a switch factor for synthesis of IgE in mice. It also promotes clonal growth of $CD4^+$ T cells and enhances class II major histocompatability complex (MHC) molecule expression and enlargement of resting B lymphocytes. Both murine and human IL-4 induces switching of B lymphocytes to synthesize IgE. Human IL-4 also induces CD23 expression in B lymphocytes and macrophages in humans. IL-4 may have some role in cell-mediated immunity.

IL-6 may act on many types of cells. A significant function is its ability to cause B lymphocytes to differentiate into cells that synthesize antibodies. IL-6 is also known as hepatocyte stimulating factor because it induces hepatocytes to form acute-phase proteins, including fibrinogen. It is the main growth factor for activated B lymphocytes late in B cell differentiation. IL-6 also acts as a co-stimulator of T lymphocytes and of thymocytes. It acts in concert with other cytokines that promote the growth of early bone marrow hematopoietic stem cells. IL-6 acts as an inducer of the acute-phase response by controlling the transcription levels of acute-phase proteins.

IFN-γ has antiproliferative and antiviral properties. IFN-γ is a powerful activator of mononuclear phagocytes, increasing their ability to destroy intracellular microorganisms and tumor cells. It causes many types of cells to express class II MHC molecules and can also increase the expression of class I MHC molecules. IFN-γ facilitates differentiation of both B and T lymphocytes. It is a powerful activator of NK cells and also activates neutrophils and vascular endothelial cells. IFN-γ is decreased in chronic lymphocytic leukemia, lymphoma, and IgA deficiency as well as in those infected with rubella virus, Epstein–Barr virus, and cytomegalovirus. Recombinant IFN-γ has been used in the treatment of a variety of conditions, including chronic lymphocytic leukemia, mycosis fungoides, Hodgkin's disease, and various other disorders. IFN-γ was also found to be effective in decreasing synthesis of collagen by fibroblasts and might have potential for the treatment of connective tissue disorders.

TNF-α is a cytotoxic monokine produced by macrophages stimulated with bacterial endotoxin. TNF-α participates in inflammation, wound healing, and regeneration of tissue. TNF-α, also known as cachectin, can induce septic shock and cachexia. It facilitates leukocyte recruitment, induces angiogenesis, and promotes fibroblast proliferation. TNF-α can combine with receptors on selected tumor cells and induce cell lysis. It mediates the antitumor action of murine natural cytotoxic cells, which distinguishes their function from that of NK and cytotoxic T cells. TNF-α was termed cachectin because of its ability to induce wasting and anemia when administered chronically to experimental animals.

These responses are necessary in order to modulate physiological processes. However, the sudden increase in the concentration of pro-inflammatory cytokines such as TNF-α, IFN-γ, IL-1β, and IL-6 that occurs from exposure to a superantigen or during a massive infection is also called a "cytokine storm," and results in host system shock. Overshooting activation of IL-4 could be linked to allergic asthma and atopic dermatitis. Several cancer types have been associated with production of high levels of IL-6. Furthermore, inflammation may lead to atherosclerosis and be closely correlated to additional human disease processes.[25,41–43] Therefore, inhibition of pro-inflammatory cytokines by *Chlorella* powder could have profound therapeutic benefit.

Chlorella powder was found to inhibit B and T cell proliferation in mouse splenocytes and thymocytes, respectively. Lymphocytes are small white blood cells that bear

lymph nodes. Their results confirmed that *Chlorella* enhanced immunoglobulin (Ig) M antibody production by circulating lymphocytes and lymphocytes of mesenteric lymph nodes as well as IgG antibodies by splenic lymphocytes and lymphocytes of mesenteric lymph nodes.

In a previous study we found that *Chlorella* powder has an anti-tumor effect against mouse mammary adenocarcinoma (adenocarcinoma 755) cells subcutaneously transplanted into BDF1 mice (authors' unpublished data). In an attempt to elucidate the mechanism of action, we conducted this *in vitro* study using 52 assay systems to screen for the potential effects of *Chlorella* powder on receptor binding, enzyme activities, and immunomodulatory activity.

MATERIALS AND METHODS

Materials

Chlorella powder from *Chlorella pyrenoidosa* (SUN CHLORELLA strain) was produced (lot number SCA2216) by Sun Chlorella Corp. (Kyoto, Japan). The powder was prepared by crushing the cell walls in a Dyno mill (WAB, Inc., Maschinenfabrick, Basel, Switzerland) and spray drying. The *Chlorella* powder was weighed and dissolved in 100% dimethyl sulfoxide (DMSO) to make a 75 mg/mL stock solution and stored at −20°C, and serial dilutions were prepared with DMSO on the day of assays. Chemicals used in the receptor binding, enzyme, and cellular assays were purchased from the following commercial suppliers: active matrix metalloproteinase (MMP) enzymes MMP-1, -3, -7, and -9 from Calbiochem (La Jolla, CA); MCA (7-methoxycoumarin-4-yl)-Pro-Leu-Gly-Leu-DPA [*N*-3-(2′,4′-dinitrophenyl)-L-2,3-diaminopropionyl]-Ala-Arg-NH_2 (Bachem, Bubendorf, Switzerland); caspases 1, 3, and 8 and acetyl-YVAD-7-amino-4-methylcoumarin (AMC) fluorogenic substrate for caspase 1, acetyl-DEVD-AMC fluorogenic substrate for caspases 3 and 7, and acetyl-IETD-AMC fluorogenic substrate for caspase 8 from Biomol (Plymouth Meeting, PA); caspases 6 and 7 (Pharmingen, San Diego, CA); acetyl-VEID-AMC fluorogenic substrate for caspase 6, poly(Glu,Tyr) (4:1), 3-(*N*-morpholino)propanesulfonic acid (MOPS), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), lipopolysaccharide (LPS), concanavalin A (Con A), and phorbol 12,13-dibutyrate (PDBu) from Sigma Chemical Co. (St. Louis, MO); Fyn (p55fyn), protein tyrosine phosphatase (PTP) 1B, PTP1C, and tyrosine phosphopeptide from UBI (Lake Placid, NY); [^{3}H]PDBu and [^{3}H]thymidine from Perkin Elmer (Boston, MA); T-cell-PTP (TC-PTP) (New England Biolabs, Beverly, MA); enzyme-linked immunosorbent assay (ELISA) kits for interleukin (IL)-1β, interferon (IFN)-γ, IL-2, IL-4, IL-6, and tumor necrosis factor (TNF)-α from R&D Systems (Minneapolis, MN); human peripheral blood mononuclear cells (PBMNs) from the Chinese Blood Services Foundation (Taipei, Taiwan); ICR mice from MDS Pharma Services (Taipei); human recombinant CD45 from MDS Pharma Services (Bothell, WA); and DMSO from Merck (Darmstadt, Germany).

Methods

In this study, we used 25 enzymatic and 27 receptor/cellular assays, but describe the materials and methods only for the assays demonstrating some activities.

PTPase assays of CD45/PTP1C

Wells were coated overnight with fyn kinase-phosphorylated substrate poly(Glu:Tyr) (4:1). The reaction was initiated by addition of *Chlorella* powder and/or vehicle and the appropriate dilution of enzyme in imidazole buffer (pH 7.2) at room temperature for 20 minutes for CD45 and 30 minutes for PTP1C.[12–15] An ELISA was used for quantitation of poly(Glu:Tyr-P).

PTPase assays of PTP1B/TC-PTP

Wells were coated with substrate tyrosine phosphopeptide overnight. The reaction was initiated by addition of *Chlorella* powder and/or vehicle and enzyme in imidazole buffer (pH 7.2) at room temperature for 30 minutes.[16,17] An ELISA was used for quantitation of tyrosine phosphopeptide.

Peptidase (MMP) assays of MMP-1, -3, -7, and -9

Chlorella powder and/or vehicle was preincubated for 60 minutes at 37°C with the active enzyme in a reaction mixture containing modified 50 m*M* MOPS (pH 7.2). The reaction was initiated by addition of MCA-Pro-Leu-Gly-Leu-DPA-Ala-Arg-NH_2 and incubated for 120 minutes at 37°C.[18–20] Enzyme activity was determined spectrofluorometrically with excitation at 460 nm and emission at 520 nm by measuring the formation of fluorescent MCA-Pro-Leu-Gly.

Peptidase (caspase) assays of caspases 1, 3, 6, 7, and 8

Chlorella powder and/or vehicle was incubated with caspase and fluorogenic substrate in modified 50 m*M* HEPES (pH 7.4) for 180, 60, 120, 60, and 60 minutes for caspases 1, 3, 6, 7, and 8, respectively at 37°C.[21–24] The proteolytic product was then read spectrofluorometrically with excitation at 360 nm and emission at 465 nm.

Cellular assays of release of the mediators IL-1β, IL-6, and TNF-α

Chlorella powder and/or vehicle was incubated with 25 ng/mL LPS-stimulated human PBMNs in pH 7.4 medium overnight at 37°C.[25] Cytokine levels in the conditioned medium were then quantitated using a sandwich ELISA kit.

Cellular assays of release of the mediators IFN-γ, IL-2, and IL-4

Chlorella powder and/or vehicle was incubated with 20 μg/mL Con A-stimulated human PBMNs in pH 7.4 medium overnight at 37°C.[25] Cytokine levels in the conditioned medium were then quantitated using a sandwich ELISA kit.

B-cell + LPS proliferation assays

Chlorella powder and/or vehicle and 10 μg/mL LPS-stimulated B lymphocytes isolated from the spleen of BALB/c mice were incubated with [^{3}H]thymidine for 48 hours at 37°C.[26,27] Bound radioligands were separated from free by vacuum filtration using a Micro96 harvester (Skatron Instruments, Inc., Tranby, Norway). Quantitation of [^{3}H]thymidine incorporation in the filter was determined using a scintillation counter.

T-cell + Con A proliferation assays

Chlorella powder and/or vehicle and 3 μg/mL ConA-stimulated T lymphocytes isolated from the thymus of BALB/c mice were incubated with [^{3}H]thymidine for 48 hours at 37°C.[26,27] Bound radioligands were separated from free by vacuum filtration using a Micro96 harvester. Quantitation of [^{3}H]thymidine incorporation in the filter was determined using a scintillation counter.

Receptor binding assay using [^{3}H]PDBu

An aliquot of mouse brain membrane prepared in modified Tris-HCl buffer was incubated with 3 n*M* [^{3}H]PDBu for 60 minutes at 25°C. Nonspecific binding was estimated in the presence of 1 μ*M* PDBu.[28] Membranes were filtered and washed, and residual radioactivities on the filters were counted to determine specifically bound [^{3}H]PDBu.

Equipment

A Spectrafluor Plus (Tecan, Grodig, Austria) fluorometer was used for spectrofluorometric and ELISA quantitation. [^{3}H]Thymidine incorporation and amount of [^{3}H]PDBu specifically bound were quantitated by counting on a Microbeta scintillation counter and Betaplate scintillation counter (both from Wallac, Turku, Finland), respectively.

Calculation and data analysis

Chlorella powder was tested at 300 μg/mL in duplicate. A concentration–response study was conducted if activity at 300 μg/mL was >50% inhibition. The percent inhibition was calculated as follows:

$$\text{Inhibition (\%)} = \left[1 - \frac{\text{Counts of sample} - \text{Counts of minimum control}}{\text{Counts of maximum control} - \text{Counts of minimum control}}\right] \times 100\%$$

The 50% inhibitory concentration (IC_{50}) values were estimated by non-linear regression using Data Analysis Toolbox™ (MDL Information System, Inc., San Leandro, CA).

RESULTS

We used 52 *in vitro* assay systems to screen for 25 enzyme and 27 receptors/cellular activities. *Chlorella* powder exhibited inhibitory activity in the following assays, with IC_{50} values ranging from 0.678 to 301 μg/mL.

Potent inhibitory activities were observed for the following: CD45 and PTP1C PTPs (IC_{50} of 0.678 and 1.56 μg/mL, respectively). The inhibitory ability of *Chlorella* powder was stronger than those of sodium orthovanadate (IC_{50} of 2.6 and 77 μg/mL, respectively) and ammonium heptamolybdate (IC_{50} of 17 and 1,235 μg/mL, respectively), which are known to be potent inhibitors of PTP.

Moderate inhibitory activities were observed for the following: production of the cytokines TNF-α, IL-2, IFN-γ, IL-6, IL-1β, and IL-4; proliferation of LPS-stimulated B cells and Con A-stimulated T cells; and enzyme activity of MMP-7, PTP1B, the protein tyrosine kinase Lck, TC-PTP, MMP-1, phorbol ester receptor, and MMP-3 (IC_{50} range, 11-185 μg/mL).

Weak inhibitory activities were observed for the following: the peptidases caspase 1, caspase 3, caspase 6, caspase 7, and caspase 8; MMP-9; and the transcription response action of nuclear factor of activated T cells (IC_{50} range, >200 μg/mL).

The moderate and weak activities of this powder in the assays mentioned above were weaker than reference standard compounds tested in each assay, but are briefly described since the inhibitory activity is still significant.

The *Chlorella* powder did not show any significant inhibition in other enzyme and receptor binding/cellular assays (data not shown). Tables 1–6 summarize the results.

TABLE 1. *CHLORELLA* POWDER INHIBITION OF PTP ACTIVITY OF HUMAN RECOMBINANT CD45, PTP1B, PTP1C, AND TC-PTP

PTP assayed	IC_{50} (*μg/mL*)
CD45	0.678
PTP1B	65.3
PTP1C	1.56
TC-PTP	114

Chlorella powder was tested in duplicate at serial concentrations from 300 to 0.3 μg/mL in 1% DMSO for each enzyme assay. IC_{50} values were calculated by non-linear regression analysis.

TABLE 2. *CHLORELLA* POWDER INHIBITION OF MMP ACTIVITY OF HUMAN RHEUMATOID SYNOVIAL FIBROBLAST MMP-1 OR HUMAN RECOMBINANT MMP-3, MMP-7, AND MMP-9

Peptidase MMP assayed	IC_{50} (*μg/mL*)
MMP-1	127
MMP-3	185
MMP-7	18.1
MMP-9	237

Chlorella powder was tested in duplicate at serial concentrations from 300 to 0.3 μg/mL in 1% DMSO for each enzyme assay. IC_{50} values were calculated by non-linear regression analysis.

15. Hirose M, Ito T, Takahashi S, *et al*: Prevention by synthetic phenolic antioxidants of 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx)-or activated MeIQx-induced mutagenesis and MeIQx-induced rat hepatocarcinogenesis, and role of antioxidant activity in the prevention of carcinogenesis. Eur J Cancer Prev 7: 233-241, 1998.
16. Takekoshi H, Suzuki G, Chubachi H and Nakano M: Effect of *Chlorella pyrenoidosa* on fecal excretion and liver accumulation of polychlorinated dibenzo-*p*-dioxin in mice. Chemosphere 59: 297-304, 2005.
17. Hsu SM, Raine L and Fanger H: Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: A comparison between ABC and unlabeled antibody (PAP) procedures. J Histochem Cytochem 29: 577-580, 1981.
18. Kato T, Ohgaki H, Hasegawa H, Sato S, Takayama S and Sugimura T: Carcinogenicity in rats of a mutagenic compound, 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline. Carcinogenesis 9: 71-73, 1988.
19. Wakabayashi K, Ushiyama H, Takahashi M, *et al*: Exposure to heterocyclic amines. Environ Health Perspect 99: 129-134, 1993.
20. Dashwood RH: Protection by chlorophyllin against the covalent binding of 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) to rat liver DNA. Carcinogenesis 13: 113-118, 1992.
21. Negishi T, Arimoto S, Nishizaki C and Hayatsu H: Inhibitory effect of chlorophyll on the genotoxicity of 3-amino-1-methyl-5H-pyrido[4,3-b]indole (Trp-P-2). Carcinogenesis 10: 145-149, 1989.
22. Wattenberg LW: Inhibition of carcinogenesis by minor dietary constituents. Cancer Res 52: 2085-2091, 1992.
23. Dashwood RH and Guo D: Inhibition of 2-amino-3-methylimidazo[4,5-f]quinoline (IQ)-DNA binding by chlorophyllin: studies of enzyme inhibition and molecular complex formation. Carcinogenesis 13: 1121-1126, 1992.
24. Yun CH, Jeong HG, Jhoun JW and Guengerich FP: Non-specific inhibition of cytochrome P450 activities by chlorophyllin in human and rat liver microsomes. Carcinogenesis 16: 721-727, 1995.
25. Hasegawa R, Hirose M, Kato T, Hagiwara A, Boonyaphiphat P, Nagao M, Ito N and Shirai T: Inhibitory effect of chlorophyllin on PhIP-induced mammary carcinogenesis in female F344 rats. Carcinogenesis 16: 2243-2246, 1995.
26. Hakkak R, Korourian S, Shelnutt SR, Lensing S, Ronis MJ and Badger TM: Diets containing whey proteins or soy protein isolate protect against 7,12-dimethylbenz(a)anthracene-induced mammary tumors in female rats. Cancer Epidem Biomar Prev 9: 113-117, 2000.
27. Constantinou AI, Lantvit D, Hawthorne M, Xu X, Pezzuto JM and van Breemen RB: Chemopreventive effects of soy protein and purified soy isoflavones on DMBA-induced mammary tumors in female Sprague-Dawley rats. Nutr Cancer 41: 75-81, 2001.
28. Kanamoto R, Azuma N, Miyamoto T, Saeki T, Tsuchihashi Y and Iwami K: Soybean resistant proteins interrupt an enterohepatic circulation of bile acids and suppress liver tumorigenesis induced by azoxymethane and dietary deoxycholate in rats. Biosci Biotechnol Biochem 65: 999-1002, 2001.
29. Alam BS and Alam SQ: The effect of different levels of dietary beta-carotene on DMBA-induced salivary gland tumors. Nutr Cancer 9: 93-101, 1987.
30. Tsuda H, Uehara N, Iwahori Y, Asamoto M, Iigo M, Ngao M, Matsumoto K, Ito M and Hirono I: Chemopreventive effects of beta-carotene, alpha-tocopherol and five naturally occurring antioxidants on initiation of hepatocarcinogenesis by 2-amino-3-methylimidazo[4,5-f]quinoline in the rat. Jpn J Cancer Res 85: 1214-1219, 1994.
31. Van Beresteyn EC, Van Schaik M and Mogot MF: Effect of bran and cellulose on lipid metabolism in obese female Zucker rats. J Nutr 109: 2085-2097, 1979.
32. Ibusuki K and Minamishima Y: Effect of *Chlorella vulgaris* extracts on murine cytomegalovirus infections. Nat Immun Cell Growth Regul 9: 121-128, 1990.
33. Takaba K, Hirose M, Yoshida Y, Kimura J, Ito N and Shirai T: Effects of n-tritriacontane-16,18-dione, curcumin, chlorophyllin, dihydroguaiaretic acid, tannic acid and phytic acid on the initiation stage in rat multi-organ carcinogenesis model. Cancer Lett 113: 39-46, 1997.
34. Pinkel D: The use of body surface area as a criterion of drug dosage in cancer chemotherapy. Cancer Res 18: 853-856, 1958.
35. Guo D and Dashwood RH: Inhibition of 2-amino-3-methylimidazo [4,5-f]quinoline (IQ)-DNA binding in rats given chlorophyllin: dose-response and time-course studies in the liver and colon. Carcinogenesis 15: 763-766, 1994.
36. Arimoto S, Fukuoka S, Itome C, Nakano H, Rai H and Hayatsu H: Binding of polycyclic planar mutagens to chlorophyllin resulting in inhibition of the mutagenic activity. Mutat Res 287: 293-305, 1993.

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Effects of *Chlorella* on Activities of Protein Tyrosine Phosphatases, Matrix Metalloproteinases, Caspases, Cytokine Release, B and T Cell Proliferations, and Phorbol Ester Receptor Binding

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ABSTRACT A Chlorella powder was screened using 52 *in vitro* assay systems for enzyme activity, receptor binding, cellular cytokine release, and B and T cell proliferation. The screening revealed a very potent inhibition of human protein tyrosine phosphatase (PTP) activity of CD45 and PTP1C with 50% inhibitory concentration (IC_{50}) values of 0.678 and 1.56 μg/mL, respectively. It also showed a moderate inhibition of other PTPs, including PTP1B (IC_{50} = 65.3 μg/mL) and T-cell-PTP (114 μg/mL). Other inhibitory activities and their IC_{50} values included inhibition of the human matrix metalloproteinases (MMPs) MMP-1 (127 μg/mL), MMP-3 (185 μg/mL), MMP-7 (18.1 μg/mL), and MMP-9 (237 μg/mL) and the human peptidase caspases caspase 1 (300 μg/mL), caspase 3 (203 μg/mL), caspase 6 (301 μg/mL), caspase 7 (291 μg/mL), and caspase 8 (261 μg/mL), as well as release of the cytokines interleukin (IL)-1β (44.9 μg/mL), IL-2 (14.8 μg/mL), IL-4 (49.2 μg/mL), IL-6 (34.7 μg/mL), interferon-γ (31.6 μg/mL), and tumor necrosis factor-α (11 μg/mL) from human peripheral blood mononuclear cells. *Chlorella* also inhibited B cell proliferation (16.6 μg/mL) in mouse splenocytes and T cell proliferation (54.2 μg/mL) in mouse thymocytes. The binding of a phorbol ester, phorbol 12,13-dibutyrate, to its receptors was also inhibited by *Chlorella* with an IC_{50} of 152 μg/mL. These results reveal potential pharmacological activities that, if confirmed by *in vivo* studies, might be exploited for the prevention or treatment of several serious pathologies, including inflammatory disease and cancer.

KEY WORDS: • ***caspases*** • **Chlorella** • ***cytokines*** • ***matrix metalloproteinases*** • ***phorbol ester*** • ***protein tyrosine phosphatase***

INTRODUCTION

CHLORELLA is a nearly spherical unicellular green algae with a diameter of 3–8 μm, and is believed to have survived for more than 2 billion years, since the original eukaryotic organisms first came into existence on earth. Taxonomically speaking, it belongs to the genus of *Chlorella*, which is a member of the Class Chlorophyceae; the *Chlorella* genus is further subdivided, and currently several tens of species are known.[1] Owing to its protein content, as high as 60%, research was started in Japan soon after World War II on its utilization as a food resource. Currently no research continues for that purpose, but since the latter half of the 1960s *Chlorella* has been used as a health-promoting nutritional supplement and health food, contributing nutrients such as protein, vitamins, minerals, fiber, and chlorophyll as well as bioactive phytochemicals. Numerous human and animal experiments have documented a variety of pharmacological effects of *Chlorella* and *Chlorella* extract, including improving hypertension[2,3] and lipid metabolism,[4] enhancing anti-tumor[5–7] and antibacterial[8] activities, and promoting dioxin excretion.[9] Tanaka *et al.*[5] subcutaneously transplanted Meth A tumor cells into BALB/c mice and then injected *Chlorella* extract into the tumors at four dosage levels of 2, 10, 50, and 250 mg/kg post-transplantation. They found that the injections dose-dependently prevented increases in tumor weight and prolonged survival. When they repeated the same experiments on athymic nude mice, they found the benefits of *Chlorella* extract to be reduced or eliminated, thereby suggesting that T cells and macrophages might be involved in the anti-tumor effect of *Chlorella*.

Komiyama *et al.*[10] treated female *ddy* mice with an acidic polysaccharide extracted from *Chlorella*, and confirmed the positive augmentation of macrophage activities and reticuloendothelial functions.

Furthermore, Kanouchi *et al.*[11] orally treated male Sprague–Dawley rats with *Chlorella* powder to study its effects on serum antibody levels and antibody production by splenic lymphocytes and by lymphocytes of the mesenteric

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