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AN EXTRACT OF THE FERN *POLYPODIUM LEUCOTOMOS* INHIBITS HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS PROLIFERATION *IN VITRO*

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Abstract—An alcoholic extract of the fern *Polypodium leucotomos* (PLE) has been empirically used as an immunosuppressor for the treatment of several autoimmune diseases. In this paper, we investigated the effects of PLE on activation and proliferative responses of peripheral blood mononuclear cells (PBMNC) from healthy donors to T lymphocyte polyclonal mitogens. PLE shows a significant inhibitory effect on the proliferative response of PBMNC to stimulation with phytohaemagglutinin (PHA) or anti CD3 monoclonal antibodies ($P < 0.05$). In contrast, PLE did not modify the proliferative response of PBMNC to phorbol esters ($P > 0.05$). The inhibitory effect of PLE upon mitogen induced PBMNC proliferation is time dependent and can be overcome by the exogenous addition of interleukin-2 to the culture medium ($P < 0.05$). The decreased proliferative response of PBMNC to PHA stimulation in the presence of PLE is not associated with a significant modification of expression of the α chain (CD25) of the IL-2 receptor ($P > 0.05$). In conclusion, PLE shows an inhibitory effect on the polyclonal proliferative response of PBMNC to T lymphocyte mitogens that interact with cytoplasmic membrane molecules. © 1997 International Society for Immunopharmacology.

Keywords: *Polypodium leucotomos* extract, T-specific immunosuppression

Since remote times PLE has been empirically used in Central America to treat phlogistic disorders. More recently, a methanolic extract of PLE has been used to treat several inflammatory disorders such as psoriasis (Capella-Pérez & Castells Rodellas, 1981), atopic dermatitis (Jiménez *et al.*, 1986), vitiligo (Mohammad, 1989), where autoimmune mechanisms are probably involved. Rationale for its use was based on certain observations suggesting the ability of PLE to modify the distribution of some T lymphocyte subsets with an increase in the number of T suppressor lymphocytes (Jiménez *et al.*, 1986; Vargas *et al.*, 1983). Other recent data show the capacity of PLE to inhibit the synthesis and activities of molecules involved in the regulation of the immune and inflammatory responses, such as platelet activating factor (PAF) and leukotriene B4 (LTB4) (Tuominen *et al.*, 1992). Furthermore, *in vivo* experiments indicate that PLE delays skin graft rejection in mice (Tuominen *et al.*, 1991).

The activation and proliferation of T lymphocytes are complex processes involving several surface molecules, intracytoplasmatic enzymatic systems and cytokines, both monokines and lymphokines. When the antigen comes in contact with the clonotypic T cell receptor (TCR) in the presence of the appropriate molecule of the major histocompatibility system, activation begins (Altman *et al.*, 1990). This activation can be mimicked *in vitro* by the interaction of different polyclonal mitogens, such as vegetal lectins, with molecules of the T lymphocyte surface (Hadden, 1988). These different molecular interactions provoke activation of several intra-cytoplasmatic metabolic pathways which induce the activation and translocation of protein-kinase C (PKC). Once phosphorilated by this enzyme, several proteins induce the initiation of the cascade of gene activation leading T lymphocytes to progress through the cell cycle. This protein-kinase C activation can also be induced *in vitro* by phorbol esters (Truneh *et al.*, 1985). The progression of the activated T lymphocytes along the cell cycle, with the

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subsequent proliferation, is mainly regulated by the secretion of interleukin-2 (IL-2) and the cell surface expression of the IL-2 receptors (Depper *et al.*, 1985). Along this IL-2 pathway, other cytokines can also be involved in the regulation of the T lymphocyte proliferation (Arai *et al.*, 1990). Several immunosuppressor drugs such as Cyclosporine A, FK506 and glucocorticoids can inhibit these cellular processes (Sigal & Dumont, 1992).

In this paper, we investigated the effect of PLE on the activation and proliferative response of peripheral blood mononuclear cells (PBMNC) from healthy donors to T lymphocyte polyclonal mitogens, including phytohaemagglutinin (PHA), anti-CD3 monoclonal antibodies and phorbol esters, in the presence or absence of interleukin-2 (IL-2).

EXPERIMENTAL PROCEDURES

Donors

Twenty-five volunteers, healthy donors of both gender were studied. The ages of the study population ranged from 20 to 40 yr.

Cell separation

PBMNC were obtained by Ficoll-Hypaque (Lymphoprep Nyegaard and Co., Oslo, Norway) gradient and suspended in RPMI 1640 (Whitaker Bioproducts, Walkerville, U.S.A.) containing 10% fetal bovine serum (Biochrom KG, Berlin). After counting, cells were resuspended in RPMI 1640 supplemented with 10% heat activated fetal bovine serum, L-Glutamine (2 mM Flow Lab, Irvine, U.K.), Hepes (0.5% Flow Lab) and 1% penicillin–streptomycin (Difco Lab, Detroit, MI, U.S.A.), this will be referred to as complete medium. Cell viability was checked by trypan blue exclusion.

Proliferation studies

PBMNC (50 000 cells/well) were cultured in complete medium on 96 flat-bottomed culture plates with one of the following: phytohaemagglutinin (PHA, 10 μg/ml, Difco Lab, MI, U.S.A.) plastic immobilized anti-CD3 (5 μg/ml, Ortho-inmune, Orthodiagnostic System) or 12-0-tetradecanoylphorbol 13-acetate (TPA, 100 ng/ml, Sigma, MO, U.S.A.) either in the presence or absence of recombinant interleukin-2 (IL-2, 100 IU/ml, Hoffman-LaRoche, NJ, U.S.A.). Each reagent was tested in dose–response titrations before use. Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO2 for 3, 5 or 7 days. Cell viability in cultures maintained for 3, 5 or 7 days was similar whether in the presence or absence of PLE. Twenty-four hours before the end of the incubation, 1 μCi of (3H)-thymidine (Dupont, Boston, MA, U.S.A.) was added to the culture medium. Cells were harvested and results expressed as mean counts per minute (CPM) of triplicate cultures $\pm$ estimated error.

Staining of cells and flow cytometry analysis

A direct immunofluorescence technique was used. The cells (5×10 in 50 μl of PBS with 2% human albumin) were incubated with 5–15 μl of monoclonal antibodies for 30 min at 4°C. The cells were washed 3 times with PBS and then fluorescence was analyzed with a FACSCAN (Becton-Dickinson, San Jose, CA, U.S.A.) as previously described (García-Suarez *et al.*, 1993).

Statistical analyses

All cultures were made in triplicate, and median counts per minute (cpm) used for all calculations. Data from groups were compared with a Wilcoxon test for paired samples. A P value of less than 0.05 was considered to indicate a significant difference between groups.

Plant and extracts

Leaves were collected from adult *Polypodium leucotomos*, syn. (PLE) plants from the cultures at Lago Yojoa, Honduras. After drying, the leaves are finely ground, defatted with n-Hexane, air dried overnight and extracted with the mixture alcohol/water (7:3). Evaporation of the aqueous/methanolic solution at temperature lower than 40°C and under reduced pressure, followed by ion exchange chromatography purification and treatment with active charcoal, gives a final PLE as a stabilized syrup containing 20% water. PLE (Difur^{R}) was gently supplied by Laboratorios Cantabria S.A. (Spain).

RESULTS

PLE inhibits the proliferative response of PBMNC to PHA and can be overcome by IL-2.

The proliferative response of PBMNC to suboptimal concentrations of PHA in the presence or absence of PLE were investigated. As shown in Fig. 1A, the presence of PLE at concentrations in the range of 1mg/ml–0.01 pg/ml induced a significant decrease in the blastogenesis of the PHA stimulated PBMNC at 3 days of culture ($P < 0.05$). Similar suppressor effects were observed after 5 days of culture ($P < 0.05$) (Fig. 1B).

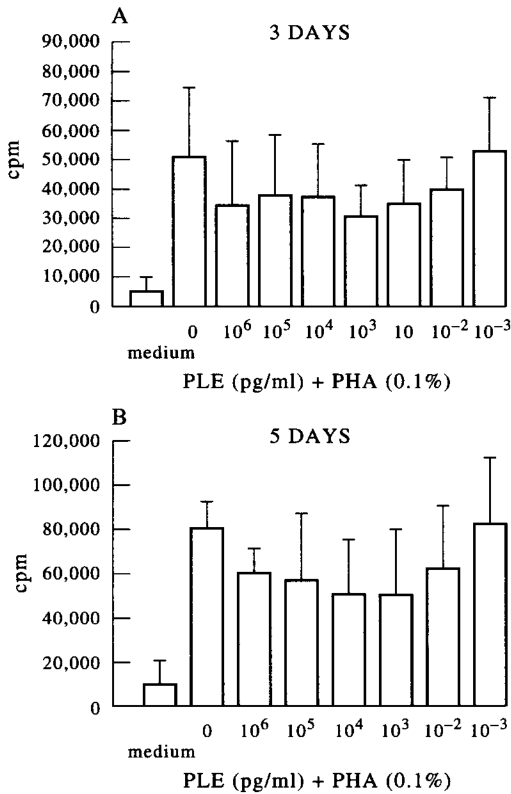

Fig. 1. The effect of PLE on the proliferative response of PBMNC from 25 healthy controls to PHA (0.1%) stimulation. PBMNC (50,000 cells/well) were cultured in the presence of PHA (0.1%) and of the indicated concentrations of PLE for 3 (A) and 5 (B) days. The results are expressed as mean $\pm$ SD.

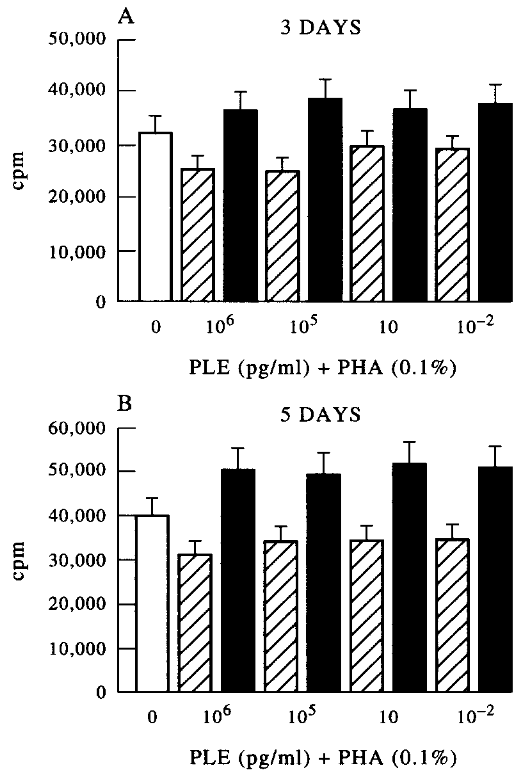

Fig. 2. The time-dependent effect of PLE on the proliferative response of PBMNC from six healthy controls to PHA 0.1% stimulation. PBMNC (50,000 cells/well) from eight healthy controls were cultured in the presence of PHA (empty bars) and of the indicated concentrations of PLE added at the beginning of the culture (coarse bars) or 24 h before the end of the 3 (A) or 5 (B) days of culture (filled bars). The results were expressed as mean counts per minute (cpm) of triplicate cultures $\pm$ SD.

In contrast, there was no significant diminution of the blastogenic response of PBMNC in the presence of PLE after 7 days of culture (data not shown).

Next, we tested the possible relationship between the suppressor effect of PLE upon the proliferation of PBMNC and the time of its addition to the culture medium. The exogenous addition of PLE to the medium during the last 24 or 48 h of the 3 or 5 days cultures was not associated with a significant inhibition of the PHA stimulated PBMNC proliferation ($P > 0.05$), in contrast to that found with its presence from the beginning of the cultures ($P < 0.05$) (Fig. 2A and 2B).

Then, we investigated the proliferative response of PBMNC to optimal concentrations of PHA (1%) in the presence or absence of different concentrations of PLE. We did not observe that the presence of PLE in the culture medium was associated with significant variations in the PHA stimulated (^{3}H) thymidine incorporation by PBMNC after 3 and 5 days of culture (data not shown). The addition of PLE to the culture medium did not induce significant modifications upon the low spontaneous proliferation of PBMNC observed after 3 or 5 days of culture (data not shown).

The effect of the exogenous addition of IL-2 to the culture medium upon the proliferative response of PBMNC to PHA in the presence of PLE was investigated (Fig. 3A and 3B).

We found that the exogenous addition of IL-2 significantly enhanced the blastogenic response of PBMNC to PHA in the presence of PLE ($P < 0.05$). The proliferative response of PBMNC to PHA in the presence of PLE and IL-2 was similar to that observed with PHA alone ($P > 0.05$).

We also studied the effect of PLE on the distribution of the lymphocyte populations of PBMNC defined by the expression of CD3, CD4, CD8, CD16, CD19, CD56 antigens. PHA stimulated PBMNC were cul-

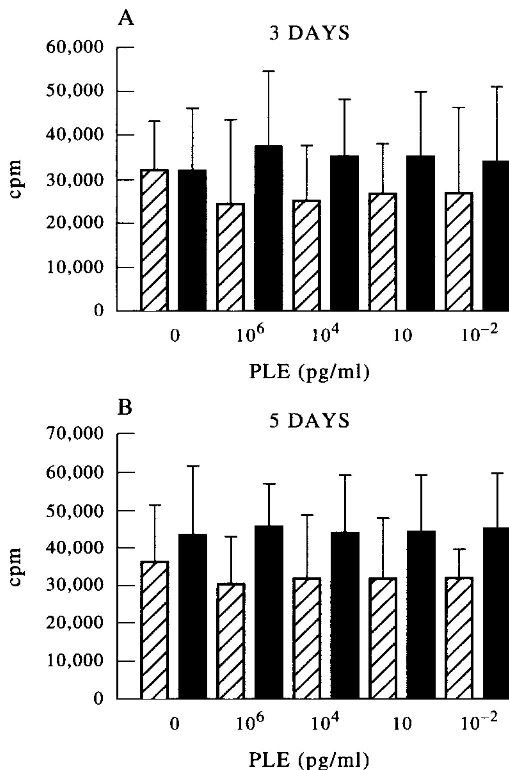

Fig. 3. The effect of exogenous addition of IL-2 on the inhibitory effect of PLE on the proliferative response of PBMNC from six healthy donors to PHA 0.1% stimulation. PBMNC (50,000 cells/well) were cultured in the presence of PHA 0.1% (coarse bars) or PHA 0.1% + IL-2 (100 U/ml) (filled bars), in the presence of the indicated concentrations of PLE for 3 (A) and 5 (B) days. The results were expressed as mean counts per minute (cpm) of triplicate cultures ± SD.

Table 1. Percentage of antigen expression on PBMNC from healthy controls after PHA 0.1% stimulation in the presence or absence of PLE

	BASAL	PHA 0.1%	PHA 0.1% +PLE
CD3	74.5±5.6	86.1±7.6	80.3±6.7
CD4	48.2±8.8	65.7±6.8	63.0±5.6
CD8	27.1±3.8	26.5±5.3	30.0±6.3
CD25	15.1±1.3	72.1±15.3	76.4±10.2
HLA-DR	14.9±4.0	35.1±10.2	30.0±4.0

PBMNC from 12 healthy controls were cultivated with PHA 0.1% in the presence of PLE 1 mg/ml in basal conditions and after 72 h of culture.

tured in the presence or absence of PLE for 5 days. The presence of PLE in the culture medium did not significant modify the percentage of positive cells for the different antigens analyzed in the PHA stimulated PBMNC, with respect to those observed in its absence ($P > 0.05$) (Table 1).

Furthermore, the presence of PLE did not decrease the expression of the a chain of the IL-2 receptor CD25 and HLA-DR molecules on PHA stimulated PBMNC ($P > 0.05$).

PLE shows an inhibitory effect upon the proliferative response of PBMNC to anti-CD3 monoclonal antibodies.

The effect of PLE on the proliferative response of PBMNC to anti-CD3 monoclonal antibodies was studied. As seen in Fig. 4, at days 3 and 5 of culture, there was a significant decrease in the proliferative response of PBMNC to anti-CD3 monoclonal antibodies in the presence of a range of concentrations of PLE between 1 mg/ml and 0.01 μg/ml ($P < 0.05$). We also found that the exogenous addition of IL-2 to the culture medium significatively enhanced ($P < 0.05$) the proliferative response of PBMNC to anti-CD3 mono-

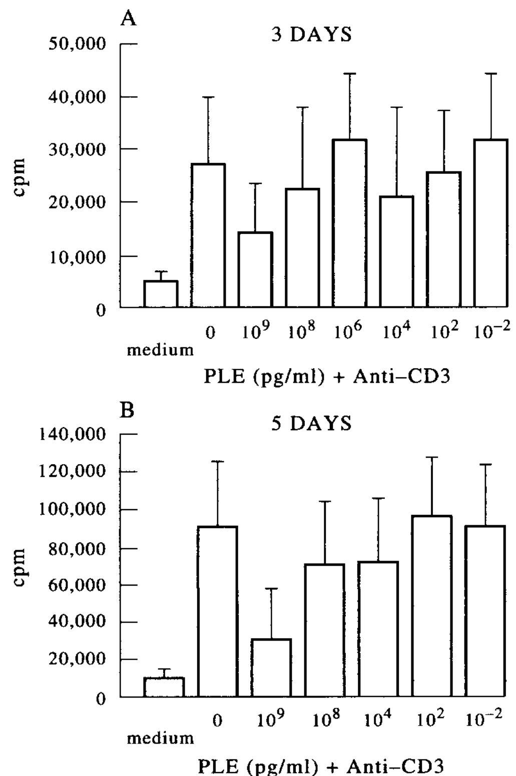

Fig. 4. The effect of PLE on the proliferative response of PBMNC from eight healthy controls to anti-CD3 stimulation. PBMNC (50,000 cells/well) were cultured in the presence of anti-CD3 and the indicated concentrations of PLE for 3 (A) and 5 (B) days. The results were expressed as mean counts per minute (cpm) of triplicate cultures ± SD.

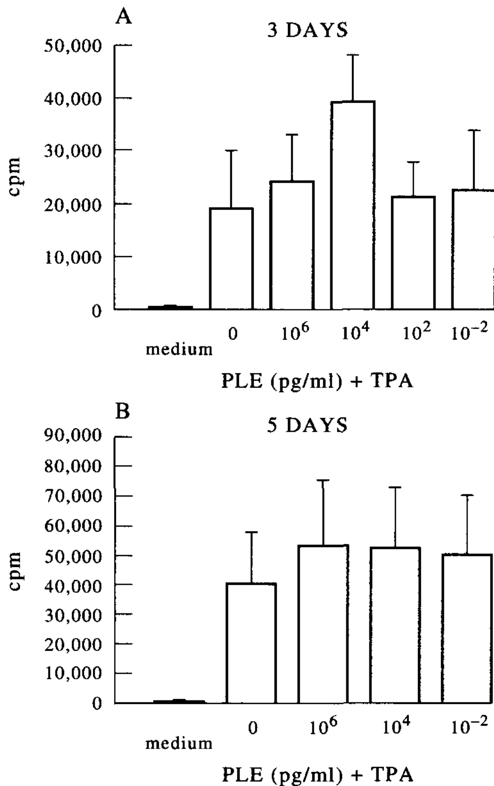

Fig. 5. The effect of PLE on the proliferative response of PBMNC from eight healthy controls to TPA stimulation. PBMNC (50,000 cells/well) were cultured in the presence of TPA and of the indicated concentrations of PLE for 3 (A) and 5 (B) days. The results were expressed as mean counts per minute (cpm) of triplicate cultures ± SD.

clonal antibodies in the presence of the suppressor concentration of PLE and reached values similar to those found with anti-CD3 monoclonal antibodies alone ($P > 0.05$) (data not shown)).

PLE does not show immunomodulator effects upon the proliferative response of PBMNC to TPA.

The effect of PLE on the proliferative response of PBMNC to TPA was analyzed (Fig. 5A and 5B).

We found that the presence of the different concentrations analyzed of PLE in the culture medium was not associated with a decreased proliferative response of PBMNC to TPA ($P > 0.05$).

DISCUSSION

In this paper we have demonstrated that PLE has an inhibitory effect upon the polyclonal proliferative response of PBMNC to mitogens that interact with cytoplasmatic membrane molecules. This suppressor effect of PLE upon the mitogenic induced proliferation of PBMNC can be overcome by IL-2.

Our data demonstrate that the inhibitory effect of PLE upon the proliferation of PBMNC is not secondary to a toxic effect. In fact, the viability of the PBMNC cultures was not changed with the presence of PLE in the culture medium. Furthermore, the suppressor effect of PLE upon the proliferation of PBMNC was only observed under some experimental conditions of mitogenic stimulation, and it was reversible by the exogenous addition of IL-2 to the culture medium.

PLE shows the inhibitory effect upon the proliferative response of PBMNC to mitogenic signals, that interact with membrane molecules, inducing T lymphocyte proliferation (PHA and anti-CD3 monoclonal antibodies). However, this suppressor effect is not found after stimulation of PBMNC with a phorbol ester that directly activates protein kinase C. These results indicate that PLE has an inhibitory effect upon the mechanisms of signal transduction of T lymphocytes, functionally located between the CD3 complex, or other surface receptors with which PHA can also interact, and protein kinase C. This inhibitory effect of PLE upon PBMNC proliferation can be reverted by the exogenous addition of IL-2 to the culture medium. According to the pivotal role of IL-2 in the proliferation of T lymphocytes, PLE appears to provoke a decrease in the IL-2 production by PHA and anti-CD3 monoclonal antibody stimulated PBMNC. This statement is also supported by the absence of any effect of PLE on the expression of the a chain of the IL-2 receptor by the mitogenic stimulated PBMNC. The production of IL-2 by T lymphocytes is dependent on the secretion of IL-1 by the accessory cells (Manger *et al.*, 1985). Thus, the inhibitory effect of PLE upon the proliferative response of PBMNC to mitogens may be related to either a direct effect on T lymphocytes and/or an indirect effect through macrophage inhibition.

Taken together these data show PLE has an inhibitory effect upon the IL-2 dependent T lymphocytes proliferative pathway. These results agree with the previous demonstration of an enhancing effect of PLE on the prevention of allografts rejection in animal models (Tuominen *et al.*, 1991). This immunomodulator effect of PLE might also be involved in its mechanism of action observed in the treatment of vitiligo patients (Corrales *et al.*, 1994). It remains unknown if this suppressor effect of PLE upon the T lymphocyte proliferation is mediated by a direct effect on the IL-2 pathway and/or to an indirect effect

through the induction of inhibitory T lymphokines such as IL-4, IL-10 or transforming growth factor.

The immunosuppressor effect of PLE upon the proliferation of PBMNC demonstrated in this work may be involved in the therapeutical activity observed with its administration in some patients with autoimmune diseases.

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