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Extracts from *Polypodium* ferns upregulate the expression of CD95 in human peripheral blood lymphocytes

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SUMMARY

There are several data in the literature indicating a great variety of pharmacological activities of Polypodium genus, which exhibit antiinflammatory and immunomodulatory activities. Since one of our main interests is to obtain natural immunoregulatory agents devoid of pharmacological adverse effects, we used flow cytometry analysis to highlight relative contributions of a water-soluble fraction of different concentrations of *Polypodium* rhizome extracts on lymphocyte subpopulations, NK and LAK activity. To measure their potential immunoregulatory activity a T cell proliferation assay in response to phytohemaglutinin (PHA) and mixed lymphocyte reactions were chosen. As a confirmatory bioassay we studied the effect of our extracts on CD45RO and CD95 antigen expressions. The results indicate that CD95 expression dramatically increases after peripheral blood lymphocyte activation and treatment with *Polypodium leucotomus*, *cambricum* and *vulgare* extracts, suggesting a powerful intrinsic pro-apoptotic effect.

Key words: *Polypodium* ferns; MLR; CD95; CD45RO; Apoptosis

INTRODUCTION

The current therapy for several diseases such as systemic lupus erythematosus (SLE), multisystem vasculitis or the prevention of allograft rejection is based on combination therapies that nonspecifically suppress the patients immune system (El-Miedany, 2002; Kalunian *et al.*, 2002; Saydain *et al.*, 2002). Such therapies can be profoundly potent, and combination therapies that incorporate calcineurin-binding agents (such as cyclosporin and tacrolimus) dramatically reduce the rate of acute rejection of solid organ transplants (Krook *et al.*, 2002; Lazzaro *et al.*, 2002; Waller *et al.*, 2002; Kuiper-Geertsma and Derksen, 2003).

There is also a general agreement that the majority of antitumor drugs used in cancer chemotherapy are immunodepressive. However detailed analyses of the effects of these agents in various experimental conditions points out that antitumor drugs-induced modulation of the immune system is rather complex.

T-cell recognition of alloantigens supplemented by appropriate costimulatory signals triggers the scenario of acute rejection, which includes cytokine production, killing of the allograft by cytotoxic T cells, and macrophage/neutrophil inflammatory response within the graft (Haque *et al.*, 2002; Segel *et al.*, 2002; Zweifel *et al.*, 2002).

Many types of ferns have been used in traditional medicine around the world (Reixach *et al.*, 1999; Gomes *et al.*, 2001; Sempere-Ortells *et al.*, 2002). *Polypodium vulgare* is a common fern indigenous to the forests of Europe which has held a place in herbal medicine there for centuries. Most ferns, including the European *P. vulgar* and the South American *P. decumanum* are considered to have important properties for numerous respiratory problems as well as rheumatism and skin problems (Vasange-Tuominen *et al.*, 1994; Vasange *et al.*, 1997). In recent years there has been a great deal of scientific interest in *polypodium* ferns and

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several bioactive compounds, including flavonoids, tannins, polyphenols and alkaloids have been extracted, isolated and studied (Bernd *et al*., 1995; McCutcheon *et al*., 1995).

The potential of plant extracts as sources of immunomodulators (Álvarez *et al*., 1997; Fernández-Novoa *et al*., 1997; Álvarez *et al*., 2000; De Smet, 2002; Fujii, 2002; Reynolds and Meggitt, 2002; Vickers, 2002) has been previously shown. The final goal of this work was to design a method that would permit a concomitant enumeration of the absolute number and phenotype of cells that proliferate or undergo apoptosis in different culture conditions, with the final objective of studying the apoptotic contribution of *Polypodium leucotomus*, *cambricum* and *vulgare* extracts to the development of efficient or nonefficient inhibition of proliferation of PBMCs and CD95 antigen expression.

MATERIALS AND METHODS

The plant materials were collected on the Vigo area, Spain. Voucher specimen numbers from rhizome of *Polypodium leucotomos* (PLE1; 5-02-55), *Polypodium cambricum* L. ssp. (PLE2; 7-02-88) and *Polypodium vulgare* L. (PLE3; 8-02-121) were deposited at the Herbarium of the College of Pharmacy, Vigo University, Spain. Samples were dried at 40ºC for 8 h and pretreated by means of the following method: the rhizome, after removing all the villosite was stirred on a magnetic-stirrer 10 minutes with sodium hypochlorite solution 5%, sterile distilled water, isopropyl alcohol 70%, and sterile distilled water in three times cycles. All treatments were performed under a vacuum pump (at 25 mm of Hg) to help penetration of the disinfectants into plant tissues. After last washing, rhizhomes were frozen over night at -20ºC and vacuum dried in a liofilizer during a 72 hours cycle process. The powered plant material was percolated sequentially with 500 ml of the following solvents: petroleum ether, dichloromethane, ethanol, and water. The various extract were carefully evaporated to dryness under reduced pressure and were stored at -20ºC until used. To test the biological activity, the dried crude extracts were dissolved in dimethyl sulfoxide (DMSO, Sigma) to a concentration of 10 mg / ml, and at the time of the assay dilutions were prepared in cell culture medium starting from a concentration of 0.2 µg / ml to 4.0 µg / ml.

Media and reagents

RPMI 1640 culture medium was supplemented with 10% heat inactivated FCS, 2 mM glutamine, 5 mM HEPES, 5×10^{-5} M 2-ME, 100 µg / ml penicillin, 20 µg / ml gentamycin (pH 7.2-7.4). Ficoll hypaque, RPMI-1640, phytohemoaagglutinin-L (PHA-L), and all other reagents were purchased from Sigma Chemical Co. Monoclonal antibodies were purchased from Becton and Dickinson, and recombinant IL-2 (rIL-2) was purchased from Genezyme. All plastic ware were obtained from Costar and Falcon.

Preparation of human mononuclear cells

Blood was collected from 20 normal, healthy volunteers in 4.5 ml EDTA BD Vacutainer™ tubes. 4 ml of blood samples were gently layered over equal quantities of Ficoll-Hypaque density gradient and centrifuged at 1800 rpm for 30 minutes. PBMCs from the interface were collected, washed twice in Hank's Balanced Salt Solution by centrifugation at 1500 rpm for 15 minutes. The washed PBMCs were suspended in complete RPMI medium and adjusted to a volume of 10×10^{6} cells / ml.

Long term stimulation studies

2.5×10^{6} PBMCs were cultured in a total volume of 500 l complete medium in 48-well tissue culture plates in the presence of 5U / 10^{6} cells of IL-2 (a), 50U / 106 cells of IL-2 (b), 5U+50U / 106 cells of IL-2+IL-12 (c), 50+125 U / 106 cells of IL-2+IL-12 (d), 50 U / 106 cells of IL-12 (e), and 125 U / 106 cells of IL-12 (f). These cells were harvested and washed on days 2, 4, 7, 9 , 11, and 14. Chromium release assay was performed and samples were also analyzed by flow-cytometer at all time points including day 0 (fresh PBMCs).

Flow cytometry phenotyping

0.25×10^{6} cells / 400 µl medium were added to three tubes and 5 µl isotype control, leucogate and monoclonal antibody anti-CD3-FITC, anti-CD56-PE, anti-CD45RO-FITC and anti-CD95-PE conjugate were added to the respective tubes. The tubes were

incubated for 15 minutes at RT in dark and washed with PBS at 1200 rpm for 5 minutes. The stained cells were fixed with 0.5 ml 1% paraformaldehyde and kept (covered with foil) at 4ºC until further analysis on a flow-cytometer (FACScan, Becton Dickinson) using Cell Quest software.

Mixed lymphocyte reaction

Cells acting as stimulators were treated with mitomycin-C at the final concentration of 10 µg / ml. A mixed population of T and B lymphocytes (2×10^5 cells / well) was incubated for 96 hours in the presence of 1 µg / ml of PHA-L in complete RPMI 1640 according to the following protocol:

(a) 0.1 ml responder lymphocytes (2×10^5 cells / well) +complete medium;

(b) 0.1 ml stimulator lymphocytes (2×10^5 cells / well) +0.1 ml mitomycin-C treated cells (4×10^5 cells / well);

c) 0.1 ml responder lymphocytes (2×10^5 cells / well) +0.1 ml mitomycin-C treated stimulator lymphocytes (4×10^5 cells / well)+0.2 µg / ml of *polypodium* extract;

(d) 0.1 ml responder lymphocytes (2×10^5 cells / well) +0.1 ml mitomycin-C treated stimulator lymphocytes (4×10^5 cells / well)+2 µg / ml of *polypodium* extract; and

(e) 0.1 ml responder lymphocytes (2×10^5 cells / well) +0.1 ml mitomycin-C stimulator lymphocytes (4×10^5 cells / well)+4 µg / ml of *polypodium* extract.

All experiments were run in triplicate and 6 hours before termination of culture, plates were pulsed with isotope-labelled thymidine (^{3}H-TdR) and harvested 6 hours later. The proliferation of responder cells, which depends on the degree of histocompatibility difference between cells, was measured by counting the level of (^{3}H-TdR) incorporation.

^{51}Chromium release assay

1×10^6 target U937 cells in 100 µl medium were labeled 100 µci ^{51}Cr in a sterile screw-capped 4 ml falcon tube and incubated for 1 hr at 37ºC in a CO_2 incubator. Target cells were then washed twice by centrifugation at 1200 rpm for 10 minutes and suspended in complete medium at a concentration of 0.1 million cells / ml. 0.01×10^6 radio-labeled target cells in 100 µl were mixed with 0.5×10^6 lymphocytes in 50 µl to give an E:T ratio of 50:1 in triplicates in a U-bottom 96-well tissue culture plate. Total volume was made up with complete medium and adjusted to 250 µl / well. Control wells containing only target cells were also processed simultaneously. For spontaneous cytotoxicity, only set of triplicates was set up in which volume was adjusted to 250 µl with medium alone. In another set, 100 µl of Triton X and 150 µl medium was added and this was considered as maximum lysis. The 96-well plate was centrifuged at slow speed (100 rpm) for 5 minutes to enable contact between effectors and targets. Incubation was carried out for 4 hours at 37ºC in a humidified CO_2 incubator. Plates were then centrifuged and 150 µl supernatant was carefully removed and transferred to a plastic vial. Counts were recorded in a Gamma counter and percentage cytotoxicity was calculated as follows:

$$\%\text{Cytotoxicity} = \frac{\text{counts in test-counts in spontaneous lysis}}{\text{counts in maximum-counts in spontaneous lysis}} \times 100$$

RESULTS

Before to test the effect of distinct *Polypodium* extracts on MLR reactions and CD95 expression, a preliminary study was undertaken to see the effect of IL-2 and / or IL12 stimulation on a long term basis and correlate the phenotypic changes of the proliferating population corresponding to their cytotoxic abilities after in vivo culture of the PBMCs. LAK cells were generated as described and at regular time points the cells were harvested. The chromium release cytotoxic assay was performed by the standard procedure and compared with the corresponding phenotypic characteristics of the cells, by flow cytometry. A representative histogram plot of unstimulated and stimulated cells is shown in Fig. 1. In Table 1 the results of these preliminary tests are summarized. The total cell counts were analyzed after appropriately applying the gate on the corresponding histogram plots. It can be seen that IL-2 at 50U / 10^6 cells is very effective in inducing proliferation of NK ($CD56^+$) cells and simultaneously augmenting cytotoxicity. The percentage of $CD56^+$ cells rises from a basal value of 17±3% of the total lymphocytes on day zero to 54±7% of the total lymphocytes on day 14. Simultaneously percentage cytotoxicity also rises from a basal value of 16±4% on day zero to 80±6%

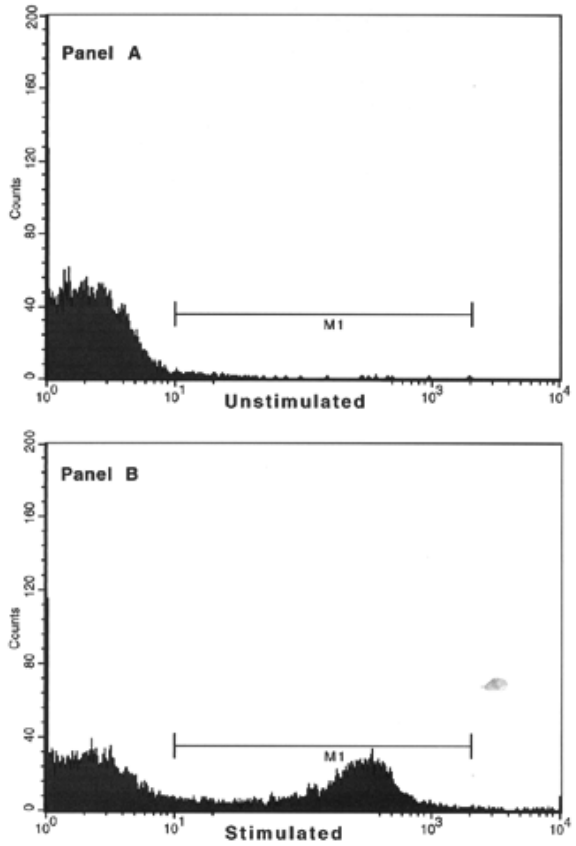

Fig. 1. Representative histograms showing the effect on cell viability. Cells were incubated with RPMI as negative control (panel A) and with 2.0 μg/ml of PLE1 for 72 h at 37ºC in 95% humidity atmosphere with 5% CO_2. Following incubation, cells were treated for 10 minutes with trypan blue (0.4% in normal saline) prior to the flow cytometric analysis. Gated lymphocytes (M1 region) were analyzed based on their fluorescence properties. Viable lymphocytes were identified as non-fluorescent cells, whereas dead lymphocytes presented high fluorescence intensity on FL2 channel (M1 region, panel B).

on day 14 concurring with the increase in $CD56^+$ cells. When IL-2 at a concentration of 50 U / 10^6 cells was added along with IL-12 at a concentration of 125 U / 10^6 cells, there was an increase of NK phenotype up to 45±4% on day 11 and a corresponding maximum NK activity of 95%±8%. Equally impressive augmentation of NK activity was seen even at lower concentrations of IL-2 and IL-12 combination. At IL-2 concentration of 5 U / 10^6 cells along with IL-12 concentration of 50 U / 10^6 cells, there was an increase of NK phenotype up to 37±4% on day 9 and a corresponding maximum NK activity of 61±7%. But when IL-2 alone was added at the same low concentration, percentage of NK cells was only 11±3% after 7 days. However IL-12 by itself at concentrations of 50 U or 125 U / 10^6 cells was unable to cause neither an increase in the NK phenotype nor in their cytotoxic ability. From the data summarized in the table it is evident that initially on day 2 even though there is a transient increase in the CD56 positive T cells up to 25-45% the corresponding increase in the chromium release values against U937 target cells was negligible even at a high dose of IL-2. In contrast, at later time points when the cytotoxicity goes up to 60-95%, there were very few CD56 positive T cells as compared to the NK phenotype.

PLE1, PLE2 and PLE3 were evaluated for their ability to inhibit PBMCs proliferation at concentrations between 0.2 and 4.0 μg / ml. After stimulation with PHA and incubation for 96 h, all three extracts inhibited the PBMCs proliferation as measured by [^{3}H]-thymidine incorporation by the DNA from these cells (Table 2). To analyze whether the Fas-mediated pathway of apoptosis induction was functionally affected by *Polypodium* extracts, MLRs were performed as described. Results of CD95 positive cells is shown in Table 3. The percentage of CD95 expressing lymphocytes increased from 8±2 (control) to a maximum value of 88±9 (p<0.001) when MLR were treated with 4 μg / ml of PLE3 with IL-2 and IL-12, 874 (PLE1 with IL-2 and IL-12) and 81±5 (PLE2 with IL-2 and IL-12). Increase of CD95 expression occurred also after incubation with PLE1 alone (66±6), PLE2 alone (56±5) and PLE3 alone (62±6), though less pronounced. As Fas is upregulated upon cell activation we analyzed the relation between expression of Fas and the expression of activation markers on the various lymphocyte subsets. Percentages of activated B-lymphocytes ($CD38^+$), $CD4^+$ T-lymphocytes ($CD25^+$) and $CD8^+$ T-lymphocytes ($HLA\text{-}DR^+$) are shown in Fig. 2. Percentages of activated B cells were significantly increased in MLRs treated with all *Polypodium* extracts. Besides an increase in the percentage of CD45RO expression, results were comparable between controls and treated groups (Table 4). As we found Fas-induced apoptosis increased in MLRs treated with all extracts, we wondered whether differences in percentages of apoptotic lymphocytes could be related to

Table 1. NK activity (^{51}Cr release) and phenotypic characteristics (CD3 and CD56 expression) after in vitro stimulation of PBMCs with different concentrations of IL-2 and IL-12 during a period of 14 days

5U IL-2	DAY 0	DAY 2	DAY 4	DAY 7	DAY 9	DAY 11	DAY 14
CD3	65±5	59±4	67±4	66±5	0	0	0
CD56	15±4	7±3	8±3	11±3	0	0	0
$CD3^+/CD56^+$	11±6	22±4	9±3	5±2	0	0	0
^{51}Cr release	12±5	0	27±4	33±3	0	0	0
50U IL-2	DAY 0	DAY 2	DAY 4	DAY 7	DAY 9	DAY 11	DAY 14
CD3	64±4	42±3	47±3	61±6	55±4	48±5**	37±4***
CD56	17±3	11±2	15±2	24±6**	31±3**	33±3**	54±7***
$CD3^+/CD56^+$	9±4	40±5***	31±2***	14±4	8±4	8±2	15±3
^{51}Cr release	16±4	19±4	37±3**	59±4***	62±2***	66±5***	80±6***
5U IL-2+50U IL-12	DAY 0	DAY 2	DAY 4	DAY 7	DAY 9	DAY 11	DAY 14
CD3	65±6	58±4	67±5	58±4	49±5	63±5	0
CD56	13±5	2±1**	5±2**	22±4	37±4**	14±2	0
$CD3^+/CD56^+$	9±3	42±5***	15±4	7±3	12±3	14±3	0
^{51}Cr release	22±4	20±2	28±5	47±4**	61±7***	42±4**	0
50U IL-2+125U IL-12	DAY 0	DAY 2	DAY 4	DAY 7	DAY 9	DAY 11	DAY 14
CD3	64±5	38±4**	51±4	57±5	56±4	37±5**	58±3
CD56	14±3	9±3	11±4	23±5	24±5	45±4***	26±3
$CD3^+/CD56^+$	8±2	37±4**	22±3	11±5	14±6	16±4	18±2
^{51}Cr release	15±3	18±3	44±4***	47±7***	63±3***	95±8***	53±5***
50U IL-12	DAY 0	DAY 2	DAY 4	DAY 7	DAY 9	DAY 11	DAY 14
CD3	64±6	38±5**	77±4	75±6	76±9	0	0
CD56	17±3	15±3	1	1	1	0	0
$CD3^+CD56^+$	16±3	42±4	0	0	0	0	0
^{51}Cr release	15±4	1	0	0	0	0	0
125U IL-2	DAY 0	DAY 2	DAY 4	DAY 7	DAY 9	DAY 11	DAY 14
CD3	64±6	36±5***	81±8**	78±6**	80±5**	0	0
CD56	17±2	1	0	0	0	0	0
$CD3^+CD56^+$	15±3	44±6**	41	1	1	0	0
^{51}Cr release	19±4	2	0	0	0	0	0

Results are expressed as Mean±SD (n=3). **p<0.01, ***p<0.001, p values were calculated using the Mann-Whitney U test (2-tailed).

inhibition of proliferation. The results illustrated in Table 5 show that:

(a) pretreatment of MLR with PLE1, PLE2 and PLE3 produced a marked reduction of cell proliferation (cpm values ranged from 2066 to 4956, at 4.0 μg/ml); (b) pretreatment of MLR with extracts plus IL-2 and IL-12 determined a reduction of cell proliferation (cpm values increased from 4388 to 5855, at 4.0 μg/ml); and c) although at a lesser extent, pretreatment of MLR with lower concentrations of PLE extracts (0.2 μg/ml and 2.0 μg/ml) influenced also the proliferation in MLR assays (cpm values ranged from 21543 to 25388 and 7561 to 8758, respectively).

DISCUSSION

Although apoptosis is a physiologic process involved in cellular selection and elimination, its regulation has been associated with several pathologic situations, such as immunologic response. Therefore, the investigation of novel methods to study the mechanisms that regulate apoptosis and cellular proliferation would help in reaching adequate diagnosis and perhaps define more specific therapeutic approaches.

Exploitation of new small molecules from natural sources such as microbial metabolites has contributed to the discovery of useful medicines as

Table 2. Demonstration of inhibitory activity of PLE1, PLE2 and PLE3 on PHA-induced peripheral blood mononuclear cells proliferative response

	%Prolif.	0.2 μg / ml	2.0 μg / ml	4.0 μg / ml
PLE1	-	87±3	94±4	98±7
PLE1+5U / IL-2 & 50U IL-12	-	55±2***	69±5**	84±3
PLE2	-	75±4	96±4	98±5
PLE2+5U / IL-2 & 50U IL-12	-	62±3**	74±6	83±6
PLE3	-	82±4	95±4	97±4
PLE3+5U / IL-2 & 50U IL-12	-	79±4	83±5	85±5
5U / IL-2 & 50U IL-12	98±6	-	-	-
5U / IL-2	89±7	-	-	-
50U IL-12	75±4	-	-	-
PBMC control	100	-	-	-

Numbers indicate the percent of inhibition of the proliferation after 96 h incubation at 37ºC, 95% relative humidity and 5% CO_2 atmosphere. The proliferation was estimated by measuring [^{3}H]-thymidine incorporation into DNA and compared with the control without extracts and with IL-2 alone, with IL-12 alone and with a combination of both. Results are expressed as Mean±SD (n=3). **p<0.01, ***p<0.001, p values were calculated using the Mann-Whitney U test (2-tailed).

well as the basic research in chemistry and biology. In the *in vitro* antiproliferative screening program we routinely use several methods of microculture assays for the evaluation of extracts of natural products, for the identification of their target(s) and possible biological activity.

The first objective of this study was to assess the ability of *Polypodium leucotomos*, *Polypodium cambricum* L., and *Polypodium vulgare* L. extracts to modulate the expression of several lymphocyte antigens. Our results demonstrated that under different stimulation (PHA, cytotoxicity, proliferation and MLR) and culture conditions (*Polypodium* treatment), all the three *Polypodium* extracts, displayed excellent immunomodulatory activities, as observed on the percentage of CD4, CD8 and CD19 positive cells (Fig. 2). Percentages of activated B-lymphocytes ($CD38^+$), $CD4^+$ T-lymphocytes ($CD25^+$) and $CD8^+$ T-lymphocytes ($HLADR^+$) were always increased in the treated groups comparing with the IL-2 and IL-12 groups. In addition when CD95 expression was correlated with CD4, CD8 and CD19 expression, an increased percentage of Fas expressing both B and T lymphocytes was observed upon activation and *Polypodium* treatment (Fig. 2).

Since antigen-dependent immune responses and the synthesis and release of different cytokines often require the activation-driven expansion of reactive T lymphocytes, a methodologic strategy that quantifies lymphocyte subsets that proliferate or undergo apoptosis in MLR models was developed in combination with appropriate amounts of IL-2 and IL-12 cytokines.

The growth-promoting property of IL-2 is the best characterized and perhaps most important function of this cytokine, while activities of IL-12 include induction of IFN-γ production by resting and activated T and NK cells, enhancement of T cell proliferation in combination with costimulation, some enhancement of NK cell proliferation, and enhancement of NK and T cell cytolytic activity (Das and Khar, 2002). For induction of CD95, IL-12 synergizes strongly with IL-2, an effect largely due to stabilization of the induced mRNA (Wigginton *et al.*, 2001).

In the second stage, and among the different experimental conditions used for apoptotic measurements, we chose the expression of CD95 because it offers several advantages: (a) it labels nonfixed living lymphocytes that can be subsequently sorted and used to study their function, (b) it allows a more complete immunophenotyping when it is combined with appropriate mAbs, and (c) it detects apoptotic bodies in the flow cytometry analysis. A significant relation between membrane Fas expression and the state of activation for the T lymphocyte subpopulations analysed was demonstrated with the *Polypodium* extracts in combination with IL-2 and IL-12. As shown in the analysis of CD95 expression in Table 3, a kinetic

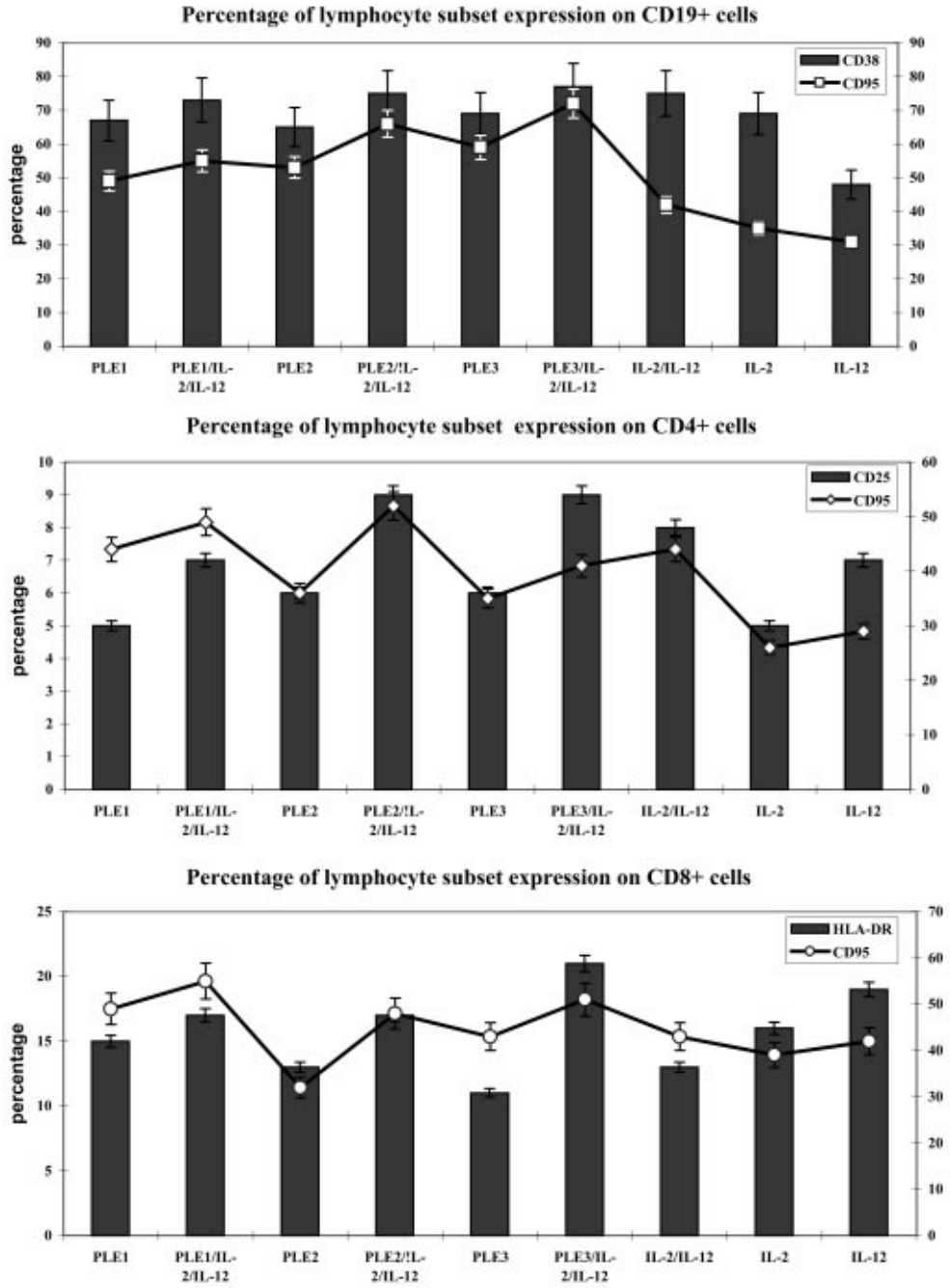

Fig. 2. Percentages of lymphocyte subsets and expression of activation markers and Fas (CD95) within these subsets in MLRs treated with Polypodium extracts. Percentages are expressed as percentages of total lymphocytes. Activation markers and CD95 expression are expressed as percentage of positive cells within the $CD19^+$, $CD4^+$, and $CD8^+$ subpopulations.

pattern of apoptosis was detected in accordance with the increasing amount of extracts used.

The generation of the recognition repertoires of T and B lymphocytes is dependent on the apoptotic deletion of cells with inappropriate specificities. Activation of T lymphocytes after encounters with cognate antigen / major MHC results in activation and concomitant upregulation of Fas ligand (Akashi and Kuwano, 2002; Ford *et al.*, 2002; Pinkoski *et al.*, 2002). Subsequent interactions between Fas ligand and Fas induces apoptotic death of the activated T cells, thereby downregulating the immune response.

Table 3. Effect of *Polypodium* extracts on CD95 expression induced in PHA-mediated MLR. Results are depicted as the Mean of three samples±SD from two experiments expressed as percentage of CD95 positive cells

	% of CD95	% of CD95 with 0.2 μg / ml	% of CD95 with 2.0 μg / ml	% of CD95 with 4.0 μg / ml
PLE1	-	34±4**	55±4***	66±6***
PLE1+5U / IL-2 & 50U IL-12	-	42±5***	77±8***	87±4***
PLE2	-	25±4**	55±5***	56±5***
PLE2+5U / IL-2 & 50U IL-12	-	38±3**	65±7***	81±5***
PLE3	-	27±5	59±6***	62±6***
PLE3+5U / IL-2 & 50U IL-12	-	26±4	71±6***	88±9***
5U / IL-2 & 50U IL-12	32±5	-	-	-
5U / IL-2	25±4	-	-	-
50U IL-12	23±4	-	-	-
PBMC control	8±2	-	-	-

**p<0.01, ***p<0.001, p values were calculated using the Mann-Whitney U test (2-tailed).

Table 4. Effect of *Polypodium* extracts on PHA-mediated MLR. Results are depicted as the Mean of three samples ±SD from two experiments expressed as percentage of CD45RO expression

	% of CD45RO	% of CD45RO with 0.2 μg / ml	% of CD45RO with 2.0 μg / ml	% of CD$5RO with 4.0 μg / ml
PLE1	-	44±4	43±8	37±7
PLE1+5U / IL-2 & 50U IL-12	-	36±5	38±5	35±6
PLE2	-	35±4	37±5	34±7
PLE2+5U / IL-2 & 50U IL-12	-	42±3	41±4	38±3
PLE3	-	35±6	38±6	44±4
PLE3+5U / IL-2 & 50U IL-12	-	45±5	40±5	41±7
5U / IL-2 & 50U IL-12	44*±4	-	-	-
5U / IL-2	42*±5	-	-	-
50U IL-12	39*±6	-	-	-

*Control untreated cultures

Table 5. Effect of PLE1, PLE2 and PLE3 extracts on proliferative response MLR assays

	% prolif.	% inhibition 0.2 μg / ml	% inhibition 2.0 μg / ml	% inhibition 4.0 μg / ml
PLE1	-	21543±554	7561±328	2066±553
PLE1+5U / IL-2 & 50U IL-12	-	25566±669	8444±590	4388±319
PLE2	-	24659±775	19985±658	2856±287
PLE2+5U / IL-2 & 50U IL-12	-	26556±694	21365±846	4467±178
PLE3	-	25388±668	8758±439	4956±447
PLE3+5U / IL-2 & 50U IL-12	-	27456±882	10446±387	5855±541
5U / IL-2 & 50U IL-12	30651±886	-	-	-
5U / IL-2	26544±985	-	-	-
50U IL-12	12565±584	-	-	-
PBMC control	35720±819	-	-	-

Data are expressed as average counts per minute (cpm). The proliferation was estimated by measuring [^{3}H]-thymidine incorporation into DNA and compared with the control without extracts and with IL-2 alone, with IL-12 alone and with a combination of both. Results are expressed as Mean±SD (n=3).

The third important contribution of this work was the use of MLR assay to obtain an accurate estimation of the lymphocyte proliferation / inhibition. *Polypodium leucotomos* (PL1), *Polypodium cambricum* L. ssp. (PL2) and *Polypodium vulgare* L. (PL3) extracts at a concentration ranging between 0.2 μg / ml and

4.0 μg/ml, showed a remarkably potent ability to induce the CD95 expression. As shown in Table 5, the inhibition of [^{3}H]-thymidine incorporation was highly significant with both PLE1 (cpm value: 2066) and PLE2 (cpm value: 2856) at a concentration of 4.0 μg/ml.

In summary, this report describes a useful method allowing the study of the kinetics of proliferation and apoptosis in allogenic cultures with the advantage of simultaneous determination of the absolute number of apoptotic and proliferative cells tested with plant extracts.

CONCLUSIONS

The remarkable technical advancement in flow-cytometric analysis and cell sorting during the past decade have provided tools that have substantially enhanced our understanding of the human immune system. The use of flow cytometry as a tool in correlating the functional capacity of various subpopulations of mixed lymphocyte cultures to their phenotype has been demonstrated. The active extracts from *Polypodium leucotomos*, *Polypodium cambricum* L. ssp. and *Polypodium vulgare* L., by showing dose-dependent pro-apoptotic activity in the range between 0.2 and 4 μg/ml, appear to be a particularly potent and effective CD95 expression inducer/promoter. Further studies intended to confirm the activity in other experimental models, the mechanisms of pro-apoptotic action, as well as isolation of the active principles responsible for such activity, are currently being conducted.

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