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Genistein, a Selective Protein Tyrosine Kinase Inhibitor, Inhibits Interleukin-2 and Leukotriene B_4 Production from Human Mononuclear Cells[1,2]

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In this study, genistein, a selective protein tyrosine kinase (PTK) inhibitor, inhibited peripheral blood mononuclear cell (PBMC) proliferation and interleukin-2 production from cultures that were stimulated with phytohemagglutinin (PHA), phorbol 12-myristate 13-acetate (PMA) plus A23187, or PHA plus PMA, and genistein effectively blocked the PHA plus IL-2-induced PBMC proliferation. Further, we also found that genistein inhibited LTB_4 production from A23187-stimulated cultures whereas H-7, a PKC inhibitor, had no effect on LTB_4 production. Our results suggest that PTK may be necessary for the synthesis of LTB_4.

INTRODUCTION

The leutrokrienes are formed by the oxygenation of arachidonic acid (AA)[3] at the C-5 position (1). This process results in the production of an unstable epoxide, leukotriene A_4, which can be converted enzymatically by hydrolysis to LTB_4 or to LTC_4 with the addition of glutathione (1). Reports from several laboratories show that LTB_4 exerts significant and diversified effects on mononuclear cells. LTB_4 was shown to induce suppressor cell activity (2–4) or to enhance lymphocyte proliferation (4–6). Further, roles for lipoxygenase metabolites of AA in IL-1 and IL-2 production were described (7–9).

Protein tyrosine kinases (PTK) are among the molecules that have been implicated in the control of cell growth and differentiation. High levels of PTK activity have been described in normal and transformed T cells and monocytes of humans (10–13). T cells stimulated by mitogenic lectins have been shown to cause increased phosphorylation of membrane (12) and cytosolic (14) proteins on tyrosine residues, suggesting a role for PTK activity in T-cell activation or proliferation.

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[2] Part of this data was presented at the annual ASBMB/AAI Joint Meeting, June 3–7, 1990, New Orleans, LA.

[3] Abbreviations used: AA, arachidonic acid; LT, leukotriene; PTK, protein tyrosine kinase; PKC, protein kinase C; IL-1, interleukin-1; IL-2, interleukin-2; PBMC, peripheral blood mononuclear cells; PHA, phytohemagglutinin A; PMA, phorbol 12-myristate 13-acetate; PBS, phosphate-buffered saline (pH 7.4); A23187, calcium ionophore; CsA, cyclosporin A.

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We and others have shown that genistein, an isoflavonoid compound, specifically inhibits PTK (15–17).

Protein kinase C (PKC) is a ubiquitous enzyme involved in the signal transduction of cell proliferation (18). Addition of PHA to human T cells resulted in a rapid, transient association of PKC with the particulate fraction (19). Using H-7, a specific PKC inhibitor, several laboratories have shown the role of PKC in the proliferation of T cells or T cell lines, IL-2 production, and IL–2R expression (20–23). Further, it was shown that H-7 potentiated the release of arachidonic acid from human neutrophils (24).

In the present study we designed experiments to compare the effects of genistein and H-7 on A23187-stimulated LTB_4 production and the effects of genistein on PBMC proliferation and IL-2 production. We found that genistein inhibited LTB_4 production, but that H-7 had no measurable effect on LTB_4 production. Genistein inhibited [^{3}H]thymidine incorporation of PBMC in a dose-dependent manner. Addition of exogenous IL-2 had no effect in reversing the inhibition caused by genistein. Our results suggest that PTK but not PKC may play an important role in regulating LTB_4 production.

MATERIALS AND METHODS

Peripheral blood mononuclear cell isolation. Peripheral venous blood was obtained from each subject into syringes containing preservative-free heparin. Peripheral blood mononuclear cells were isolated by Ficoll–Hypaque centrifugation (22) was washed three times with phosphate-buffered saline (PBS). The PBMC were then used in the assays as described below.

Cell cultures. PBMC at 10^5 cells in 200 μl of RPMI 1640 medium supplemented with 10% fetal calf serum, L-glutamine, and penicillin–streptomycin were cultured in microtiter plates as described (22). Briefly, PHA, PMA, calcium ionophore (A23187), or genistein was added directly to the wells upon initiation of the cultures. In some experiments, genistein was added at various times after the initiation of cell cultures. Tritiated thymidine was added to the cell cultures 66 hr after their initiation, and the cells were harvested at 72 hr onto glass wool filter paper and counted in a liquid scintillation counter. All cultures were performed in triplicate. The percentage inhibition caused by genistein was calculated and results are expressed as

$$\text{percentage inhibition} = \frac{1 - A - C}{B - C} \times 100,$$

in which A is the cpm in cultures with genistein, B is the cpm in cultures without genistein, and C is the cpm in nonstimulated cultures.

PBMC at 1×10^6 cells in 1.0 ml medium were cultured in 12 × 75-mm culture tubes for measuring interleukin-2 (IL-2) production. PHA, PMA, A23187, or genistein was added at the initiation of cell cultures. IL-2 activity was measured from 24-hr culture supernatants by using CTLL-2 cells as described previously (22).

Radioimmunoassay for LTB_4. PBMC at 1×10^6/ml were incubated with A23817

and/or genistein or H-7 for 30 min at 37°C, and supernatants were assayed for LTB_4 directly or after extraction using radioimmunoassay as described previously (25). The results were calculated using PC software (Quickcal Q, Arbor Immunalysis, Ann Arbor, MI). The RIA employed (Amersham, Arlington Heights, IL) was sensitive to 1.6 pg/ml and had the following cross-reactivities: LTC_4 and LTD_4 <0.05%; 5-, 12-, and 15-HETE <0.05%; and 20-OH-LTB_4 0.4%.

Culture additives. PHA was purchased from Burroughs-Wellcome (Research Triangle Park, NC). A23187 was purchased from Calbiochem (LaJolla, CA). PMA was purchased from Sigma Chemical Co. (St. Louis, MO). Both A23187 and PMA was dissolved in DMSO. Genistein was obtained from Bio-Mol (Philadelphia, PA) and dissolved in DMSO at 10 mg/ml and stored at −20°C. PKC inhibitor H-7 was purchased from Seikagaku America Inc. (St. Petersburg, FL) and dissolved in distilled water to yield a 10 m*M* stock solution. All culture additives were appropriately diluted in RPMI 1640 medium immediately before use.

RESULTS

Figure 1 presents the results of experiments with three subjects, measuring the dose–response of genistein on [^{3}H]thymidine incorporation in PHA-stimulated PBMC proliferation. Genistein inhibited PBMC proliferation in a dose–response manner. Further, we found that genistein inhibited PMA plus A23187—stimulated PBMC proliferation (Fig. 2). In this study, PMA or A23187 by itself did not cause any proliferation of PBMC (data not shown).

Next, we asked whether genistein was inhibiting PBMC proliferation via inhibition of IL-2 production from these cultures. Genistein inhibited IL-2 production from PHA plus PMA or PMA plus A23187-stimulated cultures (Table 1). Further, we were interested to see whether IL-2 could reverse the genistein effect on PHA-stimulated PBMC proliferation. The presence of exogenous IL-2 (25 U/ml) caused an increased proliferation of PBMC and had no effect in reversing the inhibitory effect of genistein (Table 2).

Next, we performed experiments to measure the kinetic effect of genistein on

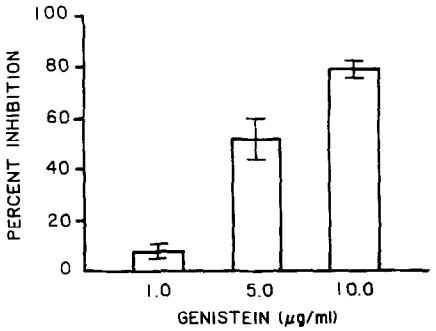

FIG. 1. Effect of genistein on PHA-stimulated PBMC proliferation. PBMC were cultured with PHA and/or genistein (various concentrations) for 3 days to measure [^{3}H]thymidine incorporation as described under Materials and Methods. Culture additives are used as follows: PHA, 2.0 μg/ml; genistein, 1.0, 5.0, and 10.0 μg/ml. Data represent mean ± SEM of three experiments using different donors.

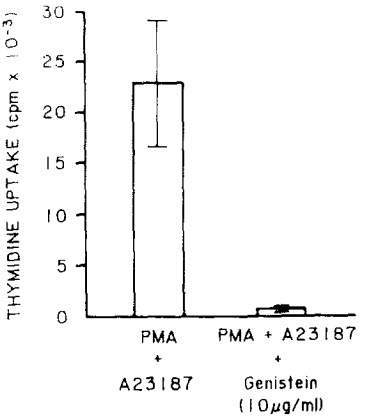

FIG. 2. Inhibition of PMA plus A23187-stimulated PBMC proliferation. PBMC were cultured for 3 days to measure [^{3}H]thymidine incorporation. Culture additives are used at the following concentrations: PMA, 10.0 ng/ml; A23187, 0.5 μM/ml. Data represent mean ± SD of two experiments using different donors.

[^{3}H]thymidine incorporation, when 10.0 μg/ml of genistein was added to the PHA-stimulated PBMC at various times after culture initiation. Genistein inhibited the late stage activation of PBMC proliferation (Fig. 3).

Because LTB_4 has been described as playing an important role in IL-2 production (9), we investigated whether genistein at the same concentration that inhibited IL-2 synthesis had any effect on LTB_4 production from PBMC cultures. Data presented in Table 3 show the levels of LTB_4 from the supernatants of PBMC cultured with A23187 and/or genistein was measured by RIA. A23187 caused an increase in LTB_4 production, and genistein inhibited >50% of LTB_4 production. Recently, we have shown that H-7 (10 μM) inhibited IL-2 production from PHA plus PMA-stimulated human T cells (22). However, in the present study, H-7 at the same concentration showed no effect on A23187-stimulated LTB_4 production (Table 3).

Because of these inhibitory effects by genistein in cell cultures, we measured PBMC viabilities. Genistein at concentrations that inhibited PBMC proliferation,

TABLE 1
EFFECT OF GENISTEIN ON IL-2 PRODUCTION FROM PBMC CULTURES

Culture conditions	IL-2 production (U/ml)[a] Expt 1	Expt 2
PBMC	<0.1	0.1
PBMC + PHA + PMA	15.0	13.2
PBMC + PHA + PMA + genistein	<0.1	<0.1
PBMC + PMA + A23187	39.0	34.0
PBMC + PMA + A23187 + genistein	7.0	3.5

[a] Results are expressed as units of interleukin-2 produced from 24-hr culture supernatants from two different experiments using different donors. Similar results were obtained using three other experiments using different donors. Culture additives are used at the following concentrations: PHA, 10.0 μg/ml; PMA, 10.0 ng/ml; A23187, 0.5 μM/ml; genistein, 10.0 μg/ml.

TABLE 2
EFFECT OF GENISTEIN ON PHA-STIMULATED [^{3}H]THYMIDINE INCORPORATION IN THE PRESENCE OR ABSENCE OF INTERLEUKIN-2

PHA	GTN[a]	IL-2[b]	Counts per minute[c] Expt 1	Expt 2	Expt 3
—	—	—	1,281	1,712	1,828
+	–	–	20,696	16,342	22,876
+	+	–	4,383	2,862	2,789
+	–	+	47,807	29,572	30,473
+	+	+	9,093	5,198	8,678

[a] Genistein was added at 10.0 μg/ml.
[b] Purified human IL-2 was added at 25 U/ml.
[c] Results are expressed as counts per minute from triplicate determinations of each experiment using different donors. Similar results were obtained using two other experiments using different donors.

IL-2 synthesis, and LTB4 production from stimulated cultures had no effect on PBMC viabilities, as measured by the trypan blue dye exclusion test (Table 4).

DISCUSSION

The results of the present investigation can be summarized as follows. Genistein, a selective PTK inhibitor, inhibited PHA or PMA plus A23187-stimulated PBMC proliferation; at the same concentration, it also inhibited IL-2 synthesis from PHA plus PMA or PMA plus A23187-stimulated lymphocytes; further, it inhibited A23187-stimulated LTB_4 production.

T-cell proliferation is an autocrine process characterized by the sequential expression of IL-2R, synthesis and secretion of IL-2, and finally interaction of the growth factor with its receptor (26). T-cell receptor (TCR) activation by antigen, lectins, or anti-TCR mAb has been shown to cause rapid stimulation of PTK

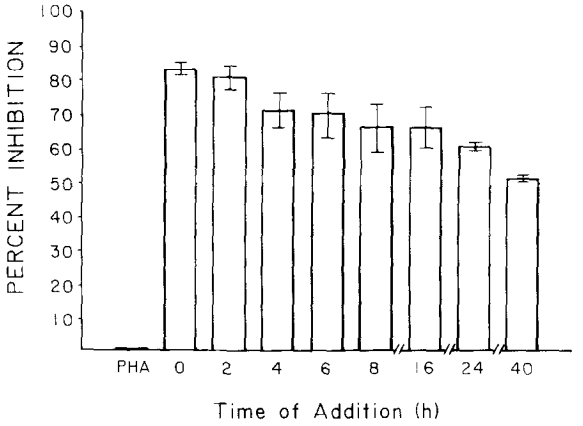

FIG. 3. Kinetic study of the inhibitory effect of genistein (10.0 μg/ml) on PHA-stimulated human PBMC proliferation. PBMC were cultured for 3 days to measure [^{3}H]thymidine incorporation as described under Materials and Methods. Genistein (10.0 μg/ml) was added at various times after initiation of cultures. Data represent mean ± SD of two experiments using different donors.

TABLE 3
EFFECTS OF GENISTEIN OR H-7 ON LTB_4 PRODUCTION

Culture conditions	LTB_4 production (pg/100 μl) Expt 1	Expt 2	Expt 3	Expt 4
PBMC	1.6	2.0	2.0	—
PBMC + A23187	136	180	146	122
PBMC + A23187 + GTN	50	22	7.4	50.0
PBMC + A23187 + H-7	N.D.[a]	190	180	N.D.

Note. Results are expressed as units of LTB_4 produced in culture supernatants, from four different donors. Similar results were obtained using six other experiments using different donors. Culture additives are used as follows: A23187, 1.0 μ*M*/ml; GTN 10.0 μg/ml; H-7, 10.0 μ*M*/ml.

[a] Not done.

activity, as demonstrated by observing TCR-ζ chain tyrosine phosphorylation (27–30). Considerable interest has developed regarding TCR activation of tyrosine kinase activity, because there is substantial evidence to implicate tyrosine–kinase in the control of mitogenesis, cell cycle growth, and development (31, 32). Recently, using genistein, we have shown the inhibition of PTK- (15), PHA-, and anti-CD_3 mAb-induced TCR-ζ phosphorylation (33). Similar results were reported by Mustelin *et al.* (17). Further, they found that genistein also inhibited phorbol ester plus A23187-stimulated T-cell proliferation (cited as unpublished observation in Ref. (17)). Our data are consistent and confirm their findings (Fig. 2). One could argue that genistein may be nonspecifically inhibiting PKC, thus causing the immunosuppression reported herein. We found that genistein at this concentration (10.0 μg/ml) had no effect on CD_{69} (33), the early activating antigen induced by PMA (34). Similarly, cyclosporin A was shown not to have any effect on PMA-induced CD_{69} (35), but CsA is still a very important immunosuppressive agent (36).

The role of lipoxygenase products of AA in IL-1 and IL-2 production have been described (7–9). Using lipoxygenase inhibitors, Dinarello *et al.* (7) reported a decrease in IL-1 production. Another laboratory reported an increase in IL-1 and IL-2 synthesis from human monocyte and T-cell cultures in the presence of exogenous LTB_4 and LTD_4 (8, 9). Moreover, AA has already been proposed as another potential second messenger in T lymphocytes (37). The data presented in this study show that genistein at the concentration that inhibited LTB4 synthesis also inhibited IL-2 production (Tables 1 and 3). Earlier, we showed that genistein

TABLE 4
EFFECT OF GENISTEIN ON PBMC VIABILITIES

Culture additives	% Viable cells[a] Expt 1	Expt 2	Expt 3
PHA	79	80	75
PHA + GTN (10.0 μg/ml)	78	80	73

[a] Cell viabilities were determined using the trypan blue dye exclusion test from 48-hr cultures of three experiments using different donors.

at this concentration (10.0 μg/ml) inhibited the mRNA for IL-2 from PHA plus PMA or PMA plus OKT_3-stimulated human T-cell cultures (33). Although the role of PKC in T-cell proliferation is well documented, its role in the AA metabolite(s) pathway is not clear. Activation of PKC apparently is not sufficient to induce LTB_4, but its action appears to be required to couple a rise in intracellular Ca^{2+} to induce LTB_4 (38). However, H-7, a PKC inhibitor, was shown to enhance the A23187-stimulated release of AA from human neutrophils (24). Gouvello *et al.* (39) showed that PMA neither stimulated nor modulated CD_2-induced AA release, and they suggested that CD_2 triggering stimulates phospholipase A_2 activity in T lymphocytes via an extracellular Ca^{2+}-dependent and PLC- and PKC-independent mechanism (39). The data presented in Table 3 show that PKC has no role in regulating LTB_4 synthesis, although H-7 inhibited PHA-induced IL-2 production and cell proliferation (data not presented). Preliminary data obtained from another study show that endogenous LTB_4 appears necessary in regulating IL-2 production at the message level (D. Atluru, unpublished data).

The kinetic study of the addition of genistein to the PHA-stimulated cell cultures showed that genistein, when added at any time, still inhibited lymphocyte proliferation (Fig. 3). Similar results were reported by Mustelin et al. (17), where they found that genistein inhibited the early and late stage activation of human T-cell proliferation. Further, we found that genistein inhibited the purified human T-cell proliferation of both early and late stage activation (data not presented). These inhibitory effects caused by genistein were not due to its toxic effects on PBMC (Table 4). Genistein was shown to inhibit the mammalian DNA topiosomerase II (40), and we recently showed that genistein is able to block anti-CD_{28} monoclonal antibody-stimulated human T-cell proliferation (41), which is resistant to CsA or FK-506 (41–43). Preliminary data obtained from our laboratory show that CsA has no effect on LTB_4 synthesis (S. Atluru, unpublished data).

In summary, our data suggest that PTK may be necessary to regulate LTB_4 production. These data also show that the PTK inhibitor, genistein, may be used as an immunosuppressive or anti-inflammatory agent.

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