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ANTI HLA CLASS I MONOCLONAL ANTIBODY EFFECT ON PKC KINETICS IN PHA ACTIVATED HUMAN PERIPHERAL BLOOD MONONUCLEAR AND E' CELLS

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SUMMARY: Cytoplasmic protein kinase C (PKC) has been studied in phytohemagglutinin (PHA) activated peripheral blood mononuclear cells (PBMC) and macrophage depleted E' cell culture. Within 10' after contemporanous addition of PHA and anti HLA class I monoclonal antibody 01.65 (MoAb) PKC is depleted in both cell types. Enzyme activity recovers in the following hours however at 72 hours is at control values in E' cultures while in PBMC cultures it is still depleted at 68% of the control. Anti HLA class I MoAb induced tritiated tymidine (^{3}H-TdR) incorporation inhibition appears to be related to low levels of PKC activity.

Anti HLA class I antigens monoclonal antibodies (MoAbs) inhibit the proliferation of T lymphocytes triggered by ligands active on Ca^{2+} dependent pathways such as antigens, lectins, agonistic MoAbs anti T3-T1 complex and ionofores. They are inffective, howewer, on T cells stimulated by phorbol esthers that activate protein kinase C (PKC) in a Ca^{2+} independent pathway. It has been assumed that HLA class I antigens regulate an early event in the Ca^{2+} dependent pathway of T cell activation (1-5).

ABBREVIATIONS: PKC, Protein Kinase C; PBMC, Peripheral blood mononuclear cells; E', Sheep red cells rosettes purified T cells; PHA, Phytohemagglutinin; MoAb, Monoclonal antibody; SRBC, Sheep red blood cell.

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According to some reports (3-4) and in our own experience no inhibition is observed in macrophages depleted PHA activated T cell cultures (6). We have provided evidence however that anti HLA class I MoAbs do not affect in activated peripheral blood mononuclear cells (PBMC) early events such as $(Ca^{2+})_i$ rise, and inositol phosphate level, considered essential for PKC activation (6). On the other hand we could show that anti HLA class I MoAb down regulates native protein kinase C (PKC) of PHA stimulated T cell cultures. Within 10' from the addition of PHA and anti HLA class I MoAb to E' cells, PKC is depleted to 80% of the normal value, and in the following 24 hours is 58% of control (7). Furthermore we could show that when HLA Class I MoAb is added to a PHA activated PBMC culture the cell cycle progression is delaied and the number of cell divisions is reduced. As a consequence the number of dividing cells in the MoAb treated cultures is lower and the ammount of ^{3}H-TdR incorporated comparatively decreased (8).

We present in this report cytoplasmatic PKC kinetics during the 72 hours of culture in MoAb treated PHA activated PBMC and E' cells. The data furthermore suggest a direct relationship between cytoplasmatic PKC values and inhibition percent of ^{3}H-TdR incorporation by anti HLA class I MoAb.

MATERIALS AND METHODS

DEAE-cellulose (DE-52) was obtained from Whatman. Phosphatidylserine (bovine brain), Dioleoylglycerol, Histone IIIS, Leupeptin, Phenylmethylsulphonyl fluoride and ATP were purchased from Sigma. (γ-^{32}P) ATP (3000 Ci/mmol) was from Amersham, PHA was from Burroughs Wellcome. MoAb 01.65(15) anti HLA Class I antigens (HLA-A, B and -C) and Mo1 anti monocytes were kindly provided by Dr. F. Malavasi (9).
All other chemicals were reagent grade.

T cell preparation

Pheripheral blood lymphocytes were isolated from buffy coats of normal adults by centrifugation over Fycoll/Hypaque density gradient at 1000xg for 20' at 20° C. Cells were washed twice with RPMI 1640 medium and suspended in RPMI 1640 containing 5% fetal calf serum. Then, cells were incubated in Petri dishes at 37° C for 1 hour in 5% CO_2, for depletion of monocytes/macrophages. The non-adherent cells were colleted in RPMI 1640 at the concentration of $6x10^6$ cells/ml. E rosettes were formed with sheep red blood

cells (SRBC) treated with Neuraminidase (4 ml SRBC treated/100x10^6 cells) and incubated at 37° C for 15'. The mixture was centrifuged at 1000xg at room temperature and incubated on ice for 1 hour. The pellets were resuspended gently and centrifuged over Fycoll at 1000xg for 20'. The B cells were recovered from the interface, and the E^+ cells were obtained from the pellet by lysis of the SRBC with 5 mM Ammonium Cloride followed by centrifugation at 700xg for 5'. E^+ cells were resuspended in RPMI 1640 added with antibiotics. E^+ cell preparation contained less then 3% of Mo1 positive cells.

Cell culture incubation
Cells were suspended at 1x10^6cells/ml concentration in RPMI medium containing 5% fetal calf serum and antibiotics and incubated at 37° C with PHA (2 ug/ml) and/or MoAb 01.65 (0.1 mg/ml) at different times. Following incubation, cells were washed and lysed by sonication as described below.

Isolation of Soluble and Particulate Fraction
Lysates were obtained by sonicating the cell suspension (15x10^6 T cells/ml in 0.25 M sucrose, 10 mM Hepes pH 7.5, 5 mM EDTA, 10 mM 2-mercaptoethanol, 2 mM phenylmethyl- sulphonylfluoride and 0,01% leupeptin) with six 10-sec. bursts from a MSE model MK2 sonicator, followed by a centrifugation at 100,000xg for 20'. The supernatant was collected as cytosolic fraction and the pellet was suspended in the same buffer containing 0.1% Triton X-100 as particulate fraction.

Partial purification of PKC from T cells
PKC was partially purified from the cytosolic fraction by a chromatography on a DEAE-cellulose column equilibrated with 10 mM Hepes pH 7.5, 10 mM 2-mercaptoethanol and 5 mM EDTA.
PKC was eluted with a 70 ml linear 0-0.4 M NaCl gradient.

PKC activity
PKC was assayed according to Melloni et al. (10). In the incubation mixture for certains experiments Ca^{2+} and phospholipids were replaced by 5 mM EDTA. PKC activity is expressed as nmol ^{32}P incorporation into Histone under experimental conditions.

Protein determination
According to Lowry (11) using bovine serum albumin as standard protein.

RESULTS

PHA activated PBMC (Fig. 1)

Native cytosolic PKC in the initial 10' after lectin addition is depleted to 47,2 % of total control activity. However the recovery is almost complete at 24 hours and enzymatic activity is completely restored at 72 hours.

In PHA activated PBMC cultured in presence of MoAb 01.65 the drop of cytosolic activity within the initial 10' is observed to 36% of control. The native cytosolic PKC recovers during the following

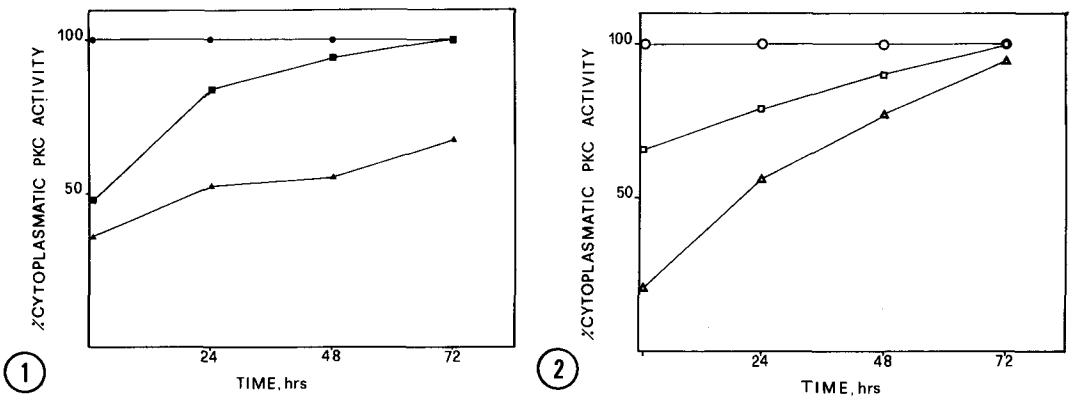

FIG. 1 : TIME COURSE OF THE EFFECT OF PHA AND PHA+MoAb 01.65 TREATMENT ON CYTOSOLIC PKC IN PBMC CULTURE.

Cells were incubated at 1X10^6/ml concentration with PHA (2 ug/ml) or PHA plus MoAb 01.65 (0.1 mg/ml). Cytosolic PKC activity was determined at different times in PBMC only (●) as control and in PHA (■) or in PHA+MoAb 01.65 (▲).

FIG. 2 : EFFECTS OF PHA AND PHA+MoAb 01.65 ON CYTOSOLIC PKC ACTIVITY IN E$^+$ CELLS.

E$^+$ cells were incubated with PHA in presence or absence of MoAb 01.65 at the same conditions described in the legend of Fig. 1. E$^+$ only (○), PHA (□), PHA+MoAb (△).

hours to about 50% of activity at 24 and 48 hours and 68% of activity at 72 hours.

PHA activated E+ cells (Fig. 2)

Native cytosolic PKC after 10' from the addition of PHA drops to 65% of control and is completely recovered at 72 hours.

When E$^+$ cells are cultured in presence of MoAb 01.65 the activity drops at 20% of control in the initial 10 minutes progressively recovers to 56% at 24 hours and 94% at 72 hours.

Fig. 3 shows PKC kinetics in PHA activated MoAb 01.65 treated PBMC and E$^+$ cell cultures.

In PBMC culture PKC depletion at 10' after the addition of lectin and MoAb is lower than in E$^+$ cells. However the recovery is much faster in E$^+$ cell cultures where in 48 hours PKC value increases from 20% to 80% of control. PKC recovery in PBMC culture is much slower in the first 24 hours and essentially null between 24 and 48

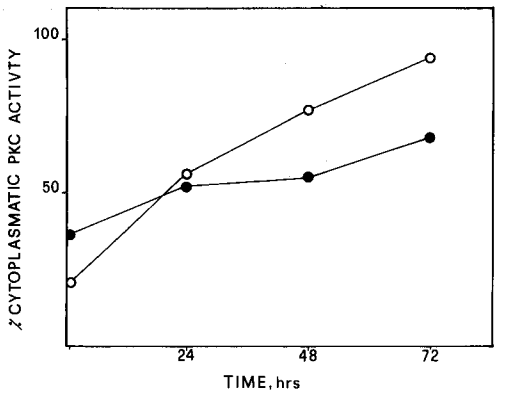

FIG. 3 : TIME KINETIC COMPARISON OF CYTOSOLIC PKC ACTIVITY IN PHA+MoAb 01.65 TREATED CULTURE OF PBMC (●) OR E' (○) CELLS.

hours. Between 48 and 72 hours the rate af increase of cytosolic PKC is apparently similar in the two types of cultures.

DISCUSSION

PKC kinetics in anti HLA class I MoAb treated PBMC and E' cell preparations is different.

Within ten minutes from the addition of PHA to either PBMC or E' cell preparations of cytosolic PKC is depleted. In both systems the depletion is reversible and within 48 hours is back to normal values.

In presence of MoAb 01.65 PBMC remains depleted at about 50% of controls enzimatic activity, while is back to normal value within 24/48 hours in the case of E' cells.

Down regulation of PKC is induced in different cell lines by a variety of external stimula (12-22). The reason of the PKC depletion remains to be established although it can be attributed to the removal of the inhibiting effect of PKC on an unidentified cell function of a short-life activated PKC form by a proteolytic mechanism (10,23,24).

MoAb activates on lymphocytes a proteolytic system wich selectively recognizes and digestes PKC. This process is completed and in few

TABLE I

		HOURS FROM LECTIN PLUS MoAb ADDITION		
		24	30	72
PBMC	PKC depletion%	38		32
	3H-TdR inhibition%		52-57*	30-85***
E+	PKC depletion%	31		6
	3H-TdR inhibition%		53-89*	0-13**

Comparison of PKC depletion percent and 3H-TdR incorporation inhibition percent. PKC depletion is expressed as the ratio

$$\frac{\text{PKC (PHA+MoAb cultures)}}{\text{PKC (PHA cultures)}}$$

*: 3 donors
**: 5 donors
***: 24 donors

minutes ends, then PKC activity reappears in the cells. The macrophages effect may be explaned as a further and prolonged activation of this proteolytic system.

PKC plays a role in PHA stimulated T cell proliferaton (25,26) and it has been suggested that Ca^{2+}/phospholipid dependent PKC is involved in the G1 transit of epithelial cell lines during the transition G0/G1 and G1/S (27).

Our work has shown that anti HLA class I MoAb affects the cell cycle of PHA stimulated PBMC slowing down the cell cycle and induces a significant increase of cell with a G1 DNA content (2C) incorporating 5-Bromo2-deoxyuridine (8).

A possible correlation between PKC activity and prolipherative response of PHA activated T cells is suggested by the comparison of per cent enzymatic depletion and inhibition of ^{3}H-TdR incorporation (Tab. 1). Data have been obtained in independent experiments on

different donors. There is however a clear indication that at the first step in wich in vitro lymphocyte prolipheration triggered by PHA stimulation can be monitored: i.e. 30 hours PKC activity is depleted in both PBMC and E' cell cultures anti HLA class I MoAb inhibits ^{3}H-TdR incorporation. Vice versa at 72 hours of culture when PKC value is normal in E' cells preparation no ^{3}H-TdR incorporation inhibition is detectable. In PHA activated PBMC preparation, containing macrophages, at 72 hours PKC is still depleted to 35% of control and ^{3}H-TdR incorporation inhibition is observed varying in different cases from 35 to 80% (24 experiments).

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