Clin. exp. Immunol. (1987) **70**, 456–462

Impaired phorbol ester and calcium ionophore induced proliferation of T cells from old humans

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(Accepted for publication 10 June 1987)

SUMMARY

Current models of T cell activation implicate increases in intracellular free Ca^{2+} concentration and activation of the Ca^{2+} and phospholipid dependent enzyme protein kinase C (PKC) as important early events leading to interleukin 2 (IL-2) production, interleukin 2 receptor (IL-2R) expression, and subsequent cell proliferation. The present study examined the age-related defect in T cell proliferation to determine if the signals that activate PKC and increase intracytosolic free Ca^{2+} concentration might be defective. Using phorbol myristate acetate (PMA), which directly activates PKC, and Ca^{2+} ionophore A23187, which increases intracellular cytoplasmic free Ca^{2+} concentration, the induction of IL-2 secretion, IL-2R expression and cell proliferation were studied. The results demonstrate that following stimulation with PMA and A23187, purified T cells from elderly subjects demonstrate low levels of IL-2 production, IL-2R expression and cell proliferation. Exogenous purified human IL-2 did not fully correct the low proliferative responses of T cells from old donors, however, did markedly boost the response. While it appears that the inability of T cells to express IL-2R and respond to IL-2, along with a lower endogenous IL-2 production are limiting factors in cell proliferation, the inability of PMA and A23187 to correct this defect suggests that the early phases of signal transduction *per se* are probably not a primary cause of the immunodeficiency seen in ageing.

Keywords Ca-ionophore phorbol myristate acetate interleukin 2 interleukin 2 receptor

INTRODUCTION

Defective proliferative responses of lymphocytes from elderly donors to phytohaemagglutinin (PHA), concanavalin A (Con A), OKT3 monoclonal antibody and antigen have been reported in numerous studies (Adler, Jones & Nariuchi, 1977; Doggett *et al.*, 1981; Nagel, Chrest & Adler, 1982; Schwab *et al.*, 1985). Decreased levels of IL-2 production have also been observed in cultures of cells from elderly subjects and aged animals (Thoman & Weigle, 1981; Gillis *et al.*, 1981).

The molecular basis for the age related decline in T cell responses is unknown and of great interest. Recent studies demonstrate that an intermediary step between antigen or mitogen binding and cell activation is the hydrolysis of phosphatidylinositol bisphosphate (PIP_2) to produce diacylglycerol (DG) and inositol trisphosphate (IP_3) (Berridge, 1984; Isakov, Scholz & Altman, 1986). IP_3 is the putative mobilizer of Ca^{2+} from the intracellular membrane pool, while DG

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activates phospholipid dependent PKC. PKC can also be directly activated by PMA (Niedel, Kuhn & Vandenbark, 1983). Similarly A23187, a Ca^{2+} channel opening antibiotic mimicks the action of IP_3 by causing an influx of Ca^{2+} from the extracellular space into cell cytoplasm (Tsien, Pozzan & Rink, 1982). PMA and A23187, therefore, appear to bypass the early transmembrane signal pathways and directly induce the cell to secrete IL-2, to express membrane IL-2R and to eventually undergo a proliferative response (Truneh *et al.*, 1985; Chopra *et al.*, 1987).

Previously, we have observed significantly lower proliferative responses by cells from old individuals to a variety of stimuli (Nagel, 1983). Because the impaired proliferative response to mitogens by cells from old donors and laboratory animals could result from a relative inability to transduce early activation signals (i.e., increase in intracellular cytosolic free Ca^{2+} concentration and activate PKC enzyme activity) (Proust *et al.*, 1987), we used PMA and A23187, which bypass the PIP_2 metabolic pathway, to activate purified human T cells. Using these stimuli, we observed lower IL-2R expression and IL-2 production as well as decreased T cell proliferation in cells from elderly compared to young donors. These results suggest that the age related decline in cellular proliferative ability is not secondary to a defect in transmembrane signal transduction, but is associated with intracellular events that follow the transduction.

MATERIALS AND METHODS

Study subjects. Peripheral blood was obtained from participants in the Baltimore Longitudinal Study of Ageing (BLSA). Criteria of selection of healthy volunteers has been described previously (Nagel *et al.*, 1982). The young subjects were 20–40 years of age, whereas the elderly subjects were over 60 years of age.

T lymphocyte preparation. Heparinized blood was diluted 2-fold with RPMI 1640 containing 2% fetal bovine serum (FBS), and centrifuged at 400 ***g*** for 30 min over Ficoll-sodium diatrizoate (LSM, Litton Bionetics, Kensington, Maryland, USA). Cells from the interface were collected, washed three times in culture medium, and depleted of monocytes by a 90 min incubation at 37°C in plastic culture flasks (Falcon, Oxnard, California, USA). Non-adherent cells were further purified by rosetting with AET (2-aminoethylisothiouronium bromide hydrobromide; Sigma Chemical, St Louis, Missouri, USA) conjugated sheep red cells (SRBC) (Falkoff, Peters & Fauci, 1982). The SRBC on the rosetted cells were lysed with chilled 0·15 M TRIS-ammonium chloride buffer. This procedure routinely yielded a greater than 98% $CD2^+$ cell population. The purified T cells were washed four times and suspended in complete culture media consisting of RPMI 1640 (GIBCO, Grand Island, New York, USA), 10% FBS and 100 μg/ml gentamycin.

Proliferation assay. T cells (10^5/well) were cultured in a final volume of 200 μl of complete media in triplicate in 96-well flat-bottomed microculture plates. Stock solutions of PMA and A23187 (both from Sigma) were made in dimethyl sulphoxide and ethanol respectively. Further dilutions were made in complete culture medium and used at the concentrations indicated in the results. In some experiments purified human IL-2 (Collaborative Research Inc., Lexington, Massachusetts, USA) reconstituted in RPMI 1640 was added at the initiation of the culture. The plates were incubated in a 5% CO_2 and humidified environment at 37°C for 72 h. Cell proliferation was measured by ^{3}H-thymidine (1 μCi/well, Sp. act. 2 Ci/mmol, New England Nuclear, Boston, Massachusetts, USA) incorporation during the last 18 h of culture. The cultures were harvested onto glass fibre filters, washed with water, methanol and air-dried before placing in vials with scintillation cocktail for subsequent analysis.

IL-2 assay. Cell culture supernatants were collected 1 to 3 days after the initiation of culture. IL-2 was assayed by determining the ability of cell culture supernatants to stimulate proliferation of IL-2 dependent CTLL-2 cells (Gillis *et al.*, 1978). For this bioassay, 100 μl of the culture supernatant was serially diluted in complete culture medium supplemented with 5×10^{-5} M 2-mercaptoethanol. Five thousand CTLL in a volume of 100 μl were added to each well and the plates incubated at 37°C for 24 h. For the last 4 h of culture, 1 μCi of ^{3}H-thymidine was added to each well and thymidine incorporation measured as described above. A partially purified concanavalin A induced rat spleen cell culture supernatant was titrated in each experiment to provide a standard curve. The units of

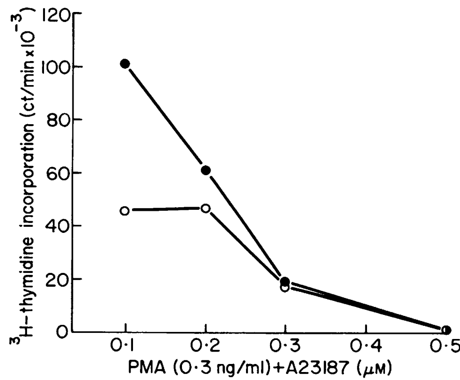

Fig. 1. ^{3}H-thymidine incorporation by purified human T cells stimulated with 0·3 ng/ml PMA plus various concentrations of A23187. Each value represents the mean of triplicate cultures. After optimization of culture conditions, this experiment was reproduced in two young and two old individuals and a similar pattern of proliferative response was observed. PMA and A23187 alone were not stimulatory to purified T cells. See Table 1 for details. (●) young; (○) old.

IL-2 were calculated by probit analysis (Gillis *et al.*, 1978). One unit of IL-2 activity was arbitrarily defined as the amount of IL-2 producing one-half of maximum thymidine incorporation by the CTLL.

Flow cytometric analysis for IL-2 receptors. The expression of membrane IL-2R was examined using FITC conjugated anti IL-2R monoclonal antibody (Becton Dickinson, Mountain View, California, USA). The culture conditions were the same as described for the proliferation experiments. After 18 h stimulation, cells from triplicate cultures were pooled, washed and stained with FITC conjugated anti IL-2R antibody and propidium iodide (Nagel *et al.*, 1981; 1983). Cell preparations were analysed on a flow cytometer with an integrated computer (Cytofluorograf 50H with 2150 Data Handling System, Ortho Diagnostics, Westwood, Massachusetts, USA). Green fluorescence histograms were generated from live cells as determined by forward scatter and green fluorescence. Only viable cells (i.e. those not stained by PI) were analysed for IL-2R expression.

RESULTS

Experiments were conducted to study the dose response characterstics of PMA and A23187 that would yield optimal levels of T cell activation using cells from young and old individuals. Activation was quantified by assaying the incorporation of ^{3}H thymidine into cellular DNA. In all experiments PMA, an activator of PKC activity, was used in combination with A23187, a Ca^{2+} channel opening antibiotic which causes a flux of Ca^{2+} from media into the cell cytoplasm. Several features of the dose response curves were common to all experiments (Figs 1, 2). First, the cells from young subjects always incorporated approximately 2-fold greater ^{3}H-thymidine in the proliferative assays than did cells from the old subjects. Second, the optimal stimulating doses of PMA and A23187 was the same for cells from both young and old donors. Third, the dose response curve showed a diminution in proliferation with all combinations of reagents when either was added at a concentration higher than needed to obtain peak stimulation. In our system the concentration of PMA necessary for peak synergism with A23187 was 0·3 ng/ml, while the optimal concentration for A23187 was 0·1 μM.

Since the level of proliferative ability of cells from old donors was consistently less than seen using cells from the young donors, and since this finding was not explained by differences in dose response characteristics, other mechanisms for this difference in proliferative ability were sought. Because PMA + A23187 induced proliferation is IL-2 dependent (Isakov & Altman, 1985), IL-2 was added to stimulated cell cultures at a concentration sufficient to insure that IL-2 would not be a limiting factor in the cells ability to proliferate. In this way a defect in IL-2 production would not be

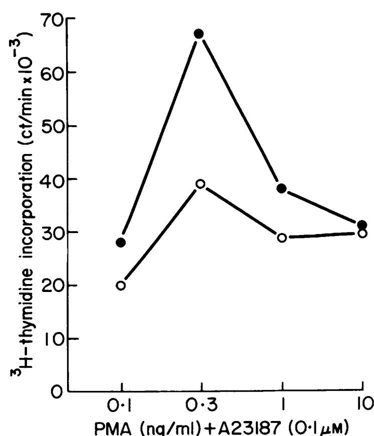

Fig. 2. ^{3}H-thymidine incorporation by purified human T cells stimulated with various concentrations of PMA and 0·1 μM A23187. Each value represents the mean of triplicate cultures. PMA up to 1 ng/ml was nonstimulatory to purified human T cells and induced a maximum of 14,000 ct/min ^{3}H-thymidine incorporation at 10 ng/ml. (●) young; (○) old.

Table 1. Effect of exogenous purified human IL-2 on ^{3}H-thymidine incorporation (ct/min) by purified T cells of young and old individuals

		Culture stimulant			
	IL-2	—	PMA	A23187	PMA+A23187
Young	−	276±28	2160±625	229±38	88563±8407
Young	+	925±238	24079±3237	919±224	103569±11720
Old	−	219±26	1738±919	220±58	36637±4323*
Old	+	1144±221	14287±1447	679±120	52392±5414*

Purified human IL-2 was reconstituted in complete RPMI 1640 medium and was added at a concentration of five half maximal units/well of inception of cultures.

* Statistically different from the untreated control by Student's *t*-test at $P<0·001$. Each value is the mean ± s.e.m. of 10 individuals.

a rate limiting step. As shown in Table 1, the proliferative response of cells from both young and old donors was boosted by the addition of IL-2. This was the case for cultures stimulated with either PMA or PMA + A23187. The addition of IL-2 did not, however, bring the level of response of cells from old donors up to that seen with the cells from young donors. Exogenous IL-2 boosted the response of the cells from old donors by 43%, while the response of the cells from young donors was only boosted by 16%. This would suggest that while an IL-2 deficiency in the cultures of cells from the old donors was a contributing factor, it was not the only aetiology for a poor response. To examine further the IL-2-producing abilities of cells from young and old donors, further experiments were carried out.

As shown in Table 2, there is a deficiency in the ability of PMA + A23187 stimulated cells from old donors to produce IL-2 when compared to cells from young donors. In these experiments the cells were stimulated using 10 ng/ml PMA with 1 μM A23187. Both of these concentrations are

Table 2. PMA and A23187 induced IL-2 production by purified T cells of young and old individuals

	IL-2 (units/ml)		
Day	1	2	3
Young	316 ± 468	1126 ± 149	3373 ± 230
Old	212 ± 247	750 ± 159	1588 ± 183*

T cells (10^5/well) were cultured with PMA (10 ng/ml) + A23187 (1 μM) for 1 to 3 days. At the end of incubation, 150 μl of supernatant from triplicate cultures was pooled, centrifuged and titrated against CTLL. One unit of IL-2 activity was calculated as the reciprocal dilution of supernatant required to give $\frac{1}{2}$ maximum proliferation of $2{\cdot}5 \times 10^4$ CTLL.

* Statistically different from the young control by the Student's t-test at $P < 0{\cdot}001$. Each value is the mean ± s.e.m. of seven young or nine old individuals.

Table 3. IL-2 receptor expression on activated T cells of young and old individuals

	IL-2R-positive cells (%)	
Group	PMA	PMA + A23187
Young	57·3 ± 1·8	62·6 ± 1·6
Old	51·1 ± 4·1	54·3 ± 4·5

The percentage of IL-2 receptor positive T cells were determined 18 h after stimulation. Culture conditions were similar to proliferation. Cells from triplicate cultures were harvested, pooled, washed and stained with FITC conjugated anti IL-2 receptor antibody. PMA was used at 0·3 ng/ml and A23187 at 0·1 μM. Background fluorescence from unstimulated culture is subtracted. A23187 alone never induced more than 3% of the T cells to express IL-2R. Each value is the mean ± s.e.m. of nine young or seven old individuals.

higher than the optimal for cell proliferation and, in fact, do not induce cell proliferation but provide optimal IL-2 synthesis. This suggests that the mechanism for the diminished IL-2 production and proliferation by the cells from old donors is not due to a defect in intracellular Ca^{2+} flux or PKC activation following mitogen or antigen binding.

The binding of IL-2 to IL-2 receptor (IL-2R) on activated T cells provides a necessary, but undetermined, signal for the induction of proliferation. To determine if IL-2R expression was

deficient and a possible contributory factor to the proliferative defect seen with cells from old donors, T cells were activated with PMA (0·3 ng/ml) and A23187 (0·1 μM) the peak proliferation inducing doses. After 18 h of culture, cells were examined for IL-2R expression. As shown in Table 3, the cell cultures from the old donors had about 8% fewer IL-2R$^+$ cells as compared to cultures of cells from the young donors. This magnitude of difference does not however reflect the marked difference seen in the 72 h proliferative abilities of cells from young and old donors.

DISCUSSION

Low levels of IL-2 production and proliferation in response to mitogen stimulation have been found in many studies of the effects of ageing on lymphocyte reactivity (for review see Nagel, 1983; Gottesman, 1987). It is currently thought that mitogen-induced proliferation strictly depends upon the T cells' ability to produce and respond to IL-2. Additional indirect evidence also suggests that the number and/or affinity of the IL-2R decreases with age (Gillis *et al.*, 1981; Gilman, Rosenberg & Feldman, 1982; Chang *et al.*, 1982). However, the precise biochemical events underlying these phenomena remain poorly understood. Because PKC activation and Ca^{2+} mobilization appear to be critical events in IL-2 synthesis and IL-2R expression, it is possible that low levels of DG or IP_3 or a defective ability of these compounds to activate PKC and increase cytosolic Ca^{2+} concentrations are causes of the low levels of T lymphocyte proliferation observed in cells from old individuals. Recent work from this laboratory has demonstrated that while basal and PMA induced levels of the enzyme PKC are comparable in 6-week-old and 24-month-old mice, Con A induced PKC translocation is reduced approximately 50% in cells from 24-month-old animals. Likewise, Con A induced similar levels of intracellular Ca^{2+} and inositol phosphate production, but since basal levels were consistently higher in the old animals, the net increase in these messengers in the elderly was reduced to half that observed in the young animals (Proust *et al.*, 1987). To further persue and expand these observations on the mechanisms of signal transduction, we used PMA and A23187 to directly trigger DG and IP_3 in greater than 98% CD2$^+$ human T cell populations (Tsien *et al.*, 1982; Nishizuka, 1984). We found that the doses of PMA and A23187 necessary to induce optimal T cell proliferation were the same in young and old donors. In addition, the cells from young, as well as old, donors displayed incremental increases in the PMA and A23187 concentrations necessary to induce optimal IL-2 production. However, despite increased PMA and A23187 concentrations, the cells from old donors proliferated less when compared to the cells from young donors. A recent study has reported that PMA and ionomycin, another calcium ionophore similar to A23187, induced low T cell proliferation by cells from old mice (Miller, 1986). However, in these experiments a higher concentration of ionomycin was able to augment the low response of the cells from the old mice in PMA costimulated cultures. We did not find this to be the case with our human studies. We found that while higher concentrations of PMA (10 ng/ml) and A23187 (1 μM) were able to induce maximal IL-2 production by cells from both young and old donors, there was still lower IL-2 production by the cells from old donors. Therefore, maximal or rapid mobilization of cytosolic Ca^{2+} does not appear to correct the low response of cells from old donors. It is possible that PMA and A23187 fail to deliver optimal signals to aged cells. Additionally, the differences between the murine and human PBL study results may be attributable to the source of cells (mouse splenic T cells vs human peripheral T cells). Perhaps the precursor cells present in the murine splenic population are not present in a purified homogeneous human CD2$^+$ cell population.

The binding of IL-2 to the IL-2R on an activated T cell drives the cell into a DNA replication cycle (Smith & Cantrell, 1985; Welte *et al.*, 1984) and therefore a deficiency of either IL-2 or IL-2R can contribute to an impaired proliferative response. IL-2 production by cells from the old donors was less than the level seen in cell cultures from young donors. The addition of exogenous IL-2 boosted the response of the cells from the old donors to a marked degree. Along with less IL-2 being produced, stimulation with PMA or PMA + A23187 induced less IL-2R positive cells in cultures of cells from the old donors. Both of these factors, decreased IL-2 production and IL-2R expression, probably contribute to a lower proliferative response of cells from old donors. Further, even though the percentage of IL-2R positive cells is not dramatically different in cultures of cells from the young

and old donors, there may be quantitative differences in the IL-2R expressed. While the mechanisms responsible for low IL-2 production, lower levels of IL-2R expression and proliferation by cells from old individuals remains obscure, the data obtained using PMA and A23187, which provide maximal stimulation of several pathways, suggests that there are other unknown mechanisms involved in the activation of lymphoid cells and which may be responsible for the diminished proliferative responsiveness observed in the elderly.

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