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Proliferative Response in Solid Culture of T Cells from Healthy Aged Subjects

Alberto J. Rivas, Esteban Kondracki, Irma Machado, Nicolás E. Bianco

Clinical Immunology National Center, SAS-UCV and WHO/PAHO-SAS Collaborative Center in Clinical Immunology, Caracas, Venezuela

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Abstract. T lymphocytes from healthy aged subjects were challenged with 12-O-tetradecanoylphorbol-13-acetate (TPA, a T-cell mitogen) in solid cultures and compared under the same experimental conditions to a group of younger controls. The aged cells showed diminished proliferation and incorporation of tritiated thymidine (^{3}H-Tdr). However, when unfractionated peripheral blood mononuclear cells or isolated T cells from the aged individuals were cultured in the presence of 2-mercaptoethanol, autologous erythrocytes or co-cultured with a non-T cell fraction, an increased proliferative response was observed. Our results suggest that the reduction in proliferative capacity of aged T cells observed in liquid culture is also elicited in solid conditions. However, under appropriate signals a TPA-susceptible T-cell subpopulation may contribute significantly in enhancing their in vitro response. This in turn would suggest that age-related cellular changes are intrinsic in nature and not fully reversible with potentiating factors.

Aging is associated with changes in immune function in both experimental animals and man. One of the most significant alterations is related to a decline in T-cell function [24] accompanied by a disturbance of the T-lymphocyte pool [8, 13], reduced proliferative responses to plant lectins and in both allogenic and autologous reactions [10, 12, 14] as well as in the generation of Con-A-stimulated T-suppresor cells [15]. Furthermore, our laboratory has reported a significant impairment of the generation of cytotoxic T cells and in the expression of cell-mediated lympholysis during immunosenescence [25]. The poor proliferative response of T cells in the aged has been attributed to a decrease of responding cells entering the cell cycle along with a reduction in the ability to divide and expand [17]. On the other hand, the function of accessory cells and the number of monocytes seems to remain intact [18, 26]. In the present investigation, we have

evaluated the response of aged lymphocytes in solid cultures in which direct intercellular contact is prevented.

Materials and Methods

Populations Studied. 14 healthy untreated individuals over 80 years of age were studied. In addition, 16 healthy young individuals (18–40 years old) were used as controls. Experiments were performed simultaneously in both groups.

Lymphocyte Purification. Heparinized blood (10 IU/ml) was diluted with an equal volume of phosphate-buffered saline (PBS) pH 7.4. Peripheral blood mononuclear cells (PBMC) were separated by centrifugation over Ficoll-Hypaque gradient (1.077 g/ml density) at 400 *g* for 40 min [5]. Cells at interphase were collected and washed three times with PBS.

Fractionation of PBMC into T and Non-T. Separation of PBMC into T and non-T cells was performed by rosette formation with sheep erythrocyte (SE) [6]. Briefly, 3 vol of the PBMC suspended in PBS at concentration of 5×10^6 cell/ml were mixed with 1 vol of 5% (v/v) of SE containing 10% SE-absorbed and heat-inactivated fetal calf serum. The suspension was incubated at 37 °C for 15 min, centrifuged at 150 *g* for 5 min and placed at 4 °C for 1 h. The cell pellets were gently resuspended, layered into Ficoll-Hypaque density gradient and then centrifuged. The non-T cells were collected from the interphase. The pellet was mixed with ammonium chloride 0.155 *M* at room temperature to lyse SE, centrifuged, washed twice and resuspended in RPMI 1640 (Gibco, Grand Island, N.Y.). The fraction had 93% E-rosette-forming cells (E-RFC) with less than 2% monocytes identified by Giemsa stain. The non-T cells had less than 3% E-RFC with 60–70% monocytes. Both preparations had more than 95% viability as assessed by trypan blue staining dye exclusion.

Preparation of Autologous Erythrocytes. Human autologous erythrocytes (HAE) were obtained from the pellet of PBMC separation, washed three times with PBS and resuspended in RPMI 1640, at a predetermined optimal concentration of 10% (v/v).

Mitogen. 12-O-tetradecanoylphorbol-13-acetate (TPA) (Consolidated Midland Co, Brewster, N.Y.) was dissolved in dimethylsulfoxide to 10^{-2} *M* and preserved at 70 °C. When in use it was diluted to working concentration with RPMI 1640. The concentration of DMSO (< 0.01%) in the cultures had previously been shown not to alter ^{3}H-thymidine incorporation. 2-Mercaptoethanol (2-ME). 2-ME (BDH, Chemicals, Poole, England) was diluted with 0.9% sodium chloride solution to 5×10^{-2} *M*.

Lymphocyte Stimulation in Solid Cultures. Lymphocyte stimulation in solid culture was performed according to *Kondracki and Milgron* [21]. Briefly, 2% (w/v) agarose (Marine Colloids, Springfield, N.J.) in distilled water was autoclaved at 121 °C for 20 min and placed in a water bath (Lab. Line Inst. Melrose Park, Ill.) at 40 °C. The agarose was then mixed with an equal volume of twice concentrated RPMI 1640, supplemented with 200 U/ml penicillin and 200 µg/ml streptomycin (Gibco, Grand Island, N.Y.) prewarmed at 40 °C. The resulting 1% agarose solution in RPMI 1640 was kept in the water bath at 40 °C. 50 µl of the 1% agarose were then added to 10 × 75 mm glass tubes (Fisher Scientific, Pa.) and the tubes were placed in the 40 °C water bath. Human leukocytes were adjusted to a concentration of 8×10^6 lymphocytes/ml in RPMI 1640. 50 µl of this leukocyte suspension were transferred to glass tubes containing 50 µl of 1% agarose in RPMI 1640. Some experiments were performed adding 10% (v/v) HAE or non-T autologous mytomicin-C-treated cells to the cell suspension at predetermined optimal concentrations. At this step the tubes contained a suspension of non-aggregated lymphocyte (4×10^5 cells/tubes in 100 µl of 0.5% agarose solid medium). The leukocyte suspension was then overlaid with 100 µl of RPMI 1640 containing the desired dose of mitogen and 20% of heat-inactivated human autologous serum; at this point, in some experiments, 2-ME was added to final concentration of 5×10^{-5} *M*. Cultures were incubated in triplicate at 37 °C in a 5% CO_2, 95% humidity chamber for 72 h. In the present study, TPA was utilized at a predetermined optimal dose of 10^{-6} *M*. 6 h prior to culture end, 0.4 µCi of ^{3}H-thymidine (1 µCi/m*M*; Commissariat à l'Energie atomique, France), in 10 µl PBS were added to each glass tube. Cultures were terminated by placing them in a –20 °C freezer where, if not processed immediately, they were stored for up to 1 month. Cultures were then processed by a modification of the method described by *Peters* [28]. Tubes with cultures were allowed to thaw at room temperature. Thereafter, 1 ml of a 3 *M* potassium iodide in 5% trichloracetate (TCA) solution was added to each tube,

Table I. ^{3}H-Thymidine incorporation by PBMC stimulated by TPA in solid cultures, effect of 2-ME

Donors		RPMI + TPA dpm × 10^{-3}	RPMI+TPA+2-ME dpm × 10^{-3}	RPMI+TPA+2-ME/ RPMI-TPA dpm × 10^{-3}
Young	1	20.2	31.1	1.54
	2	16.2	28.1	1.73
	3	35.8	57.3	1.60
	4	18.6	22.0	1.18
	5	18.5	27.0	1.46
	6	16.0	28.0	1.75
	7	19.1	37.0	1.94
Aged	1	6.8	9.4	1.38
	2	9.5	12.6	1.33
	3	3.1	6.3	2.03
	4	7.6	13.7	1.80
	5	6.2	9.0	1.45
	6	10.1	15.3	1.51
	7	11.0	22.4	2.03

Lymphocytes from aged or young donors were cultured in media, containing 10% autologous serum, 2-ME (5×10^{-5} *M*) or media containing TPA (10^{-6} *M*).

Mean cpm of triplicate cultures the SEM was always less than 10% of the mean.

and the tubes were heated to 60 °C for 2 min. Immediately after heating, the content of the tubes was stirred on a vortex mixer and centrifuged at 600 *g* for 10 min at room temperature. After centrifugation, the supernatants were removed, and the pellets washed twice with 5% TCA. After the last washing, TCA was removed and the pellets dissolved with 0.5 ml NCS of solubilizer (Amersham Searle, Arlington Heights, Ill.). The solubilized materials were transferred to vials containing 5 ml of scintillation fluid mixture (New England Nuclear, Boston, Mass.). The vials were counted in a liquid scintillation counter (Packard Instruments Co., Downers Grove, Ill.).

Data Processing. Data was transformed from counts per minute to desintegrations per minute (dpm). Mean replicate variability was less than 10%. ^{3}H-Thymidine incorporation in cell-free cultures was considered as background noise and subtracted from experimental values. Statistical analysis was performed by t test.

Results

Lymphocyte (PBMC) Responses to TPA in Solid Culture. The pattern of proliferative response of 7 aged and 13 controls to TPA (10^{-6} *M*) were evaluated. The mean ± SEM for the aged was 7.42 ± 0.70 dpm × 10^{-3} while in the young group was 32.0 ± 0.69 dpm × 10^{-3}, the difference being highly significant ($p < 0.0005$).

Influence of Autologous Erythrocytes and 2-ME on the Response of PBMC to TPA. Due to the significant reduction of proliferation from the aged in solid cultures, we explored the influence of 2-ME and autologous erythrocytes (HAE); 2-ME was added to the cultures at a final concentration of 5×10^{-5} *M*;

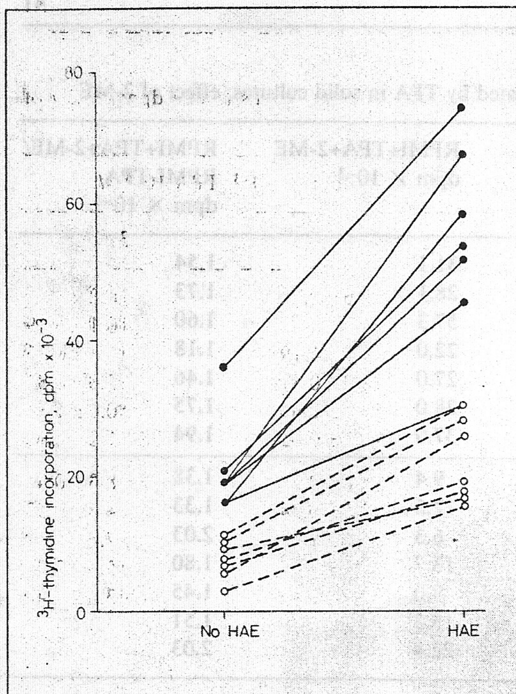

Fig. 1. ^{3}H-thymidine incorporation by PBMC stimulated by TPA. Effect of 5% autologous erythrocytes (HAE). PBMC youngs donors (●); aged donors (○). Each point represents the mean of triplicate cultures.

HAE was added at 5% (v/v). 2-ME was able to increase significantly the proliferative response to TPA of the 7 aged in solid cultures (PBMC ± TPA: 7.75 ± 2.7 vs. PBMC ± TPA ± 2-ME: 12.6 ± 5.2 dpm $\times 10^{-3}$; $p < 0.0025$). The enhancing effect was observed in both groups; however, the magnitude of the potentiation with 2-ME of cells of young and aged were comparable (1.60 ± 0.24 vs. 1.65 ± 0.30; table I). The enhancing effect of HAE is depicted in figure 1. Similar to the 2-ME effect, the difference for both groups was also highly significant ($p < 0.001$). It is important to note that ^{3}H-Tdr incorporation in unstimulated cultures in the presence of 2-ME or HAE was less than 1.11 and 1.87 dpm $\times 10^{-3}$, respectively.

Patterns of T Cell Proliferative Response to Different Stimuli in Solid Cultures. Cultures were set-up containing isolated T cells, either alone or in the presence of 2-ME, HAE or non-T cells (used as source of monocyte) and stimulated by TPA. T cells alone from aged donors were less responsive to TPA when compared with T lymphocytes from young donors ($p < 0.05$). When non-T cells were added to the cultures, the response was similar or higher than the unfractionated populations. 2-ME of HAE also significantly enhanced ($p < 0.05$) proliferative responses of isolated T cells from aged donors as well as young donors in solid cultures (table II).

Discussion

Impaired proliferative and effector T-cell responses have been linked to the process of aging [16, 25]. There is evidence that reduction in the size of the T-cell pool [8] and alteration in the mechanisms that initiate T-cell activation or a reduction in the intrinsic capacity of individual T cells to enter into cell cycle, may account for the observed T-cell dysfunction [20]. Furthermore, in regard to the altered cell response to mitogens, *Hefton* et al. [17] suggested the possibility of fewer mitogen-responsive cells due to the failure of aging cells to divide normally in culture. Since T-cell activation is dependent on several kinds of cooperative signals, research has also focused on possible changes of accessory cells, levels of thymic hormone and T-cell growth factor (IL-2) level, which may occur during senescense. Evidence, accumulated to the present, has shown that the number and function of accessory cells (monocyte-macro-

Table II. T cell proliferative response in solid culture: influence of different signals (mean dpm ± SD of triplicate cultures)

Donors[1]	Cells	^{3}H-thymidine incorporation, dpm × 10^{-3}, TPA 10^{-6} *M*		
		1	2	3
Young	PBMC	33.0 ± 3.9	18.6 ± 0.72	16.2 ± 1.40
	T cells	22.1 ± 1.42	11.2 ± 0.99	10.1 ± 0.19
	T cells+non-T cells[2]	56.9 ± 1.92	19.7 ± 1.19	18.6 ± 0.33
	T cells+2-ME	31.3 ± 0.67	18.8 ± 0.81	13.0 ± 0.81
	T cells+HAE	105.8 ± 8.70	58.0 ± 2.64	50.0 ± 1.10
	Non-T cells	1.4 ± 0.24	1.3 ± 0.05	1.8 ± 0.10
Aged	PBMC	10.6 ± 0.44	5.0 ± 0.22	8.3 ± 0.41
	T cells	6.6 ± 0.78	2.6 ± 0.34	4.5 ± 0.37
	T cells+non-T cells[2]	17.0 ± 0.79	7.1 ± 1.01	7.6 ± 0.79
	T cells+2-ME	13.4 ± 0.90	6.2 ± 0.68	15.6 ± 2.20
	T cells+HAE	28.9 ± 0.72	9.3 ± 0.60	43.6 ± 2.40
	Non-T cells	1.2 ± 0.29	0.7 ± 0.05	0.9 ± 0.04

[1] Lymphocytes from young or aged donors were incubated with TPA, or media containing 10% autologous serum, 2-ME (5 × 10^{-5} *M*) or HAE (5% v/v).
[2] T cells enriched with 10% non-T cells.

phage) remains intact [1, 26] when explored in vitro. However, the level of both thymic hormones and IL-2 decline with age [4, 11]. On the other hand, since the initial phase of T-cell proliferative response to mitogen in the aged is similar to young controls a possible alteration between the interaction of the mitogen with cell membrane has been ruled out [2]. Furthermore, neither the number of receptors to mitogens nor the affinity of these receptors seems to decline with age [7]. In the present investigation we used solid cultures, which prevent direct cell to cell contact as previously reported by *Kondracki* [22], to examine the response of the cell population to signals which may originate either directly or from accessory cells (soluble factors) or from both. The protocol also permitted the investigation of the influence of chemical (2-ME) or biological (HAE, non-T cells) signals on the proliferative response to TPA. In our experiments, aged T lymphocytes responding as single cells in solid culture showed significantly low proliferative capacity; this behavior is similar to that observed in liquid culture to various mitogens [12, 14, 27]. Remarkably, however, our results tend to indicate that aged T cells retain the ability to recognize signals of various nature and to improve significantly in their proliferative capacity. Furthermore, the recovery of T-cell response was noted either in whole unfractionated or fractionated T-cell population. *Kondracki and Milgrom* [21] and *Kondracki* [22] suggested that, in solid culture, both 2-ME and HAE may generate cooperative sig-

nals. 2-ME would also enhance the proliferative response of old mice spleen cells [19]. *Lawrence and Noelle* [23] suggested that biochemical events generated by 2-ME may facilitate in vitro activation. However, as in the case of HAE, the nature of enhanced response in the presence of TPA remains to be elucidated.

Our results showed that the addition of the non-T cell population also improved the response of separated T cells, which in turn may suggest that regulatory cooperative signals after stimulation with 2-ME or HAE may come from soluble factors released by accessory cells. In addition, as suggested by others [3] accessory cells in aged humans keep operating adequately to ensure, at least partially, proper T-cell activation. *Estensen* et al. [9] showed evidence of a TPA receptor on human peripheral blood lymphocytes. Moreover, *Nagel* et al. [27] suggested that the decline in the ability of aged mononuclear cells to be activated by TPA in liquid cultures may reflect a decrease in number or function of TPA receptors. Our results differ from such a possibility since, under appropriate signals, a susceptible TPA-aged T-cell subpopulation may augment their response without reaching the levels observed in younger cells. Furthermore, *Touraine* et al. [29] found that TPA activates a subpopulation of cells which is not only distinct from those responding to PHA or Con-A or those able to form 'high affinity' rosettes, thus probably identifying a particular T-cell population.

Our findings tend to indicate that appropriate regulatory influence may render aged T cells de novo susceptible to the stimulatory action of certain mitogens. In addition, it may indicate that age-related cellular changes are intrinsic in nature and not fully reversible with potentiating factors.

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A.J. Rivas, MSc,
Clinical Immunology National Center,
Apartado 50647,
Correos de Sabana Grande,
Caracas 1051 (Venezuela)

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The Effect of Age on the Circadian Rhythms of 23 Liver or Brain Enzymes from C57BL/6J Mice[1]

R.J. Feuers[a,c], *R.R. Delongchamp*[a], *S. Kramer*[b], *L.E. Scheving*[b], *D.A. Casciano*[a,c]

[a] Department of Health and Human Services, Food and Drug Administration, National Center for Toxicological Research, Jefferson, Ark., USA; Departments of [b] Anatomy, and [c] Biochemistry, University of Arkansas for Medical Sciences, Little Rock, Ark., USA

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Abstract. Mice were standardized to 12 h of light exposure alternating with 12 h of darkness and were fed ad libitum. The mice in the first group were 8 weeks old and in the other 104–116 weeks old. Subgroups of 7 animals from each age group were killed at 6 circadian stages. Enzyme activities of 23 enzymes from liver or brain were measured by an analysis of variance for each age group. All but 1 enzyme from young mice, and all but 9 from older mice showed significant changes over time ($p < 0.01$). The data from the 22 enzymes from young mice and the 14 enzymes from old mice were fit to the cosinor regression model to further characterize the rhythm. 15 enzymes from the young and 8 from the aged mice showed a significant regression to a 24-hour cosine curve ($p < 0.01$); of the 8, 7 were the same enzymes in both groups. Amplitude changes, where they could be compared from the cosinor data (7 enzymes), were not statistically different. When total variance was compared, 12 enzymes showed unequal variance. Of these, old mice had the larger variance in 9 enzymes. Another difference between the young and the old was changes in mean enzyme activities. 12 enzymes from aged mice had decreased mesors, 2 had increased mesors, and 9 were unchanged. In general, our data suggest that some enzyme activity rhythms were lost, others were altered and a few were not affected by aging. In the case of many enzymes, older mice have a diminished ability to synchronize to the light signals.

Aging has been described as a continuum of growth and development with progressive and unfavorable loss of adaptation [*Hayflick*, 1980]. There is a variable decline in the ability to perform several diverse functions [*Strehler*, 1980]. The 'error catastrophe' theory of aging suggests that transcription and translation become increasingly susceptible to error yielding faulty enzyme and protein molecules [*Orgel*, 1963]. This has not been

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