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Role of Regulatory T Cells on the Age-Related Increase in Mitogen-Induced IgG Production *in Vitro* by Human Lymphocytes*)

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ABSTRACT

When *in vitro* IgG production by mitogen-stimulated peripheral lymphocytes from healthy aged (70–93 year old) and young (20–29 year old) subjects was compared, it was found that the level of IgG production was elevated in the aged subjects. Co-culture studies were performed to determine whether the increase was due to changes in T or non-T cells. IgG production was significantly higher when reference non-T cells from normal young adults were mixed with T cells from aged subjects than with T cells from young adults. In contrast, no significant difference in IgG production was observed when reference T cells from normal young adults were mixed with non-T cells from either young or aged subjects. The suppressor activity of Tγ cells and helper activity of non-Tγ T cells of young and aged subjects were then determined. The results revealed that the suppressor activity of Tγ cells of aged individuals was significantly lower than that of young adults, but the helper activity of non-Tγ T cells of young and aged subjects was comparable. These results indicate that the increase in production of IgG by the peripheral lymphocytes of aged individuals is due in part to changes in the T cells which are related to a decrease in suppressor activity of Tγ cells.

INTRODUCTION

It is well known that aging in humans is associated with changes in humoral immunity [as manifested by an increase in serum immunoglobulin (Ig)[20,31] and frequency of autoantibodies[8,22]] and changes in cell-mediated immunity [as manifested by a decrease in delayed hypersensitivity reaction[21,30] and lymphocyte response to mitogens[8,27]]. Associated with age-related changes in immunologic activities is the increase in susceptibility to infectious diseases, autoimmune-immune complex diseases, and cancer[17]. Recently, T suppressor cells were found to be functionally altered in patients with common variable immunodeficiency[29], Hodgkin's disease[28], fungal infection[25], and systemic lupus erthematosus (SLE)[1]. Conceivably, alteration in T suppressor cell activity could also be contributing to the pathogenesis of certain age-related diseases. It would therefore seem desirable to investigate the activity of T suppressor cells in aged individuals, for the information may contribute to our understanding of their increased susceptibility to these diseases.

Accordingly, the present study was undertaken to investigate the influence of T suppressor cells of young adult and aged subjects on IgG production by mitogen-stimulated B

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cells. The results show that IgG production is elevated in aged subjects and that this elevation is associated with a decrease in suppressor T cell activity.

MATERIALS AND METHODS

Donors. Two groups of volunteer subjects were used in this study, one group comprised of 34 healthy old individuals (ranging in age from 70 to 93 years) and the other of 41 young adults (ranging from 20 to 29 years).

Isolation of lymphocytes. Lymphocytes were separated from fresh heparinized blood (20 units heparin per ml of peripheral blood) by Ficoll-Hypaque density gradient centrifugation as previously described.[26]

After washing three times with Hanks' Balanced Salt Solution (HBSS) containing 2.5% heat-inactivated fetal calf serum (FCS), the cells were resuspended in Eagle MEM (Nissui Seiyaku, Tokyo, Japant containing 2 mM L-glutamine, 10% heat-inactivated FCS, and 60 μg/ml of kanamycin.

Separation of T and non-T cell fractions. Four ml of lymphocyte suspension ($5-10\times10^6$/ml) were mixed with 4 ml of 1% 2-aminoethylisothiouronium bromide (AET)-treated sheep red blood cells (SRBC) in FCS, according to Kaplan's method[8]. The mixture was then centrifuged at $120\times g$ for 5 minutes and incubated at 4°C for an additional one hour. After incubation, the cells were dispersed, layered over Ficoll-Hypaque, and centrifuged at $400\times g$ for 30 minutes. Cells at the interface, which constituted the non-T cell fraction, consisted of 49-80% B lymphocytes bearing complement receptors, 9-22% peroxidase positive monocytes, and less than 1% SRBC-rosetting T cells. The pelleted fraction contained more than 96% SRBC-rosetting T cells. The rosettes were disrupted by exposing them to a 0.83% NH_4Cl tris buffer, pH 7.4, and a sufficient volume of HBSS containing 2.5% FCS was added immediately after RBC lysis. T and non-T cell fractions were washed three times with HBSS and resuspended in the culture media.

Separation of Tγ and non-Tγ cells. Tγ cells (IgG Fc-receptor bearing T cells) and non-Tγ cells were obtained by using the insolubilized antigen-antibody coated plastic surface method of Alexander and Henkart[11].

FCS (10 ml) was added to cover the bottom of plastic tissue culture flasks (Corning, New York) and, after 15 minutes of incubation at room temperature, it was poured off and the flasks rinsed three times with phosphate buffered saline (PBS). Picryl sulfonic acid (1 mM in PBS) was then added, the flasks incubated for 15 minutes at 37°C, at which time the acid was poured off and the flasks rinsed three times with PBS. Four ml of rabbit anti-DNP-BSA reagent (Miles Laboratories, Elkhart, Indiana) prediluted 50-fold in PBS were then added, and the flasks were incubated for 30 minutes at room temperature. The antiserum was poured off, and the flasks rinsed tyree times with PBS. Four ml of T cells ($4-10\times10^6$/ml) were then added and the flasks incubated for 45 minutes at room temperature to allow the T cells to settle on the antigen-antibody complexed surface. The nonadherent T cells were poured off and the flasks rinsed three times with HBSS containing 2.5% FCS. Less than 1% of these nonadherent cells rosetted with anti-ox-RBC IgG antibody sensitized ox RBC. Ten mM EDTA and one mM DNP-lysine in PBS were added to each flask, and the flasks incubated for 30 minutes at 37°C. After incubation, the adherent cells were dispersed manually and removed. More than 90% of these T cells rosetted with anti-ox-RBC IgG antibody sensitized ox RBC.

IgG and anti-IgG. Human IgG was purified from the serum of a normal subject by precipitation with 33% ammonium sulfate and then by passage of the solubilized precipitate through a DEAE cellulose column equilibrated with 0.01 M phosphate buffer, pH 7.4. Antiserum against human IgG was obtained by immunizing rabbits twice with 5 mg of IgG in complete Freund's adjuvant.

In vitro culture system. Five$\times10^5$ lymphocytes were added into each well of Microtest-plate-II (Falcon Plastics, Oxnard, Calif., U. S. A.) When either T cells or non-Tγ T cells were mixed beforehand with non-T cells, the ratio was fixed at 7 : 3. A 7 : 3 ratio was selected because preliminary experiments showed that the effects of T suppressor cells are best demonstrable at ratios greater than 1 : 1 and because 7 : 3 is the ratio we observed in the peripheral blood of normal subjects.

Lymphocytes were cultured for 7 days in the presence of 5 μl/ml of pokeweed mitogen

(PWM, Gibco, Grand Island, New York, U. S. A.). A concentration of 5 μl/ml of PWM was selected because preliminary experiments showed that optimal IgG production occurred in the dose range of 1 to 10 μl/ml PWM. At the termination of the culture on day 7, the plates were centrifuged at $400 \times g$ and the culture supernatant harvested with an automatic pipette (Micromedic System, Winter Road, Horsham).

Measurement of the amounts of IgG. IgG was determined by an inhibition radioimmunoassay described by Platts-Mills and Ishizaka[19]. One-tenth ml of 8-fold diluted culture supernatants and 0.1 ml of reference control IgG (800 ng/ml to 6.25 ng/ml) were mixed. This mixture was mixed with 0.1 ml of 10^5-fold diluted rabbit anti-human IgG. One-tenth ml of ^{125}I-labeled IgG (Radio Chemical Centre, Amersham, England) was then added and allowed to stand overnight at 4°C. Then, 0.1 ml of goat anti-rabbit IgG serum or 0.1 ml of normal rabbit serum (Daiichi Radio Isotope, Tokyo, Japan) were added and allowed to stand overnight at 4°C. The precipitate was washed three times with PBS. A gamma counter (RAW-600, Shimazu Seisakusho, Kyoto, Japan) was used to determine precipitate radioactivity. The magnitude of inhibition was calculated as follows: percent suppression = [1-(ng IgG/ml in culture with suppressor cells/ng IgG/ml in culture without suppressor cells)] $\times$ 100.

Analysis of data. The data were subjected to the standard Student *t* analysis.

RESULTS

PWM-induced IgG production. Unfractionated lymphocytes obtained from young and old subjects were stimulated with PWM and the amounts of IgG produced *in vitro* determined. As shown in Fig. 1, the average amount of IgG produced by lymphocytes of old subjects [1,581$\pm$990 ng/ml (mean$\pm$SD)] was slight but significantly greater than that of young subjects (1,009$\pm$729 ng/ml) as judged by the Student *t* test ($p<0.05$).

Interaction of T cells and non-T cells in PWM-induced IgG *production.* To determine the extent to which T cells influence PWM-induced IgG production, T cells and non-T cells were stimulated with PWM separately or after mixing them at a 7 : 3 ratio, and the amounts of IgG determined 7 days later. The results (Table 1) revealed that T cells and non-T cells separately stimulated produced 5$\pm$9 ng/ml and 99$\pm$49 ng/ml, respectively. In contrast, the 7 : 3 mixture produced two orders of magnitude more IgG (1,425$\pm$127 ng/ml) than either T cells or non-T cells alone. These results demonstrate that synergistic interaction

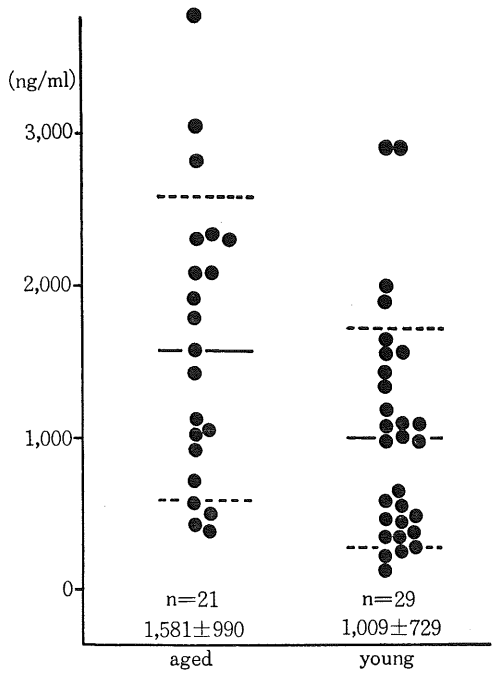

Fig. 1. *In vitro* PWM-induced IgG production of lymphocytes from aged and young subjects.
Lymphocytes (5×10^5) from aged and young subjects were cultured for 7 days in the presence of PWM.
Concentration of IgG in the supernatant of cultures was measured by radioimmunoassay ($p<0.05$).

Table 1. PWM-induced IgG production by T and non-T cells of normal adult subjects and by their mixtures[a]

Cell type (5×10^5/culture)	Level of IgG (ng/ml) Mean$\pm$S. D.
T	5$\pm$9
Non-T	99$\pm$49
T+Non-T	1,425$\pm$127

[a] T cells are AET-treated SRBC rosette-forming cells; non-T cells are non-rosette forming cells; mixed cell cultures contained 3.5×10^5 T cells and 1.5×10^5 non-T cells.

of T cells and non-T cells is required for maximum PWM-induced IgG production.

Comparison of PWM-induced IgG production between autologous and allogeneic T cell: non T cell mixed cultures. The influence of potential allogeneic effect on IgG production was determined by assessing the IgG producing capacities of autologous and allogeneic T cell: non T cell mixtures from 4 individuals. The results, as summarized in Table 2, revealed no significant difference in IgG production between autologous mixed cultures (1,516±570 ng/ml) and allogeneic mixed cultures (1,432±632 ng/ml).

Influence of age of donors of non-T and T cells on PWM-induced IgG production. Non-T cells from 4 aged subjects and 4 young subjects were mixed individually with reference T cells from aged and young donors and their PWM-induced capacity to form IgG assessed. The results (Table 3) revealed that the age of non-T cells has minimal influence on IgG

Table 2. PWM-induced IgG production by mixtures of autologous and allogeneic T and non-T cells of normal adult subjects[a]

Donor of T Cells (Age in yr)	Donor of Non-T Cells				
	None	T.I. (32)	M.T. (31)	K.G. (29)	I.F. (35)
			ng/ml		
None		466± 3	118± 17	694±25	353±121
T.I.(32)	194±60	712± 89	1,591±45	2,203± 71	684±294
M.T.(31)	43± 43	976±150	1,989±407	2,138±364	1,364±267
K.G.(29)	129± 84	827± 97	1,088±296	1,841±113	767±296
I.F.(35)	94± 41	1,104±292	1,889±374	2,555±103	1,522±322

[a] Cells per well, 3.5×10^5 T cells plus 1.5×10^5 non-T cells; IgG production expressed as mean±S.D. of triplicate cultures. Underlined values are autologous combinations. Level of IgG production by autologous T and non-T cell mixture, 1,516±570 ng/ml; level of IgG produced from allogeneic T and non-T cell mixture, 1,432±632 ng/ml.

Table 3. Influence of age of donors of non-T cells on PWM-induced IgG production (ng/ml)[a]

Donor of Non-T Cells (Age in yr)	Donor of T Cells				
	None	S.N. (74)	K.U. (77)	T.T. (24)	I.H. (25)
None		127±21	96± 35	110± 15	149± 44
S.N.(74)	221±10	2,103± 94	3,992±347	502±104	742±260
K.N.(75)	132±57	547± 94	1,472±274	392± 43	414± 48
S.S.(73)	N.D.	348± 59	127± 51	228± 35	269± 30
K.U.(77)	149±20	538±143	441± 45	261± 83	168± 17
Mean±S.D. (A)		893±810	1,508±1,753	346±126	398±250
O.K.(23)	187±56	2,267±273	2,296±242	349± 21	637±114
T.T.(24)	183±52	358± 31	523±298	189± 48	289± 45
I.H.(25)	131±35	114±232	1,080±348	219± 63	256±128
M.F.(23)	229±54	1,751±477	1,214±224	210± 38	374± 71
Mean±S.D. (B)		1,123±1,050	1,278±742	242± 73	389±173
p value (A vs. B)		N.S.	N.S.	N.S.	N.S.

[a] Cells per well, 3.5×10^5 T cells plus 1.5×10^5 non-T cells; IgG production expressed as mean±S.D. of triplicate cultures; underlined values, autologous combination; N.D., not done; N.S., not significant ($p>0.05$).

production (i. e., IgG production of cell mixtures containing non-T cells of aged subjects was comparable to those containing non-T cells of young subjects). This would suggest that the increased PWM-induced IgG production with age could be due to changes in the T cells. Comparison of columns 3 (S. N.) and 4 (K. U.) (T cells of aged subjects) vs. columns 5 (T. T.) and 6 (I. H.) (T cells of young subjects) supports this notion, as the former mixtures produced more IgG than the latter.

A more definitive experiment was then carried out by analyzing the influence of T cells of aged and young subjects on reference non-T cells from aged and young subjects. The results (Table 4) showed that the age-related increase in IgG production is indeed related to the T cells and not to non-T cells (i. e., IgG production of cell mixtures containing T cells of aged subjects is significantly higher than those containing T cells of young subjects regardless of the age of donor non-T cells).

Influence of Tγ cells on PWM-induced IgG production. The suppressor effect of Tγ cells on IgG production was investigated by determining the influence of Tγ cells on the production of IgG by mixtures of T cells deficient in Tγ cells (non-Tγ T cells) and non-T cells from 2 normal donors. The results (Table 5) revealed that the magnitude of suppression by autologous and allogeneic Tγ cells were comparable (41.6% vs. 41.2% and 47.2% and 45.7%).

Influence of age of donors of non-Tγ T cells on PWM-induced IgG production. Non-Tγ T cells from 4 aged and 4 young subjects were mixed with reference T cells of an aged or young subject and their PWM-induced IgG production determined. The results (Table 6) revealed that the non-Tγ T cells of aged subjects are comparable to those of young subjects (1,469±343 vs. 1,691±31 ng/ml and 1,619±516 vs. 1,136±751 ng/ml).

Influence of age of donors of Tγ cells on PWM-induced IgG production. The suppressor activity of Tγ cells from individual aged and young subjects were compared by adding them to a reference mixture of T cells deficient in Tγ cells and non-T cells from an adult subject (K. F.) stimulated with PWM. The results (Table 7) revealed that Tγ cells from the aged were less effective in their suppressor activity than those of young subjects (i. e., 25.8 ±8.4% suppression versus 43.3±2.7% suppression) ($p<0.01$).

Table 4. Influence of age of donors of T cells on PWM-induced IgG production (ng/ml)[a]

Donor of T Cells (Age in yr)	Donor of Non-T Cells				
	None	S.N. (74)	I.H. (25)	O.K. (23)	T.T. (24)
None		321± 10	131± 35	149± 20	183± 52
S.N. (74)	171±59	2,103±590	1,114±232	538±143	358± 31
K.N. (75)	137±36	1,573±245	1,907±215	1,882±438	368± 90
S.S. (73)	N.D.	1,143± 75	431± 40	324± 1	N.D.
K.U. (77)	105±29	3,992±347	1,080±348	441± 45	523±298
Mean±S. D. (A)		2,203±1,256	1,133±604	796±729	416± 93
O.K. (23)	51± 27	959±238	724±175	238± 20	276± 47
T.T. (24)	110± 15	502±104	219± 63	261± 83	189± 48
I.H. (25)	149± 54	742±260	256±128	248± 63	289± 45
M.F. (23)	46± 19	398± 69	187± 24	168± 17	186± 38
Mean±S. D. (B)		650±251	347±253	229± 42	235±55
p value (A vs. B)		$p<0.05$	$p<0.05$	$p<0.05$	$p<0.05$

[a] Cells per well, 3.5×10^5 T cells plus 1.5×10^5 non-T cells; Ig production expressed as mean±S. D. of triplicate cultures; underlined values, autologous combination; N. D., not done.

Table 5. Influence of Tγ cells on the PWM-induced IgG production by mixtures containing T cells deficient in Tγ Cells (Non-Tγ T cells) and Non-T cells[a]

Donor of Tγ Cells	Donor of Non-Tγ T Cells	Donor of Non-T cells	Level of IgG (ng/ml)	Percent Suppression
(—)	K.S.	(—)	159± 90	
(—)	S.I.	(—)	<10	
(—)	K.S.(29 yr)	K.S.	4,427±333	
K.S.	K.S.	K.S.	2,587±361	41.6
(—)	K.S.	S.I.	544±162	
K.S.	K.S.	S.I.	320± 23	41.2
(—)	S.I.(26 yr)	K.S.	1,893±122	
S.I.	S.I.	K.S.	987± 46	47.9
(—)	S.I.	S.I.	243± 51	
S.I.	S.I.	S.I.	132± 51	45.7

[a] Tγ cells per well, 0.5×10^5; T cells deficient in non-Tγ T cells per well, 3.5×10^5; Non-T cells per well, 1.5×10^5; Ig production expressed as mean±S.D. of tryplicate cultures; (—), none; percent suppression, [1-(level of IgG in cultures with Tγ cells/level of IgG in cultures without Tγ cells)]×100.

Table 6. Influence of age of donors of Non-Tγ T Cells on PWM-Induced IgG Production (ng/ml)[a]

Donor of Non-Tγ T Cells (Age in yr)	Non-T Cells		
	None	Y.H. (26)	S.U. (70)
None		219±37	147± 20
S.U.(70)	43±21	1,973±214	2,082±131
S.M.(73)	69±16	1,388±260	1,196± 6
S.S.(77)	22±13	1,298±164	2,048±333
A.F.(75)	72±25	1,217±255	1,150±178
Mean±S.D. (A)	None	1,469±343	1,619±516
S.E.(24)	55±28	1,420±400	737±313
F.H.(23)	36±12	1,534±134	658±116
Y.H.(26)	41±20	1,680±203	898±105
M.O.(24)	71±22	2,130±338	2,252±583
Mean±S.D. (B)		1,691±311	1,136±751
p value (A vs. B)		N.S.	N.S.

[a] Non-Tγ T cells per well, 3.5×10^5; non-T cells per well, 1.5×10^5; Ig production expressed as mean ± S.D. of triplicate cultures; underlined values, autologous combination; N.S., not significant ($p>0.05$).

DISCUSSION

Alterations in activity of T suppressor cells with age have been observed. In some instances, the T suppressor cell activity has been found to *decrease* with age, e.g., in short-lived autoimmune-susceptible (NZB×NZW)F_1 and NZB mice[4,7]. In other cases, e.g., long-lived mice[16,23,24], an *increase* with age of T suppressor cell activity has been observed. Both a decrease and an increase in T suppressor cell activity with age have also been detected in humans, but the change in activity is not related to obvious differences in life expectancy[1,9,15].

The various positive and negative correlations between age and the activity of T suppressor cells emphasize the complexity of the mechanism(s) controlling them. In an attempt to obtain a better understanding of this age-related phenomenon, we investigated the influence of T suppressor cells involved specifically in PWM-induced IgG production. This system was selected because the cells involved in IgG production can be analyzed[12,13,18,20,31].

In confirmation of earlier findings[6,10,12,13], it was established that 1) both T cells and non-T cells are required for IgG production and 2) allogeneic interaction between T cells and non-T cells of different individuals, if it occurs, exerts a minimal influence on IgG production (i.e., autologous mixture, 1516±570 ng/ml; allogeneic mixture, 1432±632 ng/ml). The component cells were then analyzed with respect to age, and the results revealed that the age-related increase in PWM-induced IgG production is related primarily to changes in T cells.

Table 7. Suppressor effect of Tγ cells of aged and young subjects on the IgG production of a reference mixture containing non-Tγ T cells and Non-T cells[a]

Donor of Tγ Cells	Age of Donor of Tγ Cells	Donor of Non-Tγ T Cells	Donor of Non-T Cells	IgG Level (ng/ml)	Percent Suppression
None		K.F.	K.F.	897± 65	0
M.I.	74	K.F.	K.F.	757± 18	15.6
T.T.	70	K.F.	K.F.	629± 9	29.9
Y.K.	80	K.F.	K.F.	693± 81	22.7
S.T.	79	K.F.	K.F.	584± 11	34.9
Mean±S.D. (A)				666± 76	25.8±8.4
E.K.	23	K.F.	K.F.	533±109	40.6
A.F.	25	K.F.	K.F.	499± 12	44.6
K.F.	25	K.F.	K.F.	523± 67	41.7
N.K.	26	K.F.	K.F.	480± 55	46.5
Mean±S.D. (B)				509± 24	43.3±2.7
p value (A vs. B)				$p<0.01$	$p<0.01$

[a] Tγ cells per well, 0.5×10^5; non-Tγ T cells per well, 3.5×10^5; non-T cells per well, 1.5×10^5; Ig production expressed as mean±S.D. of triplicate cultures; percent suppression, [1-(level of IgG in cultures with Tγ cells/level of IgG in cultures without Tγ cells)]×100.

To determine whether age-related changes in T cells reflect an alteration in T helper cells and/or T suppressor cells, studies were performed on T cells, Tγ, and T cells deficient in Tγ cells. These studies show that the increase in PWM-induced IgG production with age is related to a decrease in the suppressor cell activity of Tγ cells (aged, 25.8±8.4% suppression; young, 43.3%±2.7% suppression). No significant correlation was observed between age-related increase in PWM-induced IgG production and both non-T cells and T cells deficient in Tγ cells. However, this does not necessarily rule out the possibility that age-related changes in these cells could also contribute to the increase in IgG production. The reason for this reservation is the large variation observed between individual cultures of the same group, suggesting that cells other than Tγ cells could also be contributing to the production of IgG.

In spite of these variations, we are encouraged to investigate the extent to which these component T cells modulate production of autoantibodies by cells of elderly individuals, with respect to the magnitude of production, class of immunoglobulin, and antigenic specificity.

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