

## IMMUNOLOGICAL STUDIES OF AGING

### III. Cytokinetic Basis for the Impaired Response of Lymphocytes from Aged Humans to Plant Lectins\*

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While it is well established that lymphocytes from persons over 65 yr of age incorporate less thymidine than do cells from young subjects when cultured with plant lectins (1-5), the explanation for this deficiency has not been determined. This defective response could be due either to there being fewer mitogen-responsive lymphocytes in mononuclear preparations from old persons and/or a failure of initially responding lymphocytes to expand into a pool of proliferating cells. Some investigators (4, 6, 7) have suggested that a relative deficiency of T lymphocytes might explain the impaired response to plant lectins of lymphocyte preparations from aged persons. However, whether the postulated deficiency represents a decreased number of T cells or a decrease in the proliferative expansion of an initially normal number of cells has not been determined.

In the present studies we have undertaken to characterize at the cellular and cytokinetic levels the cause of the defective thymidine incorporation observed with lymphocyte preparations from old subjects. In agreement with other workers (8) we have found that there was no decrease in the concentrations of blood T lymphocytes of old persons. In addition, we find that even purified T-lymphocyte preparations from old persons have an impaired response to phytohemagglutinin (PHA). We have characterized the nature of this defect by determining the number of initial responding units and the kinetics of proliferation after stimulation. The number of responding units was determined by limiting dilution assays and by the vesicular stomatitis virus (VSV)<sup>1</sup> plaque assay. The proliferative expansion of the initially stimulated cells was studied by colchicine block experiments. It was concluded based upon the three independent assays that the defect in the T-cell preparations of aging humans is due to both a decrease in the number of mitogen responsive units and to a deficiency in the expansion of those responsive cells which are present. The relationship of these two defects of lymphocyte function in vitro to the involution of the thymus gland and Hay-

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<sup>1</sup> *Abbreviations used in this paper:* Con A, concanavalin A; FBS, fetal bovine serum; HBSS, Hanks' balanced salt solution; MEM, minimal essential medium; VSV, vesicular stomatitis virus.

flick's hypothesis that normal cells have limited proliferative capacity is discussed.

## Materials and Methods

*Subjects.* Blood was obtained from healthy laboratory personnel between the ages of 20 and 30 yr and from old persons between the ages of 65 and 97. Care was taken to select old persons who were free of diseases and not taking any drugs known to effect the reactivity of lymphocytes in culture. Most of the older subjects had atherosclerotic cardiovascular disease and related disorders. None of the older subjects were acutely ill. This research project was approved by the Human Rights in Research Committee of The New York Hospital-Cornell Medical Center.

*Lymphocyte Preparations Cultured with PHA.* 30 ml of venous blood from healthy volunteers were drawn into a plastic syringe containing 10 U of heparin (Upjohn Company, Kalamazoo, Mich.) per ml of blood. The blood was diluted with an equal volume of calcium and magnesium-free Hanks' balanced salt solution (HBSS, Microbiological Associates, Bethesda, Md.). 35–40 ml of the diluted blood were layered over 12 ml of a sterile mixture of 4-*vol* Ficoll (Pharmacia Fine Chemicals, Piscataway, N. J.) and 1-*vol* sodium diatrizoate (Hypaque, Winthrop Laboratories, New York). Tubes containing the diluted blood layered on Ficoll-Hypaque were centrifuged at 400 *g* for 40 min at 20°C. The cells removed from the interface were washed three times with HBSS containing 10% heat-inactivated fetal bovine serum (FBS, Microbiological Associates) and were collected by centrifugation at 150 *g* for 10 min at 20°C. If the mononuclear cells were to be cultured they were then resuspended in RPMI 1640 (Microbiological Associates) with 100 U penicillin/ml, 100  $\mu$ g streptomycin/ml, L-glutamine 2 mM (Microbiological Associates), and 10% human AB serum. If the mononuclear cells were to be fractionated into T- and B-enriched populations they were placed into some media but containing 20% FBS.

*Fractionation of T- and B-Cell Populations.* Sheep erythrocytes (SRBC) obtained from Flow Laboratories Inc., Rockville, Md. were washed two times with HBSS. The washed SRBC were suspended in HBSS at a concentration of  $1.5 \times 10^6$  cells/ml. Equal volumes of human lymphocyte and SRBC suspensions were mixed and centrifuged at 50 *g* for 5 min at 20°C and maintained at 4°C for 16 h as described by Steel et al. (9).

The pellets were then gently resuspended, and 35 ml of the SRBC lymphocyte suspension were layered over 12 ml of Ficoll-Hypaque in 28 x 106-mm polycarbonate tubes (Arthur H. Thomas Co., Philadelphia, Pa.). The cells were centrifuged at 400 *g* for 40 min at 4°C. Rosetted T lymphocytes in the pellet beneath the Ficoll-Hypaque were washed twice with 0.83% ammonium chloride 0.17 M Tris buffer, pH 7.2 to lyse the SRBC and were collected by centrifugation (150 *g* for 10 min at 20°C). The unfractionated and T-lymphocyte preparations were washed three times with HBSS with 10% FBS and resuspended in the final culture medium: RPMI 1640 with 100 U penicillin/ml, 100  $\mu$ g streptomycin/ml, 2 mM glutamine, and 20% heat-inactivated human AB serum at a concentration of  $10^6$  mononuclear cells/ml.

*Lymphocyte Proliferation Stimulated by PHA.* Unfractionated or purified T lymphocytes were cultured in triplicate in sterile multiwell round bottom plates (Linbro IS-MRC-96-TC, Linbro Chemical Co., New Haven, Conn.) in a total vol of 0.2 ml and incubated in the absence and presence of 0.5  $\mu$ g of purified PHA (Burroughs-Wellcome Co., Research Triangle Park, N. C.). This dose of PHA has been found to stimulate maximal thymidine incorporation. Cultures were incubated in a 5% CO<sub>2</sub>/95% humidified air environment. DNA synthesis during the last 24 h of culture was assessed by thymidine incorporation. 1  $\mu$ Ci of methyl [<sup>3</sup>H]thymidine (sp act 2 Ci/mM, Amersham/Searle Corp., Arlington Heights, Ill.) in 1  $\mu$ l was added to each culture well. At the end of the incubation period, the lymphocytes were aspirated from the wells, transferred to glass fiber filter paper (Reeve-Angel, Inc., Clifton, N. J.), and washed with water using an apparatus based on the design of Hartzman et al. (10). The glass fiber disks were placed into 15 x 45-mm vials and 2.5 ml of Aqueous Counting Scintillant (Amersham/Searle Co.) were added. These mini-vials were counted in a Searle ambient temperature liquid scintillation counter. The average thymidine incorporation in counts per minute of the replicate cultures is given. The counting efficiency for tritium under these conditions was 34%.

*Limiting Dilution Analysis.* 24 replicate cultures each containing the same number of lymphocytes were established. One half of the lymphocyte cultures were incubated with PHA and one half without PHA. A series of such 24 replicate cultures was set up, each with a different number of lymphocytes ranging from 10<sup>3</sup> to 10<sup>4</sup> cells per well. The samples were cultured with PHA and

harvested as described above. An individual culture was considered to have a positive response to PHA when thymidine incorporated was greater than two standard deviations above the mean of thymidine incorporated by the 12 cultures containing the same number of lymphocytes incubated without PHA. The percent negative responses at each lymphocyte level was determined and least squares regression analysis used to obtain the plot relating the percentage of negative responses to the number of lymphocytes in the cultures. The percentage of negative responses depends on the frequency of responding units and the size of the aliquot as given by the formula  $P = e^{-fa}$  ( $P$  = percent negative responses;  $f$  = frequency of responsive units;  $a$  = number of lymphocytes in culture). When  $f = a$ , the percentage of negative responses would equal 37%.

*Kinetic Analysis of T-Lymphocyte Proliferation Stimulated by PHA.* The number of responding T lymphocytes and the time of their entry into DNA synthesis was estimated using colchicine to arrest mitosis as previously described (11).

Briefly, T lymphocytes were cultured as described above. 0.02 ml of 5  $\mu$ g/ml colchicine (Sigma Chemical Co., St. Louis, Mo.) was added to the cultures at the outset of the incubation period or at 24-h intervals thereafter. [ $^3$ H]thymidine was added 24 h after the addition of the colchicine and harvested 24 h later. Incubation periods ranged from 48 to 168 h. T lymphocytes assessed in this manner were also cultured in the standard fashion with PHA. Other cultures were incubated with colchicine medium from the outset and thymidine incorporation measured during the last 24 h of incubation which ranged from 120 to 192 h.

*Enumeration of Concanavalin A (Con A) Responsive Lymphocytes by Virus Plaque Assay.* The number of Con A-responsive cells was assessed by enumerating the number of lytic foci on an L-cell monolayer produced by a lymphocyte preparation exposed to VSV as described by Jimenez and Bloom and Sutcliffe et al. (12, 13). 30 ml of venous blood from both old and young donors were defibrinated and mixed with a 3% solution of gelatin (Difco Laboratories, Detroit, Mich.) in 0.15 M NaCl and allowed to sediment for 1 h at 37°C. The gelatin-serum-lymphocyte mixture obtained was incubated in a nylon-wool column (Associated Biomedic Systems, Buffalo, N. Y.) which had been prewet with RPMI 1640 containing 15% fetal bovine serum (Microbiological Associates, Bethesda, Md.) for 30 min. The effluent cells were treated with 0.83% ammonium chloride 0.17 M Tris to lyse erythrocytes (14), were washed three times with HBSS, and were collected by centrifugation at 50  $g$  for 10 min at 20°C. After the third wash, the cells were resuspended in RPMI 1640 containing 6% heat-inactivated FBS, 100 U penicillin/ml, 100  $\mu$ g streptomycin/ml, and 2 mM L-glutamine. 2 million cells were incubated in 2 ml of culture medium in the presence or absence of 20  $\mu$ g of Con A (Sigma Chemical Co.) in loosely capped plastic 12 x 75-mm tubes (Falcon Plastics no. 2054) at 37°C in a 5% CO<sub>2</sub>/95% humidified air environment for 72 h.

VSV harvested from chicken embryo fibroblasts was added to the lymphocytes at the end of the culture period at a multiplicity of approximately 20 to 1. The infected cultures were incubated for 2 h at 37°C. After incubation the cultures were centrifuged and free virus neutralized by incubation in a potent guinea pig anti-VSV serum for 1 h at 4°C. The cells were then washed extensively in minimal essential medium (MEM) supplemented with 6% FCS to remove antibody and antibody-virus complexes. Viable counts were obtained on each culture using trypan blue exclusion before plating.

Indicator monolayers were prepared from mouse L cells. The infected lymphocytes were plated in MEM supplemented with 6% FCS, antibiotics, and glutamine in 1% agar above the L-cell monolayer in 60-mm Petri dishes. Three log dilutions of lymphocytes, each in duplicate, were made for each culture. The plates were then incubated in humidified air in 5% CO<sub>2</sub> at 37°C for 36 h, fixed in 10% formalin in saline, and stained with crystal violet. Plaques are counted, and the number of plaque-forming cells per million plates was calculated.

*Statistical Analysis.* Statistical analysis of data comparing the function of lymphocytes from old or young persons made use of the paired Student's  $t$  test or the sign test (15).

## Results

*Limiting Dilution Assay of PHA-Responsive Units in Lymphocyte Preparations from Old and Young Donors.* The impaired response to PHA of lymphocytes from old persons reported in our previous studies (3) did not appear to be the result of a decreased number of T lymphocytes in the lymphocyte preparation. This earlier observation was confirmed. Monocytes were assayed by the

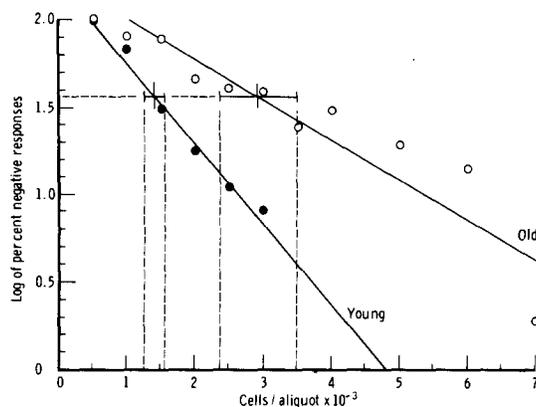


FIG. 1. Fluctuation analysis was performed on limiting dilutions of lymphocytes from 9 young and 10 old subjects. Replicate cultures containing a range of lymphocyte concentrations were cultured in the presence or absence of PHA. The percent of negative responses at each lymphocyte concentration for young and old populations was determined. The mean response of the old or young populations at each cell concentration is shown. Thus, the total number of positive responses in cultures from all young or from all old persons was divided by the total number of cultures with PHA established at this cell concentration. Least squares regression analysis was used to obtain the linear plot shown which relates the percent negative responses to lymphocyte number. The 95% confidence interval on the reciprocal of the aliquot size at which 37% of the cultures were unresponsive is given for each population.

staining for peroxidase granules (16), and T cells were assayed by SRBC rosetting (9). Based upon assays of 10 paired lymphocyte preparations from old and young subjects, monocytes made up 15–20% and T lymphocytes made up 45–50% of the mononuclear cell preparation. Old and young donors did not have significantly different numbers of monocytes or T lymphocytes in the mononuclear preparations. Blood from young donors yielded a larger, but not statistically significantly increased number of mononuclear cells per ml of blood: young  $1.42 \pm 0.24$ ; old  $1.09 \pm 0.15 \times 10^6/\text{ml}$  blood. In the present studies we have determined the frequency of mitogen-responsive units in lymphocyte preparations from young and old subjects by fluctuation analysis of PHA response at limiting dilutions. In this all or none assay, statistical theory allows the frequency of responsive units to be calculated. The frequency of responsive units equals the reciprocal of the number of cells which in replicate cultures gives 37% negative responses, as determined from a semilog plot relating the percent of negative responses to the number of cells cultured (Fig. 1). The percentage of negative responses at several lymphocyte concentrations from 9 young and from 10 old persons was determined and the number of responding units calculated from the semilogarithmic plot of the data. From the cumulative data illustrated in Fig. 1, it can be seen that the frequency of PHA responsive units is 1 per 1,400 mononuclear cells from young persons and 1 per 2,950 mononuclear cells from old persons, and it is clear that the difference is highly significant.

*Enumeration of Activated Lymphocytes from Young and Old Persons.* Resting lymphocytes are refractory to VSV infection, while activated T lymphocytes support the multiplication of VSV. The number of activated lymphocytes in a population can be enumerated by counting the number of lytic foci

TABLE I  
*Virus Plaque Formation by Young and Old Lymphocytes Cultured in Medium with or Without Con A*

Experiment number	Age of donor	PFU/10 <sup>6</sup> lymphocytes		$\Delta$ PFU per 10 <sup>6</sup> lymphocytes	Old PFU/young PFU
		Con A			
		Absent	Present		
1	22	3,402	297,845	294,443	0.134
	86	4,253	43,667	39,414	
2	24	67	16,518	16,451	0.004
	75	68	138	70	
3	38	525	25,683	25,158	0.190
	75	870	5,654	4,784	
4	25	393	5,849	5,456	0.510
	84	1,502	4,288	2,786	
5	28	73	139,617	139,554	0.053
	65	144	7,501	7,357	
6	23	1,782	28,603	26,847	0.450
	65	3,634	15,725	12,091	
Mean .....					0.224

Lymphocytes from young and old individuals were incubated for 72 h with or without Con A. These cells were exposed to vesicular stomatitis virus, washed, and exposed to anti-VSV antibody. Aliquots of the virus-infected cell suspension were poured over an L-cell monolayer. Lytic foci in the monolayer were enumerated after 48 h of incubation. The number of lytic foci (plaques) per 10<sup>6</sup> lymphocytes plated are reported.

on an L-cell monolayer produced by a lymphocyte preparation infected with VSV. The number of activated lymphocytes per million viable cells from old or young persons were compared at the end of a 72-h culture period in the presence or absence of Con A (Table I). Six experiments were performed. In the absence of Con A, the number of virus-producing cells in cultures from old persons was greater than in cultures from young persons. This difference was found in five out of the six experiments and is statistically significant ( $P < 0.02$ ). The survival of cells from old or young persons in culture without Con A was comparable: 74% of young lymphocytes and 72% of old lymphocytes were viable by trypan blue exclusion after 72 h in culture. Although there was an increased number of activated lymphocytes in cultures from old subjects without mitogen, Con A activated fewer lymphocytes in cultures from old persons. In each of the six experiments performed, Con A activated more lymphocytes in cultures from young than in cultures from old subjects. This difference is statistically significant ( $P < 0.04$ ). The number of Con A-activated lymphocytes from old persons was approximately one-fifth the number of Con A-activated lymphocytes from young persons. In the presence of Con A, the survival of lymphocytes from old persons (46%) was significantly less than the survival of cells from young persons (67%). Consequently, if the results had been expressed as the number of

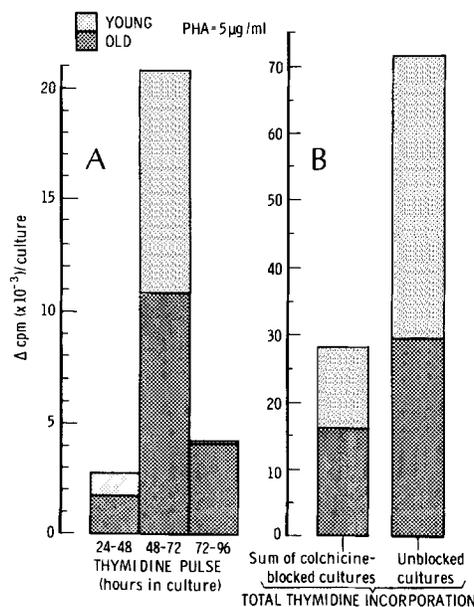


Fig. 2. PHA-induced thymidine incorporation by lymphocytes from young or old subjects was measured in culture. In all cases thymidine incorporation was measured during a 24-h period. In the absence of colchicine unblocked cultures, thymidine incorporation was measured during the 4th day of lymphocyte culture. In colchicine-blocked cultures, colchicine was added to achieve a final concentration of  $0.5 \mu\text{g/ml}$  24 h before the addition of thymidine. Total thymidine incorporated by colchicine block cultures was calculated by adding the amount of thymidine incorporated on the 2nd, 3rd, and 4th day of culture.

activated lymphocytes per initially cultured cells, the difference between young and old persons would be even greater.

*Cytkinetics of the PHA-Induced Thymidine Incorporation by T Lymphocytes from Old and Young Persons.* T-lymphocyte preparations from both old and young persons contained 90–95% cells which formed rosettes with sheep erythrocytes. Thymidine incorporated by T lymphocytes from old subjects incubated with PHA for 96 h was less than that incorporated by T cells from young subjects (Fig. 2B). In each of seven experiments, PHA-induced thymidine incorporation by T lymphocytes from old persons was less than that by T lymphocytes from young persons. Thymidine incorporation by T lymphocytes from old subjects cultured without colchicine (unblocked) averaged 42% of that of lymphocytes from young subjects. This difference is statistically highly significant ( $P < 0.003$ ).

To assess the number of lymphocytes initially responding to PHA, thymidine incorporation was measured 24 h after the addition of colchicine to prevent cells from entering a second round of thymidine incorporation. Under these conditions (11) the [<sup>3</sup>H]thymidine incorporation measured during the first 96 h of culture reflects activation of cells newly triggered into DNA synthesis. Thus, it was found (data not presented) that the amount of thymidine incorporated by lymphocytes during each 24-h period after a 24-h incubation with colchicine was identical to that incorporated during the same 24-h pulse when colchicine was present from the initiation of the culture. It is clear that if one assumes that

nucleotide transport and pool size are comparable, thymidine incorporation under these conditions, in the presence of colchicine, reflects the number of first generation responding cells passing through the S phase of the cell cycle during the labeling period. Based upon colchicine block and thymidine pulse experiments (see details in Fig. 2), it was found that first generation responding T lymphocytes from old or from young persons entered DNA synthesis at the same time. In all seven experiments, maximal thymidine incorporation by first generation responding T lymphocytes occurred between 48 and 72 h. The rate of entry of first generation responders into DNA synthesis was not altered over a 50-fold range of PHA concentrations. Thymidine incorporation by T lymphocytes from young subjects was significantly greater ( $P < 0.005$ ) during the first two 24-h labeling periods (24–48 and 48–72 h). The greatest difference between young and old T cells with regard to first generation responding lymphocytes was between 48 and 72 h of culture. There was no difference in thymidine incorporation by lymphocytes from old and young donors during the 72–96-h period.

To estimate the total number of first generation responding T lymphocytes, thymidine incorporated during each of the 24-h labeling periods between 24 and 96 h of colchicine-blocked cultures were summed. In each of the seven experiments, total thymidine incorporated by first generation responding T lymphocytes from old individuals was less than that by T lymphocytes from young individuals. The summated thymidine incorporation by T lymphocytes from old persons averaged 57% of that incorporated by T lymphocytes from young donors. This difference is statistically highly significant ( $P < 0.008$ ). The conclusion that there are fewer first generation responding lymphocytes in T-lymphocyte preparations from old persons is in accord with the results of limiting dilution analysis and the VSV plaque assay.

Although the amount of thymidine incorporated by first generation responding lymphocytes from old persons is less than that incorporated by first generation responding lymphocytes from young persons, this difference is not as great as that observed in cultures without colchicine. Thus, total thymidine incorporated by lymphocytes from old persons cultured with colchicine was 57% of that incorporated by lymphocytes from young persons, while in the absence of colchicine, lymphocytes from old persons incorporated only 39% as much as was incorporated by lymphocytes from young persons. In unblocked cultures, thymidine incorporation by second and third generation cells contribute to the total thymidine incorporated. These results imply that in cultures from old persons, the response of second or third generation lymphocytes to PHA is even more markedly impaired than is the response of first generation lymphocytes to PHA.

*Response to PHA of Lymphocytes Derived from First Generation PHA-Responding Cells.* The hypothesis that the difference in thymidine incorporation by lymphocytes from old and young persons increases with each division in culture was tested. Thymidine incorporation by increasing numbers of lymphocytes from old or young persons was compared (Fig. 3). Lymphocytes from young persons showed an exponential increase in thymidine incorporation as the number of cells in culture increased, suggesting that under these culture conditions, progeny of initially responsive cells contribute to the total thymidine incorporated. When colchicine was added to cultures of lymphocytes from young persons, a linear rise in thymidine incorporation with increasing cell number

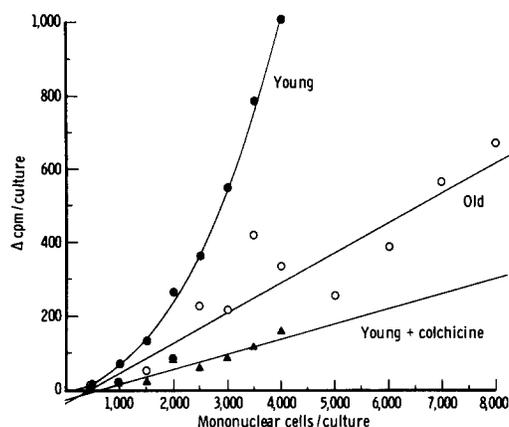


FIG. 3. PHA-induced thymidine incorporation by lymphocytes from 10 old and 9 young persons was measured in culture. Increasing numbers of lymphocytes were placed into culture with PHA and the relationship between the amount of thymidine incorporated/culture and the number of cells in culture determined. Some cultures of lymphocytes from young persons contained colchicine at a final concentration of 0.5  $\mu\text{g/ml}$ .

occurred. This supports the interpretation that exponential rise in thymidine incorporation observed in cultures of lymphocytes from young subjects results from the response of second or third generation lymphocytes to PHA. In contrast to the behavior of cells from young subjects, thymidine incorporation by lymphocytes from old persons in the absence of colchicine showed a linear rise as the number of cells in culture increased. This would imply a lack of proliferation by the progeny of initially activated cells. The observation is thus consistent with the data presented above suggesting a defective proliferation by progeny of first generation responding cells from old subjects.

*Observation of a Second Peak of Thymidine Incorporation by Colchicine-Treated Lymphocytes.* In view of the increased proliferative capacity of lymphocytes from young subjects, a search for a second wave of thymidine incorporation in culture was investigated. When thymidine incorporation was measured during consecutive 24-h periods from 24 to 168 hs, a second peak of thymidine incorporation was observed in cultures from young persons but not in cultures from old persons (Fig. 4). The late peak of thymidine incorporation was separated from the first peak by a valley. The time at which the second peak was seen varied in different individuals from the 5th to the 7th day of culture. As noted earlier, the sum of thymidine incorporation between 24 and 96 h expressed as a percent of the unblocked cultures is greater in the old subject. For example, in the experiment illustrated in Fig. 4, the colchicine-blocked thymidine incorporation was 42% of thymidine incorporation by unblocked cultures from old persons. In cultures from young persons the colchicine-blocked thymidine incorporation was 32% of thymidine incorporation by unblocked cultures. Data from seven experiments are summarized in Fig. 5. A late peak of thymidine incorporation by lymphocytes from young subjects was seen in each of seven experiments, and the increment from the valley was statistically significant ( $P < 0.03$ ). The small increment in thymidine incorporation by lymphocytes from old subjects was not statistically significant.

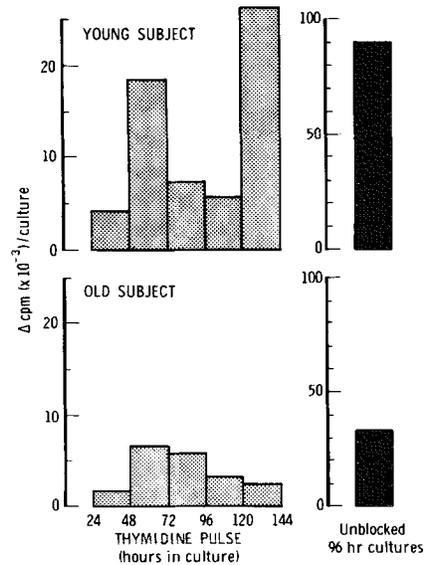


FIG. 4. PHA-induced thymidine incorporation by lymphocytes from an old and young person during the 2nd-6th day of colchicine-blocked cultures and during the 4th day of unblocked cultures was measured. In all cases, thymidine incorporation was measured during the 24-h period indicated. In the colchicine-blocked cultures, colchicine was added 24 h before the addition of thymidine.

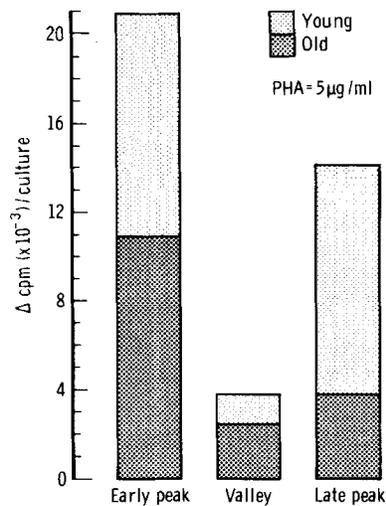


FIG. 5. Peak thymidine incorporation by lymphocytes from seven old and seven young persons during the 2nd-7th day of colchicine-blocked PHA culture is shown. The first peak of thymidine incorporation occurred on the 3rd day of culture. The average amount of thymidine incorporated by lymphocytes from young or old subjects is shown. After this first peak of thymidine incorporation the amount of thymidine incorporation declined to be followed in young subjects by a significant increase, the late peak, in thymidine incorporation.

The late peak of thymidine incorporation did not occur when colchicine was added at the initiation of the culture. This implies that the late peak reflected DNA synthesis by cells derived from the proliferation of first generation responding cells. The failure to see a second peak of thymidine incorporation with cells from old subjects provides additional support for the hypothesis that with old persons the response of progeny of the initial responding cell is more impaired than is the response of the initially responsive lymphocytes.

### Discussion

This study investigated the basis of the impaired thymidine incorporation by lymphocytes from old persons. Two mechanisms of this defect were considered: (a) a deficiency of mitogen-responsive lymphocytes in old persons, and (b) a failure of mitogen-responsive lymphocytes to expand into a pool of proliferating cells. Three independent methods were used to quantitate the number of mitogen-responsive lymphocytes: (a) VSV plaque formation, (b) limiting dilution analysis, and (c) incorporation of thymidine by first generation responding lymphocytes. All three assays showed that lymphocyte preparations from old persons had fewer mitogen-responsive cells than did preparations from young persons. The percentage of mitogen-responsive lymphocytes from old persons relative to preparations from young persons was 22% by VSV plaque assay, 47% with limiting dilution analysis, and 55% by the assessment of first generation responding lymphocytes. This defect cannot be explained by a relative deficiency of T lymphocytes in cell preparations from old persons. First of all, our studies and those of other investigators (2, 8) found no decrease in the percentage of T lymphocytes in mononuclear preparations from old persons. Furthermore, in studies in which a decreased percentage of T lymphocytes from old persons was found (4, 6, 7) this reduction was not of sufficient magnitude to explain the defect we have demonstrated. Finally, the defect in thymidine incorporation by mononuclear preparations from old persons was also apparent when purified T lymphocytes from old and young persons were compared. Thus, the impaired response of lymphocytes from old persons to plant lectins reflects a qualitative defect in the T-cell population itself. Although there is no reduction in the number of T lymphocytes, the functional capacity of a T-cell subpopulation is impaired. The level of thymic hormone has been reported to decline with age (17). The capacity of thymic hormones to facilitate differentiation of functionally active lymphocytes (18) raises the possibility that the decline of thymic hormone during aging may be the basis for the loss of the mitogen-responsive subpopulation of T lymphocytes.

The age-associated impairment of thymidine incorporation by lymphocytes cultured with PHA is greater in the absence of colchicine than in the presence of colchicine. This suggests that mitogen-responsive lymphocytes from old donors fail to expand normally into a pool of proliferating cells. This interpretation is supported by the rise in thymidine incorporation by increasing numbers of lymphocytes cultured with PHA. In cultures from young donors, increasing the number of lymphocytes produced an exponential rise in thymidine incorporation. In the presence of colchicine, which prevents recruitment of progeny cells into the proliferation pool, the rise in thymidine incorporation by lymphocytes

from young donors was linear. Cultures from old donors, in the absence of colchicine, showed a linear rise in thymidine incorporation when the number of lymphocytes in culture was increased. This suggested a failure of progeny cells from old donors to enter the proliferating pool. The lack of a second peak of thymidine incorporation in cultures from old donors is also consistent with this interpretation. Therefore, the greater impairment of the second peak compared to the first peak of thymidine incorporation by lymphocytes from old donors was not unexpected because the second peak reflects thymidine incorporation by progeny cells.

Finally, the greater number of viable lymphocytes recovered in cultures with Con A from young as compared to old donors supports the conclusion that the pool of proliferating cells is reduced in cultures from old donors. The failure of lymphocytes from old persons to expand through cell division into a large pool of proliferating cells may be explained by Hayflick's hypothesis (19) that normal cells have a limited proliferative capacity. The proliferative capacity of cells taken from young subjects is greater than that of the same type of cells taken from old subjects. Thus, any age-associated defect in cell response is amplified as cells proliferate in cultures. This would lead to a greater impairment of thymidine incorporation with each proliferative cycle of cultured lymphocytes from old persons. In summary, the age-associated defect in the response of lymphocytes from humans results from a reduced number of mitogen-responsive cells and their failure to expand into a pool of proliferating cells.

### Summary

The basis for the age-associated defect in the response of lymphocytes to plant lectins has been studied. Using three independent assays we have shown that the number of mitogen-responsive cells is markedly reduced in lymphocyte preparations from old persons. In addition, studies using colchicine block and thymidine pulse techniques have revealed a failure of mitogen-responsive cells from old persons to expand into a proliferating pool of lymphocytes as is observed when lymphocytes from young persons are cultured with phytohemagglutinin. Thus, the impaired response of lymphocytes from old persons to mitogens is attributable to a reduced number of mitogen responsive cells and their failure to undergo clonal expansion.

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