

CYTOKINETIC ANALYSIS OF THE IMPAIRED PROLIFERATIVE
RESPONSE OF PERIPHERAL
LYMPHOCYTES FROM AGED HUMANS TO
PHYTOHEMAGGLUTININ*

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An age-related decline in human immunocompetence is thought to contribute to a variety of disorders that are increasingly frequent in the elderly. One important component of this decline in immune function is a loss in cell-mediated immunity (1-9). The *in vitro* stimulation of peripheral lymphocytes by various mitogens, especially phytohemagglutinin (PHA),¹ has been one of the most extensively employed systems for examining this component of immune cell response. Lymphocyte response to PHA has generally been assessed by (a) the enumeration of blastogenic cells by either morphologic criteria (10-13) or the presence of radioisotopic label indicating active DNA synthesis (11, 14), (b) determination of mitotic indices (11, 13, 14), or (c) quantifying DNA synthesis levels with tritiated thymidine incorporation (15-19). By utilizing these methodologies, it has generally been concluded that the number of PHA-responsive cells declines in peripheral lymphocyte cultures as a function of donor age (11-18).

It had been suggested that these experimental results could be the result of a decline in the absolute number of T cells in the elderly. However, although some investigators have observed a decrease in T-cell number with age (16, 20, 21), others have been unable to substantiate this claim (17, 22-24). Furthermore, the age-related decline in mitogen reactivity does not appear to result from differences in tissue-culture requirements (11, 17, 25, 26), cell survival (25, 26), the number of mitogen receptors (25, 27), or to mitogen-receptor binding affinities (25, 27). One important aspect of this experimental system that has not been adequately examined is whether an alteration in the cell-cycle duration of responding lymphocytes from aged individuals could contribute to the observed decline in mitogen responsiveness.

The studies presented here utilize a new cytogenetic technique to examine both the rate of PHA stimulation as well as the resultant proliferation kinetics for lymphocytes cultured from young and aged individuals. The technique utilized for these studies, BISACK,² involves the growth of cellular populations in the presence of noninhibitory

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¹ Abbreviations used in this paper: BrdU, bromodeoxyuridine; PHA, phytohemagglutinin.

² Bromodeoxyuridine incorporation system for the analysis of cellular kinetics.

concentrations of bromodeoxyuridine (BrdU) and the subsequent analysis of BrdU incorporation patterns in metaphase cells as a function of time (22, 28, 29).

Materials and Methods

Culture Conditions. Heparinized venous blood was obtained from four young (19–23 yr) and four aged (74–76 yr) male donors. The aged donors were healthy, nonhospitalized volunteer members of the Longitudinal Study, Baltimore Gerontology Research Center, National Institutes of Aging, whereas the young donors were healthy laboratory personnel. Whole blood was cultured as described previously (29, 30). The level of BrdU which permitted normal cellular proliferation was determined for both young and aged lymphocyte cultures by incubating PHA-treated (3.75% PHA-M, Grand Island Biological Co., Grand Island, N.Y.) cultures from young and aged individuals in the presence of increasing concentrations of BrdU (7.5–300 μM , Sigma Chemical Co., St. Louis, Mo.) for 72 h. Also, the effect of PHA concentration on young and aged lymphocyte proliferation kinetics was compared by incubating cultures containing 25 μM BrdU with increasing levels of PHA-M (1.00–6.25%) for 68 h. Subsequent cellular kinetic experiments utilized cultures incubated continuously with 3.75% PHA-M at 25 μM BrdU.

Culture Termination and Cytogenetic Analysis. 1 h before termination, Colcemid (Grand Island Biological Co.) was added to a final concentration of 0.1 $\mu\text{g}/\text{ml}$. Cells were harvested by centrifugation, resuspended in 0.075 μM KCl for 20 min, and then fixed twice in 3:1 methanol-glacial acetic acid. The fixed cells were air-dried on microscope slides and then stained with either acridine orange or Hoechst 33258 (American Hoechst Pharmaceutical Co., Kansas City, Mo.) as described in detail elsewhere (22). Either staining technique was used with equal facility with no difference in results. 100 consecutive metaphase cells were examined in each culture and identified as having replicated for one, two, or three and more generations based on their fluorescent labeling patterns (30). In brief, cells which have divided once in the presence of BrdU contain chromosomes at metaphase which fluoresce uniformly bright, cells which have divided twice contain chromosomes at metaphase in which one sister chromatid fluoresces dull, and the other sister chromatid bright although after three divisions, some chromosomes at metaphase fluoresce completely dull. 500 consecutive interphase cells were examined for fluorescent intensity in each culture to determine the cumulative proportion of cells which had completed at least one round of DNA synthesis in the presence of BrdU. Completion of one round of DNA synthesis was indicated by a readily observable uniform decline in the fluorescent intensity of interphase nuclei (22, 29). 1,000 consecutive cells were examined in each culture to determine mitotic indices. All measurements were made on coded slides without knowledge of the age of the donor.

Results

Effect of Increasing BrdU Concentrations on Cellular Kinetics in Lymphocyte Cultures from Young and Aged Individuals. The effect of increasing concentrations of BrdU on the frequencies of first generation metaphase cells in young and aged lymphocyte cultures terminated 72 h after PHA stimulation are seen in Fig. 1. Inhibition of cellular proliferation is reflected in both young and aged lymphocyte cultures by an increasing proportion of first generation metaphase cells at BrdU concentrations $> 35 \mu\text{M}$. Below 35 μM BrdU, the constant proportion of first generation metaphase cells in both young and aged lymphocyte cultures indicates a lack of inhibition by BrdU on cellular kinetics. This finding is in agreement with previous observations for human lymphocytes proliferating under these in vitro conditions (28). Related experiments also suggest that the degree of inhibition is related to BrdU-thymidine pool size and not to incorporated levels of BrdU into the cellular DNA (R. R. Tice and E. L. Schneider, unpublished data). Therefore, all further experiments involving lymphocytes from both young and aged individuals were performed at 25 μM BrdU.

Effect of PHA Concentration on Cellular Kinetics in Lymphocyte Cultures from Young and

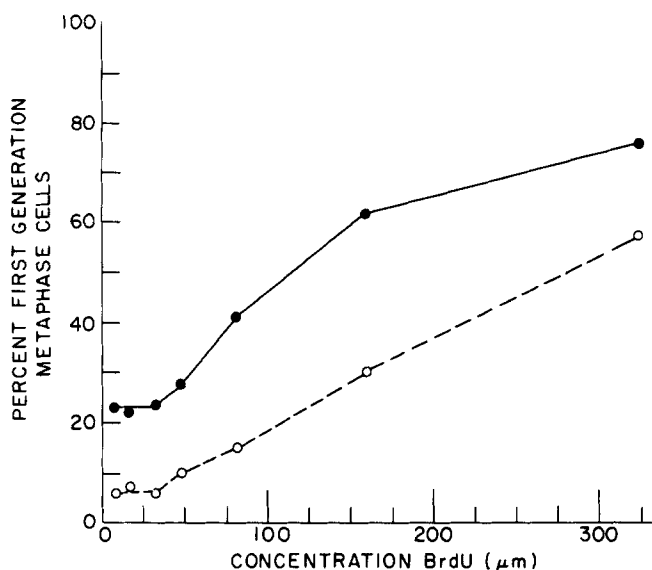


FIG. 1. Frequency of first generation metaphase cells as a function of BrdU concentration in young (○) and aged (●) 72-h lymphocyte cultures. Data points represent average values derived from two young and two aged individuals, each having duplicate cultures.

Aged Individuals. The effect of different concentrations of PHA on the frequency of first generation metaphase cells, mitotic indices, and percent replicated cells is presented in Fig. 2. In both young and aged lymphocyte cultures the frequency of first generation metaphase cells remained constant over the range of PHA concentrations examined. In contrast to the stability of the frequency of first generation metaphase cells, both the mitotic index and percent replicated cells increased and then decreased with increasing concentrations of PHA. For each parameter the replicative response of aged lymphocyte populations was significantly reduced at each concentration of PHA when compared to young lymphocyte populations. However, the maximum response to PHA was elicited in both cell populations over the same PHA concentration range (2.50–3.75%). Subsequent studies on cellular kinetics in this *in vitro* system were therefore conducted by using a PHA concentration of 3.75%.

Cellular Kinetics. The individual distributions of first, second, and third or subsequent generation metaphase cells as a function of time after PHA addition for four young and four aged individuals are presented in Fig. 3, although the combined kinetic distributions for young and aged populations are presented in Fig. 4. The proliferation kinetics of PHA-stimulated lymphocytes from young individuals (Fig. 3A–D) appears to be relatively homogeneous when compared to the variability observed in the aged group (Fig. 3E–H). This occurrence of increased intersample variability in the elderly is a well-known observation in aging studies (31). Because care was taken to select healthy-aged individuals, it is quite possible that if these studies had used individuals selected from a hospitalized or clinic population, greater variability would have been observed. In both young and aged lymphocyte cultures, second generation metaphase cells first appeared at 48 h and third generation metaphase cells at 60 h after culture initiation. However, although only second and subsequent generation metaphase cells are present 96 h after PHA stimulation in

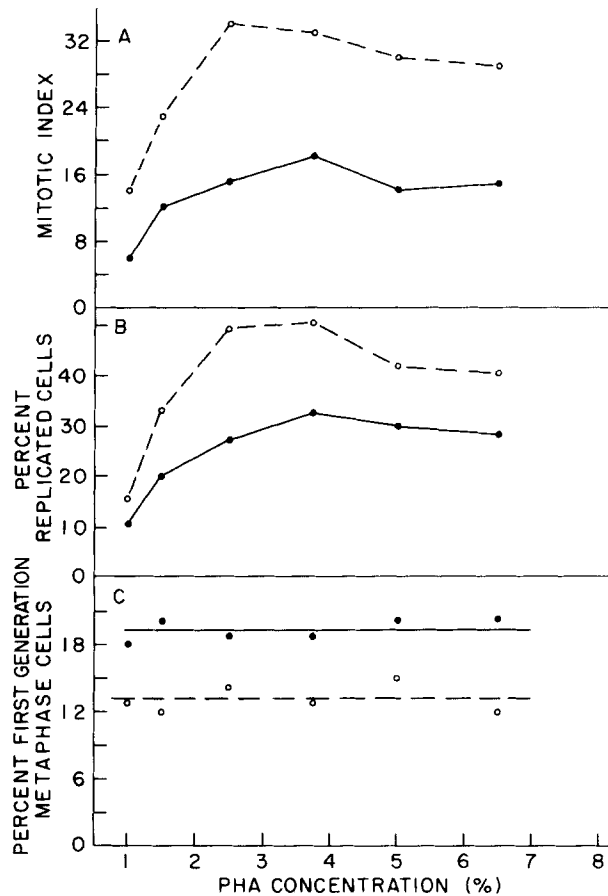


FIG. 2. Mitotic index, percent replicated cells, and proportion of first generation metaphase cells in young (○) and aged (●) lymphocyte cultures as a function of PHA concentration. A. The number of mitoses per 1,000 interphase cells. B. The proportion of interphase cells exhibiting uniformly depressed fluorescence per 500 interphase cells. C. The proportion of first generation metaphase cells per 100 metaphase cells. Data points represent average values derived from two young and two aged individuals, each having duplicate 68-h cultures.

young lymphocyte cultures, first generation metaphase cells still comprise 10–20% of the metaphase population in aged lymphocyte cultures. Also, in contrast to the relatively sharp peak of second generation metaphase cells observed in young lymphocyte cultures, the second generation peak in aged lymphocyte cultures was much more dispersed.

The identical appearance in time of second and third generation metaphase cells in both young and aged cell cultures indicates the existence of cells with the same proliferation kinetics in both populations. However, the dispersion of the peak of second generation metaphase cells combined with the late continued appearance of first generation metaphase cells in aged lymphocyte cultures suggests that a significant proportion of lymphocytes in these cultures respond at a reduced rate to PHA and/or have significantly longer cell-cycle durations. Semilogarithmic analysis of the appearance of second and third generation metaphase cells (the reciprocal of the disappear-

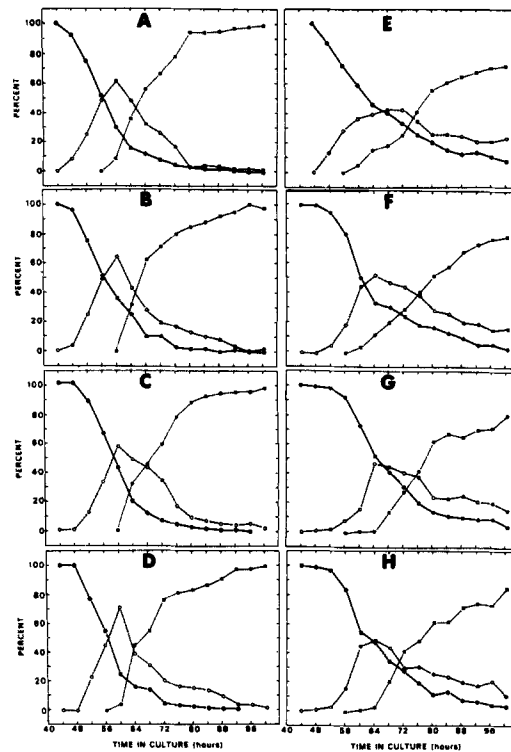


FIG. 3. Distribution of first generation (●), second generation (○), and third or subsequent generation (■) metaphase cells in young lymphocyte cultures (A-D) and aged lymphocyte cultures (E-H) as a function of time (40–100 h) after PHA and BrdU addition.

ance of first and first plus second generation metaphase cells respectively) clearly illustrates the difference between the proliferation response of young and aged lymphocytes to PHA (Fig. 5). Linear regressions for the appearance of young and aged lymphocyte populations in their second or third generation mitosis extrapolate back to 0% (or 100% for first and first plus second generation mitosis respectively) frequency at nearly identical intercepts (49.7 h vs. 50.7 h for the appearance of young and aged second generation mitosis; 60.3 h vs. 61.2 h for the appearance of young and aged third generation mitosis). Consequently, the minimal cell-cycle duration (as estimated from the differences in time between 0% frequency intercepts for second and third generation metaphase cells) remains essentially identical for both young (10.6 h) and aged (10.5 h) cell populations. However, a significant difference is observed between young and aged cell populations in the slopes of these regression lines: -0.052 (young) vs. -0.026 (aged) for the disappearance of first generation metaphase cells and -0.044 (young) vs. -0.018 (aged) for the disappearance of first plus second generation metaphase cells. The slope of the regression line for the disappearance of first generation metaphase cells also reflects the rate of PHA stimulation (29). Thus, comparison of these slopes between young and aged cell populations indicates that the rate of PHA stimulation of aged lymphocytes is approximately one-half of that for young lymphocyte population.

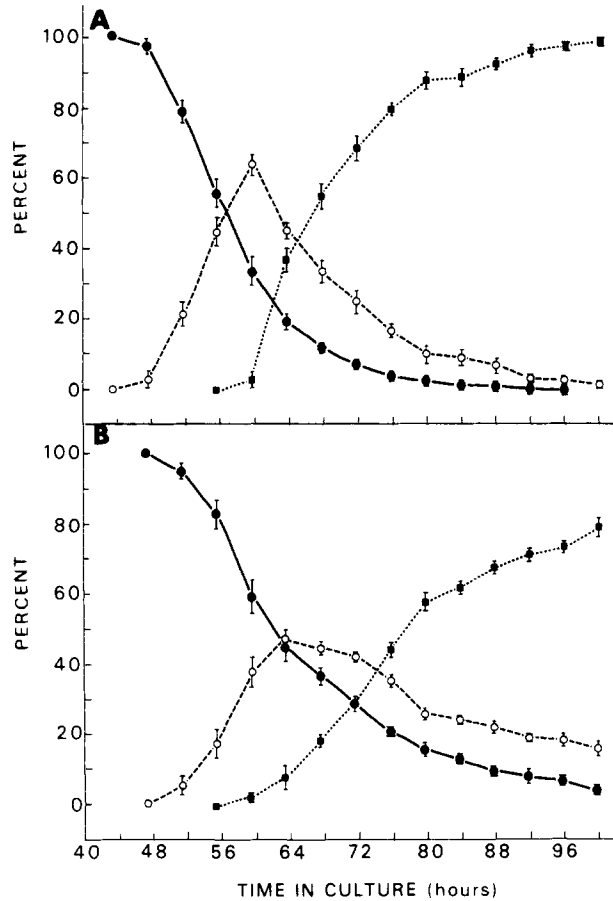


FIG. 4. Combined distribution of first generation (●), second generation (○), and third or subsequent generation (■) metaphase cells in young (A) and aged (B) lymphocyte cultures as a function of time (40–100 h) after PHA addition. Each data point indicates the mean and standard error of the mean for four individuals.

Cell-cycle durations for young and aged proliferating lymphocytes were determined by computer simulation analysis described in detail elsewhere (29). In brief, this model assumes that cells enter the proliferation pool exponentially, that once entered into this pool, cells never become inactive, and that cell cycle times are distributed in an exponential fashion. By using this computer model, cell-cycle durations for aged PHA-stimulated lymphocytes were estimated to range from 10.0 to 25.0 h, with a mean of 15.0 h although cell-cycle duration for young PHA-stimulated lymphocytes ranged from 10.6 to 15.6 h, with a mean of 12.3 h (29). Also the rate of PHA stimulation for aged lymphocytes was estimated by computer analysis to be 2.8%/h of the remaining unstimulated population versus 4.5%/h for young lymphocytes (Table I).

Mitotic Index and Percent Replicated Cells. To further examine the differences in response between young and aged lymphocytes to PHA, the mitotic index and percent replicated cells were measured as a function of culture time (Fig. 6). Although the mitotic peak occurred 72 h after culture initiation in young-cell cultures, the mitotic

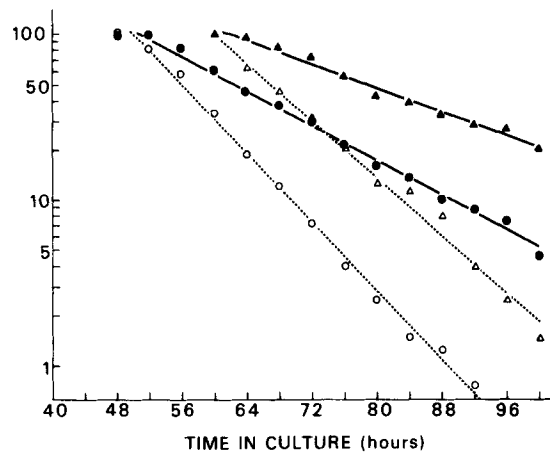


FIG. 5. Semilogarithmic plot of the frequency of first generation (O, ●) and first plus second (Δ, ▲) generation metaphase cells in young (O, Δ) and aged (●, ▲) lymphocyte cultures as a function of time (40–100 h) after PHA stimulation. The decrease of first generation metaphase cells with time is the reciprocal of the appearance of second generation metaphase cells whereas the decrease of first plus second generation metaphase cells is the reciprocal of the appearance of third generation metaphase cells. Each data point is the mean from four individuals. Dashed and solid regression lines were calculated by least squares analysis, each line having a correlation coefficient $r > 0.97$.

TABLE I
Comparison of PHA Responsiveness and Cell-Cycle Duration for Lymphocytes from Young and Aged Individuals

| | PHA Stim- ulation Rate | Cell-cycle duration | | |
|--------------|------------------------------|---------------------|------|---------|
| | | Minimum | Mean | Maximum |
| | %/h | | hr | |
| Young donors | 4.5 | 10.6 | 12.3 | 15.6 |
| Aged donors | 2.8 | 10.0 | 15.0 | 25.0 |

peak in aged cultures occurred at 96 h (Fig. 6 A). However, even at 96 h the magnitude of the mitotic peak in aged lymphocyte cultures appears to be depressed in comparison to the mitotic peak at 72 h in young lymphocyte cultures.

Although differentially labeled metaphase cells provide considerable information on cell proliferation kinetics, a more comprehensive view on population kinetics can be obtained by analysis of the interphase population as a whole. Interphase cells that have replicated in the presence of BrdU can be easily identified by a loss in fluorescent intensity. Examination of the percent replicated cells in young and aged cell cultures indicates that the accumulation of these cells is roughly parallel but apparently displaced in time (Fig. 6 B). Re-examination of these data plotted as a semilogarithmic function of the proportion of interphase cells which had not completed one round of DNA synthesis with time permits a better comparison between the proliferative response in aged and young cell cultures (Fig. 7). Determination of linear regressions by least square analysis indicates that the increase in percent replicated cells (reciprocal of the decline in nonreplicated cells) is significantly slower ($P < 0.005$) in aged cell cultures than for young-cell cultures ($\approx 20\%$ slower), presumably a result of increased cell cycle durations (mean cell-cycle time $\approx 22\%$ slower).

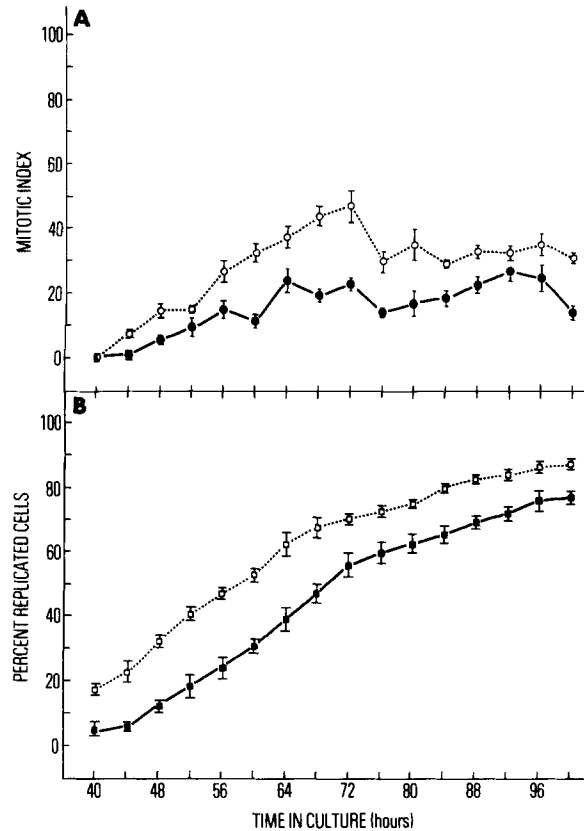


FIG. 6. The mitotic index (A) and percent replicated cells (B) in young (○, □) and aged (●, ■) lymphocyte cultures as a function of time (40–100 h) after PHA stimulation. Mitotic index is the number of mitoses per 1,000 interphase cells whereas the percent replicated cells is the number of interphase cells indicating the completion of at least one round of DNA synthesis in the presence of BrdU. Each data point is the mean and standard error of the mean calculated from four individuals.

Discussion

Thymus-dependent lymphocytes are an absolute requirement for cell-mediated immunity and the *in vitro* response of these cells to PHA has been used to assess *in vivo* mature T-cell activity (32, 33). The decline in PHA responsiveness with age is apparently not the result of a decline in T-cell number (17, 19, 23, 24) but rather the result of some intrinsic defect(s) in the ability of T cells to respond to mitogens (11–17, 24, 25–27). It has not been established whether or not the T cells which do respond to PHA subsequently proliferate at the same rate and/or to the same extent in both young and aged lymphocyte cultures.

Analysis of PHA responsiveness for lymphocytes from young and aged individuals utilizing the BrdU differential staining technique, BISACK, indicates that aged lymphocytes enter the stimulated pool more slowly approximately one-half the rate of young lymphocytes. Cell-cycle analysis of young and aged cell populations leads to the conclusion that although minimum cell-cycle duration remains the same, mean and maximum cell-cycle durations were significantly increased in aged lymphocyte populations. The identical temporal appearance of second and third generation

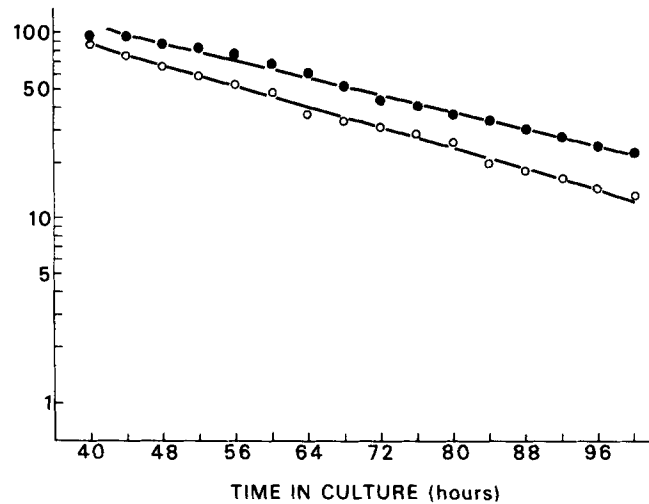


FIG. 7. Semilogarithmic plot of the frequency of interphase cells which have not undergone proliferation in response to PHA as a function of time (reciprocal of the appearance of interphase cells having completed at least one complete S period) in young (○) and aged (●) lymphocyte cultures. Dashed and solid regression lines were calculated by least squares analysis, each line having a correlation coefficient $r > 0.99$.

metaphase cells after PHA stimulation in both young and aged cell cultures correlates well with previous studies on the kinetics of DNA synthesis as well as the appearance of mitosis (14, 24). It has recently been suggested that aged human lymphocytes also fail to proliferate to the same extent as do young lymphocytes in culture (24). However, it is also possible that the increased cell-cycle durations observed here for aged lymphocytes may have contributed to this observation. This possibility is supported by the observation that the difference in the rated increase of percent replicated cells in young and aged cultures may be accounted for by the difference in mean cell-cycle duration.

Comparison of mitotic indices in young and aged cultures at maximal PHA concentrations indicates that not only is the magnitude of the mitotic peak depressed in aged cultures, in agreement with other published reports (9-14), but that the peak appears to be displaced in time, appearing later in aged cultures. Presumably, the total yield of mitosis is related in a direct manner to the total number of mitogen responding cells. The observation that after 72 h the mitotic yield declines in young cultures suggests a decline in proliferative activity. Because nutrient requirements for human lymphocyte cultures are not well established at present, perhaps quicker medium depletion in young cultures as a result of the greater number of proliferating cells present could account for the observed temporal difference in the mitotic peak.

Our finding of diminished entry of cells into the stimulated population and increased cell-cycle duration for lymphocytes from aged individuals is of particular interest in that it complements studies in other aging cell systems. First, an age-related increase in cell-cycle duration is well documented for a number of *in vitro* and *in vivo* cellular systems. *In vivo*, cell-cycle duration has been observed to increase with age for several typical cell renewal populations (34-37) and *in vitro* for human fibroblasts as a function of passage level (38, 39) or as a function of donor age cultures (40).

Second, an age-related decline in the number of responsive cells has also been observed for other quiescent cell populations. *In vivo*, regenerating liver (41), regenerating kidney tissue (42), and isoproterenol-induced stimulation of salivary gland tissue (43) all exhibit an age-related decrease in the number of responding cells. *In vitro* late passage (or senescent) confluent human embryonic lung fibroblast cultures exhibit diminished cell proliferation in response to the addition of fresh serum (44).

The exact cause of this diminished proliferative response observed in general for aging cells, and in particular for aged lymphocytes, remains unresolved. However there is some evidence to suggest that for quiescent cell populations the age-related decline may be related to the length of time cells remain in a nonproliferative state. The longer that human embryonic lung fibroblast cultures are kept in a quiescent state, the longer the mean prereplicative phase and the fewer the number of responding cells in response to a new stimulus (44, 45). This decreased replicative ability as a consequence of increased length of time in a nonproliferative state correlates well with the impaired response of aged lymphocytes (a normally quiescent population) to PHA, fewer cells responding with greater mean times. This correlation is further supported by the following observations: human lymphocytes recently stimulated *in vitro* by PHA exhibit a shorter prereplicative phase when subsequently stimulated a second time (46) and an augmented *in vitro* response to PHA is observed for lymphocytes from both young and aged individuals previously exposed *in vivo* to another antigen, the augmentation being greater in cultures from aged individuals (47).

Cellular aging has been described as a progressive conversion of proliferating cells from a cycling to a noncycling state (48). We would like to add that there also appears to be a progressive inability of normally quiescent cell populations to respond to a proliferating stimulus coupled with increased cell-cycle durations. The data presented here does not permit any conclusion being made as to whether the increase in cell-cycle duration is due to a general slowing of all phases of the cell cycle or whether one particular part of the cell cycle is critically affected. In investigations where the relative durations of each phase of the cell cycle has been determined in aging cell populations, the general finding has been that although the duration of both S and G₂ have remained relatively constant, the length of G₁ has increased with age (35, 38, 39). These observations and the findings presented here may perhaps be best explained by a model in which the aging of cell populations capable of proliferation results from alterations in transition probability, a mathematically ascertainable term defining the ability of a cell to initiate a proliferative response somewhere in the G₁ phase of the cell cycle (49, 50). Regardless of which model is most appropriate for cellular aging, the age-related decline in proliferative ability observed for lymphocyte populations *in vitro* may well reflect an important feature of the age-related *in vivo* loss of T-cell response. These studies clearly demonstrate the ability of BISACK to examine cell proliferation kinetics *in vitro* and provide insights into age-related alterations in cell kinetics not obtainable through other cell-cycle techniques. Further research will be directed at utilizing BISACK to examine age-related changes in cell proliferation kinetics *in vivo* with appropriate animal models.

Summary

The effect of donor age on the rate of cell entry into the proliferating pool and subsequent cell cycle duration for peripheral lymphocytes stimulated by phytohe-

magglutinin (PHA) were examined by using the bromodeoxyuridine incorporation-differential staining technique. Distribution curves for the appearance of metaphase cells in successive generations as a function of culture time were obtained and analyzed both graphically and by a computer simulation model. Peripheral lymphocytes from aged individuals (≈ 75 yr) were stimulated by PHA at approximately one-half of the rate of peripheral lymphocytes from young individuals (≈ 21 yr). Subsequent cell-cycle durations were estimated to range from 10.0 to 25.0 h for aged individual lymphocyte cultures and 10.6–15.6 h for young individual lymphocyte cultures. The possible significance of these findings to aging in general is discussed.

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