Decline in Phytohemagglutinin Responsiveness of Spleen Cells from Aging Mice (37524)

Y. HORI,[1] E. H. PERKINS, AND M. K. HALSALL

Biology Division, Oak Ridge National Laboratory,[2] and The University of Tennessee—Oak Ridge Graduate School of Biomedical Sciences, Oak Ridge, Tennessee 37830

Recent studies (1, 2) with thymus-dependent antigens have conclusively confirmed earlier demonstrations (3, 4) that there is a dramatic decline in humoral immune competence during senescence. Systematic studies to determine whether a comparable decline in cell-mediated immunity occurs are needed, as previous studies are not in agreement [for review see Ref. (5)]. More recently it has been postulated, and considerable interest has been generated in the concept, that age-related failure of immune surveillance mechanisms may play a prominent role in carcinogenesis and other age-dependent disease processes (6–9). Conclusive demonstration of a decline in cell-mediated immunity is essential if this concept is to remain tenable.

It is now clearly established that thymus-dependent lymphocytes (T cells) are an absolute requirement of cell-mediated immunity (10–12). It is generally accepted that phytohemagglutinin (PHA) selectively stimulates T cells (13–15), and therefore PHA stimulation of lymphocytes is commonly used as a measure of cell-mediated immunity (16, 17). Thus, it seems apparent that the *in vitro* blastogenic response of mouse spleen cells to PHA should be useful in identifying age-related immunodeficiencies that might exist in the T-cell population of the spleen, although it is recognized that PHA responsiveness at best measures only one parameter of cell-mediated immunity.

Materials and Methods. Mice used for these experiments were a long-lived hybrid (C57BL/Cum ♀ × C3H anf/Cum ♂)F_1 (hereafter referred to as $BC3F_1$) with a mean life-span of 31 months and a maximum life-span of 45 months and the shorter-lived BALB/c strain with a mean life-span of approximately 25 months. Spleens were removed under sterile conditions. Only spleens of normal size and free of pathology were used. Subsequent histological examination proved the spleens to be pathology free. Cell suspensions from individual spleens were prepared in Hanks' balanced salt solution, counted in a hemacytometer, and adjusted to contain 10×10^6 leucocytes/ml. Routinely, the culture media consisted of 89% RPMI-1630 (Grand Island Biological Co., N. Y.), 10% fetal calf serum, and 1% penicillin–streptomycin (5000 units penicillin and 5000 μg streptomycin/ml). Five replicate cultures were prepared for each spleen cell suspension in screw-cap Falcon flasks (30 ml capacity with a bottom area of approximately 5.25 cm^2) by adding 2.5×10^6 spleen cells (0.25 ml) to each flask, which contained 3.75 ml of the complete culture medium. Cultures were loosely capped and incubated at 37° in a 5% CO_2 atmosphere at 80% humidity.

Phytohemagglutinin (PHA, Wellcome) was reconstituted with sterile distilled water to 1 mg/ml. At the start of incubation 5 μg PHA was added to three culture flasks, and the two remaining flasks were incubated as non-PHA controls. Four microCuries of

[1] Postdoctoral Investigator supported by subcontract no. 3322 from the Biology Division of Oak Ridge National Laboratory to the University of Tennessee. Present address: Department of Medicine and Hygiene, Radiation Center of Osaka, Prefecture, Shinke-Cho, 704, Sakai-Shi, Osaka-Fu, Japan.

[2] Operated by Union Carbide Corporation for the U.S. Atomic Energy Commission.

tritiated thymidine (^{3}H-dThd) was added to each flask 6 hr before the termination of incubation. Determination of incorporated radioactivity was carried out on the acid-insoluble cell residues obtained in the following manner. After 48 hr of culture the flasks were chilled in an ice bath for 1 hr, and in a cold room (4°) the cells and media were removed to conical centrifuge tubes (13 × 100 mm). The flasks were rinsed with 4 ml cold normal saline and the rinse was added to the centrifuge tubes. Centrifugation was carried out at 3000 rpm for 15 min in the cold (4°) in a Sorvall (GLC-1) centrifuge. The supernatant fluid was discarded and the sedimented cells were resuspended and washed once with 4 ml normal saline. To induce complete cell lysis, 0.2 ml 0.01 *N* NaOH was added to each tube. The following day the cell lysates were transferred to filter-paper disks. The filter-paper disks were processed as follows: twice in 5% cold trichloroacetic acid for 10 min, once in 95% ethanol for 10 min, once in a 1:1 mixture of ethanol and ethyl ether for 10 min, and once in absolute ethyl ether. The air-dried filter-paper disks were placed into scintillation vials with 5 ml of scintillation fluid (Liquifluor, New England Nuclear, 40 mg PPO plus 50 mg POPOP/liter) for counting in a Beckman liquid scintillation spectrophotometer (LS-230). Results are expressed as the difference between radioactivity incorporated into PHA-stimulated cells and non-PHA controls per minute (Δ cpm) for 2.5 × 10^6 cells.

The relative number of θ-bearing spleen cells was determined with rabbit anti-θ serum ($BC3F_1$ brain). The serum was previously absorbed 3 times with $BC3F_1$ bone marrow and red cells to eliminate nonspecific cytotoxicity. At a 1:300 dilution, the absorbed serum killed > 90% of a thymus cell suspension. Ten million spleen cells in 0.1 ml Hanks' medium containing 10% decomplemented fetal calf serum was added to 0.1 ml of a 1:10 dilution of antiserum. After incubation for 30 min at 37° in a 5% CO_2 atmosphere the cells were washed 3 times, and 0.1 ml of 1:10 guinea pig complement (C′) which had been absorbed 3 times with 0.1 g Bacto agar/ml was added. After a 30-min incubation, the percentage of dead cells was determined from eosin dye uptake. Since the complement alone gave the same background levels of killing as did the negative control of antiserum without complement, the percent of cytotoxicity was determined by the following formula:

$$\%\ \text{cytotoxicity} = \frac{(\%\ \text{killed with anti-}\theta\ \text{serum} + \text{C}') - (\%\ \text{killed with anti-}\theta\ \text{serum only})}{100 - (\%\ \text{killed with anti-}\theta\ \text{serum only})} \times 100.$$

Results. To determine the optimal concentration of PHA, the length of spleen cell culture and the requirements for fetal calf serum in the culture medium, the following experiments were performed. Young adult mouse spleen cells were cultured for 48 hr with different amounts of PHA (Fig. 1a). PHA-stimulated rates of DNA synthesis are plotted as the percentage of the maximum rate of ^{3}H-dThd incorporation against the concentration (μg/ml) of PHA in the culture. It can be seen that near-maximum stimulation occurred when the PHA concentration was 2.5 μg/ml. Above this level a broad plateau is seen, with a gradual decline noted above a level of 7.5 μg PHA/ml culture. From these results we considered 5.0 μg/ml a suitable concentration of PHA for use in subsequent experiments.

To determine the length of the culture time for maximum ^{3}H-dThd incorporation, 2.5 × 10^6 spleen cells were cultured in the presence of PHA for 12, 24, 36, 48, 60, and 72 hr and the incorporation of ^{3}H-dThd was measured. The PHA-stimulation effect was observable at 12 hr and increased in a linear fashion up to 36 hr, with only a marginal decrease at 48 and 60 hr (Fig. 1b). For convenience a 48-hr culture period was selected for subsequent experiments.

To determine the requirements for fetal calf serum in the culture medium, spleen cells were cultured in medium containing 2, 4, 6, 8, 10, and 15%. It can be seen from Fig. 1c that 10% fetal calf serum is a concentration that allows maximum ^{3}H-dThd incorporation. These culture conditions established for spleen cells from young adult mice

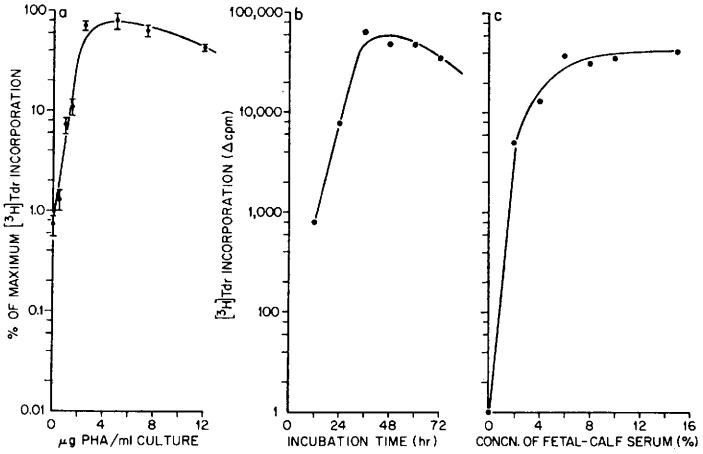

FIG. 1. Determination of optimal culture conditions for 2.5 × 10^6 mouse spleen cells. (a) Effect of PHA concentration on ^{3}H-dThd incorporation (48-hr cultures). Three separate experiments, three replicate cultures per experiment. (b) Effect of culture time on ^{3}H-dThd incorporation (5 μg PHA/ml). The mean difference between radioactivity incorporated into seven replicate PHA-stimulated cultures and three replicate nonstimulated cultures (Δcpm) is plotted. (c) Effect of fetal calf serum concentration on ^{3}H-dThd incorporation (48-hr culture). The mean difference between radioactivity incorporated into three replicate PHA-stimulated cultures and two replicate nonstimulated cultures (Δcpm) is plotted.

were tested with spleen cells from aged mice, and no significant difference was seen.

With culture conditions defined, age-dependent changes in T-cell stimulation with PHA were assessed with spleen cells from $BC3F_1$ mice ranging in age from 2 weeks to 36 months and in BALB/c mice of 4, 12, 19, and 24 months of age (Figs. 2 and 3). It can be seen in Fig. 2, as judged from ^{3}H-dThd incorporation, that the growth of a PHA-responsive T-cell population in the mouse is remarkable from birth to 1 month of age. Functional maturity appears maximal at 8 months of age, decreases gradually and then more dramatically during senescence, until at 36 months of age T-cell activity is only 2.5% of that which is seen at 8 months of age. In BALB/c mice a similar pattern is seen, although only four age groups were tested. The peak value is at 4 months, and a marked decrease is seen at 19 and 24 months of age (to 11.5 and 8.5%, respectively). This reflects a marked age-dependent decrease in T-cell activity in both strains. It should be noted that ^{3}H-dThd incorporation by non-PHA controls was relatively constant throughout the life-span, with a modest decrease seen among the very oldest groups.

To determine whether the decrease in PHA

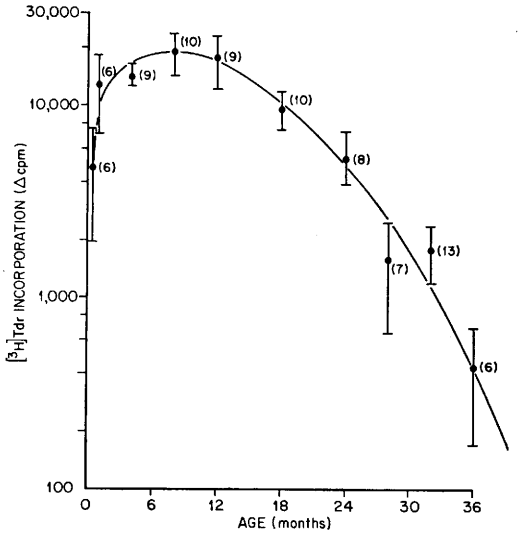

FIG. 2. The responsiveness of 2.5 × 10^6 spleen cells of individual $BC3F_1$ mice to PHA (5 μg/ml, 48-hr culture). Incorporation of ^{3}H-dThd is plotted as the mean difference between three PHA-stimulated and two nonstimulated cultures for each mouse. Numbers in parentheses equal number of mice.

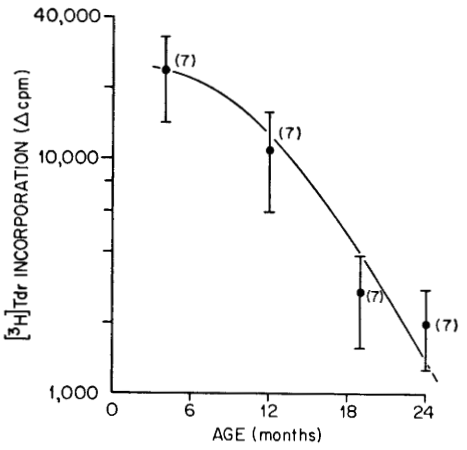

FIG. 3. The responsiveness of 2.5×10^6 spleen cells of individual BALB/c mice to PHA (5 μg/ml, 48-hr culture). Incorporation of ^{3}H-dThd is plotted as the mean difference between three PHA-stimulated and two nonstimulated cultures for each mouse. Numbers in parentheses equal numbers of mice.

responsiveness with age was due to decreased reactivity of the T-cell population or a marked drop in the relative number of T cells, the percentages of θ-positive cells in the spleens of $BC3F_1$ and BALB/c mice were determined. The data in Table I show no decrease in the relative number of θ-positive cells in the spleen. Hemacytometer counts

TABLE I. Effect of Age on Relative Numbers of Θ-Positive Lymphocytes in the Spleen.[a]

Strain	Age (mo)	Cytotoxicity with anti-Θ[b] (%)
$BC3F_1$	3	33.1, 27.2[c]
	26	38.6, 40.4[c]
	29	39.6
	32	43.6
	34	47.9
BALB/c	3	40.0
	22	40.0
	27	38.8

[a] Pooled data from three disease-free spleens.

[b] % cytotoxicity =

$$\frac{(\%\text{ killed with anti-}\Theta + C') - (\%\text{ killed with anti-}\Theta\text{ only})}{100 - (\%\text{ killed with anti-}\Theta\text{ only})} \times 100.$$

[c] Duplicate values represent results from two separate experiments.

demonstrated that there was no marked difference in the total number of nucleated cells in the spleens of young and old mice.

Discussion and Summary. The present data show a dramatic age-related decrease in cell-mediated immunity as measured by *in vitro* ^{3}H-dThd incorporation by PHA-stimulated mouse spleen cells. Thus, not only is there a marked decline in humoral immune responsiveness during senescence (5), but it now appears that a significant decrease in cell-mediated immunity also occurs. It has been recently reported from this laboratory by Peterson *et al.* (18), that spleen cells from aged animals are less effective in producing acute mortality in the classical graft-versus-host reaction. Concurrently with these studies, Goodman and Makinodan (19) have observed a marked decrease in cell-mediated immune responsiveness assessed by *in vivo* allogeneic tumor resistance and *in vitro* cytolytic activity. Walford and associates (20) have also recently reported a decline in cell-mediated immunity as measured by the *in vitro* mixed lymphocyte reaction. The present and aforementioned systematic studies were all carried out in inbred mice, where variation between individuals of a given age group is minimized, hence significant quantitative changes can be accurately determined. Although each study assessed cellular immune competence by a different immunologic parameter, all noted a significant decline in cell-mediated immunity. Combined, they provide evidence that cell-mediated immunity declines with age in long-lived inbred mice. It is of course recognized that these findings must be confirmed in other species and cannot necessarily be extrapolated to man; nevertheless, they lend credence to the concept that an age-related decline of cell-mediated immunity may play an exacting role in carcinogenesis and other age-related diseases.

It should be noted that the present results confirm the data reported by Rodney *et al.* (16) showing an age-related decline in the *in vitro* spleen cell response to PHA in three strains of mice that are generally susceptible to autoimmune disease but stand in sharp contrast to their findings in autoimmune in-

susceptible CBA mice. No decline in activity was seen when they compared spleen cells from 2- and 21-month-old CBA mice. CBA mice are considered to be a long-lived strain, so if the peak response to PHA occurred later in the first year of life (*e.g.*, at 8 months of age as in long-lived $BC3F_1$ mice) then the level of activity seen at 21 months of age could indeed represent a substantial decline in PHA responsiveness.

The dramatic decrease in PHA responsiveness of spleen cells from aged animals was not correlated with a drop in the relative number of θ-positive cells in their spleens. Stutman (21) found no change with age in CBA mice up to 600 days ($\sim$ 20 months), the oldest age tested, but a decrease in θ-positive cells in autoimmune susceptible NZB mice was seen as early as 200 days. In the present investigation, as no decline was seen in the relative number of thymus dependent T cells in the spleens with age, the decrease in PHA responsiveness must be due to a decreased reactivity of individual T cells.

This finding of a decreased proliferative capacity (as measured by ^{3}H-dThd incorporation) of the T cells responsible for cell-mediated immunity is reminiscent of the finding of Price and Makinodan (2) of a proliferative defect in the B lymphocytes from aged mice responding to a thymus-dependent antigen (sheep RBC). While those experiments demonstrated [for review see ref. (22)] that the basic defect was the inability of B lymphocytes to generate the maximum number of antibody-forming progeny, it was not ruled out that defective helper T-cell function or interaction was responsible for the observed results. Indeed a 10-fold reduction in the ability of T cells to proliferate in response to antigen was suggested (2). Therefore, the present findings of a proliferative defect in the T-cell population responsible for cell-mediated immunity may indicate a basic defective mechanism (T-cell proliferation) common to both cell-mediated and humoral immunity in aged mice. This may be an oversimplification, for heterogeneity of the T-cell population has been observed, *i.e.*, there are different subpopulations of T cells (23). It has also been demonstrated that there is variable responsiveness in T-cell subsets, and that certain subsets may not even be stimulated by PHA (24). Furthermore, it remains to be established whether the helper T cell in the thymus-dependent humoral immune response is the same as or different from the effector T cell of the cell-mediated immune response.

Finally, for immunocompetence to be a significant basic mechanism related to senescence it must be demonstrated that immunocompetence correlates with mean life span. Comparing the PHA responsiveness of long-lived $BC3F_1$ (mean life span 31 months) and medium-lived BALB/c mice (mean lifespan 25 months) it can be seen that the earlier peak of maximum spleen cell activity and its earlier decay in BALB/c mice may reflect ontogenic and life-span difference. These mice were aged under identical environment and hygenic conditions and a correlation between mean lifespan and this parameter of immunocompetence is readily apparent.

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1. Makinodan, T., Chino, F., Lever, W. E., and Brewen, B. S., J. Gerontol. **26**, 508 (1971).

2. Price, G. B., and Makinodan, T., J. Immunol. **108**, 403 (1972).

3. Makinodan, T., and Peterson, W. J., Develop. Biol. **14**, 96 (1966).

4. Makinodan, T., and Peterson, W. J., Develop. Biol. **14**, 112 (1966).

5. Makinodan, T., Perkins, E. H., and Chen, M. G., Advan. Gerontol. **3**, 171 (1971).

6. Walford, R. L., "The Immunologic Theory of Aging." Munksgaard, Copenhagen (1969).

7. Burnet, F. M., Progr. Exp. Tumor Res. **13**, 1 (1970).

8. Keast, D., Lancet **2**, 710 (1970).

9. Allison, A. C., Brit. Med. J. **4**, 419 (1970).

10. Miller, J. F. A. P., Marshall, A. H. E., and White, R. G., Advan. Immunol. **2**, 111 (1962).

11. Meuwissen, H. J., Stutman, O., and Good, R. A., Seminars Hematol. **6**, 28 (1969).

12. Bloom, B. R., Advan. Immunol. **13**, 101 (1971).

13. Doenhoff, M. J., Davies, A. J. S., Leuchars, E., and Wallis, U., Proc. Roy. Soc. London Ser. B **176**, 69 (1970).

14. Janossy, G., and Greaves, M. F., Clin. Exp. Immunol. **9**, 483 (1971).

15. Blomgren, H., and Suedmyr, E., J. Immunol.

106, 835 (1971).

16. Rodney, G. E., Good, R. A., and Yunis, E. J., Clin. Exp. Immunol. **9**, 305 (1971).

17. Carr, M. C., Stites, D. P., and Fudenberg, H. H., Cell. Immunol. **5**, 21 (1972).

18. Peterson, W. J., Proc. Congr. Gerontol. **9**, 46 (1972).

19. Goodman, S. A., and Makinodan, T., Gerontologist **12**, 29 (1972).

20. Konen, T. G., Smith, G. S., and Walford, R. L., J. Immunol. **110**, 1216 (1973).

21. Stutman, O., J. Immunol. **109**, 602 (1972).

22. Perkins, E. H., and Makinodan, T., *in* "Proceedings of the First Rocky Mountain Symposium on Aging, Colorado State University" (in press).

23. Stobo, J. D., Rosenthal, A. S., and Paul, W. E., J. Immunol. **108**, 1 (1972).

24. Stobo, J. D., Paul, W. E. and Henney, C. S., J. Immunol. **110**, 652 (1973).

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