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LYMPHOCYTE AGING IN ALLOPHENIC MICE

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Abstract—Allophenic mice are chimeras which are produced from the aggregation of two genotypically distinct embryos. In this study, embryos from the C57BL/6J and A/J strains were used to produce C57BL/6J ⟷ A/J allophenic mice. These strains were chosen because of their markedly different lifespans, their different glucose phosphate isomerase (GPI) isozymes, and their different *H-2* haplotypes [C57BL/6J: long-lived, *Gpi*-1^b, *H-2*^{b}; A/J: short-lived, *Gpi*-1^a, *H-2*^{a}]. The mice were bled at two month intervals and the composition of their peripheral blood lymphocytes determined at each point from analysis of GPI isozymes and *H-2* antigens. It was found that the proportion of long-lived (C57BL/6J) lymphocytes tended to increase with age. Moreover, the total lifespan of the allophenic mice was directly related to the percentage of long-lived lymphocytes in their peripheral blood.

INTRODUCTION

THE IMMUNE system plays an important, perhaps crucial, role in the aging process. It is well documented that aged animals show disturbances in humoral and cellular immune responses, T cell/B cell ratio, ability to respond to lectins, and a myriad of other immune functions (Kay and Makinodan, 1981). It is unknown whether these abnormalities are the cause or the consequence of the aging process. Aberrant immune function in aged animals can lead to death from infectious agents, cancer, or autoimmune disease.

The aging process, leading to a particular aging phenotype, has both genetic and environmental components. In the mouse, a species in which many inbred strains are available, it is clear that different strains have markedly different lifespans (Storer, 1969; Altman and Katz, 1979; Heiniger and Dorey, 1980). Some of the reported differences in lifespan are in strains of animals raised in the same environment, leading to the conclusion that the aging process has a strong genetic component. The definition of "the same environment" is a difficult one to make. There is, however, a system in which cells from two animals of markedly different lifespans may be combined into a single animal, thus creating a "same environment" for both types of cells. This system is the allophenic mouse, first described by Tarkowski in 1961 and Mintz in 1962.

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Allophenic mice are chimeras produced by the aggregation of two embryos early in development. These animals show true mosaicism and no apparent intolerance of the two types of cells for each other. McLaren (1976) has termed allophenic mice primary chimeras because they are potentially chimeric in all their tissues and organs. This distinguishes them from secondary chimeras, such as bone marrow chimeras, which are chimeric in only the hematopoietic system.

Allophenic mice have been used in a number of immunologically interesting studies, including studies on the mechanism of tolerance (McLaren, 1976) and *Ir* (immune response) gene function (Warner *et al.*, 1973; 1976; 1977a; 1978; Gorcyca *et al.*, 1982). It has been reported that the composition of the immune system of allophenic mice changes with age (Stephens *et al.*, 1977; Warner *et al.*, 1977b; Stephens and Warner, 1980), but the mice in these studies were not allowed to get truly "old"; they were all killed to complete the studies by the relatively young age of 8 months. Abnormalities in the immune system of mice do not usually become strikingly apparent until the mice are greater than 16 months old (Kay and Makinodan, 1981).

The purpose of the present study was to combine cells from a short-lived mouse strain and a long-lived mouse strain into a single animal through the creation of allophenic mice. The mice were then bled at two month intervals until death and the composition of their peripheral blood lymphocytes analyzed. The two markers used for the analyses were the enzyme glucose phosphate isomerase (GPI) as an internal marker, and the cell surface antigen *H-2,* as an external marker. The results reported in this paper will show that the lifespan of each allophenic mouse was correlated with the percentage of long-lived lymphocytes in the particular animal.

MATERIALS AND METHODS

Mice

The C57BL/6J and A/J mice were purchased from the Jackson Laboratory, Bar Harbor, ME. The pertinent characteristics of the A/J and C57BL/6J strains are listed in Table 1. It is seen that the mean lifespan of the animals varies from lab to lab, but the A/J mice are always short-lived compared to the C57BL/6J mice. We are currently obtaining lifespan data for these two strains in our own laboratory, but the data are not yet available.

Allophenic mice (C57BL/6J ⟷ A/J) were produced as described previously (Warner *et al.*, 1973; Mintz *et al.*, 1973). Briefly, eight cell embryos were collected from superovulated female mice, the zona pellucida removed with pronase (Calbiochem), the embryos treated with phytohemagglutinin-P (Difco) after which they were bumped together and cultured for about 24 hours in an atmosphere of 5% CO_2, 5% O_2, 90% N_2. The resulting double-sized blastocysts were transferred to pseudopregnant CF1 mice (Charles River) and allowed to

TABLE 1. CHARACTERISTICS OF C57BL/6J AND A/J MICE.

					Mean Lifespan (days)		
Strain	*Coat Color*	Gpi-*1*[1]	H-2-*haplotype*[1]	*Sex*	*Source 1*[1]	*Source 2*[2]	*Source 3*[3]
A/J	albino	a	a	Virgin ♂	662 ± 20	490 ± 18	634
				Virgin ♀	688 ± 18	590 ± 19	694
C57BL/6J	black	b	b	Virgin ♂	827 ± 34	676 ± 20	841
				Virgin ♀	818 ± 21	692 ± 16	789

[1]From Altman and Katz, 1979.
[2]Storer, 1969.
[3]Heininger and Dorey, 1980.

come to term. After weaning, male and female allophenic mice were separated, and same sex littermates kept in the same cage, as virgins, throughout their lives.

Coat Color Estimation

Coat color was estimated by visual inspection at weaning, at 2 months of age and every 2 months thereafter.

Blood Collection and Lymphocyte Isolation

The allophenic mice were bled at 2 months of age and every 2 months thereafter until death. About 0.3 ml of blood was collected from the orbital venous plexus into heparinized hematocrit capillary tubes. The blood was immediately expelled into 0.7 ml of PBS (0.01 M sodium phosphate buffered saline, pH 7.0) containing 0.01% EDTA and 50 IU heparin. The blood was then divided into two 0.5 ml aliquots and layered on 1.0 ml Ficoll-Hypaque (density = 1.088) in each of two 1.5 ml microfuge tubes. The tubes were centrifuged at 10,500 × g for 3 minutes in a Beckman Microfuge 12. The white blood cell layers were collected, resuspended in PBS, and centrifuged at 6,000 × g for 2 minutes in the Microfuge. The pellets were combined, again suspended in PBS and repelleted at 6,000 × g for 1 minute. The remaining red blood cells were removed by suspending the pellets in 250 μl PBS to which 1 ml of ammonium chloride lysis solution (0.155 M NH_4Cl, 0.01 M $KHCO_3$, 0.1 mM EDTA, pH 7.4) were added. The cells were immediately pelleted again at 6,000 × g for 1 minute and washed twice with PBS under the same conditions. The final pellet was resuspended in 100 μl of PBS which gave a concentration of about 1-3 × 10^6 cells/ml.

At this point 40 μl of the cell suspension were removed for direct use in the cytotoxicity assay for *H-2* antigens, described below. The remainder was freeze-thawed twice to give the lysate that was used for isozyme analysis, as described below.

Assay for Glucose Phosphate Isomerase (GPI)

Analysis of GPI isozymes was as previously described (Balinsky *et al.*, 1983). The cell lysates were run on Cellogel electrophoresis strips, the enzyme bands stained, and then the Cellogel strips were scanned in an LKB densitometer. All assays were run at least in duplicate. The total areas under the curves were calculated and the percentage of the two isozymes calculated from the total.

Assay for H-2 Antigens

Antisera for the detection of *H-2* antigens were prepared by reciprocal immunization of the C57BL/6J and A/J mice with spleen cells according to Batchelor (1973). For convenience, these antisera are designated anti-$H\text{-}2^a$ and anti-$H\text{-}2^b$, because *H-2* is the immunodominant antigen on spleen cells. The antisera may contain antibodies to other cell surface antigens in addition to *H-2*, but this is inconsequential to these studies. For the experiments described in this paper, in which the proportion of cells of the A/J vs. the C57BL/6J lineage is being detected in allophenic mice, it is advantageous to use antisera made in reciprocal strains to eliminate any possible cross-reactivity of the antisera (Warner *et al.*, 1977c). The cytotoxicity assays were as described previously (Warner *et al.*, 1977c). A minimum of 200 cells were scored for viability by trypan blue dye exclusion. Each experiment and the controls were run in duplicate, and the results normalized to controls containing only A/J or C57BL/6J cells.

RESULTS

A total of 23 C57BL/6J ⟷ A/J allophenic mice, from 10 different litters, were produced for this study. Data on the first litter is now complete, because all the animals have died. The pertinent sex and lifespan data of this litter are shown in Table 2. The animals

TABLE 2. CHARACTERISTICS OF C57BL/6J ⟷ A/J ALLOPHENIC MICE.

Mouse Designation	*Sex*	*Lifespan (days)*
AG-0011-1	♂	483
AG-0012-1	♂	645
AG-0013-1	♂	649
AG-0014-1	♂	790

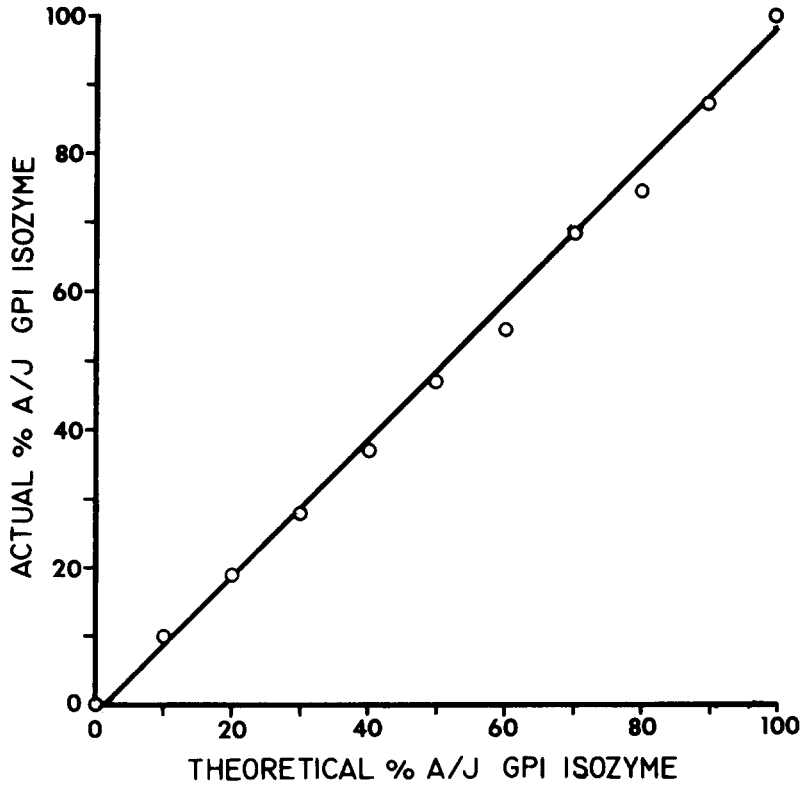

FIG. 1. The percentage of *Gpi*-1^a isozyme of the total in known mixtures of C57BL/6J and A/J spleen lymphocytes. The least squares linear regression line is shown (y = 0.98x − 1.00, r = 0.99).

were visually examined for coat color and bled at 2 month intervals throughout their lives. The isolated peripheral blood lymphocytes were tested for GPI isozyme composition and *H-2* antigen composition. To evaluate the methods used for detection of GPI isozymes and *H-2* antigens, experiments were performed in which known quantities of lymphocytes were mixed together and then analyzed. Figure 1 shows the results of mixing experiments for GPI analysis. These results have been corrected to reflect the fact that on a per cell basis, A/J lymphocytes often have more enzyme activity than C57BL/6J lymphocytes (Warner and Briggs, unpublished). Figure 2 shows the results of mixing experiments for cell surface antigen analysis. No correction was necessary for the *H-2* antigen analysis because the cytotoxicity assay was done at an antiserum concentration which would kill all the cells of one type or the other. The *H-2* analysis is therefore a direct reflection of the proportion of the two types of cells in the peripheral blood of the allophenic mice.

The results of the analysis of coat color, glucose phosphate isomerase isozymes, and *H-2* antigens, as a function of age, are shown in Figures 3, 4, and 5, respectively.

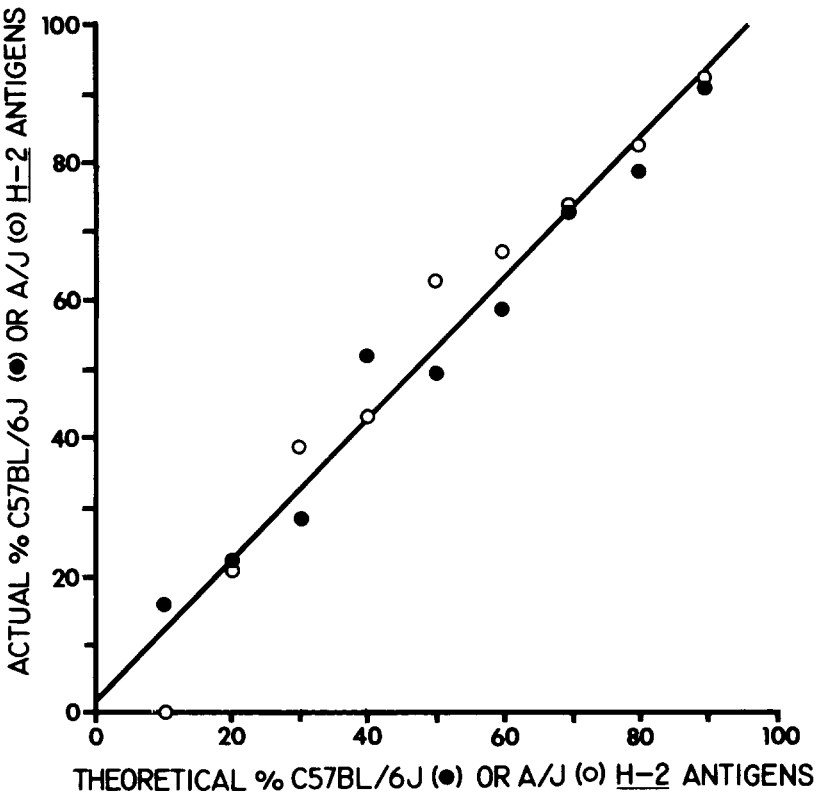

FIG. 2. The percentage of *H-2* antigen expressing cells in known mixtures of C57BL/6J and A/J spleen lymphocytes. Two independent determinations were made using either anti-$H\text{-}2^a$ (○) or anti-$H\text{-}2^b$ (●) antisera. The least squares linear regression line is shown ($y = 0.99x + 2.76$, $r = 0.98$).

DISCUSSION

This paper is the first report of the use of allophenic mice for aging research. The mice were allowed to live out their full lifespans, and were monitored for coat color and the composition of their peripheral blood lymphocytes at two month intervals. The C57BL/6J and A/J strains were chosen for these studies because of their markedly different lifespans and their different *H-2* and GPI isozyme genotypes.

The coat color of the three mice with marked chimerism at two months of age (AG-0011-1, AG-0012-1, and AG-0013-1), showed a steady increase in albino hair with age (Figure 3). (Inbred C57BL/6J mice remain black throughout their lives.) A study by Gearhart and Oster-Granite (1981) has shown that BALB/cByJ ⟷ C57BL/6J allophenic mice also get progressively whiter as they age, even though the animals were observed only up to 10 months of age. This infers that there is a progressive loss of black melanocytes as allophenic mice age.

The composition of the peripheral blood lymphocytes was evaluated as a function of

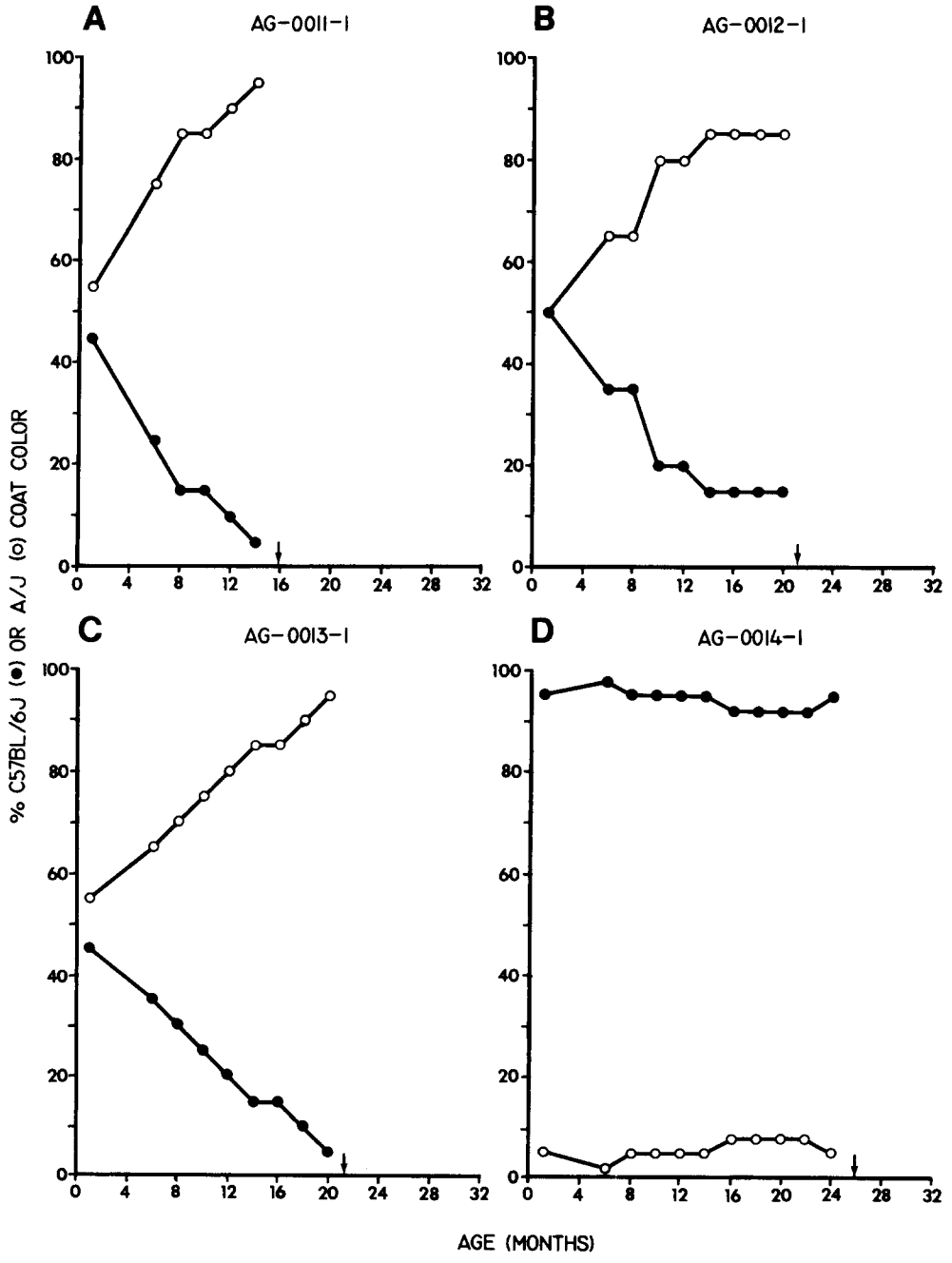

FIG. 3. Change in coat color composition of allophenic mice with age. The percentage of albino (○) or black (●) hair, and the age at death (↓) are shown.

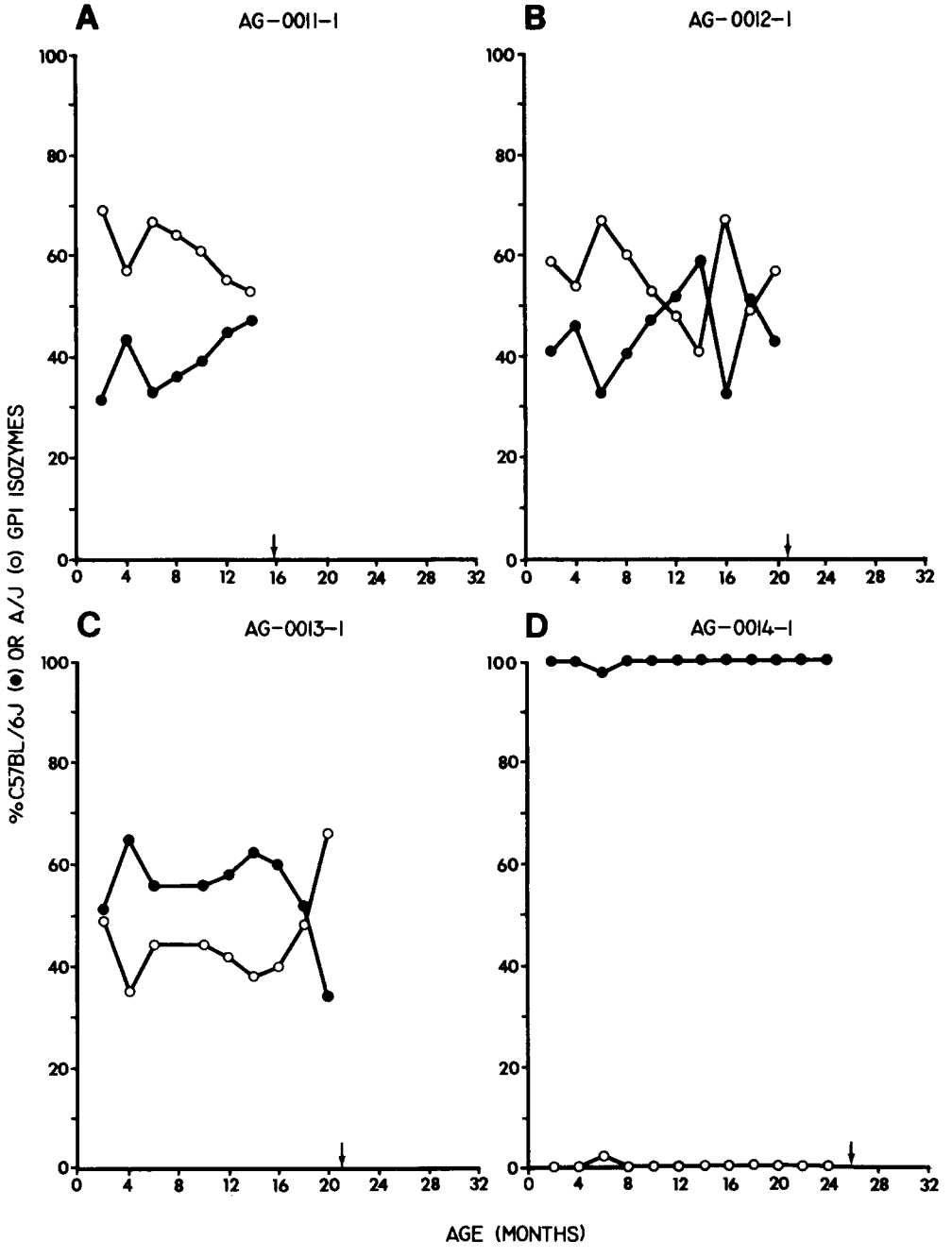

FIG. 4. Change in GPI isozyme composition of peripheral blood lymphocytes of allophenic mice with age. The percentage of *Gpi*-1^a (○) or *Gpi*-1^b (●) isozymes, and the age at death (↓) are shown.

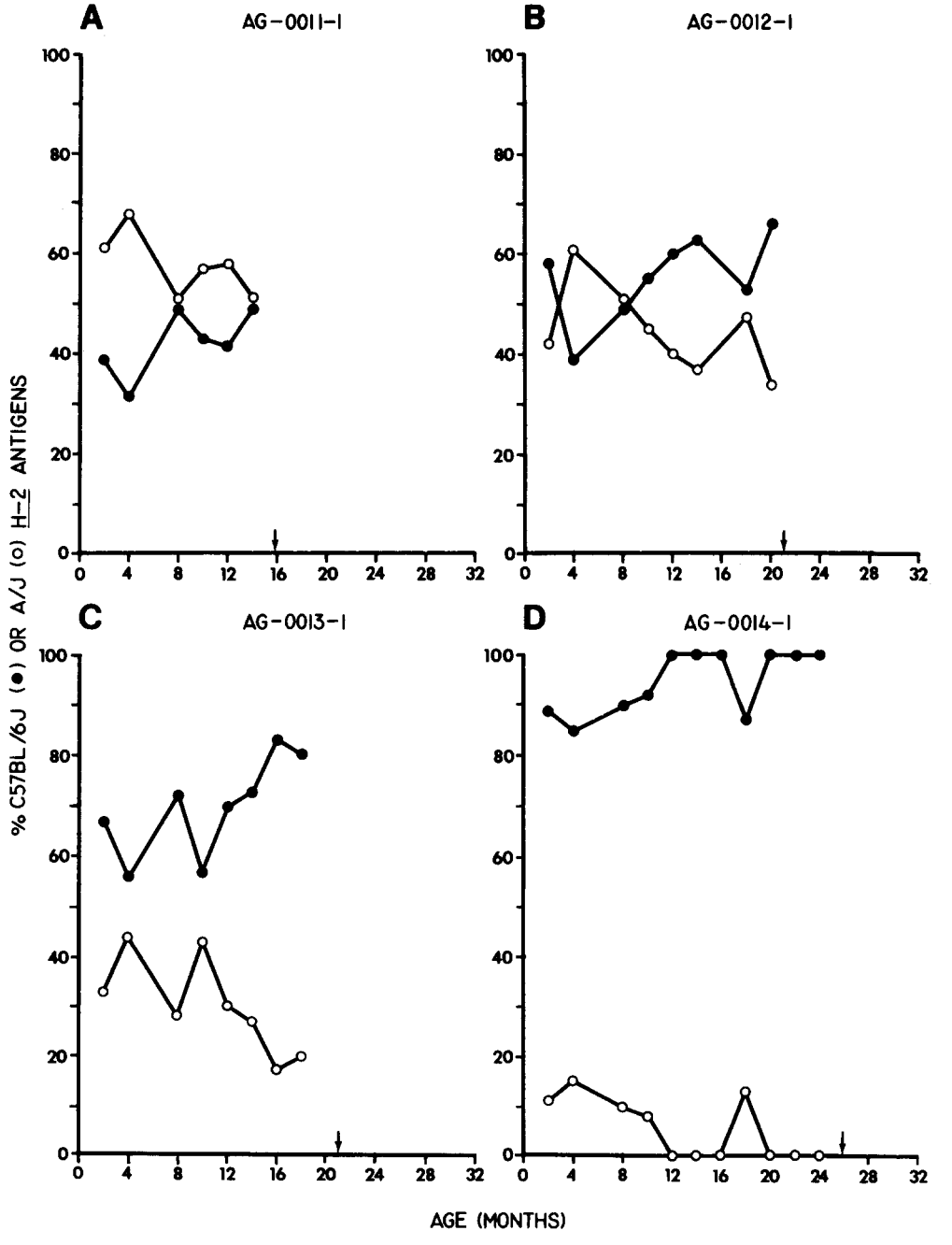

FIG. 5. Change in *H-2* antigen composition of peripheral blood lymphocytes of allophenic mice with age. The percentage of *H-2*^{a} (○) or *H-2*^{b} (●) cells, and the age at death (↓) are shown.

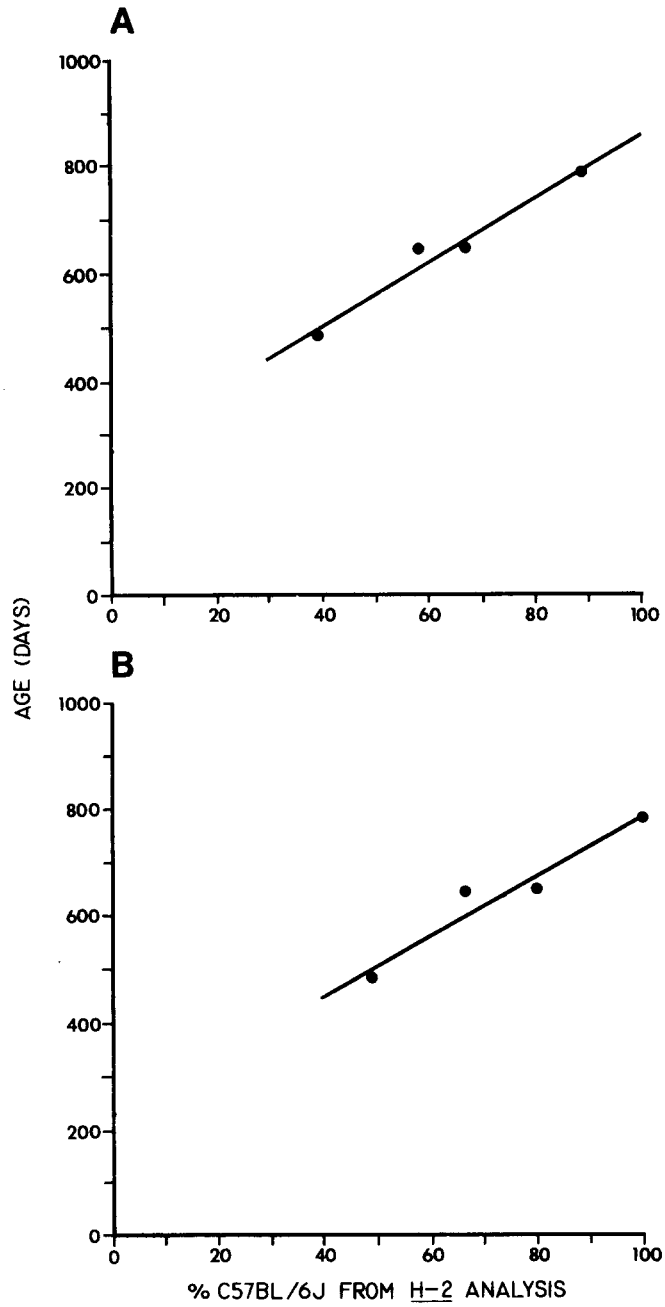

FIG. 6. Correlation of age at death with % C57BL/6J peripheral blood lymphocytes. Data in panel A are from *H-2* analysis at 2 months of age; data in panel B are from *H-2* analysis just prior to death. The equation for the least squares linear regression line in panel A is $y = 5.94x + 266$, $r = 0.98$; the equation for the least squares linear regression line in panel B is $y = 5.60x + 229$, $r = 0.96$.

age. Both GPI isozyme analysis (Figure 4) and *H-2* antigen analysis (Figure 5) indicate that the relative proportions of the two types of lymphocytes, C57BL/6J and A/J, were unstable as a function of age. This instability has been termed "chimeric drift" (Stephens *et al.*, 1977) and has been observed previously for both red blood cells (Mintz and Palm, 1969; West, 1975, Stephens *et al.*, 1977; Warner *et al.*, 1977b; Stephens and Warner, 1980) and white blood cells (Stephens *et al.*, 1977; Warner *et al.*, 1977b; Stephens and Warner, 1980) in allophenic mice. A recent paper by Behringer *et al.* (1984) suggests that chimeric drift only occurs in mice which differ in *H-2* haplotype. The mice in this study do, of course, differ in their *H-2* haplotypes. The most interesting trend which we have observed in this study is the tendency of the proportion of C57BL/6J (long-lived) peripheral blood lymphocytes to increase with age. This is clearest in Figure 5 (*H-2* analysis) which, as discussed previously, is the most accurate reflection of the true proportion of the two types of parental cells in the peripheral blood of the allophenic mice. There is no correlation of changes in the peripheral blood composition to changes in coat color (compare Figures 3 and 5).

In Figure 6, the age at which each of the allophenic animals died is plotted as a function of the percentage of long-lived (C57BL/6J) cells at the initial time point tested (2 months) and at the last time point tested before death. The percentage of C57BL/6J cells is based on the data in Figure 5. The least squares linear regression lines have been plotted in Figure 6. The line in panel A (2 month data) has a correlation coefficient, $r = 0.98$, and the line in panel B (last point before death) has a correlation coefficient, $r = 0.96$. In fact, similar correlations exist for all the ages tested. Thus, at two months of age it could be predicted which mouse would live the longest and which would live the shortest, based on the relative contribution of short-lived and long-lived cells to the peripheral blood lymphocyte population.

In summary, analysis of a litter of four C57BL/6J ⟷ A/J allophenic mice has shown that as they aged their coat color got whiter, the relative proportion of C57BL/6J (long-lived) peripheral blood lymphocytes increased, and their ultimate lifespan was a function of the proportion of long-lived lymphocytes in their blood. The allophenic mouse should be a useful tool for future studies on the aging of genotypically distinct cells in a common environment.

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