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Model Approach to Immunological Rejuvenation of the Aged

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Reconstitution of a damaged or exhausted immune system by injection of genetically compatible immunocompetent cells (immunologic rejuvenation) is a promising approach for restoration of immune activity. By using this model, spleen cells from young-adult mice, previously immunized with *Salmonella typhimurium*, were transferred to either young-adult or old, syngeneic recipients before or after storage at −196 C. The susceptibility of recipient mice was then determined by challenging them at increasing time intervals after reconstitution with lethal doses of the virulent organisms. The findings, although preliminary in nature, demonstrate that (i) immunological rejuvenation of mice is possible with immunocompetent cells from specifically immunized donors; (ii) prolonged "takes" of these cells can occur even in nonirradiated recipient mice, and (iii) storage at −196 C does not impair their protective capacity.

The marked increase in the incidence of cancer and autoimmune diseases and the dramatic decrease in humoral and, to some extent, cell-mediated immune capacity in aged mice are convincingly established (13, 17, 21). Based on these and other data, Walford (21, 22) and Burnett (4) have introduced an immunological theory of aging—an extension of the somatic-mutation theory of aging—wherein they suggest that, with advancing age, there is a gradual accumulation of mutated cells among the immunocompetent or other somatic cells of the body, or both, that can lead to immunological diseases. According to the theory, mature adults remain healthy and are free of these self-destructive autoimmune diseases because, presumably, immunological surveillance by a normally functioning, highly efficient immune system continually protects against infectious agents and aberrant (cancer) cells (5, 20, 21). If follows, then, that rejuvenation of a reduced or even exhausted immune system by infusion of genetically compatible immunocompetent cells should enhance general health and vigor by increasing resistance to infections and to growth of malignant or benign cancer cells, thus markedly improving the quality of the final quarter of life. This approach is especially feasible with inbred laboratory animals where one is not confronted with formidable histoincompatibility problems.

For immunologic rejuvenation to be effective, the infused immunocompetent cells must (i) persist for an extended period without loss of functional capacity, (ii) perform maximally, and (iii) not produce histoincompatibility reactions against the host. If possible, the prospective recipient should not be subjected beforehand to physical or chemical insult. Based on these criteria and with the following questions in mind, a model test system has been developed in mice. (i) Can immunocompetent cells persist for an extended period of time in both young and aged syngeneic recipients and yield significant protection to an *unirradiated* host? (ii) Are immunocompetent cells from immunized donors superior to those from normal (nonimmunized) donors? (iii) Can immunocompetent cells be stored at liquid nitrogen temperature (−196 C) and thawed without appreciable loss of activity? The last question is unnecessary when using inbred laboratory animals, but is pertinent when studying species where inbred animals are not available. Thus, when using noninbred animals, a realistic approach is storage of the animal's own immunocompetent cells with subsequent retrieval and infusion to meet needs with advancing age.

To answer these questions, we have assessed the ability of infused spleen cells from young-adult mice immunized against a virulent strain of *Salmonella typhimurium* to protect both young and old mice from a parenterally induced infection with the same organism. This was thought to be an appropriate model test system because mortality due to infection generally increases

with advancing age and benign infections are often difficult to control in the aged.

MATERIALS AND METHODS

Mice and housing. $BC3F_1$ hybrid mice (C57BL/Cum ♀ × C3H/Anf Cum ♂) of both sexes, reared in either a conventional (clean) or substandard (dirty) environment as previously reported in reference 7, were used in this investigation. No difference in life expectancy, incidence of old-age-associated diseases, or immunological activity had been observed in mice reared in these two environments (7, 11). After intravenous injection with donor spleen cells, and 1 or 2 days prior to experimental challenge with virulent *Salmonella* organisms, the mice were transferred from the farms to special isolation facilities. After challenge, the mice were recaged 10 per pan and allowed free access to food and clean, but not sterile, water. Pans and water bottles were changed once a week and pans were checked daily for 14 days for dead mice. The incidence of mortality at 14 and 30 days did not differ significantly.

Challenge organisms. A mouse-virulent strain of *S. typhimurium* (Utah SHD) obtained from the Communicable Disease Center, Atlanta, Georgia, was used in this study. Stock cultures of the organism on nutrient agar slants were stored at −18 C and maintained by transfer culture at 6-month intervals. To obtain standardized doses of *S. typhimurium* for challenge experiments, organisms from a stock slant were streaked on a nutrient agar plate and incubated overnight at 37 C. From this plate a second nutrient agar plate was streaked and incubated at 37 C for 6 hr. Challenge organisms were harvested from the second plate with sterile saline, filtered through sterile glass wool, and standardized to 66% transmittance in a Coleman Junior spectrophotometer at 700 nm. Then dilutions of the standard suspension were quickly made in sterile saline containing 1% gelatin, and mice were immediately challenged by intraperitoneal injection of an appropriate dilution of organisms. Plate counts made through the course of these experiments revealed less than 3% variation in the number of organisms in the challenge inocula.

Vaccine preparation. A heat-killed, formaldehyde-preserved vaccine was prepared from *S. typhimurium* cultured for 18 hr on nutrient agar plates and harvested in sterile saline. The suspension was filtered through sterile, glass wool and the organisms were killed by heating them in a 56 C water bath for 1 hr. The vaccine was standardized in a Coleman Junior spectrophotometer to 82% transmittance at 700 nm. Formaldehyde was added to give a final concentration of 0.5%, and the vaccine was stored in a refrigerator at 5 C. Sterility was demonstrated by replicate plating of 0.5-ml samples of the vaccine on nutrient agar plates, followed by incubation at 37 C.

Spleen cell preparation and administration. Spleen cell suspensions were prepared in Hanks balanced salt solution (HBSS) containing 1% normal mouse serum from nonimmunized 12-week-old mice. Similarly, spleen cell suspensions were prepared from 16-week-old mice which had been immunized 4 weeks earlier with a single 0.5-ml intravenous injection of *S. typhimurium* vaccine. The latter treatment resulted in markedly enlarged spleens, and only spleens weighing between 300 and 600 mg were used. Spleen cells were counted in a hemocytometer and adjusted to desired concentrations. Heparin (final concentration of 5 units/ml) was routinely added just prior to intravenous injection of 5×10^7 spleen cells into nonirradiated mice. However, an essential feature of the experimental design in "cell banking" experiments was injection of spleen cells into sublethally irradiated (600 R) mice.

Irradiation. Mice received a single whole-body exposure from a G.E. Maxitron X-ray machine. Irradiation conditions were 300 kvp, 20 ma; 175 R/min at a target-object distance of 70 cm; half-value layer, 0.4 mm of Cu; inherent filtration, 4.75 mm of Be; added filtration, 3 mm of Al.

Freezing, storage, and thawing of immunocompetent cells. A 20% suspension of dimethylsulfoxide in HBSS was slowly added, with continuous mixing, to an equal volume of a pooled spleen-cell suspension containing 4×10^8 cells/ml. Samples of 1-ml were transferred to glass ampoules which were sealed with a flame and then chilled in ice. The temperature was decreased at a rate of 1 C/min from 4 to −50 C, and the ampoules were stored at −196 C in liquid nitrogen. Two weeks later, cell suspensions were removed from the liquid nitrogen and quickly thawed by placing the ampoules in a 37 C water bath. Cells were diluted fourfold with HBSS prior to intravenous injection into recipient mice.

RESULTS

Three replicate experiments were carried out over a 6-month period. In each experiment, three groups of 10 to 15 mice each received decreasing numbers of *S. typhimurium* organisms per group. Minimal variation was found among the three experiments, so the data were pooled and mean lethal dose (LD_{50}) of 1.3×10^5 organisms was estimated by the method of Berkson (3) for young-adult (12-week-old) $BC3F_1$ mice following intraperitoneal injection (Table 1). It should be emphasized that small increases in the number of challenge organisms introduced intraperitoneally produced marked increases in mortality, and it was a routine observation that challenge of mice with three LD_{50} resulted in 100% mortality.

TABLE 1. *Estimation of 14-day LD_{50} for S. typhimurium following intraperitoneal infection of young-adult mice*[a]

No. of challenge organisms	No. of deaths per no. challenged	Percent mortality
52,500	1/30	3.3
105,000	20/50	40.0
210,000	43/52	82.7

[a] The LD_{50} was estimated by the method of Berkson (3) and equals 132,800 *S. typhimurium* organisms per mouse.

The protective capacity of spleen cells stored in liquid nitrogen. Albright et al. (2) have previously demonstrated that the antibody-synthesizing capacity of spleen cells to sheep erythrocytes is in no way impaired by freezing and prolonged storage. However, it was of paramount importance to demonstrate that this could be done in the present experimental system where both adoptively transferred cellular and humoral immunity could contribute to survival of challenged recipient mice. Accordingly, for this experiment, prospective recipient mice were X-irradiated (600 R) to destroy their ability to generate an immune response (12). These animals were then injected with 5×10^7 fresh or stored spleen cells from immunized or normal (nonimmunized) donors and 1 day later were challenged with appropriate doses of *S. typhimurium* organisms. A preliminary investigation with fresh spleen cells injected into X-rayed recipients had established an appropriate challenge-inoculum dose range.

It can be seen from Fig. 1 that comparable 14-day mortalities were obtained in animals given stored (−196 C) or fresh spleen cells. That the protective capacity of stored spleen cells, from either previously immunized (Fig. 1A) or normal (Fig. 1B) mice, was as effective as that of fresh cells is further emphasized by the strikingly similar cumulative mortality curves seen when recipient mice were challenged with the same number of *S. typhimurium* organisms.

The enhanced protective capacity of spleen cells from immunized donors was dramatic when compared with that of normal spleen cells (compare Fig. 1A with Fig. 1B). Sublethally irradiated (600 R) recipients injected with fresh spleen cells from normal mice were highly susceptible to infection; challenge with a 0.25- or 0.50 LD_{50} of organisms resulted in 40 and 80% mortality, respectively. In marked contrast, recipients injected with fresh spleen cells from immunized donors and challenged with 50 times the LD_{50} of organisms resulted in mortality of only 73%.

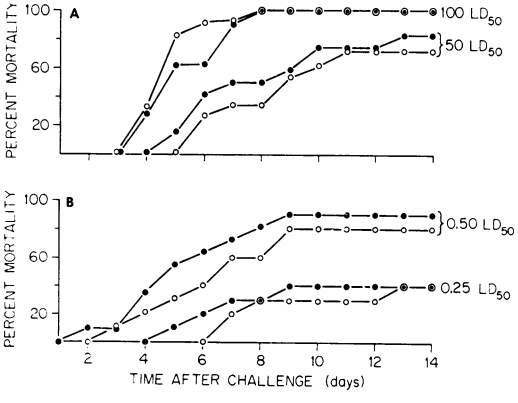

FIG. 1. *Comparable ability of fresh spleen cells* (○) *and those stored in liquid nitrogen* (●) *to protect sublethally irradiated (600 R) mice against intraperitoneal challenge with S. typhimurium. A, Spleen cells from immunized donors. B, Spleen cells from normal donors. Mice received* 5×10^7 *fresh or stored spleen cells within 2 hr after X-irradiation and were challenged 1 day later (10 to 12 mice per group).*

Increased susceptibility of aged $BC3F_1$ mice to S. typhimurium. A marked increase in susceptibility was seen among aged animals. Because only limited numbers of mice of any one age group greater than 2 years were available, results from separate small experiments were grouped according to the age of the mice to facilitate presentation of the data. Hence, the lower cumulative mortality curve (closed circle) presented in Fig. 2 represents 24 animals 109 to 118 weeks of age challenged with a 0.1 LD_{50} of organisms. The cumulative 14-day mortality was 70%.

This increased susceptibility was even more pronounced in still older animals. The upper curve (open circle) in Fig. 2 presents the cumulative mortality of 36 133- to 140-week-old mice challenged with a 0.05- or 0.1 LD_{50} of organisms. Although almost half of these animals had been challenged with only a 0.05 LD_{50} dose of organisms, 100% mortality was reached by day 13. Abrupt, early mortality (1 day) accounted for over half of the deaths among the mice of this age group that had been challenged with a 0.1 LD_{50} of organisms.

After we established the general susceptibility of young-adult and aged mice, it was necessary to determine if sufficient numbers of adoptively

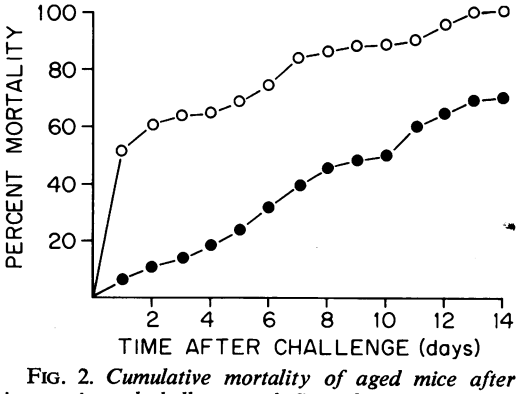

FIG. 2. *Cumulative mortality of aged mice after intraperitoneal challenge with S. typhimurium. Upper curve* (○)*: 36 mice, 133 to 140 weeks of age, challenged with either a 0.05- or 0.1-*LD_{50} *dose of organisms. Lower curve* (●)*: 24 mice, 109 to 118 weeks of age, challenged with a 0.1* LD_{50} *of organisms.*

transferred spleen cells from immunized donors would "take" in nonirradiated young-adult and aged recipient mice to confer significant protection against *S. typhimurium* infection. The data presented in Fig. 3 show that 2 to 4 weeks after receipt of 5×10^7 primed spleen cells, 95% of young-adult mice survived a challenge 100 times the LD_{50} (Fig. 3B). The protective effect of primed spleen cells in aged (133 to 140 weeks) recipients was also impressive (Fig. 3A). Although 100% of aged mice succumbed to an inoculum of 0.05 or 0.1 LD_{50}, a 10- to 20-fold greater challenge dose (1 LD_{50} for young-adult mice) 2 to 4 weeks after adoptive transfer of 5×10^7 primed spleen cells resulted in only 50% mortality.

Duration of adoptive immunity. To determine if the transferred cells survived and retained their ability to provide long-lasting immunity in nonirradiated recipients, young-adult mice were challenged at various times after receipt of primed spleen cells. It can be seen in Fig. 4 that even after 40 weeks the transferred cells still provided a highly significant degree of protection. At this time these mice were highly susceptible to a 100 LD_{50} challenge dose of organisms, indicating a decline in the level of immunity. However, a 10 LD_{50} challenge dose of organisms resulted in only 20% mortality. In comparison, a 3 LD_{50} challenge dose of organisms was fatal for all mice of a comparable age (52 to 56 weeks) that had not received spleen cells. Persistence of adoptive immunity was not a unique observation seen only among young animals. When mice 133- to 140-weeks old at the time of spleen cell transfer were challenged with 1 LD_{50} of organisms 2 weeks later, mortality was 50% ($13/26$). Challenge with the same number of organisms 10 to 12 weeks after cell transfer increased mortality to only 70% ($14/20$). In contrast, 0.05 LD_{50} of organisms resulted in 100% mortality for the 133- to 140-week-old mice that did not receive spleen cells (Fig. 2).

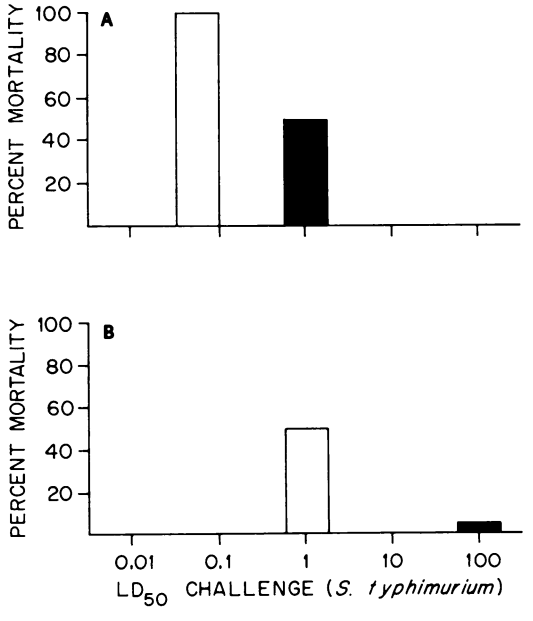

FIG. 3. *Comparative mortality of normal mice (nonimmunized and nonirradiated, □) and protected mice (given 5 × 10⁷ spleen cells from immunized donors, ■) after S. typhimurium challenge 2 to 4 weeks after spleen cell injection. A, Aged (133- to 140-week-old) recipients; B, young-adult (12-week-old) recipients (25 mice per protected group).*

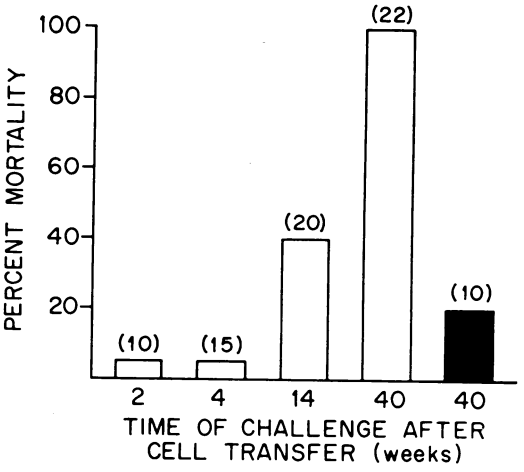

FIG. 4. *Effect of time of challenge (with S. typhimurium) on mortality of nonirradiated young-adult (12-week-old) mice receiving 5 × 10⁷ spleen cells from immunized young-adult syngeneic donors. Dose: 100 LD_{50} (□); 10 LD_{50} (■). (Number of mice per group is in parentheses.)*

Further evidence of prolonged "take" of transferred immunocompetent cells. The preceding experiments clearly demonstrated that adoptive immunity was long-lasting in nonirradiated animals. However, we thought that a direct comparison of the duration of adoptive immunity in nonirradiated and irradiated recipient mice receiving spleen cells from immunized donors should be made. Accordingly, two groups of 12-week-old $BC3F_1$ mice were selected at random. One group received 400 R of total-body X-irradiation and the other no irradiation. Both groups received 5×10^7 primed spleen cells from the same cell suspension. At 1.5, 4, and 12 weeks after spleen-cell transfer, one and one-half groups of mice were challenged with a 100 LD_{50} dose of *S. typhimurium* and 45 weeks after spleen-cell transfer with 50 LD_{50}. The results presented in

Fig. 5 show that adoptive immunity in nonirradiated recipient mice is comparable to that of X-irradiated recipient mice. This demonstrates that X-irradiation of the prospective recipient is not an essential requirement for long-lasting "take" of transferred primed spleen cells. As expected, X-irradiated recipient mice were more susceptible to challenge shortly after X-ray (1.5 weeks) for recovery of X-ray-suppressed host-defense mechanisms was apparently incomplete.

DISCUSSION

Survival and functional capacity is an essential requirement of immunological rejuvenation when transferred cells are used. Routinely, in the past, prospective recipients have been exposed to X-rays or treated with immunosuppressive drugs prior to infusion of donor cells. The underlying rationale for such treatment is that X-irradiation increases the amount of space available for transferred cells, and it is tacitly assumed that, in an X-irradiated recipient, transferred cells are able to compete on equal terms with undamaged resident cells of the host because increased numbers of proliferative sites are available. In this regard Najarian and Jolicouer (16), by using isotopically labeled spleen cells from nonimmunized donors, demonstrated that the splenic lymphoid space available for transferred syngeneic cells doubled after X-irradiation of recipient mice. Moreover, Celada (6) found that the secondary antibody-forming capacity of previously immunized spleen cells is 50 to 100 times better in X-irradiated than in nonirradiated syngeneic recipients when challenged immediately after cell transfer.

However, from the practical point of view, it is not desirable to subject the prospective recipient to deleterious X-irradiation or to immunosuppressive drugs. There are reports suggesting the possible feasibility of this approach. Thus, Micklem et al. (15) by using CBA T_6T_6 marker bone marrow cells found that donor cells could be detected in nonirradiated recipients and that they were able to proliferate in the spleen, lymph nodes, thymus, and bone marrow as late as 12 weeks after cell transfer. However, the majority of the cells (> 95%) were eliminated. Doenhoff et al. (9) have also demonstrated that the number of lymphoid cells, derived either from a neonatal thymus lobe implanted under the kidney capsule of a nonirradiated recipient or following repeated injection of dispersed thymocytes, becomes a small, but significant, part of the lymphocyte pool of the recipient (~3 and 5%, respectively). Furthermore, Najarian and Jolicouer (16) have also demonstrated that significant numbers of transferred spleen cells colonize in the spleen of nonirradiated recipient mice. They found that the subsequent "half-life" of the transferred cells in the spleen of nonirradiated recipients did not differ from cells transferred to X-irradiated (400 to 1,100 R) recipients.

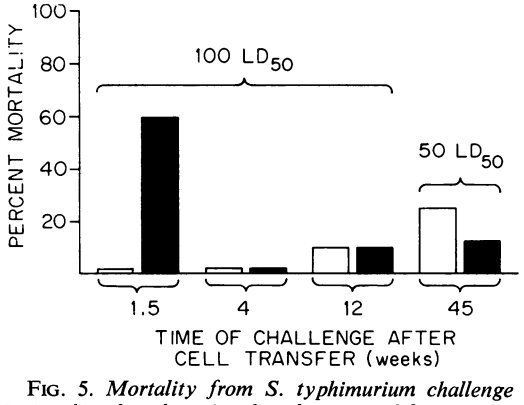

FIG. 5. *Mortality from S. typhimurium challenge in irradiated and nonirradiated young-adult mice receiving 5 × 10^7 spleen cells from immunized donors. Nonirradiated (□); irradiated (400 R, ■); 10 mice per group).*

Unfortunately, none of these studies has assessed the long-term survival and functional capacity of these cells. Hence, the present observation of long-lasting (> 45 weeks), adoptively transferred immunity against *S. typhimurium* infection is significant. Moreover, the finding that protection against an otherwise lethal infection with *S. typhimurium* was as great in nonirradiated as in irradiated recipients constitutes definitive evidence that X-irradiation is not required for survival of potentially functional cells. Perhaps the present method of assessing the biological capabilities of the transferred cells permits a more sensitive and relevant assessment of persistence of immunocompetent cells. In this regard it should be noted that intraperitoneal challenge with *S. typhimurium*, in contrast to intravenous challenge, results in rapid and extensive extracellular growth of the pathogen. Opsonization of bacteria with specific immune sera increases the rate of phagocytosis and substantially increases the number of bacteria killed immediately following infection (8). Only minute amounts of antibody are necessary (18). Although no attempts have been made to determine the relative contribution of humoral versus cell-mediated immunity in the present study, specific immunoglobulin synthesized by persisting antibody-forming cells may provide a unique, highly sensitive marker for the presence of functional immunocompetent cells. Regardless of the explanation, the pertinent observation is that adoptively transferred immunocompetent cells

conveyed high-level, long-lasting immunity to nonirradiated recipients.

The earlier attempts of Metcalf et al. (14) to enhance the humoral antibody response of aged mice to sheep red blood cell antigens with syngeneic spleen and thymus grafts from nonimmunized neonatal mice were unsuccessful. In retrospect, failure could have been associated with the following. The stimulatory dose of antigen may not have reached the subcutaneous graft sites in sufficient concentration to initiate an immune response. Spleen cells from neonatal donor mice are immature, and the aged environment may not have been conducive to optimal functional development. Limited cellular migration may have restricted essential cellular interactions between grafted thymus cells and potentially competent bone marrow-derived cells resident in the spleen and lymph nodes of the aged recipient. Perhaps of greater significance is that nonimmunized lymphoid tissue has a very limited number of antigen-sensitive immunocompetent units (10). In marked contrast, spleen from previously immunized donors contains a greatly expanded population of immunocompetent units responsive to the sensitizing antigen (10).

In contrast to the findings of Metcalf et al. (14) it should be noted that, by using an autoimmune-prone strain of relatively short life-span, Teague and Friou (19) have reported that substantial benefit can be derived from transferred nonimmunized spleen and thymus cells in nonirradiated recipients. When thymus cells from young (7-week-old) A/Jax mice were transferred to older (36- and 72-week-old) syngeneic recipients that had developed anti-2,4-dinitrophenol (DNP) antibody spontaneously (autoantibody), the anti-DNP activity decreased or disappeared from the sera of most recipients. Furthermore, injection of young (4-week-old) thymus or spleen cells prevented older (36-week-old) A/Jax mice from developing autoimmune anti-DNP antibody after specific immunization with DNP. If such autoantibodies have deleterious effects on survival and life-span, then this treatment should extend the life-span. Unfortunately, no such studies were carried out. In this regard, Albright et al. (1) previously could not extend significantly the life-span of aged (78-week-old) $BC3F_1$ mice by transfer of nonimmunized spleen and bone marrow cells. In that study (1) no assessment of immunological indices was made, and the unsuccessful effort was attributed to the inability of nonimmunized spleen cells to interrupt the progressive development of deleterious spleen factors (i.e., most probably tumor cells). In the long-living $BC3F_1$ hybrid, 80% of the animals die with tumors of which more than half are reticulum sarcoma, and an additional 10% die from infectious processes (7). Based on the findings reported here, it would appear that protection could have been afforded these aged animals by infusion of immunocompetent cells from young donors previously immunized against the causative agents. The ultimate test of this model would be to utilize such a polyvalent vaccine and assess its effect on resistance and life-span.

Finally, it should be noted that spleen cells can be frozen, stored at liquid nitrogen temperature, and later thawed without loss of functional integrity, which confirms previous observations from this laboratory (2). This finding demonstrates the feasibility of establishing reserve banks of frozen, viable, immunohematopoietic cells to meet clinical disease crises in later life or to be used to reconstitute these systems after destruction by accidental exposure to radiation, toxic chemicals, or deleterious environmental mutagens. In view of the complexity of human histoincompatibility reactions, banking a reserve of an individual's own optimally functioning immunohematopoietic cells or tissue for later immunological rejuvenation offers, hopefully, a rational and realistic approach to this complex and unresolved problem.

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