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Age-Related Decline in the Resistance of Mice to Infection with Intracellular Pathogens

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Resistance to infection with *Toxoplasma gondii* and *Listeria monocytogenes* in BALB/c female mice decreased with increasing age. The decrease was apparent as early as 9 months of age and was more marked as the animals aged further. This age-related decline in resistance was not restricted to BALB/c female mice, as male and female mice of the C57BL/6 strain exhibited similar responses. With both pathogens, aged mice showed a more marked susceptibility to the strain of lesser virulence. Transfer of normal serum from old mice to young or old mice before infection with *T. gondii* resulted in an increased susceptibility to this organism, suggesting the presence of inhibitory factors or the absence of potentiating factors that are present in the serum of young mice.

During the past decade, a large body of evidence has accumulated which demonstrates a significant effect of the process of aging on the immune response of both animals and humans. In studies on cell-mediated immunity, the capacity of lymphocytes of old mice to effect a graft-versus-host reaction (24, 26) and to proliferate when exposed to mitogens (7, 16, 26) was found to be diminished in comparison to lymphocytes from young mice. Lymphocytes from old mice have also variously been reported to be significantly less efficient (14, 22), or to be as good as those from young mice as either responders or stimulators in mixed lymphocyte culture (26). Another lymphocyte-mediated effect, primary allograft rejection, is significantly delayed in old mice (13).

Other age-related deficiencies have been shown in the production of specific T cell functions. With old mice, the level of production of cytotoxic T cells by MLC (22) or by in vivo immunization (6, 13) has been noted to be markedly less than when young mice were employed. That an age-related decline in an immune function as measured in vitro may not completely reflect the magnitude of the immunological dysfunction in vivo is suggested by observations such as those made by Goodman and Makinodan (6). These workers used the transplantable mastocytoma P-815 in young and old mice, and noted a 500-fold decrease in resistance of old mice to the tumor in vivo at a time when the production of cytotoxic T cells (tested in vitro) was only fourfold less than in young mice.

In studies on the primary humoral antibody response, the response of aged mice was found to be significantly less than that of young mice (5, 7, 8, 15). This lesser response was marked particularly by a decrease in the avidity of the antibody produced (5, 8) and by an increased number of suppressor T cells (5, 21) in the aged mice.

As cellular immunity has been shown to be of paramount importance in recovery from, and resistance to, intracellular pathogens, including bacteria (11), viruses (2), and protozoa (4), and as humoral immunity may play an important role as well (1), we considered it of interest to study whether the age-related decline in general immune function produces a significant decrease in the resistance of aged mice to intracellular opportunistic pathogens.

MATERIALS AND METHODS

Mice. Mice of the BALB/cAN and C57BL/6 strains were purchased from Simonsen Laboratories, Gilroy, Calif. Old mice were purchased at 8 to 12 months of age and maintained in our animal quarters until they attained the age desired for the experiment (9 to 18 months). Young mice of the same strains, ranging in age from 2 to 5 months, were purchased as required from the same source and were not used earlier than 2 weeks after arrival in our animal quarters.

Listeria monocytogenes. Two strains of *L. monocytogenes* were employed, and inocula prepared with each were administered to mice by the intravenous (i.v.) route in a volume of 0.2 ml of Hanks balanced salt solution (HBSS). The less virulent strain had been used previously in this laboratory (10) and had

an i.v. mean lethal dose (LD_{50}) of approximately 6×10^5 in the strain of mice used. The more virulent strain was obtained from Frank Collins, Trudeau Institute, Saranac Lake, N.Y., and had an i.v. LD_{50} of approximately 4×10^4. Stock suspensions of these strains were prepared in the following manner. A culture was grown to peak log phase (8 h) in Trypticase soy broth (Difco), washed three times in HBSS at $3000 \times g$, and resuspended in phosphate-buffered saline, pH 7.2 containing 1% gelatin. One-milliliter portions of this preparation were stored at −70°C and thawed and diluted as necessary for use. Quantitation of viable organisms in each inoculum was performed on tryptic soy agar.

In each individual experiment, mice were injected i.v. with doses of 20% LD_{50}, LD_{50}, or 5 LD_{50}.

Toxoplasma gondii. The C56 and C37 strains of *T. gondii* were used in these experiments. Trophozoites were prepared from peritoneal fluid as described previously (9), and the number of organisms was quantitated by counting in a hemacytometer. Appropriate dilutions were made in HBSS containing 10% heat-inactivated (56°C for 30 min) fetal calf serum, and the inoculum containing the desired number of organisms was injected i.v. into mice in a volume of 0.2 ml. All procedures from the time of harvest of organisms from the peritoneal cavity of mice to time of i.v. injection into mice required approximately 1 h.

Serum transfers. Donor mice, aged 17 or 3 months, were bled to death from the axilla, and blood from each age group was pooled. The blood was allowed to clot at 37°C for 30 min and centrifuged at $1,500 \times g$ for 20 min, and the serum was removed. Serum transfers were carried out within 2 h of clotting. Groups of 17- and 3-month-old recipient mice syngeneic with the donors were injected with 0.2 ml of a serum by the intraperitoneal (i.p.) route 1 h before infection.

Cell transfers. Suspensions of cells were prepared from spleens of 17- and 3-month-old mice. Spleens were minced with scissors, and then the pieces were pressed through a 60-mesh stainless-steel grid into medium 199 (Grand Island Biological Co., Berkeley, Calif.) containing 10% heat-inactivated fetal calf serum (M199-FCS). The resulting cell suspension was washed twice in M199-FCS, and clumps were removed by low-speed centrifugation. The resulting supernatant was removed and counted in a hemacytometer. Viability was determined by trypan blue exclusion. An inoculum of 0.5 ml containing 10^8 viable mononuclear cells in M199-FCS was injected i.p. into recipient 17- or 3-month-old mice 1 h before infection.

Experimental design and evaluation of results. Groups of 6 to 10 old and young mice of the same sex and strain were injected intravenously with a range of doses of either *Listeria* or *T. gondii*. Prior experience in this laboratory has indicated the time span within which mice die from the effects of the agents used. Mice were checked daily for fatalities until approximately 7 days after these expected times to death (7 days for *Listeria*, 21 days for *T. gondii*). Significance of the results was assessed by the chi-square test.

RESULTS

Effect of age on susceptibility to *Listeria* infection. Aged BALB/c female mice differed in their susceptibility to two strains of *L. monocytogenes* when compared with young mice. As can be seen in Fig. 1, when 9- and 2-month-old mice were inoculated with 6×10^5 organisms of the less virulent strain, mortality was significantly greater in the older mice. All of the older mice, but only 60% of young mice, had died by day 9 ($P < 0.02$). When 11- and 2-month-old mice were inoculated with 10^5 organisms of the more virulent strain (Fig. 2), the older mice appeared to be slightly less susceptible than the young ($P < 0.05$) at 6 days. However, 18-month-old mice in the same experiment began dying earlier and were all dead by day 6 ($P < 0.025$).

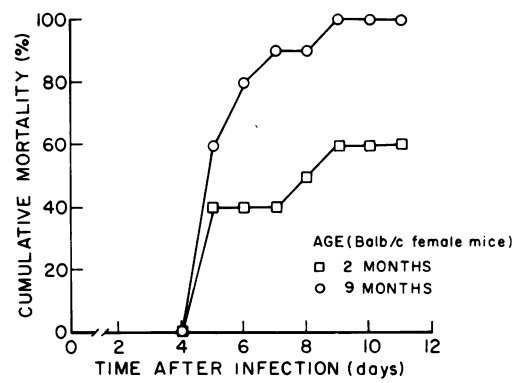

FIG. 1. *Increased susceptibility of aged BALB/c female mice after i.v. challenge with 6×10^5 organisms of the less virulent strain of L. monocytogenes ($P < 0.02$, day 9).*

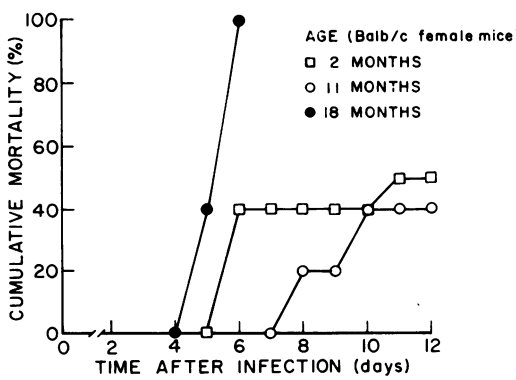

FIG. 2. *Increased susceptibility of aged BALB/c female mice after i.v. challenge with 10^5 organisms of the more virulent strain of L. monocytogenes. Six days after infection, 2-month-old mice showed significantly more mortality than 11-month-old mice ($P < 0.05$) and significantly less mortality than 18-month-old mice ($P < 0.025$).*

Response to *T. gondii* infection. Infection of BALB/c female mice with the C56 strain of *Toxoplasma* produced 100% mortality at the lowest intravenous dose tested (500 trophozoites). At doses of both 500 and 5,000 trophozoites, 9-month-old mice began dying 1 day earlier than 4-month-old mice and reached 100% mortality 1 day earlier (Fig. 3). At a challenge dose of 500 trophozoites, the older mice showed significantly greater mortality at day 7 ($P < 0.0125$) and, at a dose of 5,000 trophozoites, significantly greater mortality at day 9 ($P < 0.0125$).

The increased susceptibility of older BALB/c female mice was more clearly brought out with i.v. inoculation of the less virulent C37 strain of *Toxoplasma* (Fig. 4). A dose of 4×10^5 trophozoites of this strain killed 100% of 15-month-old mice but only 40% of 5-month-old mice ($P < 0.0025$). One-fifth of this dose (8×10^4) killed

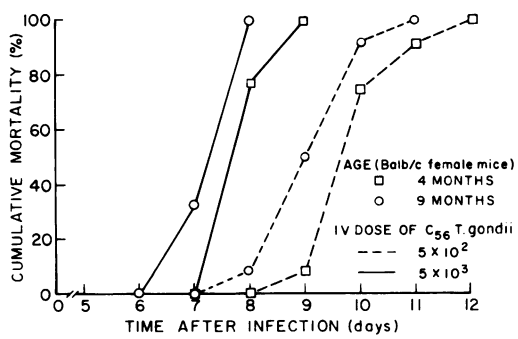

FIG. 3. *Increased susceptibility of aged BALB/c female mice after i.v. challenge with 5×10^2 or 5×10^3 trophozoites of the C56 strain of T. gondii (5×10^2, $P < 0.0125$ at day 9; 5×10^3, $P < 0.0125$ at day 7).*

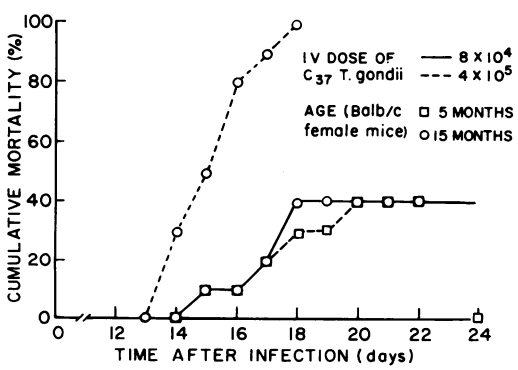

FIG. 4. *Increased susceptibility of aged BALB/c female mice after i.v. challenge with 4×10^5 or 8×10^4 trophozoites of the C37 strain of T. gondii (4×10^5, $P < 0.0025$ at 18 days; 8×10^4, $P < 0.05$ at 20 days).*

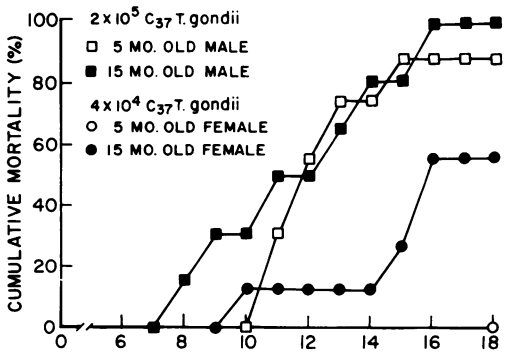

FIG. 5. *Increased susceptibility of aged male and female C57BL/6 mice following i.v. challenge with 2×10^5 or 4×10^4 trophozoites of the C37 strain of T. gondii (males receiving 2×10^5, $P < 0.05$ at 10 days; females receiving 4×10^4, $P < 0.005$ at 16 days).*

40% of 15-month-old mice by day 20; the cumulative mortality pattern in this group was nearly identical with that of 5-month-old mice that had received the higher challenge dose. All young mice injected with 8×10^4 C37 trophozoites survived the infection.

To determine whether this phenomenon was restricted to female mice or the strain of mice employed (BALB/c) or both, groups of 15- and 5-month-old male and female C57BL/6 mice were injected i.v. with a range of doses of the C37 strain of *T. gondii* (Fig. 5). Male and female C57BL/6 mice did not appear to differ significantly in their susceptibility. Age, however, did affect the outcome; 15-month-old male mice began dying 3 days earlier and had significantly greater mortality at 10 days ($P < 0.05$) than did 5-month-old males. The differences in final mortality were not significant. At a dose of 4×10^4, a significant increase in susceptibility of 15-month-old female mice was noted when compared with 5-month-old mice: 55% mortality versus 0%, respectively, at 16 days ($P < 0.005$). At the higher i.v. dose of C37 *T. gondii* (2×10^5), there was no significant difference between old and young mice (data not presented).

Effects of transfer of humoral factors and cells. To determine whether the observed increased susceptibility or resistance to *T. gondii* infection could be transferred with normal serum or spleen cells, portions of pools of serum or spleen cells from uninfected 17- or 3-month-old BALB/c female mice were injected i.p. into 3- or 17-month old mice before infection. After infection, mice were observed for 24 days.

The results are shown in Fig. 6 and 7. Figure 6 shows that injection of "young" serum into old mice conferred significant protection compared with injection of "old" serum, both in terms of

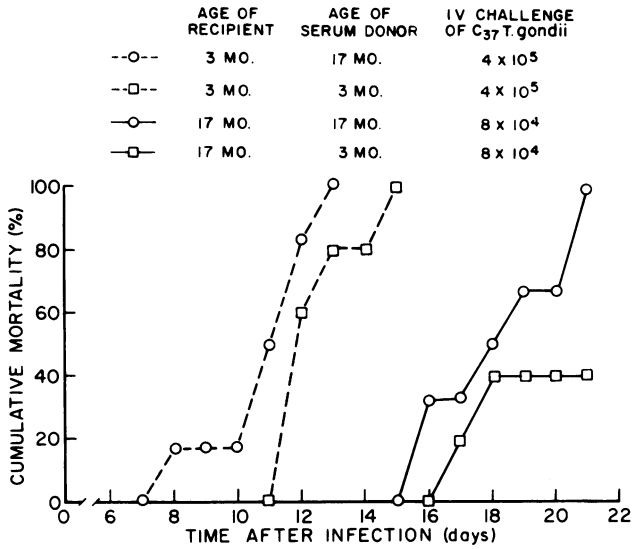

FIG. 6. *Increased susceptibility of aged and young female BALB/c mice receiving 0.2 ml of pooled serum from old mice i.p. before i.v. challenge with 8 × 10⁴ or 4 × 10⁵ trophozoites of the C37 strain of T. gondii (aged mice challenged with 8 × 10⁴, P < 0.025 at 21 days; young mice challenged with 4 × 10⁵, P < 0.05 at 11 days).*

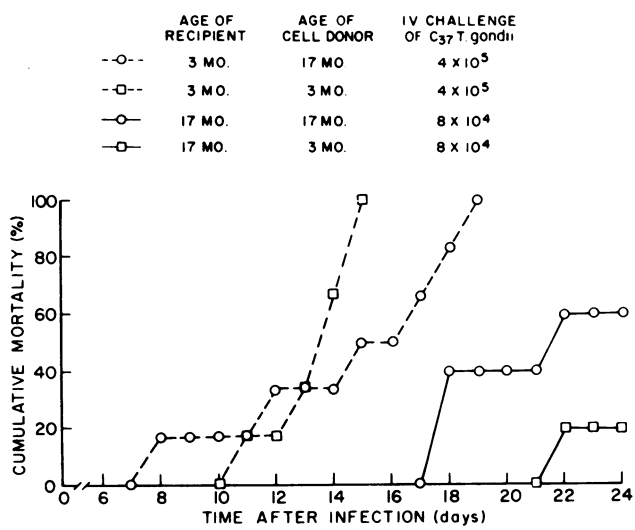

FIG. 7. *Changes in susceptibility of aged and young mice receiving 10⁸ spleen cells from young or aged donor mice i.p. before infection with 8 × 10⁴ or 4 × 10⁵ trophozoites of the C37 strain of T. gondii. Aged mice receiving "young" spleen cells had decreased mortality compared with mice receiving "old" spleen cells (P < 0.05 at 20 days). Young mice receiving "old" spleen cells showed prolonged survival, compared with young mice receiving "young" spleen cells (P < 0.025 at 15 days), but commenced dying earlier.*

delaying onset of death and in final mortality (40% compared with 100% for "old" serum, $P < 0.025$ at 21 days). Conversely, injection of "old" serum into young mice resulted in earlier death of young mice when compared with time to death in young mice injected with "young" serum ($P < 0.05$) at day 11.

Injection of "young" spleen cells into old mice (Fig. 7) resulted in an increased survival time and decreased final mortality when compared with the same parameters in old mice injected with "old" spleen cells ($P < 0.05$ at 20 days). However, the effect of "old" cells on young mice was not consistent. These mice began to die earlier than the young mice that had received "young" spleen cells. However, at day 15, 100%

of the young mice receiving "young" spleen cells had died, whereas only 50% of the young mice receiving "old" spleen cells had died ($P < 0.025$); the latter group did not reach 100% mortality until day 19.

DISCUSSION

The importance of the cellular immune response in recovery from primary infection with the intracellular bacterium *L. monocytogenes* has been clearly demonstrated (11). The situation with *T. gondii* is not as clear, since resistance to this intracellular protozoan has been successfully transferred with immune serum (9) and with immune cells (4). The latter, however, appear to play the major role in resistance to this organism (20; J. S. Remington and J. L. Krahenbuhl, *in* A. J. Nahmias and R. J. O'Reilly (ed.), *Immunology of Human Infections*, in press). Our results described above indicate that whatever the mechanisms of primary resistance, their efficiency against infection with *L. monocytogenes* and *T. gondii* decreases markedly in mice as the animals age.

The mechanism(s) of the age-related decline in resistance to infection which we observed may be multifactorial, but two factors, acting alone or together, seem most apparent. The physical and physiological degenerations, due to the process of aging per se, that create a milieu within the aged host more favorable for survival and multiplication of a pathogen have been referred to by Price and Makinodan (19) as extrinsic deficiencies. The specific deficiencies due to the aging process that are associated with the immune cells directly involved in both afferent and efferent limbs of an immune response, whether it be to an antigen or to a pathogen, were referred to as intrinsic deficiencies (18). Their studies on the age-related decline in antibody formation to sheep erythrocytes revealed that the decline in old mice was due to both extrinsic (19) and intrinsic deficiencies. The latter was reflected by a reduced capacity of thymus- and bone marrow-derived cells to grow and proliferate, as well as a decline in the efficiency of antigen processing (18).

In the present experiments, an attempt was made to examine the possible role of serum factors and spleen cells in the observed altered resistance of older mice. Transfer of serum from uninfected young mice into old mice conferred protection against subsequent *Toxoplasma* challenge when compared with an equivalent transfer of serum from old mice. In contrast, transfer of serum from old mice into young mice increased their susceptibility to *Toxoplasma* challenge when compared with an equivalent transfer of serum from young mice. Among the possible interpretations which might be set forth to explain these results are the presence of detrimental factors or lack of accessory factors (present in serum of young mice) in serum of old mice which may affect growth and differentiation of immunocompetent cells. Another possibility is that factors may be present in serum of older mice that promote, or factors in serum of young mice that inhibit, in vivo growth of the organisms themselves.

When spleen cells from young mice were transferred into old mice, a clear protective effect against *Toxoplasma* challenge was observed in comparison with old mice receiving spleen cells from old mice. The mechanism(s) whereby such protection was conferred includes the possibility that spleen cells of young mice may have provided an increased number of more efficient immunocompetent precursor cells to respond to the infection. Alternatively, the spleen cell population of the older mice may have contained an increased number of suppressor cells (5, 21). Recently, the reduction in the in vivo anti-sheep erythrocyte antibody response and the depressed response of spleen lymphocytes to mitogens in aged mice were reversed by a combined bone marrow-thymus graft from young mice (7).

Perkins achieved long-term restoration of the decreased resistance of aged mice to infection with *Salmonella typhimurium* by injection of spleen cells from young mice previously immunized with *S. typhimurium* (17). No attempt was made to compare the efficacy of spleen cells from aged immunized mice. These results are difficult to interpret in the context of our study, as spleen cells from unimmunized old or young mice were not transferred to determine whether immune capability could be restored by normal cells from young mice.

The decrease in resistance of aged mice to infection with *Listeria* and *Toxoplasma* was marked when compared with resistance in young mice. An age-related decline in resistance has previously been noted for *S. typhimurium* (17) and *Trichinella spiralis* (3) infections of mice. The subject of the age at which a mouse can be considered "old" has been raised by Walford (25), who contends that much current work on aging and the immune response is being carried out in mice that are not old by gerontological standards. The experiments presented here were performed in mice that would not fit his gerontological definition, but the clear-cut nature of the results suggests that the decline in resistance to infection with age occurs at an

earlier time than the onset of classical senescence. It is of interest that the age-related decline in resistance of mice to *Toxoplasma* and *Listeria* infections varied with the virulence of the particular strain of pathogen. In each infection, the increased susceptibility with age was more marked with the less virulent strain of each organism. It appears likely that infection with the more virulent strains overwhelmed the immune defense system and thereby did not allow subtle defects in the immune system, due to the aging process, to be expressed as easily in terms of decreased survival.

Since the immune systems of mice and humans have been shown to decline in effectiveness with age, particularly the primary T lymphocyte-mediated effector functions (12, 23), the specific immune responses to infection with *Toxoplasma* and *Listeria* are probably also diminished. The deleterious effect of transferring normal serum of older mice to both old and young mice before *Toxoplasma* infection suggests that there is some extrinsic factor in the serum of aged mice that does not allow the full expression of protective immunity after infection.

ACKNOWLEDGMENTS

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