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EFFECT OF AGE ON CELL-MEDIATED IMMUNITY IN LONG-LIVED MICE

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SUMMARY

The cytolytic ability of sensitized spleen cells to kill allogeneic target cells *in vitro*, a correlate of cell-mediated immune activity, was assessed in aging long-lived hybrid mice. There was about a 4-fold decline with age, of which about 2-fold could be accounted for by the decrease in relative numbers of immunocompetent precursor cell units. The terminally differentiated progeny cells of antigen-stimulated precursor cells of old mice were as efficient in killing target cells as were those of precursor cells of young mice. Thus, it would appear that a decrease in the proliferative and transforming capacities of antigen-stimulated precursor cells of old mice can account for the other 2-fold decline with age in the cytolytic activity.

Cytolytic activity may not be the limiting function in resistance to tumour formation. This was indicated by the observation that resistance to allogeneic tumour cell challenge declines with age by as much as 500-fold in mice, showing only a 4-fold decline in their cytolytic activity.

INTRODUCTION

Walford (1969), Burnet (1970), and others (Smith & Landy, 1970) have postulated that a decline in thymus-regulated immune surveillance (Thomas, 1959) is one of the major factors contributing to senescence. If so, there should be a decrease in cell-mediated immune activity with age and a concomitant increase in the incidence of immunodeficiency diseases. However, there is disagreement about the age-related decline in cell-mediated immunity (Makinodan, Perkins & Chen, 1971c; Krohn, 1961; Adler, Takiguchi & Smith, 1971; Chen, Price & Makinodan, 1972). The conflict may arise from several sources, including the use of mouse strains with different life-spans, and the use of assay methods that are difficult to quantitate (Makinodan *et al.*, 1971c; Krohn, 1961; Adler *et al.*, 1971; Chen *et al.*, 1972;

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Teller *et al.*, 1964; Gazdar, Beitzel & Talal, 1971; Teague *et al.*, 1970; Davis & Cole, 1968; Cole, 1962; Hargis & Malkiel, 1972; Stutman, Yunis & Good, 1968; Stjernsward, 1966; Konen, Smith & Walford, 1973). Therefore, this study was undertaken with two objectives. The first was to determine the effects of age on cell-mediated immunity, as expressed by the ability of sensitized spleen cells to kill allogeneic target cells *in vitro*, a well-defined quantitative correlate of cell-mediated immunity. The second was to determine the nature of any age-related alterations in cell-mediated immune activity.

We used long-lived hybrid mice for two reasons: (a) we feel that until the biology of aging is better understood, studies on immunity and aging of any species should be performed on model animals with a long life-span, for animals with a short life-span may not age in the usual sense but die of life-shortening diseases; (b) our model animal is a genetically defined, long-lived hybrid mouse for which we have extensive documentation of actuarial and pathological changes in relation to age-related changes in the humoral immune stem cell, and graft-versus-host activities (Chen *et al.*, 1972; Chino *et al.*, 1971; Makinodan *et al.*, 1971a, b; Price & Makinodan, 1972a, b; Chen, 1971).

MATERIALS AND METHODS

Mice

(C57Bl/Cum × C3H/Anf Cum)F1 mice (H-2^{bk}), hereafter referred to as BC3F1, were the main object of investigation. Their life-span (mean, 30 months; maximum, 47 months) probably is as long as or longer than that of any other strain. For the present studies they were used at 1–32 months of age. (BALB/c × DBA/2)F1 mice (H-2^{dd}), hereafter referred to as CD2F1, were used at 2–4 months of age as recipients for spleen cell transfer studies and for passage of P815 mastocytoma cells (H-2^{d}). Inbred DBA/2 mice (H-2^{d}) were also used on occasion to passage mastocytoma cells.

All mice were obtained from Cumberland View Farms, Clinton, Tennessee, except CD2F1, which were bred from parental strains from the same source. They were housed in groups of ten or less and allowed food and water *ad libitum*. For experiments they were classified as very young (1 month), young adult (3–4 months), middle-aged (22–24 months), or old (27–32 months).

Tumour cells

The P815 mastocytoma tumour, derived from a DBA/2 mouse, was obtained from Dr Michael Potter of NIH and passaged in CD2F1 mice in ascitic form by weekly intraperitoneal transfer for approximately 2 years. Cultured tumour cells were obtained from Dr A. A. Nordin and had been shown to be equivalent to the ascitic form (Goodman, 1973).

X-irradiation

Animals and tumour cell suspensions were irradiated with a G. E. Maxitron X-ray machine as previously described (Price & Makinodan, 1972a). Prospective recipients in the cell transfer study were given a single exposure of 600 R of X-irradiation to the total body, a dose that had been shown in preliminary studies to be optimum for the assay system.

Immunization by cell transfer

The spleens from BC3F1 mice free of gross pathology were removed asceptically, placed

in ice-chilled Hanks's balanced salt solution containing heparin, and teased by the method of Schooley (1966). The desired concentration of spleen cells was injected intravenously (i.v.) in 1 ml into CD2F1 recipients which had been given 600 R of total-body X-irradiation less than 3 hr earlier. At various times thereafter, individual spleens of recipient mice were assayed for cytolytic activity against target tumour cells *in vitro*.

In vitro assay for cell-mediated immunity

Thrice-washed ascitic tumour cells were labelled with sodium chromate ($Na_3{}^{51}CrO_4$, specific activity $\sim$2–8 mCi/μg upon receipt) according to the method of Brunner *et al.* (1970). Labelling was routinely performed with isotope that was not more than 2 weeks old (half-life, 27·8 days). Suspensions of immunized spleen cells were prepared at ice temperature as previously described (Price & Makinodan, 1972a) and incubated in 12×75 mm plastic tubes at a ratio of 100:1 (10^7 viable spleen cells/10^5 viable target cells) in 1 ml of Dulbecco's medium containing penicillin, streptomycin, and 10% heat-inactivated foetal calf serum. The loosely capped samples were incubated at 37°C in 5% CO_2 air, and samples were routinely assayed after 8 hr of incubation. The radioactivity released from labelled target cells was determined by counting a sample of the supernatant in a Nuclear Chicago automatic gamma spectrometer (Model 42–30) (Goodman, 1973). The maximum releasable radioactivity (number of counts) was determined after freezing and thawing triplicate to quadruplicate samples of labelled target cells. This value was 80–95% of the counts incorporated into the cells. Cytolytic activity (percentage maximum cytolysis) was calculated as $[(C_s - C_n)/(C_t - C_n)] \times 100$, where C_s is the number of counts released by target cells in tubes containing the sensitized killer cells, C_n the number of counts released by target cells in tubes containing unsensitized cells ($\sim$10% within 8 hr), and C_t the total number of releasable counts. Samples were assayed in triplicate generally, but in duplicate when the cell yield from individual spleens was low.

Resistance to allogeneic tumour cells

Graded doses of washed viable (Eosin Y excluding) tumour cells were injected intraperitoneally (i.p.) in 1-ml volumes into recipient mice. Cell concentrations below 10^7 cells/ml, were adjusted to 10^7 cells/ml with erythrocytes syngeneic to the recipients, to minimize loss of tumour cells through attachment. The animals were checked daily for 20 days, and all dead mice were autopsied by Dr C. P. Peter. The results are expressed as 20-day cumulative mortality.

RESULTS

Immunization of mice with viable and X-irradiated tumour cells

We began our studies on the effects of age on cell-mediated immunity by immunizing young and old mice with either viable or lightly X-irradiated (600 R) tumour cells and measuring *in vitro* the cytolytic activity of their spleen cells at the time of peak response 12 days later. However, significant numbers of optimally immunized old mice were killed even when X-irradiated cells were used as immunogens. Histological examinations showed that death was caused by proliferation of tumour cells. It is possible that tumour cells may have persisted in the spleen of some surviving old mice at the time of assay. If so, then estimates of their cytolytic activity would be lower than they should be. The reason is that

in the *in vitro* cytolytic assay the ratio of spleen cells and isotopically labelled target tumour cells is kept constant, so that the true ratio of effector cells to target tumour cells in the mixture would be lower than anticipated. As a consequence, a low cytolytic index would be registered, for the index decreases as the ratio of effector cells to target cells decreases (Goodman, 1973; Brunner *et al.*, 1970). Another limitation of this approach is that, because only mice surviving the tumour cell challenge are evaluated, the results would necessarily reflect only the most resistant mice and not the total population of old mice.

This approach was therefore abandoned. Instead, we chose to measure cell-mediated immune activity of spleen cells by injecting them into irradiated allogeneic mice, which are syngeneic to the target cells, and assessing *in vitro* the spleen cells for cytolytic activity at various intervals. This method has been well characterized and is being used extensively as a method of sensitization of lymphoid cells to histoincompatibility antigens (Cerottini, Nordin & Brunner, 1971).

Influence of age on the dose–response relationship

The time of peak response was first determined, using low (10×10^6) and high (50×10^6) doses of spleen cells of young adult and old BC3F1 mice after their transfer into irradiated young CD2F1 recipients, by assessing daily for 7 consecutive days the *in vitro* cytolytic activity of the recipients' spleen cells. The results showed that the peak response was on day 5 after transfer of high and low doses of spleen cells of young mice and high doses of spleen cells of old mice, and on day 6 after transfer of low doses of spleen cells of old mice.

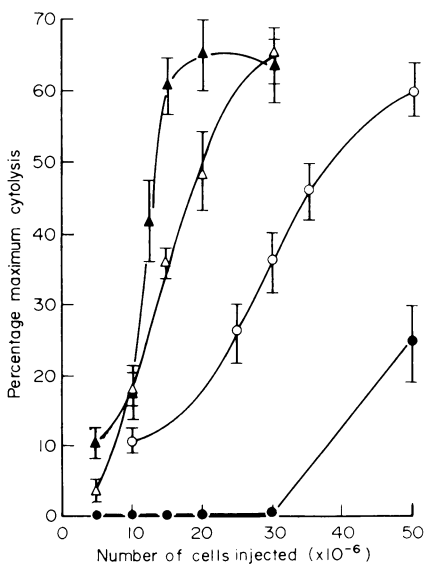

FIG. 1. Dose–response analysis of BC3F1 spleen cells from donors of various ages. Spleen cells from pools of seven to eight spleens of disease-free donors 1 (●), 3–4 (▲), 12 (△), 27 (not shown), or 32 (○) months old were injected into X-irradiated (600 R) CD2F1 recipients. The cytolytic activity of the recipient spleens was assayed 5 days later. The results for 27-month-old donors were similar to those for 32-month-old mice. Bars indicate the standard error of the mean of at least five recipient spleens.

A dose–response study was then performed, using the same cell transfer system to determine the peak cytolytic activity of spleen cells ($5–50 \times 10^6$) from mice of various ages (1–32 months). Only mice free of gross pathological manifestations after inspection of internal tissues were used as spleen cell donors. Because of the variability between experiments, especially with spleen cells of old mice, several dose–response studies were performed; one of the better dose–response curves is presented in Fig. 1. Two quantifiable indices were derived from the curves: (a) the slope of the linear portion of the curve, which expresses the cytolytic generating capacity of spleen cells; (b) the minimum number of spleen cells necessary to generate a maximum response. The cytolytic activity of spleen cells of young mice was most effective as judged by these indices. The slope (percentage cytolysis of target cells per 10^6 spleen cells) of the linear portion of the dose–response curve was 8·5 (95% confidence limits, 7·8–9·3), and the minimum number of spleen cells necessary to generate a maximum response (in this experiment a 65% cytolysis of target cells) was 20×10^6. The cytolytic activity of 12-month-old cells was also high, but not as high: the slope was 3·0 (95% confidence limits, 2·9–3·1), and the minimum spleen cell dose to generate a maximum response was 30×10^6. Spleen cells from 27- and 32-month-old mice were even less effective: the slope was 2·0 (95% confidence limits, 0·5–3·6), and it was estimated that about 60×10^6 old cells were needed to generate a maximum response. These indices could not be obtained with 1-month-old spleen cells, for no cytolytic activity was detected with less than 30×10^6 cells, and effector killer cells were generated only after transfer of 50×10^6 cells.

These studies show a 4-fold difference in the activity of spleen cells of young and old mice as judged by the slope of the linear portion of the dose–response curve, and a 3-fold difference as judged by the minimum dose necessary to generate a maximum response. It is also of interest that the cytolytic activity of spleen cells of 1-month-old mice was less efficient than that of the old mice.

Efficiency of effector killer cells of young and old mice

The 3- to 4-fold difference between the cytolytic activity of spleen cells of young and old mice may be due to a difference in the efficiency of effector killer cells. Henney (1971) has reported that this can be determined by comparing the cytolytic responses of various numbers of sensitized lymphoid cells toward a fixed number of target cells. To determine whether effector cells of old mice can kill target cells as efficiently as cells of young mice, we compared the cytolytic activity of maximally sensitized spleen cells at killer cell:target cell ratios ranging from 200:1 to 1:1. Based on earlier findings (Fig. 1), maximally sensitized spleen cells were obtained when 20×10^6 spleen cells from young BC3F1 mice or 50×10^6 spleen cells from old BC3F1 mice were transferred into irradiated young CD2F1 recipients and the recipient spleens were assayed 5 days later. Fig. 2 shows that the cytolytic activities of maximally sensitized spleen cells of old and young mice were virtually identical at all ratios tested. With both cell types the magnitude of killing of target cells increased at about the same rate as the number of spleen cells, reaching the same maximum level when the killer cell:target cell ratio was 100:1, as reported previously (Goodman, 1973). This indicates that the efficiency of target cell killing is equivalent in old and young cells; however, the frequencies of effector cells in the spleens of young and old mice may be different (Henney, 1971) as a result of decreased frequency of immunocompetent units and/or decreased burst size; i.e. number of effector cells generated from a single precursor cell.

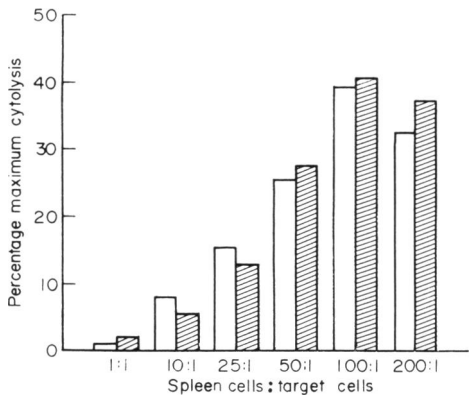

FIG. 2. Efficiency of target cell killing by spleen cells of maximally sensitized young and old BC3F1 mice. Twenty million spleen cells of young (blank columns) and 50 million cells of old (hatched columns) BC3F1 mice were injected into irradiated (600 R) young CD2F1 mice; 5 days later the spleens in each group were pooled, and the spleen cells were assayed *in vitro* at effector cell:target cell ratios ranging from 1:1 to 200:1.

Estimation of the frequency of immunocompetent units

An immunocompetent unit is defined as the minimum number of spleen cells necessary to generate a cytolytic response. Therefore it reflects the minimum configuration of precursor cells which can interact with the antigen to generate cytolytic killer cell progeny. The frequency of immunocompetent units was estimated by the limiting dilution assay, according to the method of Groves, Lever & Makinodan (1970) to determine to what extent the difference between ability of spleen cells from young and old mice to generate maximum cytolytic activity (Fig. 1) is due to a difference in the frequency of such units in the spleen. The experimental design involved assessment of the frequency of nonresponders in limiting numbers of spleen cells of young and old mice, and the frequencies of immunocompetent units were estimated from the calculated slopes of the regression lines in Fig. 3 and not from the y intercept values, for the former method is more precise. In the young adult mice there was one unit in $5{\cdot}9 \times 10^6$ spleen cells (95% confidence limits, $5{\cdot}8$–$6{\cdot}4 \times 10^6$), and in the old mice there was one in $12{\cdot}1 \times 10^6$ spleen cells (95% confidence limits, $11{\cdot}4$–$16{\cdot}5 \times 10^6$). Assuming that the 'cloning efficiency' (i.e. the frequency of cells making up the units that settle in the spleens of irradiated recipients) is about 10% for both old and young spleen cells (Bosma, Perkins & Makinodan, 1968; Chen, 1971) and assuming that there are approximately 10^8 nucleated cells in the spleen of both young and old mice, this would indicate that there are about 250 immunocompetent units in the spleen of a young BC3F1 mouse, an estimate comparable to the number of T cell-dependent immunocompetent units (Bosma, Makinodan & Walburg, 1967; Halsall & Makinodan, 1974) and about 120 in the spleen of an old BC3F1 mouse. It should be noted that this difference is comparable to that of colony-forming stem cell units in the spleen of young and old mice (Albright & Makinodan, 1966; Chen, 1971). This would suggest that the stem cells are contributing substantially to the observed difference. If so, in future studies, some effort should be focused on the effects of age on the differentiation process of stem cells into antigen-responsive immunocompetent precursors of effector killer cells. In any event, the estimated 2-fold

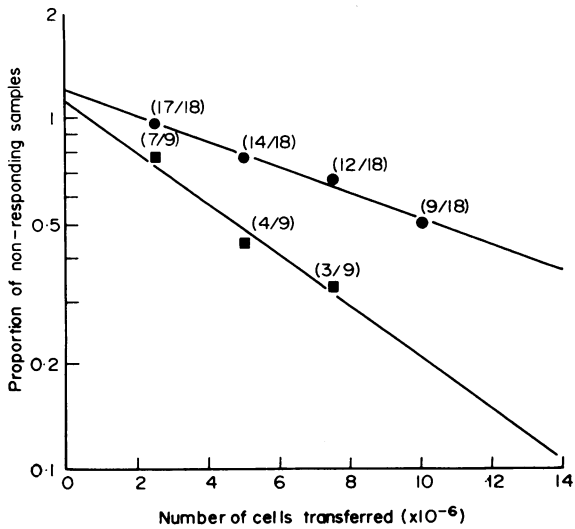

FIG. 3. Estimation of the frequency of cell-mediated immunocompetent units in the spleens of young and old mice by limiting dilution analysis. Limiting numbers of spleen cells from young (■) and old (●) mice were transferred into X-irradiated (600 R) CD2F1 recipients. The spleens of recipients were assayed 5 days after transfer of young adult spleen cells and 6 days after transfer of old spleen cells. Numbers in parentheses show the number of non-responders/number of recipients assayed.

difference in the number of immunocompetent units accounts for only about half the difference in activity observed in the dose–response curves (Fig. 1).

Resistance to allogeniec tumour cells

Our preliminary observations that old mice can succumb to a challenge with even 600 R-irradiated allogeneic tumour cells prompted us to assess relative resistance to allogeneic tumour cell challenge as a function of age. The survival curve was first assessed after i.p. injection of 3×10^7 viable mastocytoma cells into a group of sixteen old BC3F1 mice. The mice were checked daily over a period of 30 days, and necropsies were kindly performed by Dr C. P. Peter. A sigmoid cumulative mortality curve was obtained which reached a maximum of about 94% (fifteen out of sixteen dead). All deaths occurred in 15 days and were attributed to proliferation of the tumour cells. The actuarial data accumulated for this hybrid indicate that only a small percentage (<5%) of the old animals are expected to die of senescence during the 30-day period (Chino *et al.*, 1971). Accordingly, a 20-day cumulative mortality assay was employed in subsequent studies.

BC3F1 mice, 1–32 months old, were then challenged with tumour cells over a 500-fold dose range (3×10^5–$1{\cdot}5 \times 10^8$ cells), and their survival was assessed. The results (Table 1) show that mice between 3 and 12 months of age were most resistant. Mice 16 months of age were less resistant, as they were most susceptible to the highest tumour cell dose tested. Mice that were 1, 20–22, and 28–32 months of age were most susceptible.

It should be noted that $1{\cdot}5 \times 10^8$ mastocytoma cells, the highest tumour cell dose tested, killed only one out of eight (12·5%) of the young mice, but a dose of only 3×10^5 mastocytoma cells was sufficient to kill one out of seven (14·3%) of the old mice. In terms of

TABLE 1. Mortality of BC3F1 mice after intraperitoneal challenge with viable allogeneic mastocytoma cells

Age of mice (months)	Tumour cell dose: 3×10^5 Number dead / Number tested	%	3×10^6 Number dead / Number tested	%	3×10^7 Number dead / Number tested	%	$1{\cdot}5 \times 10^8$ Number dead / Number tested	%
1	0/8	0	3/8	37·5	7/8	87·5	6/6	100
3–4	n.d.	—	n.d.	—	0/10	0	1/8	12·5
12	n.d.	—	n.d.	—	1/7	14·3	0/10	0
16	0/8	0	0/8	0	1/8	12·5	7/14	50·0
20–22	0/8	0	4/9	44·4	9/10	90·0	7/7	100
28–32	1/7	14·3	2/7	28·6	15/16	93·8	n.d.	—

Data show 20-day cumulative mortality. Differences were judged significant at the 0·05 level when the difference between two values was 37·5% or greater. All deaths were attributed to tumour cell proliferation.
n.d. = Not determined.

minimum lethal dose, there seems to be about a 500-fold decrease with age in resistance to challenge with allogeneic tumor cells.

DISCUSSION

These studies show that the ability of spleen cells to kill allogeneic tumour cells *in vitro* declines with age in BC3F1 mice. A 3- to 4-fold difference was detected between the cytolytic activities of spleen cells from young and old mice, as judged by their relative cytolytic generating capacities and by the minimum number of cells necessary to generate maximum cytolytic response, respectively. The decline with age in cell-mediated immune activity of lymphoid cells can be attributed to a decrease in: (a) the cytolytic ability of effector killer cells; (b) the number of specific immunocompetent precursor cell units; (c) the proliferative and transforming capacities of antigen-stimulated precursor cells. The first possibility seems unlikely, since cytolysis of target cells elicited by spleen cells of optimally immunized young and old mice was the same at all effector cell:target cell ratios from 1:1 to 200:1 (Fig. 2). It was also found that effector cells of old mice could lyse target cells as efficiently as effector cells of young mice (Fig. 2) when the method of Henney (1971) was employed to calculate their relative cytolytic efficiencies. Limiting dilution analysis was used to demonstrate that the decline with age in cell-mediated immune activity is attributable only in part to a decrease in immunocompetent units (Fig. 3). On the basis of these findings we suspect that the third possibility, a decrease in the proliferative and transforming capacities of antigen-stimulated precursor cells, is also partly responsible for the decline. It remains to be resolved to what extent the defects are due to changes intrinsic and extrinsic to the precursor cells. With regard to the latter, it is interesting to note that cells which suppress the thymus-dependent humoral response seem to increase with age in BC3F1 mice (Halsall *et al.*, 1973).

The demonstration that resistance to allogeneic tumour cell challenge can decline with

age by as much as 500-fold in BC3F1 mice, which show only a 4-fold decline in cytolytic activity, suggests that the cytolytic activity may not be the limiting immunological function for tumour formation. Therefore, it is likely that a decline with age in tumour resistance and immune surviellance is a result of alteration of both cellular and humoral functions which cannot be assessed by a single assay system.

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