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Age-associated changes in mitogen-induced lymphoproliferation and lymphokine production in the long-lived brown-norway rat: effect of caloric restriction

I. Michael Goonewardene, Donna M. Murasko*

Department of Microbiology and Immunology, The Medical College of Pennsylvania, 2900 Queen Lane, Philadelphia, PA 19129, USA

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Abstract

We have previously demonstrated that age-related declines in Concanavalin A (ConA), induced proliferation and lymphokine production, occur in ad-libitum fed Brown Norway (AL BN) rats. Since caloric restriction (CR) extends lifespan, we expected that the age related changes in immune parameters would be delayed by CR. CR does act to delay age-related changes in proliferation in response to ConA. In addition, CR postpones the plateau in ConA induced interferon (IFN) production seen after 23 months of age in AL rats. However, CR does not postpone the age-related decline in ConA induced interleukin-2 (IL-2) production. Therefore, ConA induced IFN production maybe a good candidate as an early marker of physiologic aging, while ConA induced proliferative response is a possible candidate for a marker of late stages of aging.

Keywords: Caloric restriction; Immune parameters; Brown Norway rat

1. Introduction

The ability of immune cells to respond to various forms of stimuli changes with advancing age. Decreases in proliferative response to antigens and mitogens have

* Corresponding Author, Tel.: + 1 215 9918357; Fax: + 1 215 8482271.

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been reported in humans [1], rats [2,3 and mice [4], as well to anti-CD3 antibody in mice [5] and humans [6]. Although there have been numerous investigations of possible mechanisms of this decline in response with increasing age, including changes in lymphokine production, calcium fluxes and phosphorylation, the mechanism of the decline has not been definitively established.

The anti-aging effects of caloric restriction (CR) in rodents have been known since 1934 [7]. Mean and maximal lifespan have been shown to increase in both mice and rats on CR diets. In addition, long-lived strains of mice exhibit decreased tumor incidence on CR diets [8,9]. It has been hypothesized that CR may exert positive effects upon the immune system, which may be part of the mechanism of extended life [10]. The effects of CR on immune function were shown as early as 1971 [11]. CR has been shown to correct IL-2 production deficits in autoimmune mice, as well as to prolong their life [12]. CR ameliorates age-related declines in both PHA and ConA induced proliferation in splenic lymphocytes of mice [8,9]. CR also improves age-related declines in influenza-specific T cell proliferation in mice [13].

There are fewer studies of the effect of CR on immune response of rats than of mice. In the long-lived Wistar-Lobund (WL) rat, CR rats of all ages had increased mitogen and alloantigen induced proliferation compared to ad libitum fed (AL) rats of comparable ages, and CR partially postponed age related declines in those responses [14]. CR partially reversed age-related decreases in ConA induced proliferation in Fischer 344 (F344) rats; however level of response of young AL and young CR rats was comparable [15,16]. IL-2 and IL-2 mRNA induction by ConA were also shown to be higher in aged CR versus aged AL F344 rats [16]. However, in Sprague–Dawley (SD) rats, there was no significant difference between AL and CR rats in mitogen stimulated proliferation of splenocytes in either young or old rats [17].

All of the studies on rats used small numbers of animals. In addition, some of the studies did not use barrier maintained rats. In order to expand the information available on the effect of CR in rats, we evaluated the effects of CR on mitogen induced proliferation and lymphokine production in a large number of Brown Norway rats of various ages that were maintained throughout their lifespan under strict barrier conditions.

2. Materials and methods

2.1. Animals

Brown Norway (BN) rats of various ages were obtained from The National Center for Toxicological Research (NCTR), Jefferson, AK. All rats were nursed by ad-libitum (AL) fed mothers and after weaning were fed NIH-31 BP rodent pellets AL. At two months of age rats were separated into two groups. One group continued to feed AL and the other group was restricted to 60% of the total calories of AL rats over a four week period using NIH-31 BS pellets, supplemented by vitamins and minerals. They were maintained in AAALAC certified, specific pathogen-free barrier facilities at The Medical College of Pennsylvania for at least

one week before assays were performed. In fact, in all but one study, rats were acclimated for 2 to 3 weeks prior to assay. Autoclaved water was offered ad-libitum to both groups. Environment and diet were maintained as similar as possible to the specifications established at NCTR, including the same food which was shipped with the rats. These rats were individually housed throughout their lifetime in specific pathogen-free barrier facilities.

2.2. Preparation of splenocytes

Rats were sacrificed by guillotine and the spleens removed. Red blood cells were lysed with 0.86% NH_4Cl. Mononuclear cells were further purified by density gradient centrifugation using Histopaque 1088 (Sigma). Cells were resuspended at 2×10^6 cells/ml in growth media: RPMI 1640, 10% inactivated CPSR-5 serum (Sigma), 1% l-glutamine, 2×10^{-5}M 2-mercaptoethanol and 50 mM gentamicin.

2.3. Mitogen stimulation assays

Cells (2×10^5) were incubated in triplicate with the appropriate concentration of mitogen in 96-well round bottom plates (Flow Labs, MacLean, VA). Mitogen concentrations were final well concentrations. Plates were incubated at 37°C and 5% CO_2. Supernatants were taken just prior to radioactive labelling at 48, 72, or 96 h to assay for interferon (IFN) and interleukin-2 (IL-2) activity. Each well was pulsed with 1.0 μCi ^{3}H-thymidine for 4 h prior to harvest, then harvested with a Cambridge Cell Harvester onto glass fiber filters. Amount of ^{3}H-thymidine incorporation was determined by liquid scintillation counting and expressed as:

Net Counts per Minute (CPM) = (CPM with mitogen) − (CPM with media alone).

2.4. IFN assay

IFN was assayed by the method of Brennan and Kronenberg [18] using rat transformed kidney cells (RtK) and challenged with vesicular stomatitis virus. Cells were plated in 96-well flat bottom plates with serial two-fold dilutions of culture supernatants and incubated at 37°C, 5% CO_2 for 24 h. Cells were then challenged with virus and incubated for an additional 24 h. Plates were then scored for cytopathic effect (CPE). One unit of IFN is defined as the inverse of the dilution that gives 50% protection from viral CPE. All assays were corrected for the rat IFN standard from Lee Biomolecular, San Diego, CA.

2.5. IL-2 assay

IL-2 was assayed using the IL-2 dependent cytotoxic T-cell line CT-20, after the method of Gillis (Gillis et al., 1978). Briefly, CT-20 cells were harvested and washed free of IL-2. 4×10^3 cells/well were cultured with serial two-fold dilutions of supernatants. After 20 h of incubation (37°C, 5% CO_2), cells were pulsed with 0.5 μCi of ^{3}H-thymidine for 4 h. Cultures were harvested onto glass filter discs and ^{3}H-thymidine incorporation was determined by liquid scintillation counting. One unit of IL-2 activity was defined as the amount of the supernatant that produced 50% of the maximal proliferative response generated by the lab standard (Genzyme

rHuIL-2). Calculations of units/ml were performed by Probit analysis (parallel line assay).

2.6. Statistics

The Student's t-test of significance was used to compare groups. Determination of correlation between various parameters was evaluated by Pearsons' correlation coefficient.

3. Results

3.1. Effect of CR on survival

Fig. 1 shows the survival curves generated at NCTR for the cohorts of the BN rats used in this study. The median lifespan (50% survival) of male and female AL BN rats was 31 months. Median survival for CR males was 35.5 months, and 39 months for female CR rats. These represent increases in median lifespan of 15% for males and 26% for females. Although this increase is significant, it is not as large an increase as demonstrated by other strains (e.g., Fischer 344).

3.2. Effect of aging and CR on mitogen responsiveness

Preliminary data established that neither aging nor CR affected the dose or period of incubation required for maximal proliferative response to ConA, with all groups showing peak proliferation when incubated with ConA 4 μg/ml for 72 h. This allowed direct comparisons of all groups at one dose and time point. Fig. 2 shows two typical experiments of ConA-induced stimulation. Young (6–12 month) AL, BN rats consistently showed greater proliferative response than old ($>$ 30 month) AL, BN rats. In contrast, mean proliferation of old CR rats was not significantly different from response of young CR rats assayed in the same experiment as the AL rats. In fact, in some experiments, the response of old CR rats was greater than young CR rats. While proliferative responses to ConA was not significantly different between young AL and young CR rats, proliferation was always greater in old CR rats compared to old AL, BN rats. These data demonstrate that CR rats maintain a consistent level of ConA response throughout the ages tested compared to old AL rats, and very similar to young AL rats.

We have previously reported that age-related declines in mitogen-induced lymphoproliferation are consistently observed after 31 months in AL BN rats [3]. We wanted to examine whether CR in BN rats postponed this effect. Correlation of ConA induced proliferation to age of individual CR BN rats shows no age-related decline in proliferative ability ($r = 0.06$; Fig. 3). This is also true for PHA-induced proliferation ($r = 0.05$; data not shown). CR rats maintain proliferative ability to mitogens even at the highest ages examined (36 months).

3.3. Lymphokine production after mitogen stimulation

a. IL-2: We have previously shown that ConA induced IL-2 production in AL BN rats drops significantly after 30 months of age [3]. It is possible that maintenance of proliferative response is due, in part, to continued ability to produce IL-2

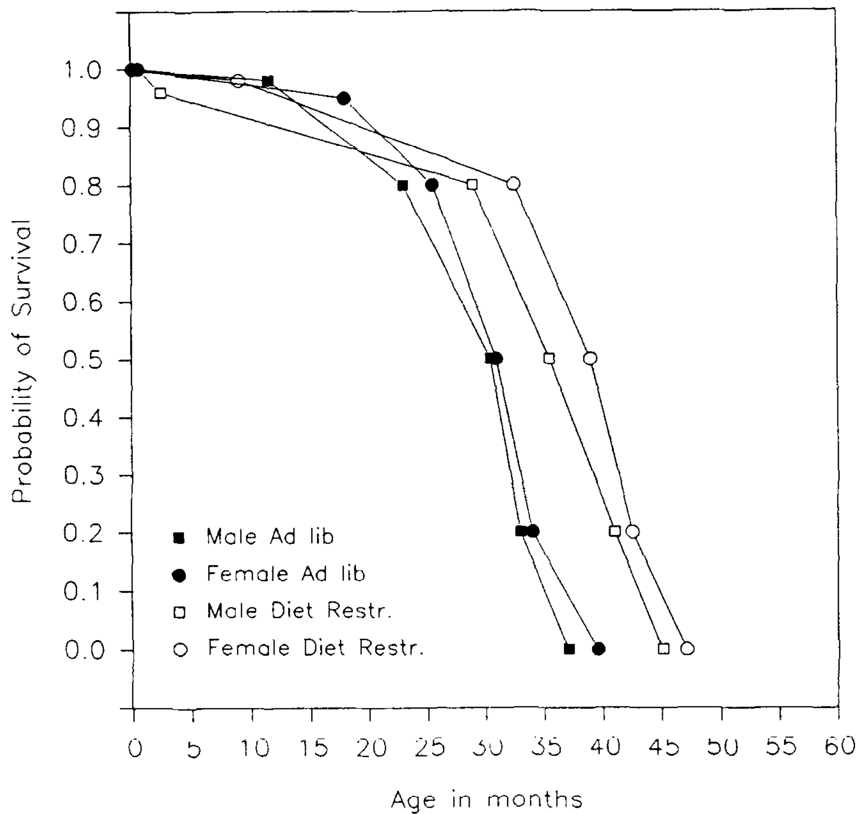


Fig. 1. Survival curve for BN rats. Graphs represent data of cohorts of those used in this study. Age is shown in months for (■) male AL, (●) female AL, (□) male CR, (○) female CR. Used with permission.

in aged CR rats. The dose of ConA and the time of incubation for peak IL-2 production was the same for all ages of both AL and CR BN rats (data not shown). We, therefore, analyzed IL-2 production from splenocytes stimulated under these peak conditions: ConA 16 μg/ml for 48 h (Fig. 4). Four age groups were selected based on regions of survival curves of AL rats: young (2–12 months; period when no deaths were observed); middle-young (13–22 months; 95–80% survival); middle-old (23–29 months; 80–50% survival); and old (30 + months; < 50% survival). IL-2 production by AL BN rats stays constant through 29 months of age and then

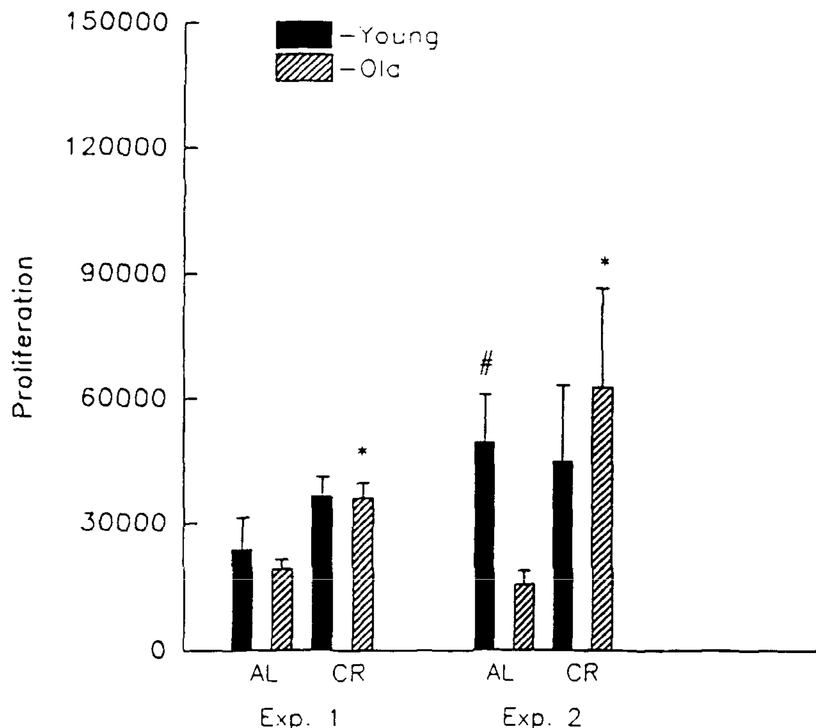


Fig. 2. ConA induced lymphoproliferation (net counts per minute) of two separate experiments comparing old (> 30 months) and young (6–12 months) BN rats after stimulation with ConA 4 μg/ml, 72 h. Bars show mean $\pm$ S.E.M. Exp.1 (n = 5 AL, $n = 6$ CR); Exp.2 (n = 5 AL, $n = 6$ CR). * = statistically significant compared to AL of same age; # = statistically significant compared to young group of same diet by Student's t-test $P < 0.05$.

drops precipitously after 30 months of age (Fig. 4). In contrast to our prediction, CR does not significantly modify IL-2 production within any age group and does not postpone or diminish the decline in IL-2 production seen at 30 + months. Since female CR rats have a longer median lifespan than male CR rats, the effect of gender was evaluated. There was no change in the pattern of IL-2 production when males and females on either diet are evaluated separately. Although CR females produce greater amounts of IL-2 at all ages than CR males, the differences between males and females were not significant in either diet (data not shown).

b. IFN-γ: We have previously shown that IFN production after ConA stimulation increases with age and then plateaus at about 22 months in AL BN rats [3]. No other studies of IFN production in aged rats have been done; however similar age-related increases have been reported for mice [19–21]. Since IFN-γ is an important immune regulator [22], and may also be affected by CR, we examined IFN production by ConA stimulated splenocytes of CR BN rats. This has not been examined in CR rats or CR mice to date. The dose and kinetics of IFN production by splenocytes stimulated by ConA is the same in AL and CR rats regardless of

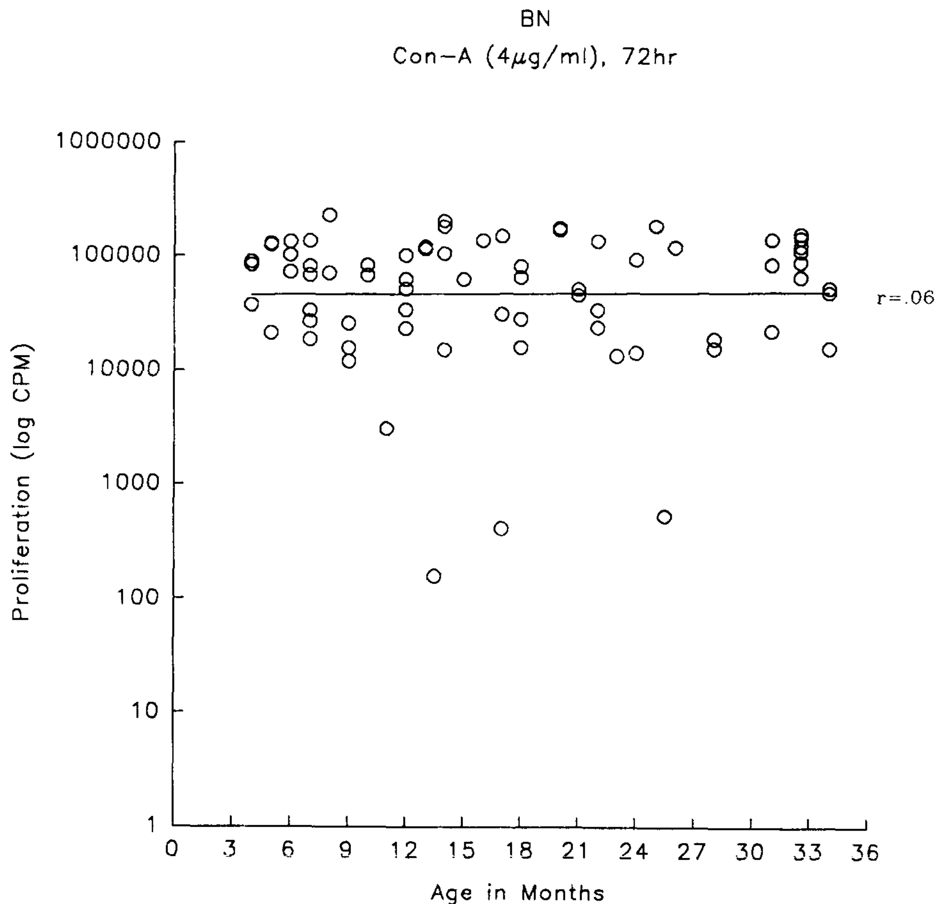


Fig. 3. Association of ConA induced proliferation to age in CR BN rats, after stimulation with ConA, 4 μg/ml for 72 h. Proliferation expressed as log CPM. Each point represents one rat. $r = 0.06$ by Pearson's correlation coefficient.

age: peak IFN production occurs at 72 h with 4 μg/ml of ConA (data not shown). These are the same conditions required for maximal proliferation with ConA. There is a significant ($P < 0.005$) increase in IFN production with advancing age in both AL and CR rats. In AL rats, IFN production levels off after 22 months of age, while there is no plateau in IFN production in CR rats with advancing age (Fig. 5). IFN production in CR rats is greater than that of AL rats of the same age in all age groups. These differences between AL and CR rats are statistically significant in the middle-young and old age groups ($P < 0.03$).

We then determined whether or not there was an association between ConA-induced proliferation and IL-2 production of individual rats. Using the optimal conditions of each parameter (proliferation: ConA 4 μg/ml, 72 h; IL-2: ConA 16 μg/ml, 48 h), a slight correlation was observed between proliferative response and

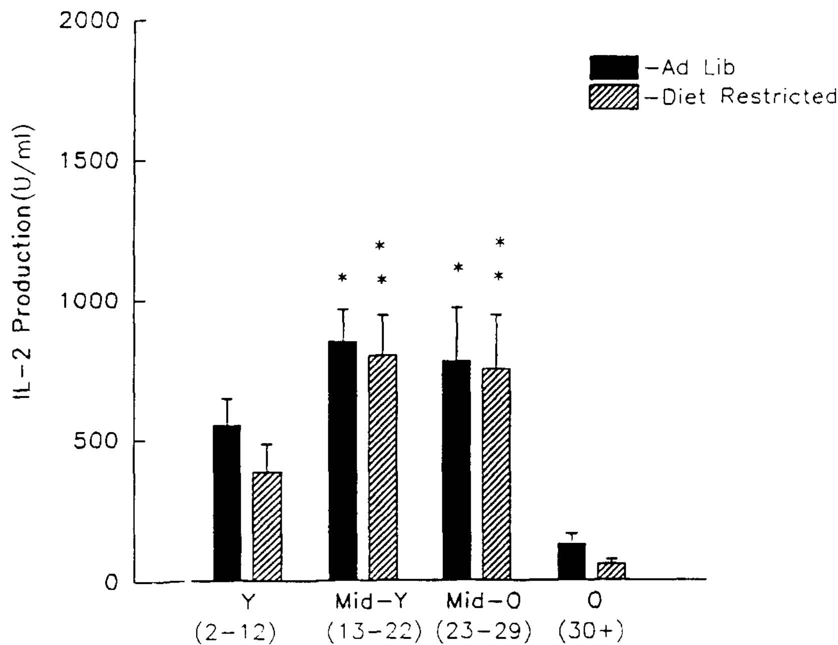


Fig. 4. IL-2 Production after stimulation with ConA. Optimal conditions of ConA stimulation of IL-2 production were used (16 μg/ml, 48 h). IL-2 is represented as units/ml. Bars show mean ± S.E.M. * = significantly different compared to 30+ AL, ** significantly different compared to 30+CR by Student's *t*-test, both at $P < 0.05$.

IL-2 for both AL ($r = 0.24$, $P < 0.05$) and CR ($r = 0.25$, $P < 0.05$) rats when all ages are included in the analysis (Fig. 6A and B). We then performed this analysis in both AL and CR rats after grouping the rats by age as described previously. The regression coefficient, direction of slope, and statistical significance is listed for both IL-2 and IFN in Table 1. Statistically significant correlations between IL-2 production and proliferation in response to ConA is noted for both AL and CR rats ages 13-22 months, and CR rats aged 23-29 months. The only statistically significant correlation between proliferation and IFN production is in the AL rats ages 13-22 months.

4. Discussion

We previously showed that BN rats exhibit an age-related decline in proliferation and IL-2 production after stimulation with ConA [3]. Both of these decreases became apparent at about the time of median survival. In contrast, IFN production in AL BN rats increases with advancing age and then plateaus at about the time of median survival. Since CR has been shown to extend lifespan, we examined the effects of CR upon these same immune parameters in BN rats.

There were three major points of our study design: (1) we defined an 'old' rat as a rat that is at or past the expected median survival time; (2) rats were examined at

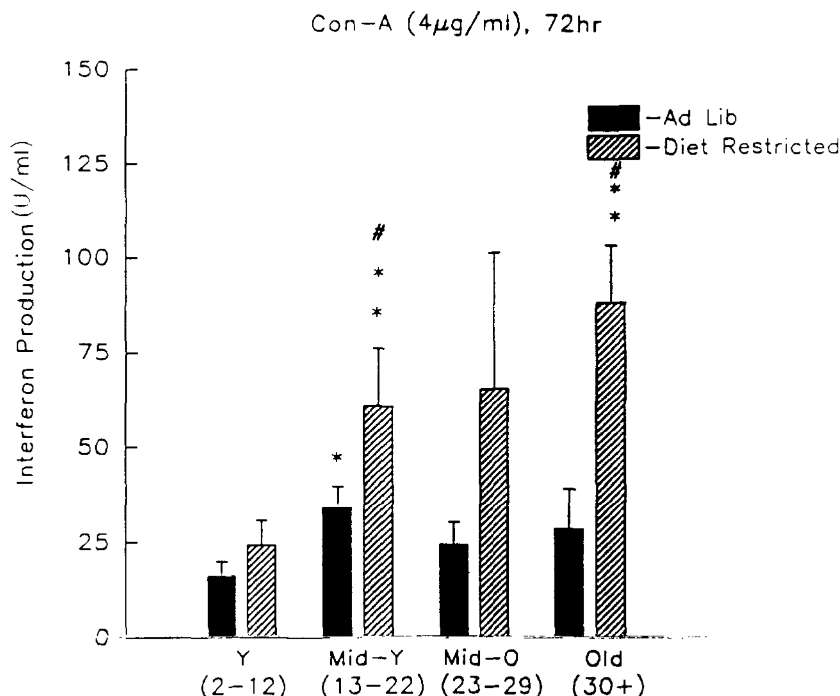
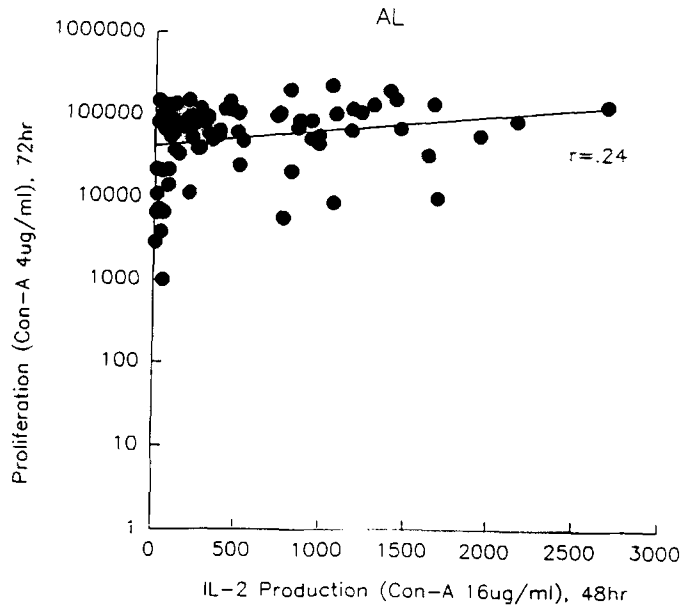


Fig. 5. Interferon production (units/ml) after stimulation with ConA 4 μg/ml, 72 h. Bars show mean ± S.E.M. * = statistically significant compared to 2–12 month AL; ** = statistically significant compared to 2–12 month CR by Student's t-test, both at $P < 0.05$. # = statistically significant compared to AL of same age, $P < 0.03$.

all ages along their lifespan, not simply young and aged; (3) rats were always assayed individually. Using this design, the declines in proliferative responses to ConA and PHA observed in old AL rats were abrogated by CR (Fig. 2). While previous work in our lab showed an age-related decline after 31 months of age in AL BN rats [3], the current study of ConA-induced proliferation of CR rats over an age range of 3 to 34 months show no age-related changes (Fig. 3). Our results are in conflict with studies of the effect of CR on mitogen-induced lymphoproliferation in Sprague–Dawley rats [17]. No significant differences between AL and CR rats in mitogen induced cell proliferation were observed in this study. This may be because the 'old' CR rats examined were younger than the median life span of the strain. However, our results are similar to studies using F-344 [15,16] and Wistar-Lobund [14]. Both studies of CR effects on immune function in F344 rats used only two age groups [15,16] which limits these studies.

Decreased IL-2 production has been hypothesized to be a major factor contributing to the changes in decreased proliferative response seen with increasing age. We reported previously, and have extended the observation here, that IL-2 production



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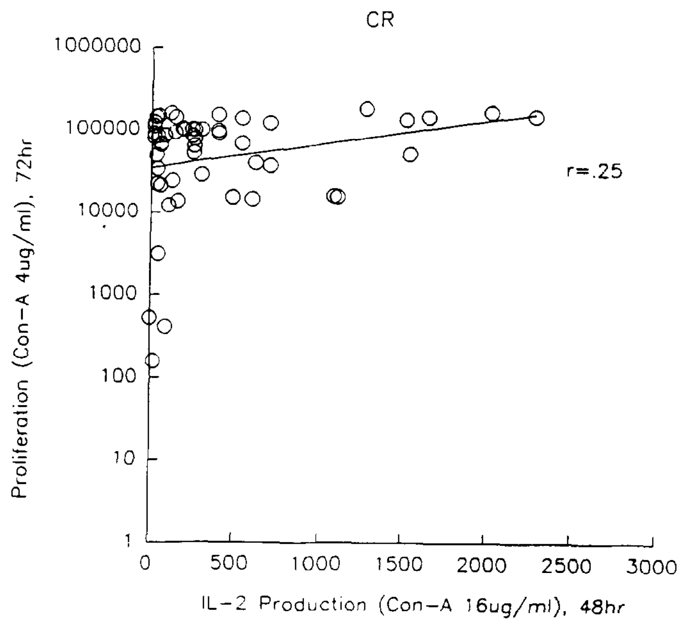

B

Fig. 6. Association of ConA induced proliferation (CPM) and IL-2 production (units/ml) in optimal time and dose for each parameter. A: AL BN rats $r = 0.245$, $P < 0.05$; B: CR BN rats $r = 0.25$; $P < 0.05$ by Pearson's correlation coefficient.

after ConA stimulation sharply declines in BN rats after 29 months of age. On the survival curve, this is the region of approximately 50% survival of AL BN rats. Based on our proliferative data and the results of Pahlavani et al. [16] with F344 rats, we hypothesized that CR would postpone this decrease in IL-2 production. In contrast to expectations, CR BN rats do not have significantly different levels of IL-2 production compared to AL rats of the same age (Fig. 4) and do not demonstrate a delay in the decline of IL-2 production. Although there is an increased survival in CR females compared to CR males, there are also no significant differences in IL-2 production between males and females, although females have higher levels of IL-2 after Con-A stimulation. This gender difference has been previously shown in AL F344 [23], and AL BN rats [3]. However, not all studies of IL-2 production from aged AL rats have provided consistent results. One study has reported increased IL-2 production with increasing age [2], another reported no change [32], while another has reported decreased IL-2 production [23]. Although the reasons for these differences are not apparent, these inconsistencies in the age-associated effects on IL-2 production in conjunction with our present data that IL-2 production in response to ConA stimulation is not delayed by CR, suggests that IL-2 production does not represent the major reason for decreased lymphoproliferation in aged AL BN rats nor is it an appropriate 'marker' of physiologic age.

This is the first study to assay IFN production after ConA stimulation in CR rats. We have previously shown an age-related increase in IFN production after ConA stimulation in AL BN rats, that plateaus at about 23 months of age. CR rats make greater amounts of IFN than AL rats of all ages, and significantly more IFN at 13–22 months ($P < 0.01$) and > 30 months of age ($P < 0.05$). The current study demonstrated that CR BN rats demonstrate an increase that continues through at least 30 months of age (Fig. 6). We suggest, therefore, that IFN production may be a sensitive marker of physiologic aging since it shows a steady increase that levels off late in life in AL rats, and this leveling is effectively

Table 1
Correlation coefficients of lymphokine production and proliferation[a]

Age range in months	IL-2		IFN	
	AL	CR	AL	CR
2–12	0.08[b] (35)	−0.11 (20)	0.21 (40)	0.23 (25)
13–22	0.36* (27)	0.49* (15)	0.39* (30)	0.31 (22)
23–29	0.49 (11)	0.68* (8)	0.29 (11)	0.30 (8)
30–34	0.1 (5)	0.28 (9)	−0.29 (5)	0.16 (9)
2–34	0.24* (78)	0.25* (52)	0.21* (86)	0.28 (64)

[a]Based on data in Figs. 6 and 7
[b]Numbers reflect Pearson's correlation coefficient with the number in parenthesis representing the number of rats assayed.
* designates $p < 0.05$.

postponed by CR. Two recent studies in mice have also demonstrated age-associated increases in IFN production after stimulation with anti-CD3 antibody [24,21]. Both studies showed that there is a concomitant increase in cell populations which have high expression of the CD-44 cell surface marker, a putative marker of memory cells. Studies of the frequency of naive T cells have been done using Fischer-344 (F-344) rats. It was demonstrated that the percentage of OX-22 positive (naive) T cells decreased significantly with age [25]. These decreases occur primarily in the CD4 + T cell population [25]. This study did not examine IFN production in these rats. It would be interesting to see whether changes in induced IFN production correlates in any manner with age related decreases in naive T cell populations. It is important to differentiate between those studies which examine age-related changes in the expression of a cell surface marker for memory cells, and those which examine cell surface markers for naive cells. Studies thus far using rat models have not examined a marker of memory cells, as has been done in mouse and human studies.

Since both IL-2 and IFN-γ are known to be regulators of T cell proliferation, we wanted to examine if there was any correlation between proliferation and either of these lymphokines. Statistically significant correlation was seen between IL-2 and proliferation for both AL and CR rats 13–22 months of age ($P < 0.05$), and CR rats 23–29 months of age ($P < 0.05$) (Table 1). This indicates that the ability to produce IL-2 is only related to ConA proliferation at time points in the survival curve before declines in proliferative ability begin to become apparent. This is an interesting finding since CR postpones the decline in proliferation, but not in IL-2 production. In contrast, while CR has the anticipated postponement of age associated changes for both proliferation and IFN-γ production, the level of IFN does not correlate with increased proliferation to ConA in any age group of CR rats, randomly at 13–22 months of age for AL rats (Table 1).

It is of interest to see if there are other lymphokines that are suitable markers of physiological aging comparable to IFN. Although they have not been examined in rats, IL-3, 4, 5, 6 and 10 have been shown to have age-associated changes in AL-fed mice. IL-4 has been shown to increase with age in AL fed mice in short term cultures [20,21,26,27]. Long term cultures of mouse CD4 + T cells from AL mice, however, have been shown to have decreased IL-4 production after stimulation with anti-CD3 antibody and exogenous IL-2 [28]. Age-related increases in IL-3 [20,21,26] and IL-5 production [21] have been demonstrated in T cell populations from AL fed mice as well. IL-10 has also been shown to increase with age in T cells from mice [27] IL-6 has been proposed as a regulatory factor in age-related changes in lymphokine production [29] and shown to increase [30] or not change [31] with advancing age in AL fed mice. However, none of these lymphokines have been examined in CR rodents to date.

We have shown that mitogen-induced production of two lymphokines, IL-2 and IFN-γ, exhibit age-related changes in BN rats. CR does not act to delay the age-related decline in IL-2 production after mitogen stimulation. CR does, however, delay the age-related peak and plateau production of IFN seen in AL BN rats. Taken together, these data demonstrate that IFN production after mitogen stimula-

tion may be a good candidate as a biomarker of aging, while mitogen-induced IL-2 production is not a suitable biomarker of aging. The validity of the hypothesis that increased IFN production is an appropriate biomarker of early aging, while decreased mitogen-induced production is a good biomarker of late aging, is currently being assessed in longitudinal studies that are quantitating mitogen induced proliferation and IFN production from 6 months of age until death in individual rats maintained on both AL and CR diets. If the hypothesis is correct, rats of three strains and of both diets will exhibit increases in IFN and decreases in proliferation at similar points within their own lifespans.

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