Abstract

Aging is often associated with a dysregulation of the immune system. We examined mitogen-stimulated production of interleukin (IL)-2 and proinflammatory cytokines, IL-1β and IL-6, in apparently healthy and generally well-nourished old versus young women. Subjects were screened for health using the SENIEUR protocol and a panel of laboratory tests for inflammation, as well as for the adequacy of nutritional status using criteria related to undernutrition, and protein, iron, vitamin B12, and folate status. Young (n=26, age: 20–40 years) and old (n=44, age: 62–88 years) cohorts did not differ on the number of circulating monocytes, granulocytes, B (CD19+) cells, and T (CD3+, CD4+, and CD8+) cells. No differences (P>0.10) were seen between the two age groups in IL-2, IL-1β and IL-6 levels in whole blood cultures at 48 h after stimulation with PHA (5 mg/l). Furthermore, no age-related differences were noted in the absolute amounts (pg) of IL-1β and IL-6 after normalizing for circulating monocytes, B cells, or T cells (P>0.10). Similarly, no age-related decline in absolute amount of IL-2 (pg) after normalizing for circulating T cells was noted (P>0.10). Thus, contrary to most previous reports, our results do not support an increase in the production of proinflammatory cytokines IL-1β and IL-6, and a reduced production of IL-2 with aging when health and nutritional status are maintained. These findings support our previous results of no change in monocyte function and few alterations in acquired immune response in a carefully selected group of healthy and well-nourished elderly women.
1. Introduction

Aging is often associated with a dysregulation of the immune system. Regulatory mechanisms involve a complex network of cytokines that are involved in differentiation, proliferation, and survival of lymphoid cells (Franceschi et al., 1995). It has been suggested that the dysregulation of cytokines may be partly responsible for the increased morbidity and mortality and the subtle presence of infection in the elderly (Ershler, 1993; Ginaldi et al., 1999; Peterson et al., 1994).

We have previously shown that several immune parameters including circulating T-lymphocyte number, natural killer cell cytotoxicity, phagocytosis, and bactericidal function, did not differ in generally healthy and well-nourished old versus young women. However, age-associated decline in lymphocyte proliferation in response to phytohemagglutinin (PHA) \((P = 0.05)\), but not to concanavalin A \((P > 0.05)\), was noted (Krause et al., 1999). Altered production of certain cytokines has been postulated to explain some of the age-related functional changes in immune response (Caruso et al., 1996; Ginaldi et al., 1999). A shift toward increased production of proinflammatory cytokines IL-1 and IL-6, and reduced production of key T cytokine IL-2, has been reported with aging in healthy old compared to younger controls (Ginaldi et al., 1999; Molteni et al., 1994). Our interest, therefore, was to examine the production of T cytokine IL-2 and proinflammatory cytokines IL-1\(\beta\) and IL-6, by stimulated mononuclear cells in apparently healthy and generally well-nourished old \((n = 44, \text{ age: } 62–88 \text{ years})\) versus young \((n = 26, \text{ age: } 20–40 \text{ years})\) women.

2. Methods

2.1. Subject recruitment

Older women ( 60 years) were recruited with the assistance of local Agencies on Aging and from local housing complexes for seniors using flyers, advertisements, and recruitment letters describing the study. Younger women (20–40 years) were recruited from the university and surrounding community through flyers and advertisements in local newspapers. Women between the ages of 41–60 years were not included due to the potentially confounding effects of hormonal changes during menopause on immune parameters (Ligthart et al., 1984; Murasko et al., 1987). Subjects received a detailed description of the study and provided written informed consent to follow the study protocol approved by the Pennsylvania State University’s Office for Regulatory Compliance.
2.2. Study protocol

The details of the study protocol have been previously described (Krause et al., 1999). Briefly, after obtaining a medical history from interested individuals (75 older and 35 younger women), a visit for blood collection was scheduled for those women who were free from systemic diseases, such as cardiac, liver, kidney and/or bone marrow disorders, and acute or chronic inflammatory conditions; were not taking any prescription or over-the-counter medications routinely as per the SENIEUR protocol (Ligthart et al., 1984); and had a body mass index (BMI) < 20 kg/m², indicative of general undernutrition, consistent with the SENIEUR protocol (Ligthart et al., 1984).

A fasting blood sample (approximately 35 ml) was obtained from 58 old and 34 young women, by a certified phlebotomist for tests of general health status, presence of inflammation/ infection, and status of various nutrients such as protein, iron, and vitamins B₁₂ and folate, and for immune function assays (Krause et al., 1999). Subjects with abnormal results on complete blood count (CBC) with differential evaluation or clinical chemistries (Chem-24 profile) suggesting liver or kidney dysfunction, and/or bone marrow proliferative disorders were excluded. Subjects with laboratory evidence of inflammation [abnormal tests results on ≥ two of four laboratory tests; alpha-1 acid glycoprotein (AGP), C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), and white blood cell (WBC) count] were dropped from analysis. After evaluation of laboratory results for general health and inflammation, six of the old subjects were excluded from analysis (Ligthart et al., 1984; Lesourd and Mazari, 1999). Further, 14 subjects (six old and eight young) were dropped from analysis because of under-nutrition, deficiencies of protein, iron, vitamin B₁₂, and/or folate, and/or iron overload, which can affect the immune response (Chandra, 1997; Mazari and Lesourd, 1998). Therefore, data on 44 older and 26 younger generally-healthy and well-nourished individuals (Krause et al., 1999) were included in these analyses.

2.3. Cell-mediated immune function

For sample volume considerations, immune function parameters were determined by whole blood assays using previously established protocols which were validated in our laboratory. B- and T-lymphocytes were quantified in heparinized blood samples (Mandy et al., 1997) by flow cytometry using fluorescently-labeled monoclonal antibodies specific for surface antigens. Monocytes were obtained from the complete blood count (CBC) with differential evaluation (Beckman Coulter, Miami, FL).
2.3.1. Cell culture with PHA

Heparinized blood was diluted (1:10) with RPMI 1640 medium containing 10% fetal bovine serum (FBS), L-glutamine (2 mmol/l), penicillin (100 000 U/l), and streptomycin (100 mg/l). For each subject, 2 ml of diluted blood was placed in a well and 2 ml of diluted blood and PHA [5 mg/l; PHA-M (L2646) was obtained from Sigma, St. Louis, MO] was placed in another well of a 24-well flat-bottom microtitre plates. Cells were incubated for 48 h (humidified, 5% CO₂, 37°C). Contents of wells were transferred into microfuge tubes, centrifuged, and supernatants were collected, aliquotted, and frozen at −80°C until subsequent assay of IL-2, IL-1β, and IL-6.

2.3.2. Measurement of cytokines

For determination of cytokines, samples from young and old subjects were assayed on the same plate and a pooled supernatant from ongoing studies was used as the internal control. IL-2 concentration in supernatants was determined by ELISA. Briefly, culture supernatants were added to anti-human monoclonal IL-2 antibody coated plates (R&D systems # MAB602) and detected with a polyclonal goat anti-human IL-2 antibody (R&D systems # BAF 202). The absolute amount of IL-2 (pg) per 1000 T cells was computed from the IL-2 concentration determined in culture supernatant (pg/ml) by expressing it per 1000 T-helper cells and per 1000 total-T cells in whole blood.

IL-1β concentration was determined by ELISA. Briefly, culture supernatants were added to anti-human monoclonal IL-1β antibody coated plates (R&D systems # MAB601) and detected with a polyclonal goat anti-human IL-1β antibody (R&D systems # BAF201). The First International Standard for IL-1β (recDNA Human type) 86/680 (National Biological Standards Board, Hertfordshire, England) was also used for quality control. IL-1β (pg) per 1000 monocytes, per 1000 B cells, and per 1000 monocytes and B cells, were computed from IL-1β concentration determined in culture supernatant (pg/ml).

IL-6 concentration was determined by ELISA. Briefly, culture supernatants were added to anti-human monoclonal IL-6 antibody coated plates (R&D systems # MAB206) and detected with a polyclonal goat anti-human IL-6 antibody (R&D systems # BAF206). The IL-6 (pg) per 1000 monocytes, per 1000 T-helper (CD4+) cells, and per 1000 T-helper plus monocytes, were computed from IL-6 concentration determined in culture supernatant (pg/ml). The lower limits of detection for all cytokines measured was 3 pg/ml. Cytokine levels in the culture supernatants from blood mononuclear cells in the absence of PHA were usually nondetectable for most old and young subjects.

2.4. Statistical analyses

Statistical analyses were performed using SAS 6.11 for Windows (SAS Institute Inc., Cary, NC). Logarithmically transformed data were used for statistical analyses because the transformed distributions were consistent with normality.
Using the exclusion criteria described above, data on 44 old (age: 62–88 years; mean age: 73.6 years) and 26 young (age: 20–40 years; mean age: 26.4 years) apparently healthy, well-nourished women from the study cohort described previously (Krause et al., 1999) were used for examining age-associated changes in cytokine production by stimulated mononuclear cells. Because of the large range in age in older women (age: 62–88 years), differences within the old group were first evaluated using the Student’s t-test by dividing older women into two groups namely, young-old (60–74 years) and old-old (≥75 years). For all variables examined, no differences were found between young-old and old-old groups ($P > 0.10$). Therefore, young-old and old-old groups were pooled as one group (old) for further statistical analyses. All subjects included in the analyses were considered healthy and generally well-nourished on the basis of the criteria used. Certain differences were observed, however, between young and old women. Old women had significantly lower serum protein and albumin and significantly greater hemoglobin and serum ferritin as compared to the younger cohort ($P < 0.05$, Krause et al., 1999). Therefore, analysis of covariance (ANCOVA) was used to evaluate the differences in cytokine production by stimulated mononuclear cells between young (20–40 years) and old (>60 years) groups using hemoglobin and serum protein, albumin and ferritin as covariates and age as the main effect.

3. Results

Young and old cohorts did not differ on the number of circulating monocytes, T (CD3+, CD4+, and CD8+) and B (CD19+) cells (Table 1). The ratio of CD4+ : CD8+ cells was, however, significantly increased in the old versus young cohort; geometric mean and geometric mean ± SD were 1.565 (1.120–2.188) and 2.309 (1.265–4.216) for young and old women, respectively ($P < 0.05$). Both groups had circulating monocytes in the normal ranges; however, there was a trend of higher monocytes in older versus younger women which was not significant (Table 1, $P = 0.08$). The old and young cohorts did not differ ($P > 0.10$) on IL-2, IL-1β or

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Young ($n = 26$)</th>
<th>Old ($n = 44$)</th>
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</thead>
<tbody>
<tr>
<td>Total lymphocyte number ($\times 10^9$/l)</td>
<td>1.733 (1.138–2.638)</td>
<td>1.935 (1.271–2.945)</td>
</tr>
<tr>
<td>Total T (CD3+) cell number ($\times 10^9$/l)</td>
<td>1.271 (0.819–1.974)</td>
<td>1.448 (0.951–2.203)</td>
</tr>
<tr>
<td>T helper (CD4+) cell number ($\times 10^9$/l)</td>
<td>0.705 (0.440–1.127)</td>
<td>0.914 (0.577–1.448)</td>
</tr>
<tr>
<td>T cytotoxic (CD8+) cell number ($\times 10^9$/l)</td>
<td>0.449 (0.273–0.741)</td>
<td>0.395 (0.208–0.748)</td>
</tr>
<tr>
<td>B cells (CD19+) cell number ($\times 10^9$/l)</td>
<td>0.117 (0.051–0.270)</td>
<td>0.135 (0.062–0.291)</td>
</tr>
<tr>
<td>Monocytes ($\times 10^9$/l)</td>
<td>0.232 (0.115–0.468)</td>
<td>0.288 (0.147–0.561)</td>
</tr>
<tr>
<td>Granulocytes ($\times 10^9$/l)</td>
<td>3.3 (2.5–4.5)</td>
<td>4.1 (3.0–5.5)</td>
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</table>

* Values are geometric mean with ±1 SD of geometric mean in the parentheses. There were no significant differences between groups.
Table 2
In vitro IL-2 production by stimulated mononuclear cells from young and old subjects*

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Young (n = 26)</th>
<th>Old (n = 44)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-2 pg/ml supernatant</td>
<td>42.966 (33.900–54.457)</td>
<td>40.994 (34.897–48.155)</td>
</tr>
<tr>
<td>IL-2 pg/1000 CD4+ cells</td>
<td>0.276 (0.211–0.362)</td>
<td>0.284 (0.239–0.338)</td>
</tr>
<tr>
<td>IL-2 pg/1000 CD3+ cells</td>
<td>0.466 (0.356–0.610)</td>
<td>0.449 (0.378–0.534)</td>
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</tbody>
</table>

* Values are adjusted geometric mean with ± 1 SE of geometric mean in parentheses. There were no significant differences between groups.

IL-6 levels in whole blood cultures at 48 h after stimulation with PHA (Tables 2–4).

No age-related differences were seen in the absolute amounts (pg) of IL-2 after normalizing for circulating T helper cell number (Table 2, *P* > 0.10). Similarly, absolute amounts (pg) of IL-1β, after normalizing for monocyte or B cell number, also did not differ between the young and old groups (Table 3, *P* > 0.10). Furthermore, there were no differences in the absolute amounts of IL-6 production, after normalizing for circulating monocyte or T helper cell number, in the young and old cohorts (Table 4, *P* > 0.10).

4. Discussion

There is a growing consensus for defining health status of participants in studies examining immune function with aging (Ligthart et al., 1984; Chandra, 1997; Corberand et al., 1986; Lesourd, 1997). Some conflicting findings on lymphocyte proliferation response to stimulation with mitogens and cytokine production with aging have been reported, however, despite the use of the SENIEUR protocol to define health status among study participants. We used nutritional status criteria in conjunction with the SENIEUR protocol to determine eligibility for examining age-related changes in immune function. The rationale was that nutritional status, as well as underlying health problems and inflammation could confound the effects of aging on immune function (Krause et al., 1999; Lesourd and Mazari, 1999; Mazari and Lesourd, 1998).

Table 3
In vitro IL-1β production by stimulated mononuclear cells from young and old subjects*

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Young (n = 26)</th>
<th>Old (n = 44)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β pg/ml supernatant</td>
<td>162.457 (126.018–209.435)</td>
<td>156.087 (133.675–182.256)</td>
</tr>
<tr>
<td>IL-1β pg/1000 monocytes</td>
<td>5.114 (3.758–6.959)</td>
<td>5.930 (4.884–7.199)</td>
</tr>
<tr>
<td>IL-1β pg/1000 monocytes+B cells</td>
<td>3.561 (2.583–4.909)</td>
<td>3.808 (3.158–4.591)</td>
</tr>
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</table>

* Values are adjusted geometric mean with ± 1 SE of geometric mean in parentheses. There were no significant differences between groups.
Table 4
In vitro IL-6 production by stimulated mononuclear cells from young and old subjects

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Young (n = 26)</th>
<th>Old (n = 44)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6 pg/ml supernatant</td>
<td>6617.116 (5412.221–8090.250)</td>
<td>5112.352 (4534.250–5764.161)</td>
</tr>
<tr>
<td>IL-6 pg/1000 monocytes</td>
<td>204.998 (155.711–269.886)</td>
<td>193.253 (163.204–228.835)</td>
</tr>
<tr>
<td>IL-6 pg/1000 CD4+ cells</td>
<td>89.121 (68.786–115.469)</td>
<td>56.599 (48.862–65.562)</td>
</tr>
<tr>
<td>IL-6 pg/ml monocytes+CD4+ cells</td>
<td>62.240 (48.424–79.998)</td>
<td>42.394 (36.745–48.911)</td>
</tr>
</tbody>
</table>

* Values are adjusted geometric mean with ± 1 SE of geometric mean in parentheses. There were no significant differences between groups.

In a cohort of healthy and well-nourished elderly women, we previously reported that no major changes in innate and acquired immune response were noted with the exception of lymphocyte hyporesponsiveness particularly to PHA (\( P = 0.05 \), Krause et al., 1999). Subjects included in the study were extensively screened for both health and nutritional status based on the use of SENIEUR protocol; and clinical tests of inflammation, namely AGP and CRP — two acute phase reactant proteins, ESR and WBC count; and a panel of laboratory tests for protein, iron, and vitamins B₁₂ and folate status. Our interest was to examine the production of IL-2 by stimulated T-cells, and whether reduced production of T cell growth factor could explain the reduced lymphocyte proliferation response we previously reported in old women (Krause et al., 1999). We were also interested in examining the production of proinflammatory cytokines IL-1β and IL-6 in stimulated cultures in this cohort of healthy and well-nourished old versus young women. A noteworthy finding was that by using a combination of health and nutrition criteria simultaneously for determining eligibility, production of IL-1, IL-2, and IL-6 was not different between young and old women.

In this cohort of generally healthy, well-nourished women aging was not associated with changes in monocyte, B cells and T cell subpopulations (Krause et al., 1999). Previous studies using SENIEUR protocol have yielded mixed findings, which may be partly related to the differences in nutritional status of subjects (Krause et al., 1999; Lesourd, 1997; Lesourd and Mazari, 1999). No age-related differences were noted in IL-2 production in the culture supernatants of in vitro stimulated lymphocytes in this cohort of women, in which health and nutritional status were comprehensively evaluated and ensured. Furthermore, the absolute amounts of IL-2 produced expressed per 1000 T-helper cells or per 1000 total T cells, did not differ between the young and old cohorts. Our results are in contrast to previously reported findings of reduced IL-2 production with advanced age in studies where SENIEUR protocol was not employed (Murasko et al., 1987; O’Leary and Hallgren, 1991) and in studies with healthy elderly (Amadori et al., 1988; Nagel et al., 1988). Molteni et al. (1994) on the other hand, found that the amount of IL-2 was greater in culture supernatants of lymphocytes from healthy elderly versus young adults. Our findings are consistent with those of Nijhuis et al. (1994) who reported no differences in IL-2 production to anti-CD3 or PHA in
healthy old versus young controls. The differences in stimulants, culture conditions, and assays, and differences in nutritional status of young and old participants may be involved in discrepancies noted in the literature among healthy elderly. In a recent study involving a similar design to the current study, where health and nutritional status were extensively evaluated prior to inclusion of subjects in the study, Mazari and Lesourd (1998) also reported no differences in IL-2 production in older versus younger adults who had adequate nutritional status. This is consistent with our findings. Furthermore, older subjects with either low serum folate or low albumin levels showed significantly reduced IL-2 production as compared to well-nourished young or old cohorts (Mazari and Lesourd, 1998), indicating the important role of nutritional status in modulating IL-2 production and other immune responses, which has not been explored in previous studies.

In our previous report, we described reduced lymphocyte proliferation response to PHA in apparently healthy, generally well-nourished old compared to young women \( (P = 0.05; \) Krause et al., 1999). The amount of IL-2 produced by lymphocytes upon culture with PHA in the same study cohort, however, was not different between young and old subjects. This suggests that the amount of IL-2 produced was not rate-limiting for DNA synthesis of lymphocytes. As in the current study, Amadori et al. (1988) did not find that the levels of IL-2 production were directly related to lymphocyte proliferation. Similarly, Molteni et al. (1994) did not observe a statistically significant relationship between IL-2 production and cell proliferation in their young controls. Furthermore, in some studies, the addition of exogenous IL-2 to lymphocyte cultures from healthy elderly did not restore the proliferative responses (Amadori et al., 1988; Schwab et al., 1990). This suggests that mechanisms other than reduced IL-2 production may be involved in lymphocyte hyporesponsiveness to certain mitogens with aging. Reduced IL-2 receptor expression and activation (Amadori et al., 1988; Nagel et al., 1988; Schwab et al., 1990; Song et al., 1993), reduced secretion of soluble IL-2 receptor (Caruso et al., 1996), and a shift from naive to memory T-cells (Mazari and Lesourd, 1998; Nijhuis et al., 1994; Song et al., 1993) are some of the mechanisms which may be involved in reduced T cell proliferation with aging. These postulated mechanisms were not examined in the current study and need to be studied further in future studies with healthy and well-nourished participants.

There are some indications that the production of proinflammatory cytokines such as IL-1\(\beta\) and IL-6, is increased in older persons (Cossarizza et al., 1997; Fagiolo et al., 1993; Ginaldi et al., 1999; Molteni et al., 1994). These proinflammatory cytokines are closely involved in mounting the acute phase response by modulating the hepatic production of acute phase reactant proteins such as AGP and CRP (Ershler, 1993; Ginaldi et al., 1999). In the current study, no age-related changes were observed in the production of IL-1\(\beta\) and IL-6 in the culture supernatants of blood mononuclear cells stimulated in vitro with PHA \( (P > 0.10) \). Furthermore, the absolute amounts of IL-6 expressed per 1000 monocytes, T-helper, or total T-cells did not differ between the young and old cohorts \( (P > 0.10) \). Similarly, no differences in the two age groups were noted in the absolute amounts of IL-1\(\beta\) expressed per 1000 monocytes, B cells, or monocytes + B cells \( (P > 0.10) \). Our findings are
consistent with those of Amadori et al. (1988) who reported no change in IL-1 content and that of Candore and colleagues (Candore et al., 1993) who did not find any age-related change in IL-6 production in healthy participants. Thus, contrary to previous reports in healthy elderly (Cossariza et al., 1997; Fagiolo et al., 1993; Molteni et al., 1994) our findings suggest that the production of these proinflammatory cytokines is not affected by aging, when both health and nutritional status are maintained. It is significant to point out that in the present study we excluded subjects not meeting the guidelines of SENIEUR protocol, and with laboratory evidence of deficiency of protein, vitamin B₁₂, folic acid, and/or iron, as well as those with more than two positive tests of inflammation including AGP and CRP. Thus, the current findings are obtained on a cohort of well-nourished subjects who were considered healthy by not only employing the SENIEUR protocol, but also by using a battery of clinical tests of inflammation and therefore the results from the current study may be more reflective of primary aging effects.

It is also significant to compare our findings with those of Mazari and Lesourd (1998) who examined IL-6 levels in supernatants from isolated mononuclear cell cultures with PHAp among young and old subjects with adequate health and nutritional status. Contrary to our finding of no change in IL-6 production in healthy and well-nourished old versus young women, these authors found an increase in IL-6 production in the old relative to young cohort. The differences in study methodologies involving different mitogens, culture conditions, and method of cytokine assay may contribute to the discrepancies in findings in age-related IL-6 production in the current study and that by Mazari and Lesourd (1998). In summary, the findings from the current study suggest that the production of proinflammatory cytokines is not affected with aging, indicating that general monocyte dysfunction may not be a primary aging effect, but rather secondary to other age-associated changes in factors such as nutrition. Thus, the results from the current study in conjunction with others (Lesourd and Mazari, 1999; Mazari and Lesourd, 1998) highlight the importance of evaluation of AGP and CRP levels in screening of subjects for participation in studies evaluating age-related changes in immune response.

In conclusion, in this cross-sectional study involving a carefully selected group of healthy and well-nourished women, no age-related changes in production of T-cytokine IL-2 and monocyte cytokines IL-1 and IL-6 were noted. These results support our previously reported findings that monocyte function is not altered in healthy, well-nourished old versus young women. The finding of no change in IL-2 production in old relative to young women when health and nutritional status are maintained suggests that additional alternative mechanisms, such as changes in the proportion of naive and memory T cells, and/or IL-2 receptor production and activation, may be involved in lymphocyte hyporesponsiveness to PHA. Future studies of immune function with aging, should examine the mechanisms for altered T cell function, as well as determine immune response in vivo with delayed type hypersensitivity testing in subjects who are not only healthy, but also generally well-nourished.
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