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Cytokine production and lymphocyte subpopulations in aged humans. An assessment during nocturnal sleep

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Abstract

The view of a general impairment of immune functions associated with aging has been challenged by recent studies including a more detailed evaluation of various cytokines and lymphocyte subsets. In the present human study, effects of age on the production of cytokines by T cells and monocytes were assessed, together with age-dependent changes in subset populations of mononuclear cells (MNC). Blood was collected every 30 min during nocturnal sleep in 16 aged (mean: 79.6 ± 7.5 years) and in 16 young controls (mean: 24.6 ± 3.1 years). Nocturnal sleep was chosen as a well-defined period within the 24-h cycle with minimal exogenous influences. The in vitro production of interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) was measured after mitogen stimulation with lipopolisaccharide from *E. coli* (LPS). Production of interleukin-2 (IL-2) and interferon-gamma (IFN-gamma) was measured after stimulation with phytohemagglutinin (PHA). Regarding MNC subsets, monocytes, lymphocytes, $CD3^+$, $CD4^+$, $CD8^+$, HLA-DR, $CD16^+$, $CD25^+$, and $CD19^+$ were determined. Advanced age was associated with a decreased number of T cells ($CD3^+$) and decreases in the major T cell subsets ($CD4^+$, $CD8^+$, $P < 0.001$). Production of IL-2 was not affected. However, production of IFN-gamma tended to be enhanced, and numbers of activated T cells (HLA-DR/$CD3^+$), natural killer cells ($CD16^+$), and T cells expressing IL-2 receptors ($CD25^+/CD3^+$) were markedly increased in the aged. While monocyte counts were unchanged in the elderly production of IL-1β and TNF-α mainly derived from these cells, was

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enhanced ($p < 0.05$). Results indicate a state of enhanced responsiveness of the T cell compartment and of monocytes in aged which may compensate for the substantial decrease in T cells.

Keywords: Elderly; Tumor necrosis factor-α; Interleukin-1β; Interleukin-2; Interferon-gamma; Lymphocyte subsets; Monocytes

1. Introduction

Aging is associated with an increased susceptibility to infections and neoplasias [1–4]. In men and animals, an age-dependent decline in immunocompetence has been demonstrated with the major alterations concerning functional changes of cell mediated immunity. In particular, proliferation of T cells has been found to be impaired in aged men and mice upon mitogen stimulation [5–9]. The T cell deficit has been related to an age-associated change in the production of cytokines, mainly of IL-2. Thus, in several studies in the aged organism, production of IL-2 was found to be significantly diminished [7,10–12]. However, a recent study using a whole-blood assay technique [13] failed to demonstrate a significant decrease in IL-2 production in elderly humans [14]. An age-dependent decrease has been reported also for the production of cytokines other than IL-2 like IL-1 and TNF [3,5,10,15,16].

The effects of age on cytokine production may strongly depend on the species investigated. An age-dependent decrease in the production of IL-2 was found in CB57BL/6J mice but not in mice from several other strains [17]. Moreover, contrasting with studies in mice, in healthy aged humans, Fagiolo and co-workers [18] observed increased amounts of IL-1β and TNF-α mainly produced by monocytes and macrophages, suggesting that monocytes in aged men are functionally intact. Thus, deficits in cytokine production with age may be restricted to T lymphocytes. Together, those findings underline the importance of examining changes in cytokine production with age directly in the human being, and to separate in this context functional alterations of monocytes and T lymphocytes. However, with the exception of a few studies [e.g. 19], effects of age on the production of cytokines other than IL-2 have not been carefully studied in humans.

Age dependent changes in cytokine production may reflect an impaired synthesis of a cytokine. Yet, they could also be a consequence of a selective decrease in MNC subsets producing a certain cytokine. Thus, MNC subset counts provide a frame for the interpretation of age related changes in cytokine production. Probably as a result of thymic involution in aged donors, numbers of T cells, including $CD4^+$ and $CD8^+$ are typically reduced [6,7,20–22]. However, numbers of activated T cells (HLA-DR) and also NK cells have been found to be increased in healthy elderly indicating a state of increased immune activation [21,23,24]. Contrasting with the distinct changes in lymphocyte subsets, the number of monocytes/macrophages appear to remain stable with age [7,18,25].

The present study aimed to examine changes in cytokine production in healthy aged humans, and to relate these changes to changes in MNC subset populations. IL-1β and TNF-α produced mainly by monocytes and macrophages, and IL-2 and

IFN-gamma produced mainly by T lymphocytes, were measured. Since production of cytokines can be strongly affected even by subclinical pathological conditions, particular attention was paid to the health status of the subjects. Also, both cytokine production and blood cell differentiation are known to be distinctly influenced by circadian rhythmicity [26–30] and stress [e.g. 31–33]. Therefore, in the present study, blood was collected during the subjects' regular nocturnal sleep which represents a well-defined time within the 24-h cycle devoid of exogenous stress.

2. Methods

Sixteen physically and mentally healthy old subjects (12 men and four women, mean age ± S.E.M.: 79.6 ± 7.5 years) and 16 young subjects (12 men and four women, age: 24.6 ± 3.1 years) participated in the overnight studies. Aged subjects included in the study were selected according to the criteria of the SENIEUR Protocol [34]. The subjects did not suffer from pathological sleep disturbances, and were not taking any medication known to alter immune and related endocrine functions and sleep. All subjects were physically examined prior to participation. Current infection was excluded by a plasma concentration of C reactive protein < 6 mg/l, and white blood cell count < 9.0/nl. All subjects gave written consent prior to participation. The study was approved by the Ethics Committee on Research involving human subjects of the University of Lübeck.

2.1. Experimental procedure

Blood was sampled at night while the subjects slept in a sleep laboratory. All subjects were adjusted to the setting by spending an adaptation night in the laboratory, prior to the experimental night. Prior to sleep, the subject was prepared for standard somnopolygraphic recordings and blood sampling. For blood sampling, a catheter was inserted into a forearm-vein and was connected to a long thin tube which enabled blood collection from an adjacent room without disturbing the subject's sleep. To prevent clotting, 400 ml saline solution was infused throughout the night.

To enable sleep, lights were turned off at the time the subject was accustomed to going to sleep which was on the average at 22.42 h (range: 22.00–23.30 h) in the young subjects and at 22.29 h (range: 21.45–23.00 h) in the aged. Blood sampling and sleep recordings started when the lights were turned off, and ended 8 h later when the subject was awakened. Blood samples were collected every 30 min and stored at room temperature (22°C) for determination of white blood cell differential counts (including lymphocyte subsets) and for mitogen stimulation (to determine cytokine production) the next morning (between 7.00 and 8.00 h).

2.2. Cytokine measurement

For cytokine determination, blood was drawn into syringes pretreated with heparine. Aliquots of 50 μl blood were resuspended under laminar airflow in 400 μl of RPMI 1640 medium (containing 2 mM glutamine, 100 U/ml penicilin, 100 μg/ml

streptomycin, 10% heat inactivated FCS; Seromed, Berlin Germany). For stimulation of TNF-α and IL-1β, 0.5 μg LPS (LPS, Sigma Chem. Co., St. Louis, MO, USA) from *E. coli* was added, dissolved in 50 μl of a medium containing 80% RPMI and 20% sterile water (final concentration: 1 μg/ml). For stimulation of IL-2 and IFN-gamma, 2.5 μg phytohemagglutinin (PHA, Murex, Dartford, UK) was added, dissolved in 50 μl of a medium containing 50% RPMI and 50% sterile water (final concentration: 5 μg/ml). Every sample was stimulated in duplicate. At the beginning and end of each measurement an unstimulated control was included to exclude contaminations of blood and reagents (unstimulated cytokine release was not detectable in any of these samples). The samples were incubated for 48 h at 37°C with 5% carbon dioxide in humidified air. The supernatants were harvested and stored at -70°C until assay.

All cytokine levels were measured by ELISA kits (R & D Systems Minneapolis, MN, USA, for determination of TNF-α, IL-1β, and IL-2; Hbt, Uden, The Netherlands, for determination of IFN-gamma). The sensitivities of the assays were 4.4 pg/ml for TNF-α, 0.3 pg/ml for IL-1β, and 6.0 pg/ml for IFN-gamma. The intra- and interassay coefficients of variation were less than 5% and 7.5%, respectively, for all assays. Each supernatant aliquot was tested in duplicate in the same assay and thawed only once. Measurements were performed according to the manufacturer's instructions, and results interpolated from the standard reference curve provided with each kit.

2.3. Hematologic investigations

For white blood cell counts and determination of lymphocyte subsets, blood samples were collected in EDTA tubes (Sarstedt Nümbrecht, Germany) and stored at room temperature for no longer than 9 h before staining. The total number of leukocytes (WBC), erythrocytes, and platelets, hemoglobin (Hb) and hematocrit was determined automatically in all samples by means of a Technicon counter (Technicon H1, Technicon, Basingstoke, UK). Also leukocyte differential counts were performed automatically by the same apparatus.

The lymphocyte subsets were determined in whole blood by FCM (Facscan, Becton Dickinson, San Jose, CA, USA) following standard methods [35]. A minimum of 10 000 cells per sample was analyzed. To analyze lymphocyte surface antigens monoclonal antibodies were directly conjugated with fluorescein isothiocyanate (FITC) and phycoerythrin (PE). After incubation over 15 min, the erythrocytes were disintegrated, and after centrifugation, the supernatants were washed by phosphate-buffered saline (PBS). Expression of the $CD3^+$ antigen defined T cells, expression of $CD19^+$ defined B cells. NK cells were determined by expression of $CD16^+$, helper/inducer T cell subsets by expression of $CD4^+$, and activated T cells by expression of HLA-DR/$CD3^+$. Moreover, IL-2 receptor (IL-2R) expressing T cells were defined by the presence of $CD25^+/CD3^+$. The $CD8^+$ T cell subset was determined by subtracting the number of $CD4^+$ and of $CD16^+/CD3^+$ from the total number of T cells ($CD3^+$).

2.4. Sleep assessment

Sleep stages were identified from the electroencephalogram vertical and horizontal electrooculogram, and electromyogram. The records were scored offline according to the standard criteria of Rechtschaffen and Kales [36]. For each night sleep onset (with reference to lights off), the absolute time, and the percentage of the total sleep time spent in the different sleep stages — S1, S2, slow wave sleep (SWS) and rapid eye movement (REM) sleep — were determined. Also, latencies of S2, SWS and REM sleep were calculated with reference to sleep onset.

2.5. Statistical analysis

For each parameter, average values were calculated for the first and second 4-h period of sleep time and for the total 8-h nocturnal period. Statistical analysis was based on analysis of variance (ANOVA) including a group factor (aged versus young) and a repeated measures factor for time (first versus second 4-h interval). Student's t-tests were used to evaluate differences between groups at a given time.

3. Results

3.1. Sleep

Subjects displayed the usual sleep patterns. Aged and young subjects were comparable with regard to sleep onset latency (mean $\pm$ S.E.M., 29.1 $\pm$ 7.3 and 19.1 $\pm$ 4.7 min respectively) and total sleep time (435.9 $\pm$ 9.9 and 445.9 $\pm$ 6.4 min). However, sleep in the aged was, as expected, less deep with an enhanced time spent awake (24.0 $\pm$ 4.1% versus 5.2 $\pm$ 1.7% in the young controls, $P < 0.01$) and in S1 sleep (19.2 $\pm$ 6.2% versus 7.8 $\pm$ 1.7%, $P < 0.01$). On the other hand, the aged subjects spent less time in S2 sleep (39.1 $\pm$ 2.7% versus 47.0 $\pm$ 2.3%), in SWS (7.7 $\pm$ 1.3% versus 20.0 $\pm$ 1.8%), and in REM sleep (10.0 $\pm$ 1.3% versus 19.9 $\pm$ 1.9%) than the young controls ($P < 0.05$, for all comparisons). SWS latency was enhanced in the aged ($P < 0.01$).

3.2. Hematologic analyses

Table 1 summarizes results from WBC counts, including leukocyte differential counts, Hb and platelet counts. Values are indicated for the blood samples collected immediately before lights were turned off, for the first and second 4-h period of the night, and for the total 8-h nocturnal sleep interval. According to the known circadian rhythm, the number of WBC decreased from the first to the second 4-h interval of the night ($P < 0.01$). Aged subjects had less WBC than young subjects ($P < 0.05$), and this decrease appeared to be more pronounced in the second rather than first 4-h interval of the night (Table 1). During the second half of the night, the number of neutrophils was also significantly lower in the aged than young subjects. The number of lymphocytes was substantially decreased in the aged throughout the night, averaging about 70% of that in young subjects. Numbers of monocytes, basophils, and eosinophils were comparable in both age groups, but Hb concentrations and platelet counts were decreased in the aged.

Table 1
Hematologic parameters of aged and young subjects during sleep Mean (±S.E.M.) white blood cell count, leucocyte differential counts, hemoglobin concentration, and platelet count in aged (left) and young subjects (right) prior to lights off, during the first and second 4-h period of the night (1.half, 2.half), and during the total 8-h nocturnal sleep period.

No. of subjects	Aged 16				Young 16			
Age (year)	Lights off 79.6 ± 7.5	1.half	2.half	Total	Lights off 24.6 ± 3.1	1.half	2.half	Total
WBC/μl	6165 ± 383	5807 ± 382[a]	5298 ± 356[a]	5553 ± 368[a]	7362 ± 502	7333 ± 509	6908 ± 419	7121 ± 445
Neutrophils/nl	3.53 ± 0.23	3.17 ± 0.22	2.86 ± 0.24[a]	3.01 ± 0.23	3.90 ± 0.41	3.81 ± 0.41	3.66 ± 0.3	3.73 ± 0.32
Lymphocytes/nl	1.81 ± 0.16[b]	1.86 ± 0.15[b]	1.71 ± 0.12[b]	1.78 ± 0.14[b]	2.54 ± 0.14	2.64 ± 0.14	2.41 ± 0.12	2.52 ± 0.13
Monocytes/nl	0.48 ± 0.04	0.45 ± 0.03	0.42 ± 0.03	0.43 ± 0.03	0.53 ± 0.03	0.49 ± 0.03	0.47 ± 0.03	0.48 ± 0.03
Eosinophils/nl	0.18 ± 0.03	0.19 ± 0.03	0.19 ± 0.03	0.19 ± 0.03	0.22 ± 0.04	0.22 ± 0.04	0.20 ± 0.03	0.21 ± 0.03
Basophils/nl	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	0.06 ± 0.01	0.06 ± 0.01
Hemoglobin g/l	126.2 ± 2.7[b]	124.5 ± 2.5[b]	125.5 ± 2.7[b]	125.0 ± 2.6[b]	139.8 ± 2.6	138.1 ± 2.6	138.9 ± 2.7	138.5 ± 2.6
Platelets/nl	181.7 ± 8.6[a]	178.7 ± 8.4[b]	175.8 ± 7.5[a]	177.3 ± 7.9[b]	215.1 ± 9.1	212.5 ± 7.4	203.1 ± 6.9	207.8 ± 7.0

[a] $P < 0.05$, [b] $P < 0.01$, for pairwise comparison with young controls.

Table 2
MNC subsets in aged and young subjects during sleep Mean (±S.E.M.) number of T cells ($CD3^+$), helper/inducer T cells ($CD4^+$), suppressor T cells ($CD8^+$), NK cells ($CD16^+$), activated T cells ($HLA\text{-}DR/CD3^+$), IL-2R expressing T cells ($CD25^+/CD3^+$), and B cells ($CD19^+$) in aged (left) and young subjects (right) prior to lights off, during the first and second 4-h period of the night (1.half, 2.half), and during the total 8-h nocturnal sleep period.

	Aged				Young			
	Lights off	1.half	2.half	Total	Lights off	1.half	2.half	Total
$CD3^+$/nl	1.36 ± 0.14[b]	1.39 ± 0.13[b]	1.27 ± 0.10[b]	1.33 ± 0.11[b]	1.94 ± 0.12	2.01 ± 0.12	1.80 ± 0.10	1.90 ± 0.11
$CD4^+$/nl	0.95 ± 0.10[a]	1.00 ± 0.10[a]	0.91 ± 0.08[a]	0.96 ± 0.09[a]	1.26 ± 0.07	1.31 ± 0.08	1.19 ± 0.08	1.25 ± 0.08
$CD8^+$/nl	0.23 ± 0.06[b]	0.21 ± 0.05[b]	0.17 ± 0.05[b]	0.19 ± 0.05[b]	0.49 ± 0.08	0.54 ± 0.08	0.48 ± 0.07	0.51 ± 0.07
$CD16^+$/nl	0.18 ± 0.02	0.17 ± 0.01	0.19 ± 0.02[a]	0.18 ± 0.02	0.18 ± 0.03	0.16 ± 0.01	0.13 ± 0.01	0.15 ± 0.01
$HLA\text{-}DR/CD3^+$/nl	0.27 ± 0.06[a]	0.26 ± 0.06[a]	0.24 ± 0.05[a]	0.25 ± 0.06[a]	0.13 ± 0.02	0.12 ± 0.01	0.11 ± 0.02	0.11 ± 0.01
$CD25^+/CD3^+$/nl	0.31 ± 0.04[b]	0.27 ± 0.03[b]	0.22 ± 0.02[b]	0.24 ± 0.02[b]	0.17 ± 0.02	0.15 ± 0.01	0.13 ± 0.01	0.14 ± 0.01
$CD19^+$ /nl	0.20 ± 0.04[b]	0.21 ± 0.04[b]	0.19 ± 0.03[b]	0.20 ± 0.04[b]	0.42 + 0.03	0.45 + 0.04	0.39 ± 0.03	0.42 ± 0.03

[a] $P < 0.05$, [b] $P < 0.01$, for pairwise comparison with young controls.

The number of T cells ($CD3^+$) decreased across the night ($P < 0.01$), and was at all times lower in aged than young subjects (refer to Table 2 for statistical results). Likewise, the number of T cells with $CD4^+$ and $CD8^+$ antigen was constantly lower in the group of aged donors. By contrast, the NK cell count ($CD16^+$) was slightly enhanced in the aged with this effect reaching significance during the second 4-h interval of the night. Moreover, throughout the night, blood samples from elderly contained about twice as many activated T cells (HLA-DR/$CD3^+$) as those of young controls. Similarly compared to the young controls, the number of T cells expressing IL-2R ($CD25^+/CD3^+$) was consistently enhanced in the elderly by about 70%. Aged subjects displayed the expected strong decrease of B lymphocytes ($CD19^+$).

3.3. Cytokine production

Production of IL-1β distinctly decreased from the first to the second 4-h period of the night ($P < 0.01$). Age enhanced production of IL-1β during the second 4-h period of the night (Table 3) whereas production of IL-1β in the beginning of the night, especially before lights were turned off, appeared to be comparable for both age groups. Production of TNF-α was significantly increased in the aged, compared to the young control subjects, throughout the night. The effect appeared to be present also in the samples collected before lights were turned off, but remained non-significant probably due to an increased variability at that time.

While age enhanced production of IL-1β and TNF-α, production of IL-2 (stimulated with PHA) was constantly lower in the aged rather than young subjects, throughout the night. However this difference vanished if IL-2 production was determined relative to the number of T cells stimulated (Table 3). Production of IFN-gamma (per stimulated T cells) tended to be enhanced in the aged during the second 4-h interval of the night.

4. Discussion

The process of aging distinctly changes immune functions. The nature of these changes is a matter for discussion. Results from a number of animal and human studies have indicated a major deficit concerning the T cell compartment in the aged organism [1,3,4,6,7]. However, age-related functional changes of monocytes have been also reported [7,8,19,21,25]. Diverging outcomes from these studies may be explained by profound differences in the aging of immune functions between humans and rodents [1]. Among human studies diverging outcomes may have been partly due to the fact that in some of the studies the health status of the elderly subjects was not carefully checked.

The present study aimed to evaluate differences in cytokine production between aged and young subjects, and to relate the differences to age-dependent changes of MNC subpopulations. All subjects were in good health, and blood sampling was performed during the night while the subjects were asleep. By this procedure, experimental stress and its potential influence on cytokine production should be minimized. Moreover, nocturnal sleep represents a well-defined, and physiologically

Table 3
Mean (± S.E.M.) cytokine production (IL-1β, TNF-α, IL-2, IFN-γ) in aged (left) and young (right) subjects prior to lights off, during the first and second 4-h period of the night (1.half, 2.half), and during the total 8-h nocturnal sleep period. Production of IL-2 and IFN-γ is indicated, in addition, relative to the number of T cells ($CD3^+$) stimulated.

	Aged				Young			
	Lights off	1.half	2.half	Total	Lights off	1.half	2.half	Total
IL-1β pg/ml	4162 ± 498	4057 ± 457	3955 ± 478[a]	4002 ± 464	4240 ± 387	3596 ± 278	2867 ± 240	3207 ± 246
TNF-α pg/ml	203.9 ± 28.4	183.3 ± 24.2[a]	180.9 ± 26.2[a]	182.0 ± 25.0[a]	163.3 ± 14.5	125.5 ± 7.4	109.4 ± 7.1	116.9 ± 6.6
IL-2 pg/ml	23.6 ± 5.7[b]	23.5 ± 4.4[b]	23.3 ± 4.1[b]	23.4 ± 4.1[b]	53.1 ± 5.8	48.7 ± 4.4	51.3 ± 4.5	50.1 ± 4.3
IL-2/$CD3^+$ pg/10^6cells	20.8 ± 5.3	20.6 ± 4.3	21.5 ± 4.1	21.1 ± 4.1	27.7 ± 4.3	25.6 ± 3.3	30.4 ± 3.8	28.0 ± 3.5
IFN-γ pg/ml	650 ± 144.6[a]	779 ± 179.7	711 ± 155.3	743 ± 164.7	1438 ± 302	1011 ± 212	762 ± 124	878 ± 152
IFN-γ/$CD3^+$ pg/10^6cells	551 ± 124.3	690 ± 184.5	668 ± 164.1	679 ± 173.4	688 ± 177.2	440 ± 108	338 ± 73.5	388 ± 84.8

[a] $P < 0.05$, [b] $P < 0.01$, for pairwise comparison with young controls.

meaningful phase of the 24-h cycle. This is important, since on the one hand pronounced circadian oscillations have been demonstrated for the major T cell subsets ($CD4^+$, $CD8^+$, $CD16^+$, HLA-DR) [27,29], and for the production of IL-1 and TNF-α [26,30]. On the other hand, aging appears to be characterized not only by a diminished amplitude of the circadian rhythm but also by a phase advance of this rhythm [37]. Correspondingly, the elderly subjects of the present study used to go to bed on average 25 min earlier than the young controls. Thus, nocturnal sleep provided the opportunity to evaluate production of cytokines and MNC subset counts in aged and young subjects during corresponding phases of their 24-h cycle.

The present results indicate — consistent with many foregoing studies [6,7,21,24] — a marked reduction of the number of T lymphocytes, and of both major T cell subsets ($CD4^+$, $CD8^+$), in the elderly as compared to the young subjects. This observation extends results from previous studies indicating age-related declines only for one of the major T cell subsets [5], and confirms a recent study in centenarians displaying a consistent decrease of the $CD4^+$ and $CD8^+$ subsets [21]. The T cell deficit in the aged has been ascribed to a progressive thymic involution [20].

The decreased number of T cells in aged subjects of the present study appeared to be accompanied by a diminished production of IL-2 known to be predominantly released by these cells. However, this difference in IL-2 production turned out to be mainly a consequence of the smaller number of T cells in the aged since it became insignificant when concentrations of IL-2 were calculated relative to the number of T cells stimulated. These findings, contrasting with results from previous reports [11,12,38] of a diminished IL-2 production of peripheral blood MNC in elderly humans, are at present difficult to integrate. One reason for the discrepancy may be that in those foregoing studies cytokine production was stimulated after separation of in fact, heterogenous samples of peripheral blood MNC (containing monocytes, T and B cells) by density gradient centrifugation. While the total number of MNC in those samples was kept constant, the MNC subset counts may have substantially varied among subjects and experimental conditions. In contrast in the present study, a whole blood assay was applied, i.e. MNC were not isolated prior to mitogen stimulation. To determine the number and type of cells stimulated, the assay was complemented by WBC counts, including the determination of lymphocyte subsets. The parallel analysis of MNC subsets allows to relate the measured cytokine production to specific MNC subsets. On this basis, it can be decided whether an age dependent change in cytokine production indeed represents an effect on the production process per se or is secondary to a change in the number of certain subset cells producing this cytokine.

Considering the different assays employed in this study (whole blood assay) and in most foregoing studies (mitogen stimulation of separated MNC), technical differences may explain part of the discrepancies among these studies. It has been reported [11], for example, that a distinctly larger number of MNC from old than young donors die, when MNC are cultured after fractionating them. Death of MNC from old donors may be reduced when kept in whole blood. In the whole blood assay, the interactions of blood cells are preserved [39] so that cytokines are

produced under approximately physiological conditions [13]. However, any judgement regarding advantages of the whole blood assay remains tentative as long as there are no studies directly comparing results from this assay with those obtained after mitogen stimulation of separated MNC in the same samples of aged and young subjects.

The number of T cells expressing IL-2R ($CD25^+/CD3^+$) was significantly enhanced in the aged. This observation fits with the failure to find any significant decrease in the production of IL-2 in the elderly of the present study, since IL-2 is known to up-regulate its own receptor [40]. Contrasting with the present results, Nagel et al. [11] found a decreased number of cells expressing IL-2R in blood from old donors. However, those authors measured cells carrying IL-2R after stimulation with PHA while the present counts refer to basal unstimulated conditions. Hence results from this foregoing and the present study may be taken together to suggest an inverse relation between expression of IL-2R under basal unstimulated conditions and following mitogen stimulation. In this case, increased receptor expression under basal conditions would be indicative of an impaired IL-2R expression in response to mitogen challenge in the aged.

T cells are also the main source of IFN-gamma. Production of IFN-gamma calculated with reference to the number of T cells tended to be increased in the elderly. While an increase in the production of IFN-gamma has been likewise observed in aged mice [41], in a foregoing human study [19], IFN-gamma production was found to be comparable in young and aged donors. Except for the different assay techniques used by those authors (stimulation of separated MNC with PHA plus PMA) and in the present study (stimulation of whole blood with PHA), the timing of blood sampling could also have contributed to the divergent results. Thus, in the present study, the increase in the production of IFN-gamma in the aged approached significance only during the second 4-h interval of the night, while IFN-gamma production in samples collected in the late evening were comparable for both age groups.

A state of increased activation in the aged within the T cell compartment was further suggested by the finding of an increased number of activated T lymphocytes ($HLA\text{-}DR/CD3^+$) and NK cells ($CD16^+$) in the elderly, with the latter effect being restricted to the second 4-h interval of the night. Both age related increases in the $HLA\text{-}DR/CD3^+$ subset and in the number of NK cells have been well documented in foregoing studies [23,42,43]. Sansoni and co-workers [43] provided evidence that this decrease selectively concentrates on cells with high NK activity ($CD16^+/CD57^-$). Those authors proposed that the age-related increase of NK cells could reflect a compensatory mechanism to cope with the general decrease of T cells, which thus can help to reach advanced age. This view is supported by findings indicating that persistently low NK activity is a predictor of infectious morbidity [44].

Contrasting with the distinct changes in T cell subsets, the number of monocytes remained unchanged in the elderly, which agrees with results from several other human studies [7,18,25]. Monocytes/macrophages represent the major source of IL-1β and TNF-α after stimulation with LPS, the production of which was

significantly enhanced in the aged. These results corroborate recent findings by Fagiolo et al. [19] of an age-related increase in the production of IL-1β, TNF-α, and IL-6, and indicate that monocytes from old donors are functionally intact. The increase in the production of these cytokines upon mitogen stimulation suggests that in the aged, monocytes — like T cells — are in a state of increased excitability. The increase in the production of IL-1β and TNF-α in the aged could be considered, in part, a consequence of the age-related deterioration of sleep characterized by diminished amounts of SWS and REM sleep, and an increased time of intermitted awakening. Acute sleep deprivation in humans has been shown to increase release of TNF-α and IL-1β after in vitro stimulation with LPS [45,46], and also production of IFN [32]. Given the poor quality of sleep in the elderly, these subjects could be considered to chronically suffer from moderate sleep deprivation activating monocytes to produce cytokines. But, it is also conceivable that, conversely, the enhanced production of IL-1β and TNF-α acts to impair central nervous sleep processes in the aged [47].

It must be emphasized that some of the signs of increased immune activation in the elderly (i.e. the increased production of IL-1β and of IFN-gamma and the increased number of NK cells) were concentrated during the second 4-h interval of the night. During this time, in young subjects production of IL-1β and IFN-gamma, and also the number of NK cells were decreased when compared to the preceding 4-h interval, which probably reflects a circadian rhythm [26,29]. It could be argued that this decrease in the young subjects is mediated through a suppressing influence of plasma cortisol concentrations markedly rising during the early morning hours, with this corticosteroid mediated suppression being less pronounced in aged subjects. However, aged subjects have been found, on the contrary, to display enhanced nocturnal cortisol concentrations [48,49]. Moreover, in young subjects, temporal oscillations of cortisol concentrations and IL-1 production were found to be uncorrelated [30,46]. However, immune functions are also strongly influenced by hormones other than cortisol like growth hormone and thyroid-stimulating hormone. Secretion of these hormones is modulated by the sleep-wake rhythm and is also subject to substantial changes with age [48,50]. Therefore, further research seems promising in deciding to what extent the immune activation observed in monocytes and T cells of aged is mediated by endocrine factors.

In summary, the present results indicate that ageing differentially affects the various MNC subsets, and also differentially affects the production of various cytokines. Hence, the process of aging may exert its essential influence via mechanisms specifically regulating production of the different cytokines and MNC subsets. The increased responsiveness of the T cell compartment and of monocytes (indicated by increases in HLA-DR/CD3$^+$, CD16$^+$, CD25$^+$/CD3$^+$, production of IFN-gamma, IL-1β, and TNF-α) could partly reflect the activation of mechanisms compensating for the substantial decrease in T cells and their proliferative capabilities in the aged. This view is not completely new and appears to complement a similar conclusion drawn in several recent studies, indicating that aging is associated with an increase in the proportion of preactivated or memory T cell phenotypes in relation to naive T cells [51–54].

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