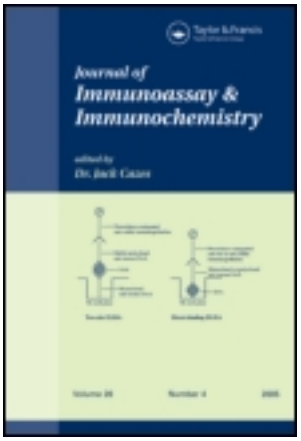

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CYTOKINE SERUM PROFILE IN A GROUP OF SICILIAN NONAGENARIANS

Marisa Palmeri,[1] Gabriella Misiano,[1] Mariano Malaguarnera,[2] Giusi Irma Forte,[1] Loredana Vaccarino,[1] Salvatore Milano,[1] Letizia Scola,[1] Calogero Caruso,[1] Massimo Motta,[2] Domenico Maugeri,[2] and Domenico Lio[1]

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□ *The aim of our study was to evaluate the possibility of using multiplex analysis of the cytokine profile as a marker for successful aging by comparing cytokine plasmatic levels of a group of Sicilian nonagenarians with those of young controls. We analyzed a panel of 17 cytokines, comprehensive of haematopoietic factors T helper 1 (Th1), Th2, inflammation regulatory cytokines, and chemokines. The assay was carried out using the Luminex system. Interleukin (IL)-6 levels ($p = 0.01$) were increased in nonagenarians, whereas no modifications of other proinflammatory cytokines and chemokines were observed. Interferon-gamma (IFN-γ) and IL-2 levels are unmodified, suggesting a substantial maintenance of relevant T cell functions. In addition, a significant increase of IL-12 serum levels in nonagenarians versus young controls that might be related to the increase of natural killer (NK) cell functions characterizing aging processes was observed. The analysis of Th2 cytokines show an increase of IL-13 and a reduction of IL-4 levels mirroring the maintenance of some effector's mechanisms of the immunoresponse in advanced ages. Our results suggest that the multiplex analysis of cytokine levels might be useful in defining a successful aging profile.*

Keywords circulating cytokine levels, immunoassay, Luminex, serum profile, successful aging

INTRODUCTION

Centenarians provide the best example of successful aging. They are people who have escaped major age-related diseases and have reached

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the extreme limit of human life in good clinical condition.[1] For example, laboratory parameters of centenarians indicate that they are characterized by reduced levels of blood glucose, transaminases, cholesterol, and platelets with respect to older subjects with an age range between 65 and 85 years.[2] In most of the cases, histories of centenarians reveal them to be free of cancer, dementia, diabetes, and cardiovascular diseases, which is surely due to a successful interaction between environmental and genetic factors.

Advancing age is correlated to an increase of inflammatory response, which is believed to be a direct consequence of the continuous attrition caused by antigenic load during the life-span—a condition that is commonly called by the authors "inflamm-aging,"[3] also sustained by the immune system remodeling, which physiologically occurs during aging ("immuno-senescence"). It is a slow but inexorable process leading to an immune system that shows peculiar features, predisposing older people to a different kind of reaction to injuries in comparison to young individuals.[4]

In particular, the dedicated immune system tissues, such as as thymus, bone marrow, spleen, and lymph nodes, undergo involution or they show regressive phenomena. The number of germinal centers is reduced, and there is a progressive loss of cell-mediated immunity, generally recognizable by a tendency toward the switch from Th1 vs. Th2 type response.[5] Moreover, in the aging process, and even in centenarians, there is a redistribution of monocytes, neutrophils, B and T subsets, and a quite normal number of T lymphocytes, even if these cells mostly show a memory phenotype, whereas a progressive reduction of virgin cells is observable, which makes the old individuals unable to respond to antigens not previously encountered.[6]

Several studies have largely demonstrated an important role of genetic background in the achievement of advanced age. A group of genes that has often been tested for association with successful aging is that which influences inflammation and immune responses. Among these, there are genes coding for interleukins. Our group has demonstrated that particular cytokine polymorphisms, especially located on the functional promoter sequence of important cytokines genes such as interleukin (IL)-1, IL-6, tumor necrosis factor-α (TNF-α), interferon-γ (INF-γ), and IL-10, may influence the susceptibility to age-associated diseases or may alternatively contribute to the genetic background associated with longevity.[7–13] These data highlight the role of cytokine production in determining the successful or unsuccessful aging phenotype. In this view, it seems of some interest to check the possibility of identifying a serum cytokine profile that might characterize successful aging. To reach this result, a technology that allows a contemporaneous and highly precise determination of a large number of cytokines should be applied.

In this article, we report data evaluation by Luminex technology of the blood levels of 17 pro- and anti-inflammatory cytokines and chemokynes, which are crucial in the orchestration of the immune response, in order to identify the circulating cytokine profile of subjects >90 years old.

MATERIALS AND METHODS

Subjects Recruitments

In our study, two groups of subjects were tested. In particular, we analyzed sera from 44 Sicilian ultra-nonagenarians (age >90 years) and 79 control subjects consisting of a group of healthy young Sicilian individuals, aged between 30 and 50 years old. None of ultra-nonagenarian subjects recruited for the study show any major age-related diseases or severe cognitive impairment (dementia or neoplastic, cardiovascular, or infectious disease), nor did they receive any drugs that influence immune functions at the time of the study. Their age was verified by archival records at the City Hall and/or church registries, verifying the concordance between reported age and personal chronologies (age of marriage and of military service for men, age of first and last pregnancy for women, age of children, etc.). The Sicilian ethnicity of all the participants at the study was established by confirming that all four grandparents were born in Sicily, in order to gain a certain guarantee of a homogeneous population, as immigration and intermarriage have historically been rare at the beginning of the last century.[14] Written informed consent for enrolling in the study and for personal data management was obtained from all subjects according to Italian laws.

Evaluation of Cytokine Blood Level Assay

Each subject underwent a fasting blood sampling. The blood samples were collected in lithium heparin additioned vacutainer tubes, immediately centrifuged at 1800 rpm for 15 min to isolate cell free plasmas that were immediately stored at −80°C.

Immediately before the cytokine assay, thawed samples were centrifuged at 12,000 rpm for 5–10 min to allow precipitation of any lipids excess that may interfere with subsequent analysis.

The samples were tested for a panel of 17 cytokines and chemokines [IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12(p70), IL-13, IL-17, IFN-γ, TNF-α, monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1β (MIP-1β), granulocyte-macrophage

colony-stimulating factor (GM-CSF), and granulocyte colony-stimulating factor (G-CSF)] using Bio-plex kit (BioRad, Milan, Italy) and following the manufacturer's instructions. The assay was carried out using the Luminex system (BioRad, München, Germany), based on the measurement of fluorescent signals released by a suspension of microspheres, bringing immobilized multiplex cytokine specific antibodies in 96-well plates. The combination of a fluorimetric signal of microspheres with that released by a secondary antibody allows us to measure cytokine concentration–related signals converted by a processor. Briefly, 50 μL of samples diluted 1:4 in a dilution buffer were incubated at room temperature in the presence of beads conjugated with specific antibodies for the different cytokines. After a wash to remove the excess of not bound serum components, an incubation with the biotin conjugated secondary antibodies was performed. Finally, after another washing step and the streptavidin-PE complex addition, the fluorimetric signal was detected by using the Luminex plate reader. The assay was performed using an eight-point standard curve for every cytokine. Samples were analyzed on a Luminex 100 device (BioRad), and the data were evaluated using the Bio-Plex Manager software (BioRad). Standards, internal controls, and samples were reported as means of duplicate measurements.

Statistics

All the data are shown as mean concentrations (pg/ml) ± standard error (SE). Differences in cytokine levels among nonagenarians and healthy controls were assessed by the Mann–Whitney test. Differences were considered significant when a p value <0.05 was obtained.

RESULTS AND DISCUSSION

As we already described in the Introduction, aging is characterized by a remodeling process of immune system, where several functions are reduced, whereas others remain unchanged or are increased.[15] A few notable instances are, surely, a persistence of a low-grade chronic inflammatory status and a consistent increase of activated cells that progressively fill the immunological space,[4] considered as the compartment occupied by the immune cell subsets and a reduced capability to cope with new immunological stimulations. In this scenario the reshaping of cytokine production network seems to play a central role.

In this article, we focus on the evaluation of the circulating cytokine profile of a group of healthy nonagenarians in an attempt to describe a plasmatic cytokine profile that might characterize successful ageing. To

reach this goal the highly sensitive multiplex Luminex technology is applied to minimize differences in cytokine evaluation due to differences of performances among methodologies useful to measure a single cytokine at a time.

The selected panel of cytokines measured included the following: (a) hematopoietic cytokines; (b) proinflammatory and anti-inflammatory cytokines and chemokines; (c) Th1, Th2, and Th17 cytokines.

As reported in Table 1, the levels of the three hematopoietic cytokines analyzed (IL-7, G-CSF, and GM-CSF) are not significantly different among young and nonagenarians groups. However, it seems relevant that IL-7, implied in lymphopoiesis, is reduced in nonagenarians with a difference with young subjects near the statistically significant threshold. As reported by Pawelec et al.[6] dysregulated hematopoiesis is seen in older individuals, raising the possibility that this could contribute to altered immune function in aged persons. Actually, data from some experimental models seems to suggest that a reduced production of IL-7 might be implied in

TABLE 1 Plasmatic Cytokine Concentration Expressed as Mean (pg/mL) ± Standard Error

Cytokines	Young Controls (79)	Nonagenarians (44)	P Value
IL-7	1.60 ± 0.10	1.0 ± 0.30	0.06
G-CSF	0.55 ± 0.15	0.30 ± 0.08	0.14
GM-CSF	5.11 ± 1.06	11.50 ± 4.41	0.16
IL-2	11.03 ± 1.49	9.30 ± 2.03	0.11
IL-12(p70)	**2.88 ± 0.6**	**7.20 ± 1.53**	**0.01**
IFN-g	10.22 ± 3.93	8.24 ± 2.69	0.27
IL-4	**0.55 ± 0.08**	**0.35 ± 0.05**	**0.03**
IL-5	0.65 ± 0.09	0.66 ± 0.06	0.76
IL-13	**0.77 ± 0.05**	**1.23 ± 0.19**	**0.02**
IL-1b	11.89 ± 4.02	11.24 ± 3.40	0.26
IL-6	**5,16 ± 3.61**	**11.18 ± 2.53**	**0.01**
TNF-a	5.80 ± 1.99	7.72 ± 1.61	0.81
IL-10	0.44 ± 0.04	0.75 ± 0.20	0.11
IL-17	7.36 ± 1.34	17.10 ± 3.32	0.09
IL-8	31.08 ± 84.9	36.10 ± 5.89	0.41
MCP-1	79.90 ± 17.06	130.80 ± 29.57	0.14
MIP-1b	125.70 ± 10.71	139.90 ± 7.54	0.47

Differences in cytokine levels among nonagenarians and healthy controls were assessed by the Mann–Whitney test.

progressive reduction of the number of naive lymphocytes associated with aging.[16]

One of the major characteristics of senescence is the constant presence of a low-grade inflammatory status characterizing aging. This subclinical inflammation produces a persistent immune system activation, resulting in continuous low-grade tissue damage, as well as in the reduction of the normal immune system response to new antigens caused by a net consuming of naive cells.[3,4,17] As reported in Table 1, nonagenarians show a significant increased level of IL-6 (p value = 0,01), which seems to suggest that an increased IL-6 level might be detected also in a successful aging phenotype to confirm the age-dependent pro-inflammatory imbalance. Moreover, increased levels of IL-6 could also be related to the increased level of IL-17 (Table 1), even if the result did not reach statistical significance ($p = 0.09$), as IL-6 acts to enhance the Th17 lymphocytes' activity by stimulating the production of IL-17, which is involved in inflammation and in the amplification of the inflammatory response. However, the concentrations of other proinflammatory and anti-inflammatory cytokines and chemokines crucial in the balance of inflammation (IL-1-beta, TNF-a, IL-8, MCP-1, MIP-1b, and IL-10), are not significantly different between the two groups of subjects, and this allows us to speculate that the cytokine profile of the studied nonagenarians is characterized by a very low grade of proinflammatory cytokine signature. In addition, the slight increase of antinflammatory IL-10 observed is in agreement with our previous description of the genetic background owned by centenarians, as we previously found among these subjects a significant increase of the frequency of carriers possessing the allele related to higher IL-10 production.[10,12]

IFN-γ and IL-2 levels are not modified in nonagenarians with respect to the controls. As reported by different groups, Th1 and IL-2 cytokine production is generally decreased in older subjects,[6] even if in selected healthy old subjects (SENIEUR protocol) these cytokines seem to be normally secreted.[6] Our data obtained in healthy nonagenarians seem to suggest that the preservation of IFN-γ and IL-2 production might be one of the components of the successful aging phenotype. In particular the maintenance of an equilibrated concentration is favorable to prevent an excessive or prolonged Th1 response. Concerning IL-2 secretion maintenance in the oldest subjects, in our previous study, no modifications of genotypic and allelic frequencies of the IL-2 functional polymorphism -330 T/G were demonstrated.[18]

In our study we found a statistically significant increase in serum levels of IL-12 in nonagenarians in comparison with controls (p value = 0.01) (Table 1). Conflicting data on IL-12 production in old subjects have been published.[6] Both increase and reduction in IL-12 secretion have been

reported in animal experimental models, as well as after in vitro stimulation of human peripheral blood cells.[6] Our data, which were obtained by evaluating IL-12 serum levels, might be related to the increase of monocytes and NK cells, which characterize aging processes, mirrored by a very well-preserved cytotoxic activity. Indeed, a well-preserved NK cell activity can be considered a factor of longevity.

It is reported that advanced-aged individuals are characterized by high plasma levels of immunoglobulins.[19] In particular IgG, IgA, and IgE levels are increased. Analysis of Th2 cytokines shows similar IL-5 levels among nonagenarians and young controls. Instead the analysis of other Th2 cytokines shows divergent results. Actually, a significant reduction of IL-4 levels ($p = 0.04$) is accompanied by a significant increase of IL-13 levels in nonagenarians versus controls ($p = 0.02$). In a recent article, we focused on B cells in the aged by studying the expression of some surface markers. In particular, in older people and in centenarians, there was an increase of CD27 + B cells with a decrease of CD27 − B lymphocytes. CD27 is considered a marker of primed memory B cells. The decrement of virgin CD27 − B lymphocytes cells and the concurrent increase of memory CD27 + B lymphocytes seem to have an impact on the antibody repertoire of older individuals.[19–21] In this view, our data might be related to the different role played by IL-4 and IL-13 in the Th2 switch and B cell response maintenance in aging. Actually the Th2 switch, where IL-4 has a major key role, is already strongly reduced in advanced aged individuals, where there is a higher ratio between differentiated memory cells and virgin naïve ones, whereas, IL13 is mainly produced by primed Th2 cells.[22] So, the contemporaneous circulating IL-4 reduction and IL-13 increase detected in nonagenarians might mirror Th2 committed cells related to increasing age. Our results taken together confirm that, although the cytokine profile is modified by aging, we were able to show that long-living persons maintain unchanged levels of some crucial cytokines useful in preserving crucial immune-system function, and this might contribute to a person reaching such an advanced age.

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