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Preferential Accumulation of Mature NK Cells During Human Immunosenescence

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Abstract The major histocompatibility complex-unrestricted, cell-mediated, constitutive anti-tumor cytotoxic function of natural killer cells is highly preserved in healthy elderly. A study of the dynamics of expression of natural killer cell–associated phenotypes during immunosenescence shows that selective, bidirectional, and disproportionate changes in certain natural killer cell subset number and ratio take place during aging. The mean natural killer cell subset ratio (%$CD16^+CD57^+$ over %$CD56^+CD57^-$) gradually increases from a young adult level of 0.7 to 4.6 with advancing age predominantly due to a tripling of %$CD16^+57^+$ cells as opposed to a moderate decrease (−54%) in %$CD56^+57^-$ phenotype. The parallel increase in natural killer phenotype ratio and cytotoxic activity might represent a shift in the maturity status of these cells. Based on these findings, a model of natural killer cell immunosenescence is proposed. It is concluded that not all immunosenescent changes need be detrimental; some may even improve the potential for survival and represent an adaptational immunosenescent change.

Key words: natural killer cells, human aging, senescence, mature phenotype, cytotoxic activity, MHC-unrestricted, cell mediated immunity, CD16 (Leu-11), CD57 (Leu-7), CD56 (Leu-19)

Natural killer (NK) cells are a heterogeneous population and a majority of them are $CD3^-$, $CD16^+$, $CD56^+$ and express only a germline configuration of the T-cell receptor genes [Trinchieri, 1989; Fitzgerald-Boscarsly et al., 1989; Ortaldo and Herberman, 1984]. We have shown that aging may have a moderate positive influence on peripheral blood NK cell system; compared to young adults (<40 years), there was a moderate but significant increase in NK cell activity (cytotoxicity against the NK sensitive tumor target, K562) in >80-year-olds [Krishnaraj and Blandford, 1984, 1987]. Using two-color immunofluorescence approach, we have also presented evidence that an NK subset, $CD16^+57^+$, can triple its number in the aged [Krishnaraj and Blandford, 1984, 1988]. But neither the cause nor significance of the latter trend [Krishnaraj, 1990a; Lighart et al., 1989; Faccini et al., 1987] was clear.

In the past, several attempts have been made to delineate in vitro NK cell differentiation pathway(s) [Phillips and Lanier, 1985; Perussia et al., 1987; Schmidt et al., 1986; Nagler et al., 1989]. Based on the expression of different densities of major NK cell antigens (viz., CD16, CD56 and CD57), as well as the cytotoxic capabilities of fluorescence-activated cell sorter-isolated adult human NK cell subsets, a model of NK cell differentiation has been proposed [Nagler et al., 1989]. It was speculated that the $CD16^+57^+56^{+dim}$ phenotype possessing a high constitutive cytotoxic activity might represent a more differentiated ("mature") stage in the NK cell lineage. Since CD57 may be expressed late in the differentiation pathway of $CD16^+$ NK cells [Phillips and Lanier, 1985; Nagler et al., 1989], most of the $CD16^+57^+$ phenotype could be deemed to be mature NK cells. On the other hand, $CD56^{+bright}16^-$ (and perhaps some $CD56^{+bright}\ 16^{+dim}$) cells do not appear to express the CD57 antigen. Since the $CD56^+16^-57^-$ phenotype was considered to represent a less differentiated NK cell with a relatively lower constitutive cytotoxic potential [Nagler et al., 1989], the $CD56^+57^-$ phenotype might be presumed to be an "immature" NK cell. Using this as a tentative model of in vivo NK cell differentiation, we have investigated if senescence of the immune system in healthy elderly is likely to be accompanied by an altered status of maturity among

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circulating NK cells. This approach provided an in vivo framework to verify the significance of potentially different maturational stages of NK cells.

METHODS

Lymphocytes

Ninety-seven venous blood samples were obtained from 20–94-year-old healthy volunteers as per the criteria described elsewhere [Krishnaraj and Blanford, 1987]. The lymphocytes obtained by Ficoll-Paque density gradient separation followed by carbonyl iron as well as plastic adherence treatments contained >95% viable cells and <1% $MO2^+$ cells [Krishnaraj and Blandford, 1987].

Chromium Release Assay

NK activity of lymphocytes suspended in complete medium with 10% bovine calf serum was measured by the standard cytotoxicity assay using [^{51}Cr] labelled K562 (erythroleukemia) tumor targets as described before [Krishnaraj and Blandford, 1987]. For simplicity of presentation, only % specific [^{51}Cr] release data are given; highly significant ($P = 0.0001$) correlations were noted previously among other parameters of expression of NK activity such as lytic units, V_{max}, and Area Under Titration Curve [Krishnaraj and Blandford, 1987].

Phenotypic Analysis

Lymphocytes were stained with monoclonal antibodies, anti-Leu-11a (CD16) and anti-Leu-7-biotin (CD57) or anti-NKH-1 (CD56) and anti-Leu-7-biotin either directly (as FITC conjugate) or indirectly (biotinylated antibody followed by avidin-rhodamine conjugate) and enumerated by two-color immunofluorescence microscopy as described elsewhere [Krishnaraj and Blandford, 1988]. A Zeiss inverted microscope (model IM35) with a mercury HBO 50 W lamp fitted with filter sets suitable for selective visualization of fluoresein (FITC) or rhodamine (TRITC) fluorescence was used. By switching the filters, single or double positive cells could be enumerated. Brightly stained cells were scored as positive cells. An immature NK cell is defined by its combined CD56 positivity and CD57 negativity. Similarly, a mature NK cell could be detected because of its co-expression of CD16 and CD57. The somewhat lower sensitivity of the microscopic procedure (as opposed to flow cytometry) as well as a cautious avoidance of ambiguity (i.e., non-inclusion of very faintly positive cells) would exclude dimly positive phenotypes. The terms *immature* and *mature* have been assigned tentatively and may need more detailed evaluation. Although a minor subset of T-cells can also express CD57, enumerating cells that co-express CD16 and CD57 ensures that we are dealing mostly with NK cells. In addition to the enumeration of relative frequency (% stained cells), the absolute number of various phenotypes was also calculated based on the total number of lymphocytes obtained from each milliliter of blood after the Ficoll step. As before [Krishnaraj and Blandford, 1988], these results (absolute number of NK subsets) followed a general trend revealed by the data on % stained cells. Former values are not shown for simplicity of presentation.

Statistical Analysis

Data were analyzed by Student's t test and regression analysis with the help of a computer software, QPRO. A 95% confidence level was generally chosen as the limit for statistical significance.

RESULTS AND DISCUSSION

Human peripheral blood lymphocytes from healthy donors were examined [Krishnaraj and Blandford, 1988] for the frequency of cells that express the NK cell–associated surface markers, CD56 or CD57 (Table I). While the mean (±SEM) expression of $CD56^+$ or $CD57^+$ cells were 9.6 ± 0.7 and 10.5 ± 0.8% of total lymphocytes for the entire study population (mean age: 47 years), there was an interesting trend in their relative expression with respect to advancing age. The frequency of $CD56^+$ cells decreased in the oldest age group in a modest (−46%) but statistically significant manner. This was in contrast to a dramatic increase (+232%) in the $CD57^+$ phenotype with aging. The moderate decline in $CD56^+$ cells was unexpected since our previous studies showed that there was a doubling of $CD16^+$ (another widely used NK cell marker) cells with aging and a selective increase in the percent and number of $CD16^+57^+$ NK subsets in these individuals [Krishnaraj and Blandford, 1988]. Hence the coexpression of CD56 and CD57 was also studied. A comparison of the $\%CD56^+57^-$ and $\%CD56^-57^+$ phenotypes in young adults (20–39 years) with that of older individuals (>80 years) reveals a statistically

TABLE I. Influence of Age on the Coexpression of CD56 and CD57 NK Antigens by Human Peripheral Blood Lymphocytes*

Age (years)	NK phenotype (% stained cells, mean ± SEM)				
	$CD56^+$	$CD57^+$	$CD56^+57^-$	$CD56^+57^+$	$CD56^-57^+$
20–39[a]	10.7 ± 1.2	7.1 ± 0.9	6.5 ± 0.8	4.4 ± 0.7	2.9 ± 0.5
40–59[b]	9.1 ± 1.4	8.7 ± 1.0	5.8 ± 1.1	3.4 ± 0.6	4.7 ± 0.8
60–79[c]	8.7 ± 1.3	13.5 ± 1.5	5.0 ± 0.8	3.6 ± 0.7	10.1 ± 1.8
>80[d]	5.8 ± 1.7	23.6 ± 2.8	3.0 ± 0.7	2.8 ± 1.3	20.7 ± 3.2
Comparison of young adults (20–39 years) with the elderly (>80 years):					
Mean change	−46%	+232%	−54%	−34%	+600%
t value	2.43[e]	5.7[e]	3.23[e]	1.09	5.48[e]
P value	<0.02	<0.001	<0.01	>0.1	<0.001

*Human peripheral blood lymphocytes were scored for the expression of NKH-1 (CD56) or Leu-7 (CD57) antigens or their coexpression. Values are mean ± SEM.
[a–d]Up to 45, 25, 17, and 10 donors were tested for each age group, respectively.
[e]Highly significant.

significant negative (−54%) and positive (+600%) trend with aging respectively. The dramatic sixfold increase in $CD56^-57^+$ phenotype with aging is qualitatively similar to a threefold increase in $\%CD16^-57^+$ phenotype reported by us previously [Krishnaraj and Blandford, 1988]. While these two may represent highly overlapping populations of lymphocyte subsets, their immunological significance, function, precursors, etc., are not clear. The double positive NK phenotype, $CD56^+57^+$, unlike the $CD16^+57^+$ phenotype, did not show a statistically significant age-related change.

Next, we tested if the ratio of $CD16^+57^+/CD56^+57^-$ cells could serve as a rough index of NK cell maturity status of a given age group. In the present study, the mean NK subset ratio (of $\%CD16^+57^+$ to $\%CD56^+57^-$) was found to gradually increase with each successive age group (Fig. 1), with a highly significant statistical difference between the youngest and oldest groups (NK subset ratios of 0.7 and 4.6, respectively, $P < 0.01$). The major reason for this increase was a dramatic increase in the $\%CD16^+57^+$ phenotype with age [Krishnaraj and Blandford, 1988; Krishnaraj, 1990b]. In contrast, the $\%CD56^+57^-$ phenotype was relatively stable up to the age 60, but thereafter showed a moderate negative trend with age in a statistically significant manner. The net result was a gradual increment in the mean NK phenotype ratio with increasing age. Figure 2 depicts photomicrographs of lymphocytes expressing CD16, CD57, or CD56, the three NK cell surface markers evaluated in this study.

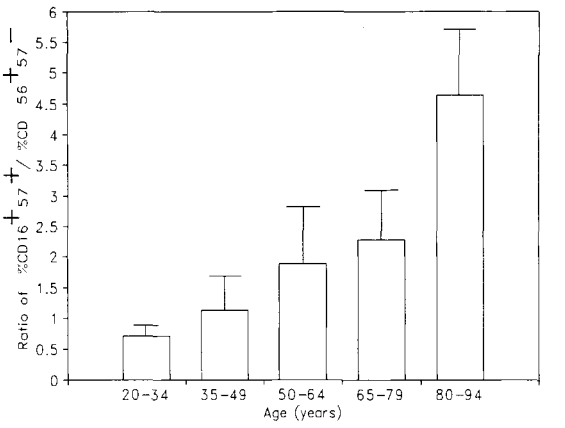

Fig. 1. Relationship between the ratio of $\%CD16^+57^+/\%CD56^+57^-$ NK subsets and age. The ratio of $\%CD16^+57^+/\%CD56^+57^-$ NK subsets was calculated for each donor (n = 31 females). Bars represent mean ± SEM ratios for indicated age groups. The mean value for young adults (20–34 years, n = 8; 4.4% $CD16^+57^+$ and 6.8% $CD56^+57^-$) is significantly different ($P < 0.01$) from the mean value for the elderly (>80 years, n = 6; 12% $CD16^+57^+$ and 2.3% $CD56^+57^-$ cells).

If the association between the expression of the above mentioned cell surface antigens by the immature and mature NK cells and their relative cytotoxic potential is valid [Nagler et al., 1989], an increased proportion of $CD16^+57^+$ cells should result in a higher mean NK activity. The higher the ratio (as seen in the old age group), the higher would be the proportion of mature NK cells expressing higher cytotoxic potential and vice versa. The NK activity of each donor was examined as a function of age, and as expected, elevated levels of NK activity with age

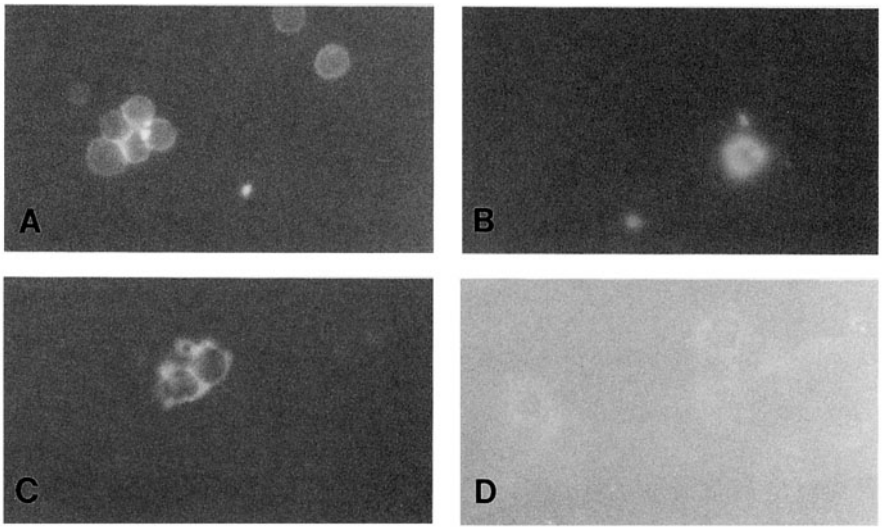

Fig. 2. Cell surface expression of various NK cell antigens shown by immunocytofluorescence microscopy. Lymphocyte preparations were stained directly or indirectly with **(A)** Leu-11a (CD16), **(B)** Leu-7 (CD57), **(C)** NKH-1 (CD56), or **(D)** isotype control monoclonal antibodies as described under Methods. Photomicrographs of formalin fixed, cytospin centrifuged cells show positive fluorescence due to FITC (A,C), positive fluorescence due to rhodamine (B), or negative cells (D). ×4,000.

were observed (Fig. 3). These data appear to suggest that an overall increase in the mature NK phenotype during aging may have been responsible for the relatively higher cytotoxic potential of NK cells from the elderly.

A comparison of some of the NK subsets during "maturational" process and aging process is presented in Table II. Our data are suggestive of a preferential accumulation of (perhaps long-lived, resting) mature NK cells over their immature precursors during senescence. Senescence invariably accompanies a decline in the proliferative potential of cells, including lymphocytes (Weksler, 1982). Interestingly, the proliferative (and cytotoxic) response to Interleukin 2 of $CD16^{bright}$ (mature) cells is lower than that of $CD16^{-}/CD16^{dim}$ or $CD56^{bright}$ (immature) NK cells [Nagler et al., 1989; Caliguiri et al., 1990]. Also, the tumor cell line–induced proliferative capacity of $CD16^{+}57^{+}$ (mature) cells is lower than that of $CD16^{+}57^{-}$ cells [Perussia et al., 1987]. It is possible that aging could have resulted in one or more of the following: an enhanced rate of maturation of immature cells of NK lineage; a reduced efficiency of removal or destruction of mature NK cells from circulation; a moderate loss of the immature NK subset; and a redistribution (migration) of NK subsets within various organs. Such age-associated changes could be either specific (e.g., due to the presence or absence of specific stimuli or altered sensitivity to such stimuli) or non-specific (e.g., cumulative effects of environmental insults). None of these can be ruled out at present.

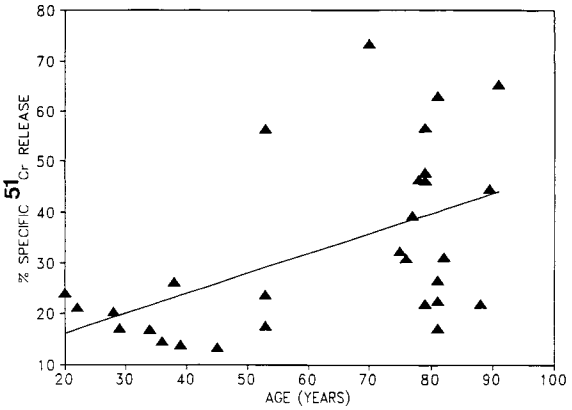

Fig. 3. NK activity of lymphocytes as a function of age. Each point represents NK activity of an individual tested by the standard [^{51}Cr] release assay using the K562 tumor cell line (see Methods). Straight line represents the regression line ($r = 0.522$; $n = 29$ females; highly significant at $P < 0.01$).

That healthy aging is accompanied by NK cell expansion is strongly supported by the data from us and others [Krishnaraj and Blandford, 1984, 1987, 1988; Krishnaraj, 1990a; Ligthart et al., 1989; Faccini et al., 1987]. It is proposed that with the progression of age there are selective, bidirectional changes of varying magnitudes in NK cell subsets (Fig. 4). Although an

TABLE II. Comparison of NK Cell Surface Antigen Expression and Density During Maturation and Immunosenescence*

NK cell differentiation[a]			NK cell number during senescence[b]		
	Immature → Mature			Young → Old	
NK activity	+	++	NK activity	++	+++
CD16	−/dim	bright	No. or % $CD16^+$	++	+++
CD57	−	+	No. or % $CD57^+$	++	++++
CD56	+	dim	No. or % $CD56^+$	++	+±
			No. or % $CD16^+57^+$	+	+++
			No. or % $CD56^+57^-$	+	±

*Young: 20–39 years; old: > 80 years.
[a] + is phenotypically brigher than dim (see text).
[b] ± is smaller in number than +, the latter being smaller in number than ++, and so on (see text).

increase in $\%CD16^+57^+$ cells with age was noted by others also, their data on NK cell function are conflicting; either a decrease or lack of a change in activity was noted [Ligthart et al., 1989; Faccini et al., 1987]. Unlike our NK phenotype-function relationship data, it would be difficult to reconcile their findings with Nagler's model of NK cell differentiation. It must be mentioned that the relationship of expression of CD16, CD56, and CD57 is highly complex and minor subsets of T-cells expressing $CD3^+16^+57^+56^-$ and $CD3^+16^-57^-56^+$ have been recorded in certain individuals [Lanier, personal communication]. Therefore, our observations can only be considered as a beginning of a search to understand the mosaic nature of NK cell immunosenescence.

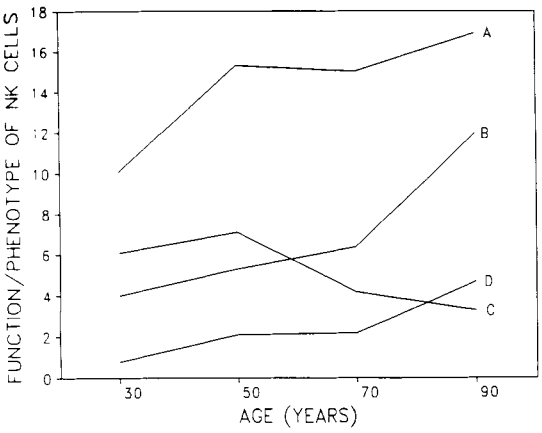

Fig. 4. Proposed model of NK cell immunosenescence. Summary of the expression of major histocompatibility complex unrestricted NK cytotoxicity of total lymphocytes and the relative frequency of expression of $CD16^+57^+$ (mature) and $CD56^+57^-$ (immature) NK phenotypes by human peripheral blood lymphocytes as a function of immune senescence, **A:** Major histocompatibility complex-unrestricted cell-mediated cytotoxicity of NK cells against K562. **B:** % Mature NK cells ($CD16^+57^+$). **C:** % Immature NK cells ($CD56^+57^-$). **D:** Ratio of mature/immature NK cells.

Although a rough correlation between the $\%CD16^+$ or $CD56^+$ or $CD57^+$ cells in unpurified peripheral blood total lymphocyte preparations and their anti-tumor cytotoxic capacity is to be expected and has been shown by some investigators, lack of a strict linear relationship is not an uncommon observation. Presence of varying proportions of NK subsets of different maturity/state of activation/differentiation and therefore, of different cytotoxic capabilities may have been a major factor responsible for such discrepancies. Various terms have been used to designate potentially different, poorly defined, perhaps overlapping stages of NK cell development (e.g., NK progenitors, NK precursors, pre-NK cells, immature NK cells, mature NK cells, resting NK cells, and activated NK cells). Most of these data were obtained by the induction of NK cell differentiation in vitro [Phillips and Lanier, 1985; Schmidt et al., 1986; Perussia et al., 1987; Nagler et al., 1989]. The study of dynamics of NK subsets during human senescence provides an additional window that could reveal the natural ratio of various NK subsets in vivo. Our approach could serve as an in vivo model of NK cell differentiation and complement the findings from in vitro models. It is devoid of artificial interferences (e.g., up- or down-regulation of markers by inductive agents during in vitro culture and alterations in the number of minor subsets due to poor survival during cell culture).

Besides providing normative reference values of NK subsets in healthy elderly, our data may contribute to the understanding of the immunological significance of NK cell differentiation in vivo under physiological conditions. The relative abundance of mature NK cells, $CD16^+57^+$, in

old age could explain a mean higher NK activity in this group. Such an immunosenescent change could be an immunobiological advantage to the elderly who may suffer from an impaired T-cell immunity during aging [Krishnaraj and Blandford, 1987; Weksler, 1982]. The relative increase in mature NK cells which function in a major histocompatibility complex-unrestricted manner may be a compensatory mechanism to overcome the age-associated partial loss of antitumor immunosurveillance capacity of cytotoxic T-cells, which operate in a major histocompatibility complex restricted manner [Bach, 1979]. In other words, when the cytotoxic T-cell generative capacity diminishes with aging, the (mature) NK cell generative capacity may be activated. Also, the nature of change (i.e., altered levels of NK subsets of certain maturity/functional capacity) may be beneficial to the elderly. Mature NK cells (besides T-cells) may be the only major immune cells that can synthesize and secrete gamma interferon [Young and Hardy, 1990], a cytokine with a variety of immunoregulatory properties. With T-cells presumably functioning at suboptimal levels in many elderly [Krishnaraj and Blandford, 1987; Weksler, 1982; Bach, 1979), mature NK cells may assume a critical, albeit non-specific role in the maintenance of certain homeostatic immune mechanisms in aged individuals.

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