

# Lymphocyte Modulation in a Baboon Model of Immunosenescence

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**The age-related modulation of lymphocyte number and function was assessed in a nonhuman primate model consisting of healthy olive baboons (*Papio cynocephalus anubis*) of ages encompassing the entire life span of this species. The objectives of this study were to characterize an animal model of immunosenescence and to assess whether or not age should be considered when designing studies for the evaluation of vaccine candidates in baboons. Specifically the following parameters were assessed in baboons from 6 months to 26 years of age: relative numbers of B lymphocytes, CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes, and T lymphocytes expressing CD28, CD25, and phytohemagglutinin-stimulated lymphoproliferative activity; and concentrations of total immunoglobulin, soluble interleukin-2 receptor  $\alpha$ , and soluble CD30 in serum. There was a statistically significant effect of age on lymphocyte numbers. As age increased, relative B-cell numbers (ranging from 6 to 50%) decreased ( $P < 0.001$ ) and relative T-cell numbers (ranging from 28 to 80%) increased ( $P < 0.001$ ). The increase in T-cell numbers involved both the CD4<sup>+</sup> and CD8<sup>+</sup> subsets. In addition, there was a significant negative correlation of age with levels of soluble interleukin-2 receptor  $\alpha$  in serum. Modulation of lymphocyte numbers appears to occur gradually during the entire baboon life span, thus suggesting the presence of an age-related developmentally regulated process. These findings indicate that baboons represent a potentially useful model to study selected phenomena related to immunosenescence. These findings also indicate that, when using the baboon model for vaccine or other experimental protocols requiring the assessment of immune responses, it would be appropriate to take into account the age of the animals in the study design.**

The immune system undergoes several functional changes during the aging process. These changes are thought to lead to an age-related increase in susceptibility to infectious diseases, autoimmune disorders, and cancer (26). Both humoral and cellular immune responses are subjected to age-related alterations, including decreased T- and B-cell responses to foreign antigens and increased responses to self-antigens (1, 26, 28, 40). Decreased responses to foreign antigens are associated with the increased morbidity and mortality from infectious diseases as well as with the low vaccine efficacy observed in individuals over 65 years of age (41). The most striking evidence of the changing T-cell compartment is provided by thymic involution, which leads to decreased production of naive T lymphocytes and therefore to limited diversity of the T-cell receptor, although thymic output is present at high levels in elderly individuals (1). The dysregulated humoral immunity is characterized by decreased production of antibodies to most foreign antigens and increased production of autoantibodies. Indeed, there is a shift in number and activity of B lymphocytes, from B2 to B1 lymphocytes (19), that produce polyreactive autoantibodies encoded by variable region genes in a germ line configuration (11). Aging is also associated with complex changes in the cytokine network (10, 18, 30, 37). To understand the mechanisms responsible for these complex changes and to evaluate strategies that can be used to manipulate the immune response in elderly individuals, it is necessary to develop ani-

mal models of human aging. Because of their genetic, anatomical, and physiological similarities to humans, and because it appears that baboon life span is under genetic control (25), these nonhuman primates have potential as models of human aging. Baboons exhibit age-related changes in autoantibody production similar to those observed in human populations (1). Most importantly, baboons are widely used as animal models for the development of several vaccines, including vaccines for infections caused by human immunodeficiency virus (22, 23, 38), *Mycobacterium tuberculosis* (32), *Hemophilus influenzae* (36), *Neisseria meningitidis* (15), group B *Streptococcus* (31), influenza virus (5), hepatitis B virus (39), and *Schistosoma mansoni* (13). Plasma biochemistry and hematology parameters for normal baboons have been established (16) and analyzed for age-related effects (17). However, there is very limited information available on the effects of age on lymphocyte populations in this nonhuman primate species. Therefore, we used healthy baboons of all ages (from the very young to the very old) to evaluate the effects of aging on immune function, with the dual objective of characterizing a baboon model of immunosenescence and assessing whether age should be considered when designing studies for the evaluation of vaccine candidates in baboons.

## MATERIALS AND METHODS

**Animals.** A total of 128 healthy olive baboons (*Papio cynocephalus anubis*) of both sexes were used in this study. A group of 85 animals (1 to 23 years of age; mean age, 123.8 months; and median age, 115.6 months), including 14 males and 71 females, was used for determination of total lymphocyte numbers, and a group of 43 animals (6 months to 26 years of age; mean age, 87.2 months; and median age, 52.7 months), including 7 males and 36 females, was used for the assessment

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TABLE 1. Effects of age on immune parameters determined by correlation analysis

Immune parameter	Result (mean $\pm$ SD)	Pearson correlation coefficient	<i>P</i> value <sup>a</sup>
Total no. of lymphocytes	11,767.06 $\pm$ 4,144.6	-0.452	0.000
% CD3 <sup>-</sup> CD20 <sup>+</sup> cells	29.21 $\pm$ 13.20	-0.861	0.000
% CD3 <sup>+</sup> CD20 <sup>-</sup> cells	56.26 $\pm$ 13.18	0.856	0.000
% CD3 <sup>+</sup> CD4 <sup>+</sup> cells	29.05 $\pm$ 7.35	0.812	0.000
% CD3 <sup>+</sup> CD8 <sup>+</sup> cells	26.05 $\pm$ 9.02	0.660	0.000
% CD3 <sup>+</sup> CD25 <sup>+</sup> cells	1.84 $\pm$ 2.49	0.024	0.879
% CD3 <sup>+</sup> CD28 <sup>+</sup> cells	40.02 $\pm$ 6.66	0.283	0.066
sIL-2R $\alpha$ concn (pg/ml)	3,320.00 $\pm$ 2,642.78	-0.561	0.000
sCD30 concn (optical density)	0.12 $\pm$ 0.14	0.010	0.578
Serum Ig level (optical density)	0.86 $\pm$ 0.07	-0.150	0.337
Proliferative activity (optical density)	0.98 $\pm$ 0.35	-0.142	0.363

<sup>a</sup> *P* < 0.01 indicates statistical significance. All *P* values are two tailed.

of all other immune parameters. Blood was collected by venipuncture under anesthesia. Animals were housed in large social groups at the Southwest Foundation for Biomedical Research, San Antonio, Tex., in accordance with federal and local guidelines.

**Determination of lymphocyte numbers.** Total lymphocyte numbers were assessed through a complete blood cell count using a Coulter MAXM hematology analyzer and a differential done from Wright Geimsa-stained blood smears. Relative lymphocyte numbers were determined by flow cytometry with a standard whole-blood staining procedure and a FACScan flow cytometer (Becton-Dickinson Immunocytometry Systems, San Jose, Calif.). A series of fluorescein isothiocyanate (FITC)- and phycoerythrin (PE)-conjugated monoclonal antibody reagents (BD) was utilized to determine the frequency of each subset. A correlated two-parameter dot plot of CD45 FITC versus CD14 PE fluorescence was used to establish the optimal light scatter gate for lymphocytes. Isotype controls were used to define the position of the negative cells and set the fluorescence markers. The cells analyzed were CD3<sup>-</sup> CD20<sup>+</sup> lymphocytes, CD3<sup>+</sup> CD20<sup>-</sup> lymphocytes, CD3<sup>+</sup> CD4<sup>+</sup> lymphocytes, CD3<sup>+</sup> CD8<sup>+</sup> lymphocytes, CD3<sup>+</sup> CD25<sup>+</sup> lymphocytes, and CD3<sup>+</sup> CD28<sup>+</sup> lymphocytes. Data were expressed as numbers of cells per cubic millimeter of blood (absolute numbers) or percent positive lymphocytes (relative numbers).

**Determination of lymphocyte proliferative activity.** Peripheral blood mononuclear cells (PBMC) were purified from heparinized whole blood by Ficoll-Hypaque gradient centrifugation. PBMC were resuspended in RPMI medium containing HEPES, glutamine, streptomycin, penicillin, and 10% human AB serum and plated in 96-well tissue culture plates. A total of 10<sup>5</sup> cells were added to each well in a volume of 150  $\mu$ l. Phytohemagglutinin (PHA) was added (5  $\mu$ g/ml) to quadruplicate wells in a total volume of 50  $\mu$ l. Plates were incubated at 37°C in 5% CO<sub>2</sub> for 72 h. Cellular proliferation was assessed by detecting 5'-bromo-2'-deoxyuridine (BrdU) incorporated into cellular DNA by using a 96-well microtiter plate cell enzyme-linked immunosorbent assay (ELISA) format (Roche Diagnostics, Indianapolis, Ind.). Briefly, following incubation with PHA, 10  $\mu$ l of BrdU was added overnight to each well of the culture plate. The culture medium was then removed. The cells were washed twice in phosphate-buffered saline (PBS) containing 10% fetal calf serum and fixed with ethanol-HCl. Each well was then incubated with a nuclease solution, washed, and incubated with horseradish peroxidase (HRP)-labeled anti-BrdU Fab fragments. Following washing, ABTS [2,2'-azino-bis(3-ethylbenzthiazolinesulfonic acid)] was added, and plates were read at 405 nm with an automated Benchmark plate reader. Data were expressed as optical density values.

**Determination of serum Ig.** Total immunoglobulin (Ig) production was assessed by ELISA. Briefly, a combination of goat antihuman IgG, IgA, and IgM antibodies (Kirkegaard and Perry Laboratories, Inc. [KPL], Gaithersburg, Md.) was used to coat microtiter wells. Baboon serum samples (diluted 1:2,000) were added to triplicate wells and incubated overnight at 4°C. Following washing, wells were incubated with the HRP-labeled goat antihuman IgG-IgA-IgM mixture (KPL) for 1 h at 37°C. The presence of reactivity was detected with ABTS. After addition of the stop solution (KPL), plates were read at 405 nm with an automated Benchmark plate reader. Data were expressed as optical density values.

**Determination of levels of sIL-2R $\alpha$  and sCD30 in serum.** Levels of soluble interleukin-2 receptor  $\alpha$  (sIL-2R $\alpha$ ) and soluble CD30 (sCD30) in serum were determined with commercially available ELISA-based kits according to the manufacturer's instructions (sIL-2R $\alpha$  from BioSource International, Inc., Camarillo, Calif.; sCD30 from DAKO Corporation, Carpinteria, Calif.). Both kits were designed for detection of human sIL-2R $\alpha$  and sCD30 and were tested for the

ability to detect the corresponding baboon molecules. Data were expressed in picograms per milliliter for sIL-2R $\alpha$  levels and in optical density values for sCD30 levels.

**Statistical analysis.** Data were analyzed by correlation and linear regression analysis, performed with SPSS version 11.5 and Excel, respectively. Statistical significance was defined as *P* < 0.01. All *P* values are two tailed.

## RESULTS

In this study, the effect of age on several immune parameters was analyzed in healthy baboons 6 months to 26 years of age in order to encompass the entire life span of this nonhuman primate species. The results of this analysis are summarized in Table 1. We used a group of 85 healthy baboons to assess whether total lymphocyte numbers decrease with increasing age. There was a statistically significant negative correlation between age and lymphocyte numbers (Pearson correlation coefficient = -0.452; *P* < 0.001). We used a different group of 43 healthy animals to ascertain whether the trend in decreasing lymphocyte numbers would differentially involve selected lymphocyte populations. The percentage of lymphocytes expressing selected cell surface markers was determined by flow cytometry with FITC- or PE-conjugated monoclonal antibodies known to cross-react with baboon antigens. The lymphocyte subsets analyzed were B cells, T cells, CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, CD28<sup>+</sup> T cells, and CD25<sup>+</sup> T cells. Figure 1 shows two-color dot-plots of lymphocyte subpopulations present in whole-blood samples collected from a representative baboon. There was a statistically significant correlation between age and B-cell numbers as well as between age and T-cell numbers. As age increased, B-cell numbers decreased (Pearson correlation coefficient = -0.816; *P* < 0.001), whereas T-cell numbers increased (Pearson correlation coefficient = 0.856; *P* < 0.001). The linear relationship between age and B- or T-cell numbers is shown in Fig. 2. The increase in relative numbers of T cells involved both the CD4<sup>+</sup> (Pearson correlation coefficient = 0.812; *P* < 0.001) and CD8<sup>+</sup> subsets (Pearson correlation coefficient = 0.660; *P* < 0.001) (Fig. 3). Although relative B-cell numbers decreased, total Ig production was not affected by age, and there was no significant correlation between relative B-cell numbers and total Ig production (data not shown). Similarly, although relative T-cell numbers increased, there was neither an effect of age on the proliferative activity of T cells stimulated with PHA nor a significant correlation between relative T-cell numbers and proliferative activity (data

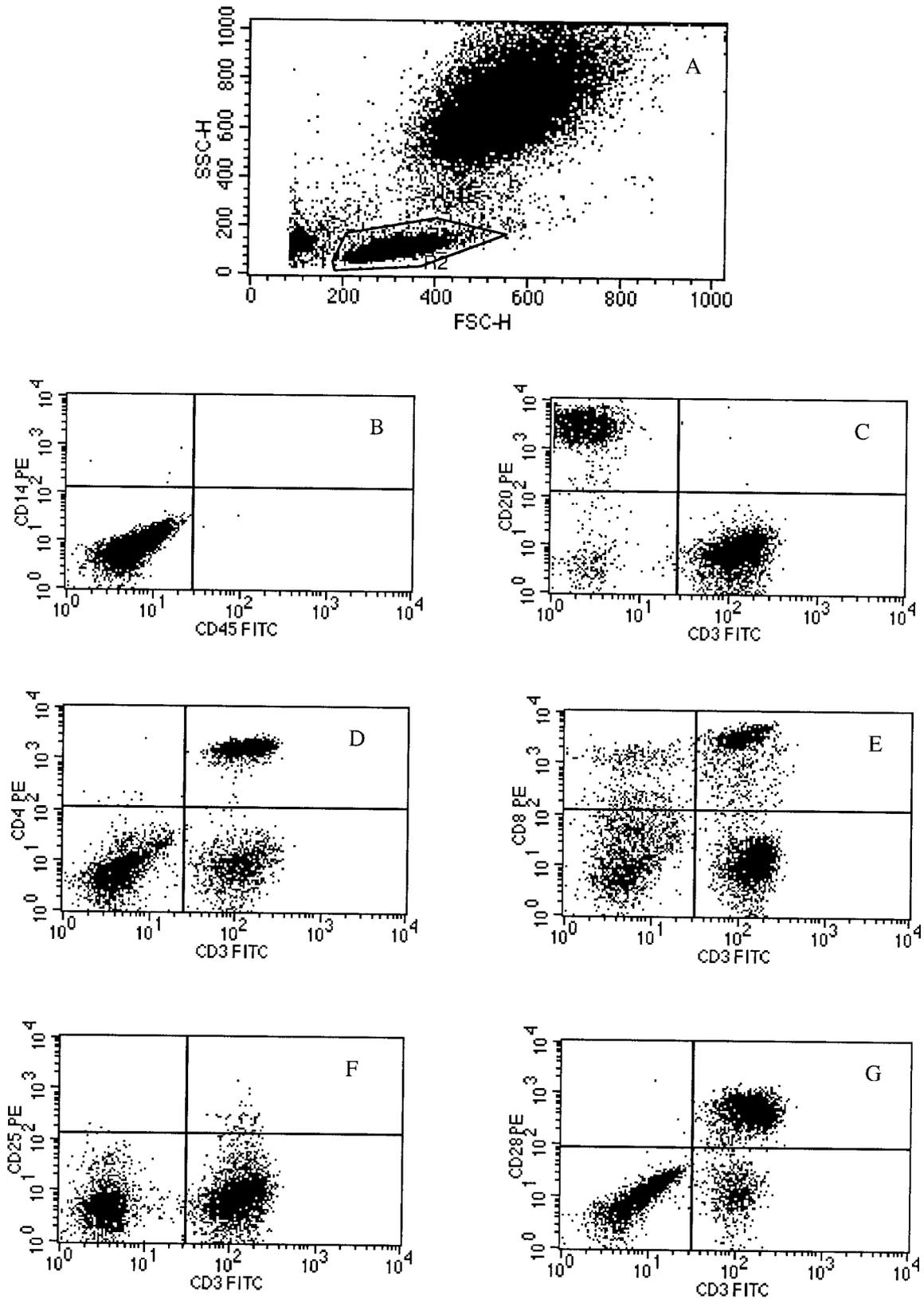


FIG. 1. Two-color dot-plots of lymphocyte subpopulations present in whole-blood samples collected from a representative baboon. (A) Forward scatter (FSC) versus side scatter (SSC). The gated lymphocyte population is circled at the bottom of the panel. (B) Isotype control. (C) B cells (32.5%) and T cells (62%). (D) CD4<sup>+</sup> T cells (38.8%). (E) CD8<sup>+</sup> T cells (22.4%). (F) CD25<sup>+</sup> T cells (2.0%). (G) CD28<sup>+</sup> T cells (47.4%).

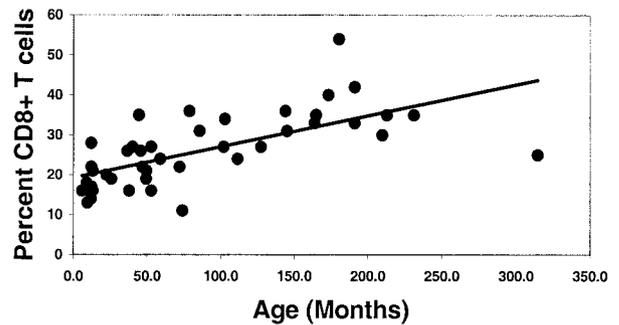
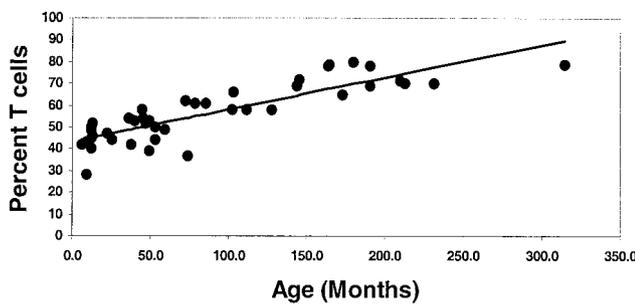
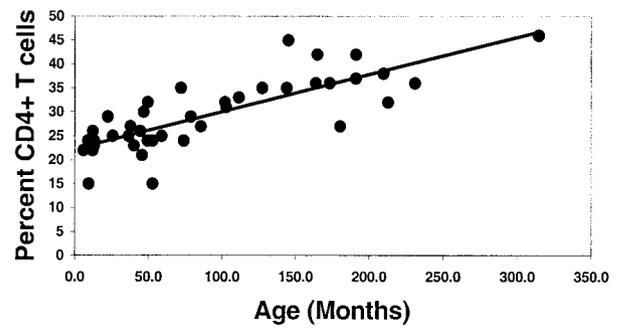
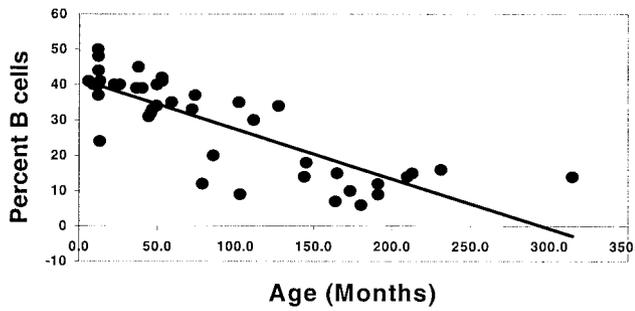


FIG. 2. Linear regression analysis showing the relationship between B-cell numbers and age and T-cell numbers and age determined by flow cytometry and expressed as percent CD3<sup>-</sup> CD20<sup>+</sup> lymphocytes and CD3<sup>+</sup> CD20<sup>-</sup> lymphocytes, respectively.

FIG. 3. Linear regression analysis showing the relationship between CD4<sup>+</sup> T-cell numbers and age and CD8<sup>+</sup> T cells and age determined by flow cytometry and expressed as percent CD3<sup>+</sup> CD4<sup>+</sup> lymphocytes and CD3<sup>+</sup> CD8<sup>+</sup> lymphocytes, respectively.

not shown). There was no effect of age on CD28<sup>+</sup> T cells or T cells expressing the activation marker CD25 (data not shown). This activation marker was expressed at minimal levels in almost all animals tested. However, there was a significant negative correlation of age with levels of IL-2R $\alpha$  in serum (Fig. 4). As age increased, levels of IL-2R $\alpha$  in serum decreased (Pearson correlation coefficient = -0.561,  $P < 0.001$ ). Levels of sCD30 in serum were minimal for all baboons. B cells, T cells, CD4<sup>+</sup> T cells, and CD8<sup>+</sup> T cells fit the linear relationship well, whereas levels of IL-2R $\alpha$  in serum seem to reach their minimum at 50 weeks.

**DISCUSSION**

It is well established that the immune system changes with increasing age. However, the nature of these changes and the corresponding underlying mechanisms are not well characterized. Therefore, it is necessary to develop well-defined animal models of human aging. Because of the similarities between baboons and humans at the genetic, anatomical, and physiological levels, these animals represent attractive models to study the immune system-related effects of the aging process. In such a model, these effects can be studied in absence of confounding factors, such as differences in diet, alcohol consumption, exercise habits, or stress levels that may hamper the interpretation of results obtained in human populations. In addition, major immunological similarities exist between humans and baboons. We have shown that baboons exhibit age-

related autoantibody production similar to that described in humans (2) and that baboon variable and constant region antibody genes are remarkably similar to their human counterparts (3, 35). Here, we report that lymphocyte populations are modulated in older baboons. There is a clear linear relationship between increasing age and relative lymphocyte numbers. Specifically, B-lymphocyte numbers decrease and T-lymphocyte numbers increase with increasing age. Because of the presence of a linear relationship found in healthy baboons living in a controlled environment, it is reasonable to assume that these are developmentally regulated alterations. Indeed, it

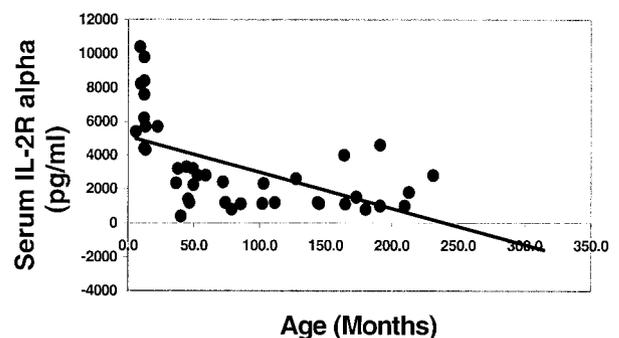


FIG. 4. Linear regression analysis showing the relationship between levels of IL-2R $\alpha$  in serum and age determined by ELISA and expressed in picograms per milliliter.

has been postulated that changes that occur progressively during the aging process may indicate the likely development of phenomena truly associated with increasing age (27). The clear decline in peripheral B-cell numbers is somewhat surprising, because it is commonly accepted that B-cell numbers are strictly regulated and do not change with age (40), although a reduced percentage of B cells was found in an elderly population when compared to a younger group (12) and an age-related effect (lower CD19<sup>+</sup> cells with increasing age) was found in adolescent males (34). Similarly, peripheral T-cell numbers are known to decrease with age (33, 43). However, relative T-cell numbers, involving both CD4<sup>+</sup> and CD8<sup>+</sup> subsets, increase in baboons.

Aging is associated with decreased T-cell activity and proliferative responses to mitogens (28, 42) as well as with complex changes in the cytokine network (10, 18, 37). The production and utilization of some cytokines decrease with age, whereas the production of other cytokines increases. Production and utilization of IL-2, the most important cytokine for T cells, decline with age (30). Results from a recent study confirm that proliferative responses to PHA stimulation are greater in young than in old individuals (7). No effects of age on PBMC proliferative responses induced by PHA could be observed in our baboon model. It could be that the inverse relationship existing between the effect of age on B- and T-cell numbers reflects a stable PHA proliferative activity because of increased T-cell numbers in the PBMC pool of older animals. Therefore, increased numbers of T lymphocytes would compensate for declined proliferative responses to PHA. However, it should be pointed out that levels of the IL-2R $\alpha$  in serum sharply decline with increasing age in our baboon model, to reach a minimum at about 5 years of age, whereas expression of its cell surface form (CD25) remains at very low levels in almost all baboons tested. The interaction of IL-2 with its corresponding cell surface receptor is necessary for T-cell activation and therefore development of cellular immune responses (4). Resting T cells express low levels of IL-2R $\alpha$ . Once activated, the expression of this receptor rapidly increases. The IL-2R $\alpha$  shed from the cell surface can be detected in serum, representing a marker for T-cell activation (29). Therefore, the finding of a sharp age-related decline in levels of IL-2R $\alpha$  in serum in conjunction with very low, stable levels of cell surface expression of CD25 indicates that T-cell function is regulated through different mechanisms at different ages in baboons. Because of the decline of IL-2R $\alpha$  in serum, we tested all animals for an increase in levels of sCD30 in serum: sCD30 is a marker indicating the anti-inflammatory activity of Th0/Th2 lymphocytes (20) and is known to be present at higher levels in healthy centenarians (14). There was no effect of age on sCD30 levels, thus suggesting lack of a major increase in Th0/Th2 activity in older baboons. However, it should be taken in account that the baboon population used in this study does not represent a model for studying immune responses in centenarians.

The progressive decline of CD28 expression on peripheral T cells is considered a consistent feature of the aging process (9). The CD28 molecule is constitutively expressed on T lymphocytes and, following interaction with B7, mediates a signal necessary for the induction and maintenance of T-cell responses (21). T cells that reach senescence in culture do not express CD28 (8). In humans, the proportion of CD28<sup>+</sup> T cells

significantly decreases from 87% in young adults to 64% in centenarians (6). Based on our results, it appears that the relative number of CD3<sup>+</sup> CD28<sup>+</sup> cells is lower in baboons (24 to 54%) than in humans. Indeed, results from a recent study show that normal baboons exhibit a lower mean percent expression of CD28 on CD8<sup>+</sup> lymphocytes than that in humans (24). There is no statistically significant correlation between age and CD3<sup>+</sup> CD28<sup>+</sup> lymphocyte numbers in baboons. Although it is possible that CD28 expression is regulated differently in baboons and humans, additional studies are necessary to address this issue.

In conclusion, results from this study indicate that several immune parameters change with increasing age in the baboon model. Modulation of lymphocyte numbers occurs gradually during the entire baboon life span, thus suggesting the presence of an age-related developmentally regulated process. These findings indicate that baboons represent a potentially useful model to study selected phenomena related to immunosenescence. In addition, these findings indicate that it is necessary to consider animal age when designing studies that require assessment of immune responses in these nonhuman primates.

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