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Detection of Elevated Antibody against Calreticulin by ELISA in Aged Cynomolgus Monkey Plasma

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Calreticulin (Crt) is a molecular chaperone ubiquitously present in the endoplasmic reticulum. In non-human primates, age-related occurrence of anti-Crt antibody has not been reported. We developed an ELISA assay for an anti-Crt antibody and determined the age-related increase in the levels of anti-Crt antibody in three groups of cynomolgus monkeys: juvenile (1.5 yr), young adults (5–10 yr) and aged adults (20–34 yr). Mean ± SD auto-antibody levels at 450 nm in juvenile, young adults and aged groups were 0.23 ± 0.18, 0.30 ± 0.28, and 0.55 ± 0.33, respectively. Statistically significant differences were noted in the autoantibody levels to Crt among the aged group and juvenile or young adults. This is the first report to demonstrate the expression of anti-Crt autoantibody in aged monkeys and indicates that cynomologous monkeys may serve as an appropriate nonhuman primate model for studies of age-related alteration of immune function in elderly humans. Though preliminary, this finding merits further investigation to determine the relationship between immunosenescence and expression of antibodies to Crt.

Key words: aging, autoantibody, calreticulin, *Macaca fascicularis*, suppressive/regulatory T cells

INTRODUCTION

Calreticulin (Crt) is a molecular chaperone protein, ubiquitously present in the endoplasmic reticulum, which assists in the correct folding of N-glycosylated proteins, and plays roles in Ca^{2+} storage and signaling, and protein processing (Johnson et al., 2001; Michalak et al., 2009). Crt has also been found to be associated with Ro/SSA and La/SSB in a ribonucleoprotein complex (Eggleton and Llewellyn, 1999). These three proteins are well characterized. The human 46-kDa Crt gene is located on chromosome 19 (Lieu et al., 1988; McCauliffe et al., 1990), whereas the human genes encoding the 60- and 52- kDa SSA/Ro and the human 48-kDa SSB/La are located on chromosome 1 (1q31) and chromosome 2, respectively (Chambers et al., 1988; Chan et al., 1994). The function of Crt on the cell surface is not well understood. In human fibroblasts, surface Crt appears to function as a receptor for fibrinogen and is essential for its mitogenic effect (Gray et al., 1995). Surface Crt may also be a receptor for specific ligands, via its ability to bind other proteins or by binding to the extracellular matrix or other glycoproteins via its lectin site (Xiao et al., 1999). It has also been shown to be very similar in sequence to the cC1q-receptor (cC1qR/Crt), the cell surface receptor that binds the collagenous domain of the first component of complement, C1q (Malhotra et al., 1993; van den Berg et al., 1998). C1q has been shown to bind Crt directly, and this binding may interfere with the binding of Crt autoantibodies to circulating Crt autoantigen (Eggleton et al., 1997).

Age-related changes in T lymphocyte functions cause various alterations in immune response in aged individuals. Decline of T cell-mediated regulatory function compromises the control of central tolerance, resulting in immune responses to self-antigen(s), including the generation of auto-antibodies. The development of various kinds of autoantibodies has been reported in human patients with several disorders (Siegert et al., 1991) and in the elderly (Nilsson et al., 2006). In humans, autoantibodies against Crt have been detected in the sera from patients with systemic lupus erythematosus (SLE) (Kishore et al., 1997; van den Berg et al., 1998), rheumatoid arthritis (RA) (Verreck et al., 1995), lupus disorders (Sontheimer et al., 1982), and Sjogren's Syndrome (Martinez-Lavin et al., 1979). Autoimmune disorders are also affected by genetic predisposition or exposure to agents that induce cross-reactive antibodies (to self), and often occur independent of the aging process. However, in non-human primates, age-related occurrence of an anti-Crt antibody has not been reported.

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We hypothesized that autoantibodies increase with aging in cynomolgus monkey due to thymus atrophy. Central tolerance occurs during lymphocyte development and operates in the thymus. Here, T lymphocytes that recognize self-antigens are deleted before they develop into fully immunocompetent cells, preventing autoimmunity, leading us to speculate that many immunological disorders, especially autoimmune diseases, are evoked by thymus atrophy.

To test the hypothesis that autoantibodies increase with age, we developed an ELISA assay for detection of autoantibody against Crt (Higashino et al., 2008). The objective of this study was to determine age-related increase in frequency and levels of anti-Crt antibody in cynomolgus monkeys aged 1.5 to 34 years. In addition, recent studies demonstrated that a forkhead transcription factor, FoxP3, is specifically expressed in most CD4+CD25+ T cells and in a small part of CD4+CD25– T cells and is required for the development of suppressive/regulatory T cells. We also investigated suppressive/regulatory T cells profiling, CD4+CD25+FoxP3+ T cells, in young adult and aged PBMC.

MATERIALS AND METHODS

Animals and materials

Cynomolgus monkeys (*Macaca fascicularis*), used in this study were bred and reared at the Tsukuba Primate Research Center (TPRC), National Institute of Biomedical Innovation (NIBIO), Japan. A cumulative total of 93 monkeys (27 males and 66 females; age range 1.5–34 years) were used; 10 juvenile (1.5 years), 20 young adults (5 to 10 years), and 63 aged (20 to 35 years).

The B-PER bacterial protein extraction reagent was purchased from Pierce, Rockford, IL. Glutathione sepharose 4B and thrombin protease were from Amersham Biosciences KK, Tokyo, Japan; Protease inhibitor cocktail set III was from Calbiochem, Darmstadt, Germany; p-aminobenzamidine-agarose beads were purchased from Sigma-Aldrich Japan, Tokyo, Japan; Block Ace was purchased from DS Pharma Biomedical Co., Ltd., Osaka, Japan; HRP-conjugated anti-monkey IgG goat antibody from Cappel, West Chester, PA; 3, 3′, 5, 5′-tetramethylbenzidine (TMB) for ELISA and Western blotting were from Kirkegaard and Perry Laboratories, Gaithersburg, MD. All other chemicals were of reagent grade.

Blood Samples

Blood Samples were obtained by venipuncture in the presence of heparin. Sampled blood was centrifuged at 2000 × g at 4°C for 30 min, and separated plasma was stored at –80°C until use.

Animal care and use guidelines

These procedures were executed in accordance with "The Guide for the Care and Use of Laboratory Animals (1985)" established by the National Institutes of Health, and compliant with national standards, such as those of the United States Public Health Service Policy on the Humane Care and Use of Laboratory Animals (Public Health Service Policy on Humane Care and Use of Laboratory Animals, Office of Laboratory Animals, National Institutes of Health, RKL. 1, Suite 1050, MSC 7982, 6705 Rockledge Drive, Bethesda, MD 20892-7982 USA); the Guide for the Care and Use of Laboratory Animals (National Research Council, National Academy Press, Washington D.C., 1996, or subsequent revisions), and the Animal Welfare Act and subsequent amendments.

ELISA analysis of auto-antibodies against Crt in cynomolgus monkeys

Ninety-six well plates (Greiner Japan, Tokyo, Japan) were coated with 100 μl of the coating 500 ng/well Crt antigen (Higashino et al., 2008) in phosphate buffered saline (PBS) and incubated overnight at 4°C. After aspiration of excess antigen and five washes with phosphate buffered saline containing 0.02% Tween 20 (PBST), 250 μl of Block Ace was added to each well. The plates were incubated overnight at 4°C. After the blocking buffer was aspirated, 100 μl of plasma samples 1000-fold diluted in Block Ace were added in each well, and the plates were incubated overnight at 4°C. The plates were then washed five times with PBST, followed by incubation with 100 μl of the HRP-conjugated anti-monkey IgG goat antibody diluted 10000-fold with Block Ace at room temperature for 2 h. After washing five times with PBST, the reaction was developed for 25 min with 100 μl of TMB at 37°C and stopped with 50 μl of 3 M H_2SO_4. Absorbance at 450 nm was measured with a Molecular Devices microplate reader (Wako, Tokyo, Japan).

Sandwich ELISA for the detection of circulating calreticulin in plasma

The calreticulin concentration in plasma was determinated by the method described by Higashino et al. (Higashino et al., 2008), using recombinant monkey calreticulin protein as a standard.

Western blotting protocol

Recombinant monkey Crt protein was separated by SDS-PAGE following Laemmli (Laemmli, 1970). After electrophoresis, Crt protein was electrophoretically transferred onto a PVDF membrane in 25 mM Tris-HCl buffer, pH 8.3, containing 20% methanol, with a semi-dry blotting apparatus. The membrane was next pretreated with SuperBlock-Dry-Blend blocking buffer, and then incubated with the anti-Crt antibody in aged monkey plasma (1:100) in Can-Get-Signal solution I for 2 h at 37°C. The membrane was extensively washed in Tris buffered saline containing 0.02% Tween 20 (TBST), and the bound antibody was labeled with HRP-conjugated anti-monkey IgG goat antibody (1:1000) in Can-Get-Signal solution II for 1 h at room temperature. After extensively washed in TBST and Tris buffered saline (TBS), the membrane was visualized with TMB for membrane.

Flow cytometry analysis

For staining with $FoxP3^+$ $CD4^+$ CD25 specific mouse monoclonal IgG, cells were suspended in 10% FBS RPMI 1640 for 30 min on ice, washed and fixed twice by FACS media, and refixed and resuspension in 2% para-formaldehyde. The cells were analyzed using a FACSCalibur flowcytometer (BD Biosciences).

Protein determination

Protein was determined by measuring absorbance at 280 nm, using BSA as a standard.

Statistical analysis

To determine the statistical significance of differences in autoantibody levels during development, the linear regression test was performed.

RESULTS

Levels of autoantibodies against Crt in plasma

We performed ELISAs with plasma from 93 (27 male, 66 female) cynomolgus monkeys, aged 1.5 to 34 years. The anti-Crt autoantibody absorbance at 450 nm in plasma samples were compared in a cumulative total of 93 plasma samples collected from three different age groups (Juvenile, Young Adult, and Aged). The mean ± SD antibody levels (absorbance at 450 nm) in juvenile (n = 10), young adult (n = 20) and aged (n = 63) groups were 0.23 ± 0.18, 0.30 ± 0.28, and 0.55 ± 0.33, respectively (Fig. 1). Statistically significant differences (Mann-Whitney *U*-test, $P < 0.01$ level) between the aged group and juvenile or young adult groups were noted.

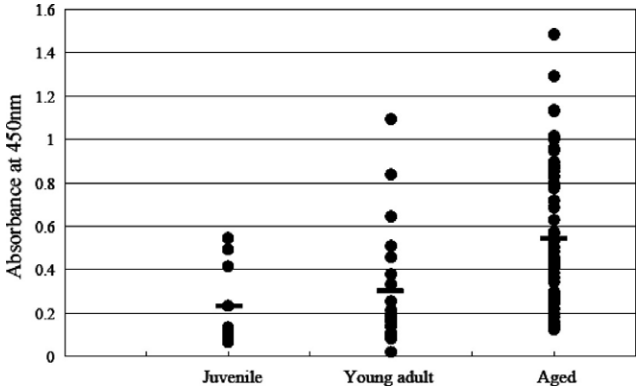

Fig. 1. Anti-Crt autoantibody absorbance at 450 nm in plasma samples derived from three different age groups; juvenile, young adult, and aged cynomolgus monkeys. Recombinant monkey Crt protein was coated and incubated with monkey plasma. Subsequently, bound IgG was measured by incubation with an anti-monkey IgG polyclonal antibody conjugated to HRP. Absorbance at 450 nm was measured and plotted. Each bar shows mean ± SD autoantibody levels at 450 nm in juvenile, young adult, and aged groups at 0.23 ± 0.18, 0.30 ± 0.28, and 0.55 ± 0.33, respectively.

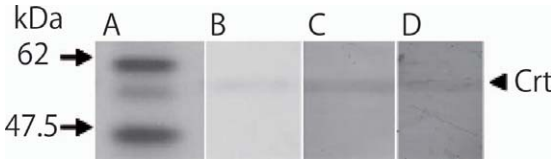

Fig. 2. Immunoblot analysis of aged cynomolgus monkey plasma reactivity with monkey recombinant calreticulin (Crt) run under reducing conditions on a 10% SDS-PAGE gel and transferred to PVDF membrane. Anti-monkey IgG-peroxidase antibody was used to detect binding of anti-Crt antibodies to calreticulin, as described in Materials and Methods. Lane A, molecular-mass standards; Lane B, recombinant Crt (3 μg). These two lanes are derived from a gel stained with Coomassie brilliant blue. Lane C, recombinant Crt transferred to a PVDF membrane was immuno-stained with aged monkey plasma and anti-monkey IgG-peroxidase antibody. Lane D, recombinant Crt transferred to a PVDF membrane was immunostained with young monkey plasma and anti-monkey IgG-peroxidase antibody. Arrowhead shows the position of Crt.

Western blotting for detection of autoantibodies against Crt

For the Western blotting analysis, the recombinant monkey Crt protein was separated by SDS-PAGE. The confirmation of anti-Crt antibody to Crt recombinant protein interactions was achieved when the young adult and aged monkey plasma shown by ELISA to be positive for autoantibody against Crt was tested for reactivity with the recombinant 55-kDa Crt protein band by Western blotting. As shown in Fig. 2, a single band was observed from the Western blotting reaction with monkey anti-Crt autoantibody, demonstrating the presence of anti-Crt autoantibody in young adult and aged monkey plasma.

The determination of Crt by ELISA in plasma

As shown in Fig. 3, the Crt levels (mean ± SEM) in plasma of juvenile, young adults, and aged cynomolgus monkeys corresponded to 66.34 ± 19.68 (n = 10), 131.35 ± 43.31 (n = 20), and 94.85 ± 19.27 (n = 63) ng/ml, respectively. There was no statistically significant difference among the juvenile, young adult, and aged groups.

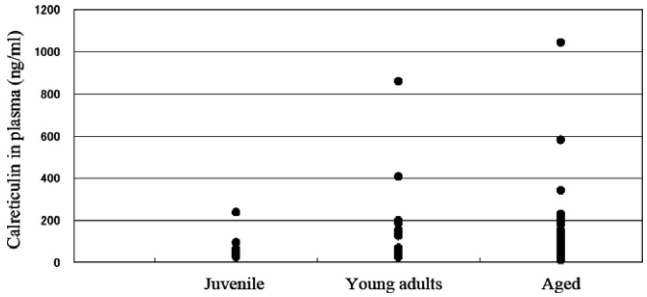

Fig. 3. Calreticulin (Crt) levels in plasma samples collected from three different age groups; juvenile, young adult, and aged cynomolgus monkeys. There is no statistically significant difference among the three age groups.

Table 1. The profile of suppressive/regulatory T cells in the PBMCs of young adult and aged cynomolgus monkeys. This table indicates mean percentages derived from flow cytometry analysis. [a]5 yr, n = 10; [b]21 yr, n = 10

Suppressive/regulatory T cell (%)	Peripheral blood mononuclear cells	
	Young adult monkey[a]	Aged monkey[b]
Fox P3	0.41 (stdev 0.16)	0.25 (stdev 0.15)
Fox P3/CD 25	1.76 (stdev 0.69)	0.78 (stdev 0.33)
Total FoxP3	2.17 (stdev 0.69)	1.03 (stdev 0.39)
CD25/CD4	3.14 (stdev 1.55)	1.51 (stdev 0.75)
FoxP3/CD25/CD4	59.15 (stdev 15.15)	54.18 (stdev 17.25)

Profiling of suppressive/regulatory T cells in young adult and aged monkey peripheral blood mononuclear cells (PBMC)

Recent studies have demonstrated that a forkhead transcription factor, FoxP3, is specially expressed in most CD4+CD25+ T cells and in a small part of CD4+CD25– T cells, and is required for the development of suppressive/regulatory T cells. We also investigated CD4+CD25+FoxP3+ T cells in the PBMCs of young adult monkeys (n = 10) and aged monkeys (n = 10). CD4+CD25+ T cells were higher in the PBMCs of young adult monkeys than in those of aged monkeys. But no apparent difference was seen in the FoxP3+CD4+CD25+ T cells between PMBCs of young adult and aged monkeys (Table 1).

DISCUSSION

In this study, we demonstrate that anti-calreticulin (Crt) autoantibody is present in aged cynomolgus monkeys. Statistically significant differences in Crt autoantibody titer between aged group and juvenile or young adults was noted. However, the Crt levels in plasma showed no significant differences in juvenile, young adult, and aged cynomolgus monkeys. From our Western blot analysis, we infer that the reactivity of the older animal's serum to Crt was nearly the same as the younger animal's serum. The value seems higher than that deduced from the amino acid composition,

which is 46 kDa. To date, the mobility of Crt in SDS-PAGE has been shown to be retarded, resulting in a high-molecular mass. Crt migrates with an apparent molecular mass of 60–63 kDa in SDS-PAGE at pH 8.8 when using the Laemmli system (Milner et al., 1991; Waisman et al., 1985), and shows 55 kDa in SDS-PAGE at neutral pH (Michalak et al., 1980; Ostwald and MacLennan, 1974). A value of 55 kDa was also obtained by sedimentation equilibrium at neutral pH (Waisman et al., 1985). An increased antibody absorbance at 450 nm against calreticulin in aged monkey plasma is presumably attributable to senescence.

The very weak correlation between groups may be attributable to the genetic non-homogeneity of individuals in different groups. Unlike in rat and mouse models where inbred individuals (for 20 generations) are used in experiments; such inbred individuals are not available for monkey species, meaning that the use of siblings and parents is the best available alternative. A better correlation might therefore be obtained if siblings and parents had been used in the present protocol.

From our results, we can infer that the aged monkey cohort may suffer from immunological disorders secondary to thymus shrinkage. The development of anti-Crt autoantibody in the aged monkey cohort may be suggestive of risk of autoimmune disease and infection in aged individuals as the multifunctional role of Crt has been implicated in infectious diseases and certain autoimmune diseases, such as systemic lupus erythematosus (SLE) (Kishore et al., 1997; van den Berg et al., 1998), rheumatoid arthritis (RA) (Verreck et al., 1995), lupus disorders (Sontheimer et al., 1982), and Sjogren's Syndrome (Martinez-Lavin et al., 1979).

During programmed cell death or apoptosis, nucleosomes containing DNA and histones, as well as endoplasmic reticulum (ER) vesicles containing calreticulin, and the Ro and La proteins, bleb off from the surface of dying cells (Casciola-Rosen et al., 1994; Eggleton et al., 1997), which are then targeted for disruption and release of their components. In addition, there is evidence that autoantibodies may directly penetrate cells and target specific host cell proteins (Alarcon-Segovia et al., 1996). However, inflammation, cell necrosis, apoptosis, and perhaps even penetration of antibodies into cells, are all normal physiological processes, which alone cannot explain autoantibody production in SLE and other autoimmune disorders.

The breakdown of immunological tolerance likely requires the contribution of additional genetic or inflammatory mediators. Moreover, the immune features that accompany the characteristic flares of SLE strongly suggest that the autoimmune response is T-cell dependent and driven by self-antigen, which has prompted the search for potential initiating process(es) that induce the release of self-antigens in a form leading to a breakdown in T-cell tolerance. The reason why Crt is targeted for immune tolerance breakdown remains unclear (Eggleton and Llewellyn, 1999). The profiles of suppressive/regulatory T cells in young adult monkeys and aged monkeys suggest a quantitative difference. Regarding the relationship between the thymus and aging, dramatic age-related changes in the thymic microenvironment, referred to as thymic involution, have long been considered as a reasonable cause for the age-related decline in the CD4+CD25+ T cell population.

Autoantibodies against cell surface cC1qR/Crt may lead to direct activation of the cells (Eggleton et al., 1997). It is not entirely clear how cC1qR/Crt is involved in signal transduction. It may be that protein kinase C (PKC), which has key regulatory roles in a wide spectrum of signal transduction pathways, interacts with Crt *in vivo* (Rendon-Huerta et al., 1999); these two proteins may operate in common signaling pathways. There are similarities in both function and structure between Crt and receptors for activated C-kinase (RACKs) (Rendon-Huerta et al., 1999; Seddiki et al., 2001). Crt has also been shown to be associated with the immune response in several ways (Eggleton et al., 1997; Kishore et al., 1997). It may be a target for circulating autoantibodies, and may contribute to the autoimmune process (Eggleton et al., 1997; Michalak et al., 1992; Treves et al., 1998).

In conclusion, we provide evidence in this study that elevated concentrations of anti-Crt autoantibody levels can be detected in aging monkey plasma, and provide evidence that cynomolgus monkey can serve as a valid non-human primate model for the study of age-related alterations in immune function. To our knowledge, this is the first report to demonstrate the development of anti-Crt autoantibody in aged monkeys. Further studies on the functions of autoantibody on immuno-homeostasis in aged cynomolgus monkeys are in progress.

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