

## Immunological abnormalities of aging: an analysis of T lymphocyte subpopulations of Werner's syndrome.

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**Research Article**

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# Immunological Abnormalities of Aging

## AN ANALYSIS OF T LYMPHOCYTE SUBPOPULATIONS OF WERNER'S SYNDROME

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**ABSTRACT** Two T lymphocyte-specific antisera, i.e. naturally-occurring auto-antibody to T cells of systemic lupus erythematosus patients (natural T cell toxic autoantibody) and heterologous antiserum against human brain tissue (antibrain-associated T-cell antigen), were used to detect cell surface antigens of human peripheral T lymphocytes.

Nylon column-purified T cells from normal aged individuals and patients with Werner's syndrome (a premature aging syndrome) were reacted with these auto- and heterologous antibodies followed by staining with appropriate fluorescence reagents. The cells were subjected to the automated analysis with fluorescence-activated cell sorter. Fluorescence profiles to T cells of both aged individuals of over 90 yr and Werner's syndrome showed a very similar pattern, with a drastic decrease in the population that had high fluorescence intensity stained with either antiserum accompanied by the relative increase in the cell population that had low fluorescence intensity. Natural T cell toxic autoantibody comparable to that detected in systemic lupus erythematosus patients was found in the serum of six out of seven patients with Werner's syndrome, whereas normal aged individuals produced no such an autoantibody. The results suggest that Werner's syndrome has a change in the lymphocyte population very similar to old individuals, and that such a change is caused by the production of autoantibodies reactive to T lymphocytes.

## INTRODUCTION

Werner's syndrome (WS)<sup>1</sup> is characterized by a variety of manifestations associated with normal aging, and thus is recognized as a clinical example of accelerated aging (1). Briefly, this syndrome is a rare, heredofamilial disorder inherited as an autosomal recessive trait. After a reasonably normal infancy and childhood, patients manifest failures of the adolescent growth spurt, teenaged graying of hair; and cataract as well as other signs of senescence with death usually in the 40's. Atherosclerosis is seen in 25% of subjects, diabetes mellitus in about 50%, hypogonadism in 80%, and malignant tumors in 10% (2, 3).

There have been found a striking shortening of the life-span and the slow elongation rate of the DNA chains of cultured fibroblasts (4, 5), the increased proportion of heat labile enzymes (6, 7), as well as the excessive excretion of urinary hyaluronic acid (8, 9), all of which are probably related to the pathogenesis of this peculiar disorder. No overt immunological abnormalities have so far been reported in WS except a slight decrease in the proportion of T cells in the peripheral blood (3).

Recently, much attention has been focussed on the immunological abnormalities associated with normal aging, which perhaps are related to the autoimmunity,

<sup>1</sup> *Abbreviations used in this paper:* ANA, antinuclear antibodies; BAT, brain-associated T-cell antigen; FACS, fluorescence-activated cell sorter; FcR<sup>+</sup>, Fc receptor-bearing; NTA, the antigen reactive to human naturally occurring auto-antibody against T cells; SLE, systemic lupus erythematosus; WS, Werner's syndrome.

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decreased resistance against infectious agents, and neoplastic proliferations (10). Because WS has several similarities to normal aging, we have attempted to investigate immune abnormalities in WS patients. This communication describes the result of analysis of peripheral T lymphocytes from WS patients by fluorescence-activated cell sorter (FACS) with antibodies against two surface markers of T-cell subpopulations, i.e. the antigen reactive to human naturally occurring autoantibody against T cells (NTA) and brain-associated T-cell antigen (BAT) (11, 12). Striking differences in the analytical patterns of T cells of WS from those of age-matched controls and the similarity to those of aged individuals suggested the presence of abnormality in the immune system of WS.

## METHODS

**Patients.** Seven patients (six males and one female) with untreated WS were examined. The age of the patients ranged from 17 to 59 yr. The criteria for the diagnosis of WS have previously been described (3). Sera and lymphocytes from apparently healthy individuals of different age groups (six in the 20's, five in the 30's, three in the 40's, six in the 50's, two in the 60's, two in the 80's, and four donors in the 90's) were used as controls. Care was taken to select the donors free from diseases and drugs known to affect lymphocytes.

**Lymphocyte preparation.** Peripheral blood lymphocytes were purified from whole heparinized blood by centrifugation over a Ficoll-Hypaque gradient (Ficoll; Pharmacia Fine Chemicals, Inc., Piscataway, N. J., Hypaque; Winthrop Laboratories, New York,) followed by passage through a nylon wool column to deplete B cells (13). The recovery of lymphocytes after the nylon wool column procedure was usually between 40 and 60% of applied total cells. Contamination of surface immunoglobulin (Ig)-positive cells (B cells) as detected by staining with fluoresceinated rabbit anti-human Ig by the method described by Blakeslee and Baines (14) was <2%.

**Antisera and fluorescence staining of peripheral lymphocytes.** Rabbit anti-BAT was obtained by the following immunization schedule: Rabbits were immunized twice with about 1 g wet wt of the human brain homogenate in Freund's complete adjuvant at a 2-wk interval. The sera were collected 10 d after the second immunization, and the pooled serum was absorbed extensively with human erythrocytes until no hemagglutination was detected. It was further absorbed with human bone marrow cells and B cell type chronic lymphocytic leukemia cells to remove anti-B cell staining activity. The specificity of this anti-BAT was further tested by both cell membrane staining and cytotoxic assay described by Terasaki and MacClelland (15).

The antiserum did not stain B-cell lymphoma cell lines such as Raji cells or other several Epstein-Barr virus transformed B cell lines. This anti-BAT eliminated about 80% of peripheral blood lymphocytes at the dilution of 1:10 ( $1 \times 10^6/0.1$  ml), with rabbit complement leaving >95% of Ig-positive cells. Concanavalin A responsiveness of peripheral blood lymphocytes was also completely abrogated after the cytotoxic treatment at the same dilution of the anti-BAT.

For the routine FACS analysis of peripheral blood lymphocytes from both normal individuals and patients, the nylon wool column-purified lymphocytes ( $1 \times 10^6/0.1$  ml) were reacted with anti-BAT at 4°C for 30 min. Normal rabbit serum or rabbit antithymocytes antiserum absorbed with

human peripheral blood lymphocytes was used as a negative control staining. After washing three times, the cells were stained with fluoresceinated goat antirabbit Ig (fluorescence:protein ratio, 2.5) at 4°C for 30 min.

A serum-containing antibody, which was capable of killing normal human T cells (NTA), was collected from a systemic lupus erythematosus (SLE) patient. The NTA used in this study for the staining was provided from Dr. T. Shirai (Department of Pathology, University of Kyoto, Japan) and was exclusively IgM antibody. The nylon wool column-purified lymphocytes ( $1 \times 10^6/0.1$  ml) were reacted with NTA at 4°C for 30 min, washed twice with Hanks' solution (Grand Island Biological Co., Grand Island, N. Y.), and were then stained with fluoresceinated rabbit anti-human Ig (fluorescence:protein ratio, 2) at 4°C for 30 min. Pooled serum from several individuals was used as a negative control staining. All the antisera used for the staining were centrifuged (15,000 rpm for 1 h) before use to delete the nonspecific binding of aggregated antibodies to Fc receptor of lymphocytes.

**Fluoresceinated immune complex and the staining of lymphocytes with fluoresceinated reagents.** The staining of Fc receptor-bearing (FcR<sup>+</sup>) T cells was performed by reacting the T cells with fluoresceinated rabbit 7S antibody serum albumin (Sigma Chemical Co., St. Louis, Mo.), complexed with bovine serum albumin at equivalence by the method described by Blakeslee and Baines (14).

**Detection of NTA in WS patients.** The nylon wool column-purified lymphocytes ( $1 \times 10^6/0.1$  ml) from WS patients as well as normal aged individuals were incubated with the autologous serum at 4°C for 30 min to detect NTA as described above. The cells were then stained with fluorescein isothiocyanate (Research Organics, Inc., Cleveland, Ohio) rabbit anti-human Ig and analyzed by the FACS (FACS II; Beckton Dickinson FACS Systems, Mountain View, Calif.). 28 serum samples from healthy donors were used as controls. The cytotoxicity test according to the method described by Koike et al. (16) was also performed to confirm the autoantibody in the serum from WS patients.

**Analysis by the FACS.** Analysis of fluorescence profile of stained cells was performed according to the method described by Herzenberg et al. (17, 18). Normal lymphocytes stained with the same reagents were analyzed under the identical condition to make comparisons. The analytical pattern was recorded by direct photography of the oscilloscope. To express fluorescence distribution of lymphocytes, control lymphocytes from normal individuals stained with the reagents were run in the FACS with a suitable gain, which gave a pattern of continuous fluorescence distribution from very dull to bright on the oscilloscope (Fig. 3). A point of fluorescence intensity at which the whole area was divided in half was set, and the analysis of samples was performed under the identical condition. The percentages of bright and dull population were calculated by measuring the areas divided by this point.

**Other immunological examining.** Detection of antibodies to double-stranded DNA and antinuclear antibodies (ANA) was carried out by the methods described by Weitzman and Walker (19) and Casales et al. (20). The number of Ig-bearing cells (B cells) was calculated by gating at the fixed point by the FACS analyzer (Table I).

## RESULTS

**Analysis of lymphocytes from WS stained with anti-BAT on the FACS.** Nylon wool column-purified T cells from WS and healthy individuals (20–96 yr olds) were stained with anti-BAT and applied to the analysis

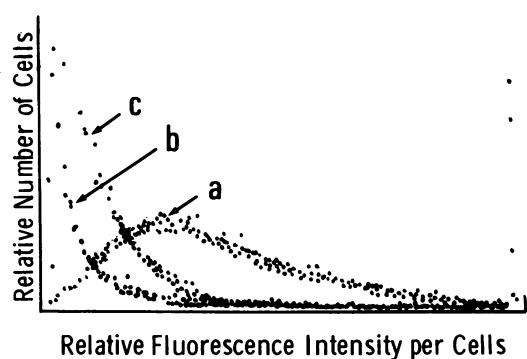


FIGURE 1 Fluorescence distribution of peripheral blood T cells labeled with anti-BAT antisera. Nylon-purified T cells (a-c) were incubated for 30 min at 4°C with anti-BAT, washed, and incubated for an additional 30 min 4°C with fluoresceinated goat antirabbit Ig. The cells were then washed and analyzed with the FACS. Distributions are based on cumulative analysis of 10,000 viable cells. (a) Control lymphocytes from normal young donors (20-36 yr olds). (b) Lymphocytes from a WS patient (T.S.). (c) Lymphocytes from healthy elderly individuals (90-96 yr olds).

by the FACS. More than 95% of nylon-purified T cells reacted with anti-BAT as analyzed by the FACS. The analytical profile of control lymphocytes from normal young donors (20-36 yr olds) distributed heterogeneously from bright to dull (Fig. 1a). If the lymphocytes from WS were analyzed under the same condition,

the fluorescence profile demonstrated a drastic decrease in the brightly stained population accompanied by a relative increase in the dullly stained cell population (Fig. 1b:T.S.).

The ratio of the brightly stained population to the total analyzed cells was calculated and presented in Table I. As the age of WS patients ranged from 17 to 59 yr, the mean value obtained from lymphocytes of healthy individuals (20-80 yr olds) was used as a control. In this age group, normal individuals showed almost the same fluorescence distribution, which was divided into 1:1 at an identical point (SD <10%). When the lymphocytes from four cases of healthy elderly individuals (90-96 yr olds) were examined, a similar profile to that of WS was observed (Fig. 1c).

*Analysis of lymphocytes stained with NTA on the FACS.* The same preparations of lymphocytes were stained with NTA. The analytical profiles on the FACS are shown in Fig. 2. Five out of seven patients (Fig. 2b: A.N.) and all aged individuals (Fig. 2c) showed a decrease in the brightly stained cell population as compared to the normal young lymphocytes (Fig. 2a). Two other patients (J.T. and C.N.), however, showed no significant change, even though these patients exhibited a significant decrease in BAT reactive cells (see above).

*The number of FcR<sup>+</sup>T cells.* The percentage of FcR<sup>+</sup>T cells in the nylon-purified T-cell population

TABLE I  
Immunological Analysis of Lymphocytes and Sera from WS Patients

Patients		B cells*	FcR <sup>+</sup> T cells*	Bright cells stained with anti-BAT†	Bright cells stained with NTA‡	NTA§	ANA¶	
Age	Sex							
		%	%	%	%			
Normal								
Young	20-36 yr¶	M	34(28-35)‡‡	11(10-14)‡‡	50	50	-	-
Aged	90-96 yr**	M	23(20-27)‡‡	10(8-13)‡‡	7(6-11)‡‡	16(14-18)‡‡	-	-
WS								
G.K.	17	M	25	7	20	38	+	Speckled 1:10
M.A.	29	F	21	14	10	26	+	-
T.S.	30	M	36	18	10	33	-	-
A.N.	31	M	33	11	30	32	+	-
T.T.	40	M	28	16	21	40	+	Speckled 1:20
J.T.	45	M	37	39	22	52	+	Speckled 1:20
C.N.	59	M	20	33	29	51	+	Speckled 1:20

\* Percentage of B cells and FcR<sup>+</sup>T cells is given by the following equations (calculation is done by the FACS analyzer): percentage of B cells = (Ig-bearing cells/total lymphocytes) × 100; percentage of FcR<sup>+</sup>T cells = (Fc receptor-positive T cells/nylon column-passed cells) × 100. (The percentage of Ig-bearing cells does not represent the absolute B cell number.)

† Percentage of bright cells is given by the following equation: percentage of bright cells = (brightly stained T cells/nylon column-passed cells) × 100.

‡ NTA is detected by the FACS.

¶ ANA are detected by the indirect immunofluorescent technique.

¶ These data are the mean values obtained from normal young donors aged from 20 to 36 yr.

\*\* These data are the mean values obtained from normal elderly donors aged from 90 to 96 yr.

‡‡ The ranges of values are indicated in parentheses.

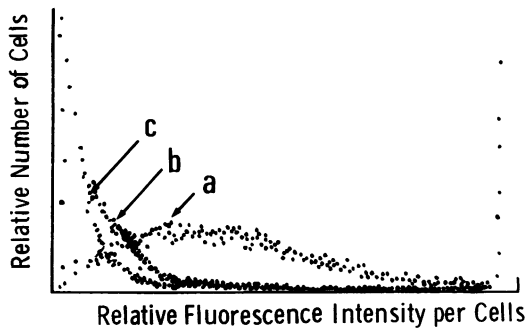


FIGURE 2 Fluorescence distribution of peripheral blood T cells labeled with NTA antisera. Nylon-purified T cells (a-c) were incubated for 30 min at 4°C with NTA antisera from a SLE patient, washed, and incubated for an additional 30 min at 4°C with fluoresceinated rabbit anti-human Ig. (a) Control lymphocytes from normal young donors (20-36 yr olds). (b) Lymphocytes from a WS patient (A.N.). (c) Lymphocytes from healthy elderly individuals (90-96 yr olds).

from two elderly patients with WS showed a significant increase as depicted in Table I. However, this was not consistent in all the cases. No significant changes in the percentage of FcR<sup>+</sup>T cells were found in healthy individuals within the range of ages from 20 to 96 yr. No minor aggregates in the antisera concerned the specific staining with anti-BAT or NTA, as the percentage of FcR<sup>+</sup>T cells in the examined patients did not correlate with the fluorescence distribution.

**Incidence of NTA in WS.** The serum samples of seven cases of WS were examined for the presence of autoantibody against T lymphocytes. Autologous T cells, as well as those from normal individuals were stained with the serum from WS patients, and analyzed by the FACS. NTA was detected in six out of seven cases (Fig. 3), the incidence of which was higher than in SLE (21). NTA was not detected in 28 serum samples from normal young and aged donors, including some in the 90's. NTA from WS reacted with nylon-purified, autologous as well as homologous T cells from healthy

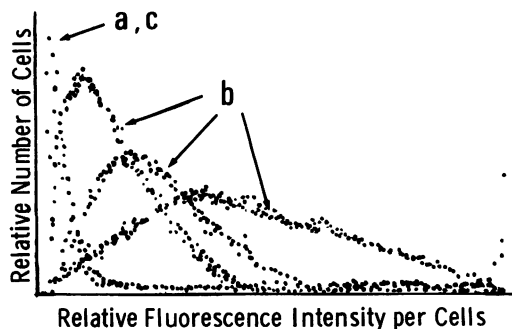


FIGURE 3 Fluorescence distribution of peripheral blood T cells labeled with autologous sera. (a and c) Normal adults (20-96 yr olds). (b) WS patients (G.K., J.T., and C.N.).

individuals in a heterogenous pattern being similar to that from SLE.

The titer of NTA from WS was generally lower than that found in SLE patients. Three (M.A., T.T.; and C.N.) out of seven WS sera examined showed about 50% killing of peripheral blood lymphocytes at the dilution of 1:1 to 1:4. No sera showed more than 50% cytotoxicity even at the dilution of 1:1.

The isotype of NTA in WS was shown to be exclusively IgM class as examined with class-specific fluorescent antisera.

**Other laboratory findings.** In the sera of four patients with WS, a low titer of ANA was detected (Table I). No rheumatoid factors or antibodies to double-stranded DNA were demonstrated. No significant changes were seen in the number of surface Ig-positive cells (B cells) and in the serum Ig levels (3).

## DISCUSSION

WS provides an ideal model for human aging though the precise etiology and pathogenesis are still unknown. Recent studies suggest that WS is a genetic disorder, and is constantly associated with a variety of progeric manifestations (2, 3), hyaluronuria (8, 9), decreased life-span of the cultured fibroblasts (4, 5), enzyme abnormalities (6, 7), and T cell deficiency (3).

In our seven patients, it was demonstrated that the drastic decrease of the population of T cells with high density of two surface antigens characteristic for T cells was detected by NTA and anti-BAT on the FACS analysis. A similar pattern was observed with T cells from apparently healthy aged donors over 90. NTA is known to preferentially kill concanavalin A and phytohemagglutinin precursor T cells, and concanavalin A-activated suppressor T cells (16). Therefore, the observed abnormal fluorescence distribution of T cells may suggest the selective loss of some T cell functions and is probably related to the age-associated changes of immunological dysfunctions in general.

In addition to the changes in T cell populations, an autoantibody against T lymphocytes (NTA) was also detected in six out of seven patients. NTA is an antibody directed at autologous T cells, and was first described by Shirai and Mellors (11, 22-25) in New Zealand Black and (New Zealand Black × New Zealand White) F<sub>1</sub> mice, which develop autoimmune diseases with aging. The human analogue of NTA has been found in some autoimmune diseases as well; the occurrence of NTA was reported in SLE (21, 26), rheumatoid arthritis (26), and juvenile arthritis (27), the pathogenetic role of which is now under an extensive investigation.

The recent report by Steinberg et al. (28) suggested that the decrease in the proportion of T cells in SLE patients is a result of cytotoxic effect by NTA in vivo.

Our present studies also suggest that NTA plays a role in the changes of T cell subpopulations in WS. Thus, one can postulate that some autoimmune process has occurred in the immune system of WS patients that brings about the premature aging of immunological functions. Some of the manifestations in WS, such as neoplasmas, diabetes mellitus, atherosclerosis, and scleroderma-like skin changes have shown to relate with the autoimmune process (29, 30). The present results suggest that WS is not merely a hereditary metabolic disorder, but involves certain autoimmune process with lymphocyte abnormalities. Further studies are now in progress to elucidate abnormalities in lymphocyte function of these WS patients in comparison with those of normal aged individuals.

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