Table 1. The prevalence of atopic dermatitis (AD) in children aged 5 years and under in nursery schools in Ishigaki City, Okinawa, Japan, in 2001

Age (years)	Boys		Girls		Total	
	Numbers examined	AD Cases (%)	Numbers examined	AD Cases (%)	Numbers examined	AD Cases (%)
1	47	2 (4.3)	41	4 (9.8)	88	6 (6.8)
2	57	3 (5.3)	56	3 (5.4)	113	6 (5.3)
3	78	5 (6.4)	52	8 (15.4)	130	13 (10.0)
4	65	5 (7.7)	68	4 (5.9)	133	9 (6.8)
5	55	4 (7.3)	46	1 (2.2)	101	5 (5.0)
Total	302	19 (6.3)	263	20 (7.6)	565	39 (6.9)

among children aged 5 years and younger.

The first set of standardized diagnostic criteria for AD arose from the work of Hanifin and Lobits, and it was revised by Hanifin and Rajka (14, 15). The Japanese Dermatological Association criteria for the diagnosis of AD were established in 1995 (16). In order to set more useful criteria for mass-screening, the United Kingdom (U.K.) Working Party furthered the development of a standardized questionnaire defining the diagnostic criteria for AD (17). This questionnaire was composed of only 5 questions that were easy to answer by parents.

The aim of the present study was to determine the prevalence of AD, serum total IgE, and specific IgE antibodies among children aged 5 years and younger living in a relatively isolated area, Ishigaki Island. An additional aim of the study was to obtain information on the predictability of the questionnaire of the U.K. Working Party diagnostic criteria for AD when used in combination with physical examination in a Japanese population.

Methods

Study population

A large-population, long-term study of residents of the Yaeyama District of Okinawa, Japan for hepatitis B virus markers has been ongoing since 1968 (18–20). The present study was done as a part of the above-mentioned epidemiologic study in 2001. We visited 15 nursery schools in Ishigaki Island, which has a population of 45,000, in the Yaeyama District of Okinawa Prefecture, Japan. Approval for the study was obtained from the Ethics Committee of Kyushu University Hospital as well as from the directors and class teachers of the schools. Informed consent to allow participation of the children was obtained from the parents and guardians. The yearly average temperature and humidity were 25.4°C and 76% on Ishigaki Island.

Six hundred and five children were originally enrolled in the study. There were 40 exclusions because of insufficient physical and laboratory examination or incomplete answers to questionnaires. The remaining 565 children were 302 boys and 263 girls, with a mean age of 3.1 years, and represented 13.7% of the 4,112 kindergarten pupils in Ishigaki City. Physical examinations with questionnaires concerning histories of symptoms and family history were completed, and venous blood samples were obtained between July 30 and August 3, 2001.

Physical and laboratory examination

The medical examinations for all children were done by two dermatologists from the Department of Dermatology at Kyushu University Hospital. AD was diagnosed according to the Japanese Dermatological Association criteria for the diagnosis of AD (16). All children were tested for total and specific IgE antibodies. Total IgE level was determined by a radioimmunoassay with a detection limit of 20 IU/ml (Shionoria IgE, Shionogi & Co., Ltd. Japan). A total IgE

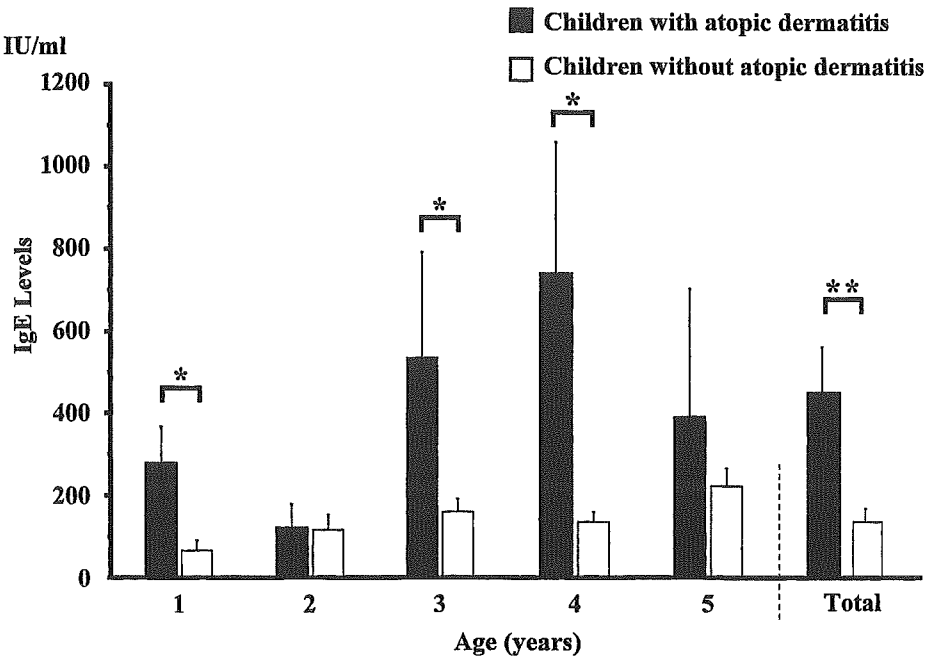

Fig. 1. Levels of total IgE in children 5 years of age and younger in nursery schools, Ishigaki City, Okinawa, Japan, in 2001.
The black bar (■) indicates children with AD and the open bar (□) shows children without AD. The standard deviations are shown by the thin, vertical bars and statistical significance is indicated by the "*" ($P<0.05$) and "**" ($P<0.001$).

Table 2. Comparison of the rates of abnormal total IgE levels between children with and without atopic dermatitis (AD) in Ishigaki City, Okinawa, Japan, in 2001

Age (years)	With AD		Without AD	
	No. tested	Abnormal IgE No. #(%)	No. tested	Abnormal IgE No. #(%)
1	6	3 (50.0)	82	3 (3.7)**
2	6	1 (16.7)	107	7 (6.5)**
3	13	3 (23.1)	117	23 (19.7)**
4	9	5 (55.6)	124	15 (12.1)**
5	5	1 (20.0)	96	19 (19.8)**
Total	39	13 (33.3)*	526	67 (12.7)

#A total IgE level of over 230 IU/ml was considered abnormal.
*A statistically significant difference was found between children with and without atopic dermatitis ($P=0.0029$)
**$P=0.0007$, calculated by use of the Cochran Armitage test.

level over 230 IU/ml was considered abnormal for statistical analysis. Specific IgE antibodies against aeroallergens such as house dust, Japanese cedar pollen, *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, *Candida*, *Malassezia*, and food allergens, such as chicken egg white,

Table 3. Comparison of positive specific IgE antibody responces of children in Ishigaki City, Okinawa, Japan, in 2001, with and without atopic dermatitis (AD)

Specific IgE antibody	AD children (n=39) Positive No. (%)	Non-AD children (n=526) Positive No. (%)	P value*
house dust	17 (43.6)	115 (21.9)	0.0038
Japanese cedar pollen	0 —	0 —	—
D. pteronyssinus	19 (48.7)	122 (23.2)	0.0008
D. farinae	19 (48.7)	98 (18.6)	<0.0001
Candida	2 (5.3)	2 (0.4)	0.0255
Malassezia	0 —	1 (0.2)	—
chicken egg white	10 (25.6)	57 (10.8)	0.0167
cow's milk	9 (23.1)	68 (12.9)	0.1233
rice	1 (2.6)	0 —	—
soy	2 (5.3)	2 (0.4)	0.0255
one or more antibodies	25 (64.1)	159 (30.2)	<0.0001

*P values represent the result of statistical comparison of children with and without atopic dermatitis.

D. pteronyssinus, Dermatophagoides pteronyssinus; D. farinae, Dermatophagoides farinae

Table 4. Comparison of positive rates for one or more specific IgE antibodies in children with and without atopic dermatitis (AD) in Ishigaki City, Okinawa, Japan, in 2001 by age

Age (years)	with AD		without AD	
	No. tested	Abnormal No. (%)	No. tested	Abnormal No. (%)
1	6	3 (50.0)	82	21 (27.6)**
2	6	5 (83.3)	107	29 (27.1)**
3	13	5 (38.5)	117	36 (30.8)**
4	9	9 (100.0)	124	32 (25.8)**
5	5	3 (60.0)	96	41 (42.7)**
Total	39	25 (64.1)*	526	159 (30.2)

*A statistically significant difference was found between children with and without atopic dermatitis (P<0.0001)

**P=0.0394, calculated by use of the Cochran Armitage test.

cow's milk, rice, and soy were tested with the Pharmacia Enzyme CAP procedure (Pharmacia CAP System Specific IgE FEIA, Pharmacia Diagnostics AB, Sweden). A level of specific IgE antibodies over 0.7 UA/ml was considered abnormal for statistical analysis.

Questionnaire

The questionnaire of The U.K. Working Party diagnostic criteria for AD was translated into Japanese by a staff member of Kyushu University Hospital. The questionnaire has 5 questions regarding the present and past history of skin conditions (17). Each one-page questionnaire was completed by parents on behalf of their children. Children with suitable positive answers were diagnosed as AD using the same evaluation method proposed by the U.K. Working Party (17).

Table 5. Responses to the United Kingdom Working Party questionnaire for children with and without AD diagnosed by clinical examination

Questionnaire		Physical examination: AD N=39	Physical examination: Non-AD N=526
AD	N=51	23 (59%)	28 (5.3%)
Non-AD	N=514	16 (41%)	498 (94.7%)

Statistical analysis

Continuous data were expressed as mean values ± standard deviation (SD) or standard error (SE) of the mean. Unpaired t-test and Mann-Whitney U-test were used to compare the means of samples between the two groups. The chi-square test or Fisher's exact test was used for categorical variables for comparisons between the two groups. The Cochran-Armitage test was used to determine the relationship between the increase or decrease in the prevalence rate of AD or the IgE abnormality rate. $P<0.05$ was considered statistically significant.

Results

Prevalence of AD

Table 1 shows the overall prevalence of AD in the study population. Out of 565 children, 39 (6.9%) were diagnosed with AD by physical examination. The prevalence peaked at age 3 (10%), and was lowest at age 5 (5%); however, the age-related difference was not statistically significant ($P=0.7146$ by the Cochran-Armitage test). No significant differences were found when boys (19 of 302, 6.3%) and girls (28 of 263, 7.6%) were compared for disease prevalence.

Total IgE levels

The mean (± SE) total IgE levels were significantly higher in children with AD (451.1 ± 120.4 IU/ml) than in those without AD (139.2 ± 14.7 IU/ml) ($P<0.001$ by Mann-Whitney U-test) (Fig. 1). The total IgE levels were quite variable in each age group, and significant differences in mean IgE levels were found at ages 1, 3, and 4 between children with and without AD (1 year old, $P=0.0026$; 3 years old, $P=0.0272$; and 4 years old, $P=0.0037$, by Mann-Whitney U-test) (Fig. 1). As shown in Table 2, the occurrence of abnormal total IgE levels of over 230 IU/ml was significantly higher in children with AD (13 of 39, 33.3%) than in those without AD (67 of 526, 12.7%) ($P=0.0029$ by the chi-square test). Interestingly, the rate of abnormal total IgE levels in children with AD did not significantly increase with age, however; the rate of abnormal total IgE levels in children without AD significantly increased with age ($P=0.0007$ by the Cochran-Armitage test) (Table 2).

Positivity of specific IgE antibodies against aeroallergens and food allergens

Antigen-specific IgE antibodies against aeroallergens and food allergens, as indicated by values over 0.7 UA/ml, were found in 184 (32.6%) of the total of 565 children. Table 3 shows the differences in specific IgE antibody between children with and without AD. A positive response for one or more specific IgE antibodies was significantly higher in children with AD (64.1%) than in those without AD (30.2%) ($P<0.0001$). Specific IgE antibody positivities, with the exceptions of Japanese cedar pollen, *Malassezia*, cow's milk and rice, were significantly higher in children with AD than those without AD (Table 3). The percentage positivity of specific IgE antibodies in children with AD did not significantly differ according to age (Table 4) (38.5% to 100%, $P=0.3618$ by the Cochran-Armitage test). However, the percentage positivity of specif-

ic IgE antibodies significantly increased with age in children without AD (Table 4) (25.8% to 42.7%, P=0.0394 by the Cochran-Armitage test).

Questionnaire

We determined the sensitivity and specificity of the translated questionnaire of the U.K. Working Party diagnostic criteria for AD (Table 5). Fifty-one out of 565 children (9%) fulfilled the criteria for AD by the questionnaire. When compared to the actual diagnosis by physical examination, the sensitivity was 59% (23 out of 39), and the specificity was 94.7% (498 out of 526). The false positive and negative rates were 5.3% and 41%, respectively (Table 5).

Discussion

Symptoms of AD began during the first year of life in 65% of the children and in 85% during the first 5 years (21); it is thus worthwhile to determine the prevalence in children under the age of 5 years. In 2000 to 2002, the research team of the Japanese Ministry of Welfare (chief researcher; Dr. S. Yamamoto) performed physical examinations of 39,755 children living in Asahikawa, Iwate, Tokyo, Gifu, Osaka, Hiroshima, Kochi, and Fukuoka (22). They reported that the national average prevalence rate of AD was 12.8% in 4-month-old children, 9.8% in 18-month-old, 13.2% in 3-year-old, 11.8% in 6- to 7-year-old, and 10.6% in 11- to 12-year-old children. In our study, the prevalence of AD (6.9%) in children aged 5 years and younger in Ishigaki Island, which is located in the subtropical zone of Japan, was lower than the average rate on the mainland of Japan. It is also interesting that the present result, like Yamamoto's study, showed that the prevalence peaked at age 3. A worldwide survey has reported that AD is increasing in the developed countries in cooler climates (8). Japanese investigators also reported that the prevalence (17.3%) of AD was significantly higher in the cooler climate of Gifu than in the warmer climate of Itoman, Okinawa (3.4%), even after controlling for genetic and environmental factors (9, 10). The reason for the lower prevalence in Okinawa (Itoman and Ishigaki) remains to be elucidated.

IgE levels have been reported to be elevated in 80 to 85% of children who developed AD (23, 24). In the present study, the total IgE levels were significantly higher in children with AD than in those without disease. The children with AD also had higher positive rates of most specific IgE antibodies against aeroallergens and food allergens than the children without AD. However, the positive percentage was lower than expected (high levels of total IgE; 33.3%, one or more specific IgE; 64.1%). None of the children had specific IgE antibody to Japanese cedar pollen, probably because there are no cedars in Ishigaki. Approximately 20% of children with AD have been reported to show allergic reactions to food constituents (25). In infancy allergic sensitization is predominantly to food. In later childhood, allergic sensitization to aeroallergens, such as house dust mites and pollen, is common (26). We also confirmed that the major allergens (specific IgE positive rates) were house dust mites, egg white, and milk in children with AD in Ishigaki. It should be emphasized that high serum levels of IgE ware detected in 12.7% (67/526) of children without AD, and that 30.2% of these non-atopic children had one or more positive specific IgE antibodies to common allergens in our study. House dust mites, milk, and egg white were also the major antigens for specific IgE production even in the non-atopic children. It is also very interesting that both the total and specific IgE levels significantly increased with age in children without AD. The tendency of age-related accumulation of total and specific IgE was not observed in the children with AD, because it had already reached high levels as early as at age 1. Nolles et al.(27) reported a similar age-related increase of IgE antibodies. These results suggest (1) that the total and specific IgE levels increase with age probably with cumulative exposure to common al-

lergens even in non-atopic individuals, and (2) that earlier and higher increases in total and specific IgE antibodies are associated with AD.

The prevalence of AD has been studied in a variety of populations throughout the world (6–13), but comparisons of prevalence is difficult because of differences in study populations and study methods. Some investigators have measured point prevalence (28, 29), while others have measured 12 months prevalence in different age groups (8). The variation problem of study designs was addressed by the International Study for Asthma and Allergies in Childhood (ISAAC) (8). The methodology was subsequently standardized by the use of a questionnaire based on the U.K. Working Party definition of AD (17). A previous validity study suggested that the questionnaire might slightly overestimate the true prevalence (30). In the present study, we translated the questionnaire of the U.K. Working Party into Japanese and analyzed the sensitivity and specificity of the translated questionnaire. Although the specificity of the translated questionnaire for AD was 94.7%, its sensitivity was only 59%. This low sensitivity may be due to some incomprehensibility in the Japanese translation and to insufficient parent cooperation. It is critical that we refine the translation to improve the parents' understanding of the translated questionnaire.

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International Immunology, Vol. 17, No. 4, pp. 383–390
doi:10.1093/intimm/dxh218

$CD27^+$ (memory) B cell decrease and apoptosis-resistant $CD27^-$ (naive) B cell increase in aged humans: implications for age-related peripheral B cell developmental disturbances

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Keywords: aging, apoptosis, B cells, memory, naive

Abstract

To investigate age-related alterations in human humoral immunity, we analyzed the quantity and quality of peripheral B cell subsets, CD27-negative ($CD27^-$) and CD27-positive ($CD27^+$) B cells, by flow cytometry analysis in 54 aged individuals (mean age ± SE, 74.6 ± 0.7 years) and 30 young individuals (mean age ± SE, 26.1 ± 0.5 years). $CD27^-$ and $CD27^+$ B cells are regarded as naive and memory B cells, respectively. CD38, Ki-67, CD95 and bcl-2 were used as activation, proliferation and apoptotic markers. Susceptibility to apoptosis was evaluated by cell size and annexin-V binding in culture cells. The percentage of $CD27^+$ B cells was significantly lower in aged (mean, 19.2%) individuals than that in young individuals (mean, 28.2%). The opposite was true for $CD27^-$ B cells (mean, 80.8% in aged and 71.8% in young) ($P < 0.01$). The absolute number of $CD27^+$ B cells in aged individuals was significantly less than the number of $CD27^-$ B cells. The $CD27^+$ B cells from aged individuals showed little susceptibility to apoptosis, although CD95 expression on the $CD27^+$ B cells was significantly higher in the aged individuals than in the young individuals ($P < 0.05$). The CD38 and bcl-2 expression on the $CD27^-$ B cells was significantly higher in the aged individuals than in the young individuals ($P < 0.05$). In addition, the $CD27^-$ B cells from the aged individuals showed a decreased susceptibility to apoptosis compared with that of the young individuals. These findings suggested that human aging leads to both quantitative and qualitative alterations in the peripheral B cell developmental system, including memory and naive B cell balance and their surface phenotypes.

Introduction

Susceptibility to infectious diseases has a serious effect on human longevity, with mortality from infectious diseases, particularly pneumonia, increasing in aged humans (1). One cause for this phenomenon is 'immunosenescence,' alteration of host defense mechanisms elicited by aging. The study of immunosenescence in humans is less developed than that in mice. In particular, humoral immunity, which is a defense system against infectious agents, has not yet been fully investigated in aged humans. The prevalence of human peripheral B cell neoplasms, such as B cell-chronic lymphocytic leukemia and B cell lymphoma, rapidly increases with age (2, 3). We have recently demonstrated that somatic mutations of Ig variable region genes accumulate in IgG B cells from aged humans (4). These findings suggest that human aging could affect peripheral B cell development, resulting in augmented B cell oncogenesis.

CD27 antigen expression on B cells primed by antigenic stimulation is important to promote the differentiation of B cells through T–B interaction (5, 6). Few CD27-negative ($CD27^-$) B cells carry somatic mutations in Ig variable region genes, whereas CD27-positive ($CD27^+$) B cells accumulate substantial numbers of somatic mutations (7). Accordingly,

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Transmitting editor: T. Watanabe

Received 25 August 2004, *accepted* 12 January 2005

Advance Access publication 21 February 2005

$CD27^-$ and $CD27^+$ B cells are regarded as naive and memory B cells, respectively (8). $CD27^-$ (naive) B cells are exclusively produced at birth and, afterwards, by the adolescent period, $CD27^+$ (memory) B cells gradually increase (9). However, little is known about how the composition of these peripheral B cell subsets is altered from the young adult period to that of old age.

Understanding how peripheral B cells are influenced by aging is important for clarifying one aspect of humoral immunity in aged humans. In particular, the analysis of B cell subsets, $CD27^-$ and $CD27^+$ B cells, is critical in addressing the alteration of the peripheral B cell developmental process caused by aging. In the present study, the peripheral levels of all B cells and B cell subsets were examined, and their biological characteristics, including activation, proliferation and susceptibility to apoptosis, were investigated in young and aged adults using cell surface and intracellular markers.

Methods

Individuals

With informed consent, 30 young individuals (15 men and 15 women) and 54 aged individuals (28 men and 26 women) were the subjects used in the present study. The ages of the young subjects ranged from 22 to 34 years, with a mean age ± SE of 26.1 ± 0.5 years. The ages of the aged subjects ranged from 68 to 87 years, with a mean age ± SE of 74.6 ± 0.7 years. All subjects were independent, were not hospitalized and were not taking any prescription medications. None of the subjects had acute infections. No chronic viral infections, including HIV-1, human T-cell leukemia virus type 1, hepatitis B virus (HBV) and hepatitis C virus (HCV), were detected in any subject. No monoclonal gammopathy was found. Blood sampling was done from March to October 2003.

Measurement of serum Ig

Serum γ-globulin, IgG, IgA, IgM and IgE levels were determined by turbidimetric immunoassay or fluorescence-enzyme immunoassay at Mitsubishi Kagaku Bio-Clinical Laboratories (Tokyo, Japan).

Flow cytometry

For phenotypic analysis of the peripheral CD19-positive cells (B cells), one- to three-color flow cytometry was done using the following FITC-, PE-, or PE–cyanin 5.1 (PC5)-conjugated mouse anti-human mAbs: CD3–PC5, CD19–PC5, CD38–FITC, CD95 (Fas)–FITC (Immunotech, Marseille Cedex, France), CD27–PE (Becton Dickinson, Bridgeport, NJ, USA), Ki-67–FITC, bcl-2–FITC (PharMingen, San Diego, CA, USA) and IgD–FITC (Southern Biotechnology Associates, Birmingham, AL). Ig isotype-matched FITC-, PE- or PC5-conjugated mouse antibodies were used as negative controls for non-specific staining. A 50-μl volume of whole blood was incubated with mAbs, lysed in 1 ml of IO Test 3 Lysing Solution (Immunotech) and prepared for analysis. This analysis was done with the following combinations of conjugated mAbs: CD19–PC5 and CD27–PE; CD19–PC5, CD27–PE and CD38–FITC, and CD19–PC5, CD27–PE and CD95 (Fas)–FITC.

PBMC were isolated from heparinized venous blood by density centrifugation. After the addition of goat serum to block Fc receptors, freshly isolated PBMC were stained with CD19–PC5, CD27–PE and IgD–FITC, and prepared for analysis.

Fixation, permeabilization and intracellular staining of PBMC were performed with the CytoStain Kit (PharMingen) according to the manufacturer's instructions. Briefly, freshly isolated PBMC were stained with CD19–PC5 and CD27–PE. After staining with mAbs, the cells were fixed, permeabilized and stained with Ki-67–FITC or bcl-2–FITC mAbs for three-color flow cytometric analysis.

The stained cells were analyzed by a flow cytometer, Cytoron Absolute (Ortho Diagnostic Systems, Raritan, NJ, USA), using ImmunoCount 2 software (Ortho Diagnostic Systems). Lymphocyte gating was performed using forward- and side-scatter parameters; up to 30 000 cells were acquired from this gate. CD19-positive cells from the lymphogate were used for each analysis.

Measurement of apoptosis

A total of 2.5×10^5 PBMC per well were cultured in 24-well plates for 24 and 48 h in 1 ml of RPMI 1640 supplemented with 10% heat-inactivated FCS, 50 U ml^{-1} penicillin and 50 μg ml^{-1} streptomycin, all supplied by GIBCO BRL (Life Technologies, Inc., Gaithersburg, MD, USA). The apoptosis levels of the B cell subsets were evaluated by two markers, small cell size and annexin-V binding, as described elsewhere (10–12). Briefly, cultured PBMC were washed, suspended and stained with CD19–PC5 and CD27–PE. After being washed, the cells were re-suspended with 500 μl of binding buffer and stained with annexin-V–FITC for 10 min on ice using the Annexin V–FITC Kit (Immunotech). The cell size of the B cell subsets was measured by the level of forward light scatter using cultured PBMC stained with CD19–PC5 and CD27–PE. Two- and three-color flow cytometry were performed after the above procedures.

Statistical analysis

The Mann–Whitney *U*-test was performed to compare differences in the analysis data between the young and aged subjects. The association between two related variables was analyzed by using Spearman's rank correlation test.

Results

Immunological characteristics in young and aged individuals

Table 1 summarizes the immunological characteristics of the subjects examined in the present study. The absolute number of white blood cells was significantly lower in the aged subjects than in the young subjects ($P < 0.01$). The absolute number of lymphocytes and CD3 cells was also lower in the aged subjects than in the young subjects ($P < 0.01$). Serum γ-globulin levels between the young and aged were comparable. The serum mean IgG and IgA levels of the aged subjects were higher than those of the young subjects, although the differences were not significant. The serum IgM levels were

Table 1. Immunological characteristics in young and aged individuals

	Aged	Young
Number of individuals	54	30
Age (years)	74.6	26.1
White blood cell (cells μl^{-1})*	5109 ± 160	6257 ± 270
Lymphocyte (cells μl^{-1})*	1620 ± 60	1995 ± 70
$CD3^+$ T cell (cells μl^{-1})*	811 ± 47	1314 ± 55
γ-Globulin (mg dl^{-1})	1210.0 ± 30.2	1200.4 ± 43.2
IgG (mg dl^{-1})	1396.5 ± 32.0	1350.7 ± 43.9
IgA (mg dl^{-1})	284.9 ± 16.4	251.5 ± 14.6
IgM (mg dl^{-1})*	89.1 ± 5.1	148.9 ± 10.2
IgE (IU dl^{-1})	168.6 ± 41.7	186.1 ± 47.3

Data are presented as mean ± SE.
*$P < 0.01$.

significantly lower in the aged subjects than in the young subjects ($P < 0.01$).

Circulating $CD27^-$ (naive) and $CD27^+$ (memory) B cells in young and aged individuals

Representative FACS analysis of PBMC stained by anti-CD19 and CD27 antibodies is shown in Fig. 1(a). The $CD27^-$ B cells were distinctively separate from the $CD27^+$ B cells. The percentage of circulating B cells in lymphocytes was lower in the aged subjects (mean, 10.9%) than in the young subjects (mean, 12.9%) ($P < 0.05$, Fig. 1b). The absolute number of B cells was also lower in the aged subjects than in the young subjects ($P < 0.01$, Fig. 1b). The percentage of circulating $CD27^-$ B cells of all B cells was significantly higher in the aged subjects (mean, 80.8%) than in the young subjects (mean, 71.8%) ($P < 0.01$, Fig. 1c). In contrast, the percentage of $CD27^+$ B cell was significantly lower in the aged subjects (mean 19.2%) than in the young subjects (mean, 28.2%) ($P < 0.01$, Fig. 1d). Although the absolute number of both the $CD27^-$ and $CD27^+$ B cells was lower in the aged subject than that in the young subjects (Fig. 1c and d), the rate of reduction in the absolute number was significantly higher in the $CD27^+$ B cells than that in the $CD27^-$ B cells ($P < 0.01$, Fig. 1e). $CD27^-$ and $CD27^+$ B cells have been reported as being naive IgD-positive (IgD^+) B cells and memory B cells, respectively (13). The percentage of circulating $CD27^-$ B cells positively correlates with the percentage of circulating IgD^+ $CD27^-$ B cells ($P < 0.01$, $r = 0.7$), suggesting that the increased $CD27^-$ B cell and decreased $CD27^+$ B cell percentages found in the aged subjects were not a result of CD27 down-regulation on memory B cells.

CD38, CD95 and bcl-2 expression on circulating $CD27^-$ (naive) and $CD27^+$ (memory) B cells in young and aged individuals

To investigate the biological characteristics of B cells in aged individuals, activation, proliferation and apoptotic markers were compared between the young and aged subjects. CD38 and Ki-67 expressions on lymphocytes are known to be activation and proliferation markers, respectively (14–16). CD95 and bcl-2 are often used as representatives of apoptotic and anti-apoptotic agents expressed by lymphocytes (17, 18).

The percentage of CD38-positive ($CD38^+$) B cells of all B cells was significantly higher in the aged subjects than that in the young subjects ($P < 0.05$, Table 2). CD38 expression was primarily observed in the $CD27^-$ B cells rather than in the $CD27^+$ B cells. The percentage of $CD38^+$ $CD27^-$ B cells of all $CD27^-$ B cells was significantly higher in the aged subjects than that in the young subjects ($P < 0.05$, Table 2). The intensity of CD38 on the $CD38^+$ $CD27^-$ B cells between the young and aged subjects was comparable. In contrast, CD38 expression on the $CD27^+$ B cells was similarly low in both the young and aged subjects (Table 2). To examine whether the elevated CD38 expression on the B cells from the aged subjects could be explained by B cell proliferation, B cell proliferation was evaluated by Ki-67 expression. The percentages of proliferative B cells between the young subjects (mean, 2.9%) and aged subjects (mean, 2.5%) were comparable. No increased proliferation of either the $CD27^-$ or the $CD27^+$ B cells was observed (data not shown).

The percentage of CD95-positive ($CD95^+$) B cells of all B cells appeared to be higher in the aged subjects than that in the young subjects, although the difference was not significant ($P = 0.15$, Table 2). CD95 expression was dominant on the $CD27^+$ B cells but was scarcely observed on the $CD27^-$ B cells. The percentage of $CD95^+$ $CD27^+$ B cells of all $CD27^+$ B cells was significantly higher in the aged subjects than in the young subjects ($P < 0.05$, Table 2). The intensity of CD95 on the $CD95^+$ $CD27^+$ B cells between the young and aged subjects was comparable. In contrast, CD95 expression on the $CD27^-$ B cells was low and comparable between the young and aged subjects (Table 2).

Bcl-2 was expressed on almost all of the circulating B cells of the subjects. The intensity of bcl-2 on the B cells was significantly higher in the aged subjects than that in the young subjects ($P < 0.05$, Table 2). Bcl-2 intensity on the $CD27^-$ B cells was significantly higher in the aged subjects than that in the young subjects ($P < 0.05$, Table 2). Bcl-2 intensity on the $CD27^+$ B cells was higher than that on the $CD27^-$ B cells (Table 2). Bcl-2 intensity on the $CD27^+$ B cells appeared to be higher in the aged subjects than that in the young subjects, but the difference was not significant (Table 2).

Susceptibility to apoptosis of circulating $CD27^-$ (naive) and $CD27^+$ (memory) B cells in young and aged individuals

To investigate whether altered CD95 and bcl-2 expression on the B cells from aged individuals affect susceptibility to B cell apoptosis, cell size and annexin-V binding in cultured B cells were examined as apoptotic markers. Representative FACS analysis of the cultured B cells is shown as the detection of apoptotic cells in Fig. 2(a). In the analysis of cell size, B cells with small cell size (small B cells) were observed only in the $CD27^-$ B cell compartment and were distinctively separate from the cells with normal cell size. Similarly, annexin-V-binding B cells were observed in the $CD27^-$ B cell compartment and few were found in the $CD27^+$ B cell compartment. A decrease in CD27 antibody-binding capacity could occur in the apoptotic cells. Thus, it is possible that some apoptotic B cells detected in the present study could come from CD27-positive cells. In the present study, the percentages of $CD27^-$ and $CD27^+$ B cells as a percentage of all B cells, 71.8 versus 28.2% in the young subjects and 80.8 versus 19.2% in the

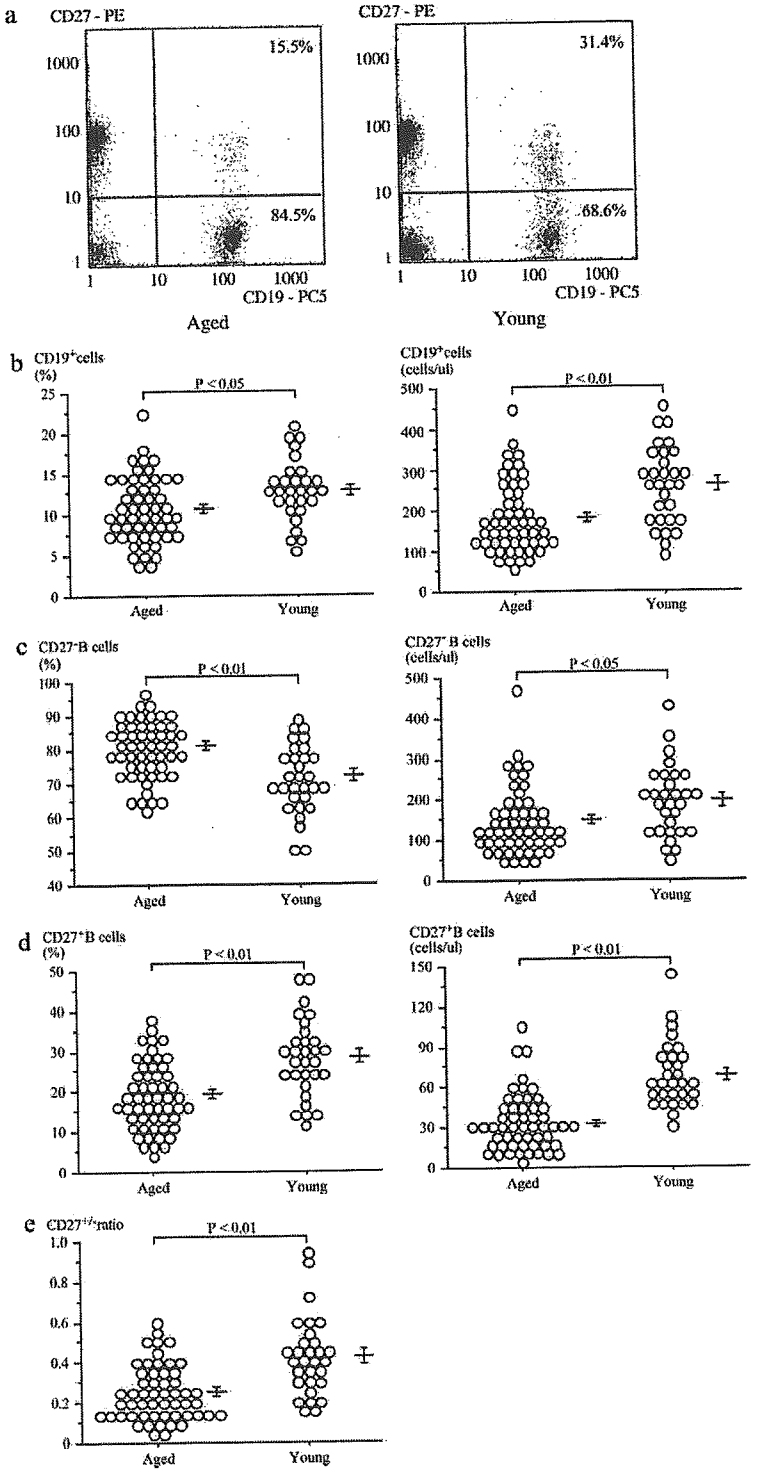


Fig. 1. (a) Representative FACS analysis of anti-CD19 and anti-CD27 stained PBMC in one young and one aged subject. Numbers in the upper and lower right quadrants show $CD27^+$ and $CD27^-$ B cells as a percentage of all peripheral B cells, respectively. (b) Percentage of peripheral $CD19^+$ cells in lymphocytes (left) and absolute number of peripheral $CD19^+$ cells (right) in young and aged subjects. (c) Percentage of peripheral $CD27^-$ B cells of all B cells (left) and absolute number of peripheral $CD27^-$ B cells (right) in young and aged subjects. (d) Percentage of peripheral $CD27^+$ B cells of all B cells (left) and absolute number of peripheral $CD27^+$ B cells (right) in young and aged subjects. (e) Ratio of absolute number of $CD27^+$ B cells to $CD27^-$ B cells in young and aged subjects. The horizontal and vertical bars represent the mean levels and SE, respectively.

Table 2. CD38, CD95 and bcl-2 expression in B cell subsets from young and aged individuals

	$CD19^+$ B cell	$CD27^-$ B cell	$CD27^+$ B cell
CD38 expression (%)			
Aged ($n = 32$)	53.3 ± 2.5*	62.1 ± 2.4*	11.6 ± 1.0
Young ($n = 30$)	44.0 ± 2.4*	54.0 ± 2.8*	13.0 ± 1.6
CD95 expression (%)			
Aged ($n = 32$)	18.4 ± 0.8	14.3 ± 0.6	38.4 ± 2.3*
Young ($n = 30$)	17.1 ± 0.7	13.5 ± 0.7	29.5 ± 1.6*
Bcl-2 expression (MFI)			
Aged ($n = 32$)	105.0 ± 1.1*	103.8 ± 1.1*	109.8 ± 1.4
Young ($n = 30$)	102.1 ± 0.9*	100.5 ± 1.0*	107.6 ± 1.1

MFI, mean fluorescence intensity. Data are presented as mean ± SE.
*$P < 0.05$

aged subjects, were stable in culture at 0, 24 and 48 h. There were no statistical differences in the percentage of $CD27^+$ B cells in the comparison of 0, 24 and 48 h of culture time. If a part of apoptotic B cells detected in the $CD27^-$ B cell compartment come from $CD27^+$ B cells, the percentage of $CD27^+$ B cells should decrease with time. The apoptotic B cells were probably derived from the $CD27^-$ B cells, and any contribution of the $CD27^+$ B cells to the apoptotic cells would be slight.

The percentage of small $CD27^-$ B cells of all B cells was significantly lower in the aged subjects than that in the young subjects ($P < 0.01$, Fig. 2b). No small $CD27^+$ B cells were detected in any of the subjects (Fig. 2b). The percentage of annexin-V-binding $CD27^-$ B cells of all B cells was significantly lower in the aged subjects than that in the young subjects ($P < 0.01$, Fig. 2c). Few annexin-V-binding $CD27^+$ B cells were detected in any of the subjects (Fig. 2c). In both the young and aged subjects, the percentage of annexin-V-binding $CD27^-$ B cells was inversely correlated with bcl-2 intensity on the $CD27^-$ B cells (Fig. 2d). No relationship was found between the percentage of annexin-V-binding $CD27^-$ B cells and the percentage of the $CD95^+$ $CD27^-$ B cells.

Discussion

In the present study, both quantitative and qualitative alterations of B cells from aged humans were found, clarifying the characteristics of B cells from aged humans by the analysis of B cell subsets, $CD27^-$ and $CD27^+$. In aged humans, the percentage of $CD27^+$ (memory) B cells was dramatically less than the percentage of $CD27^-$ B cells. $CD27^-$ (naive) B cells exhibited a reduced susceptibility to apoptosis. Interestingly, the serum Ig of aged humans were maintained at a level comparable to those of young humans.

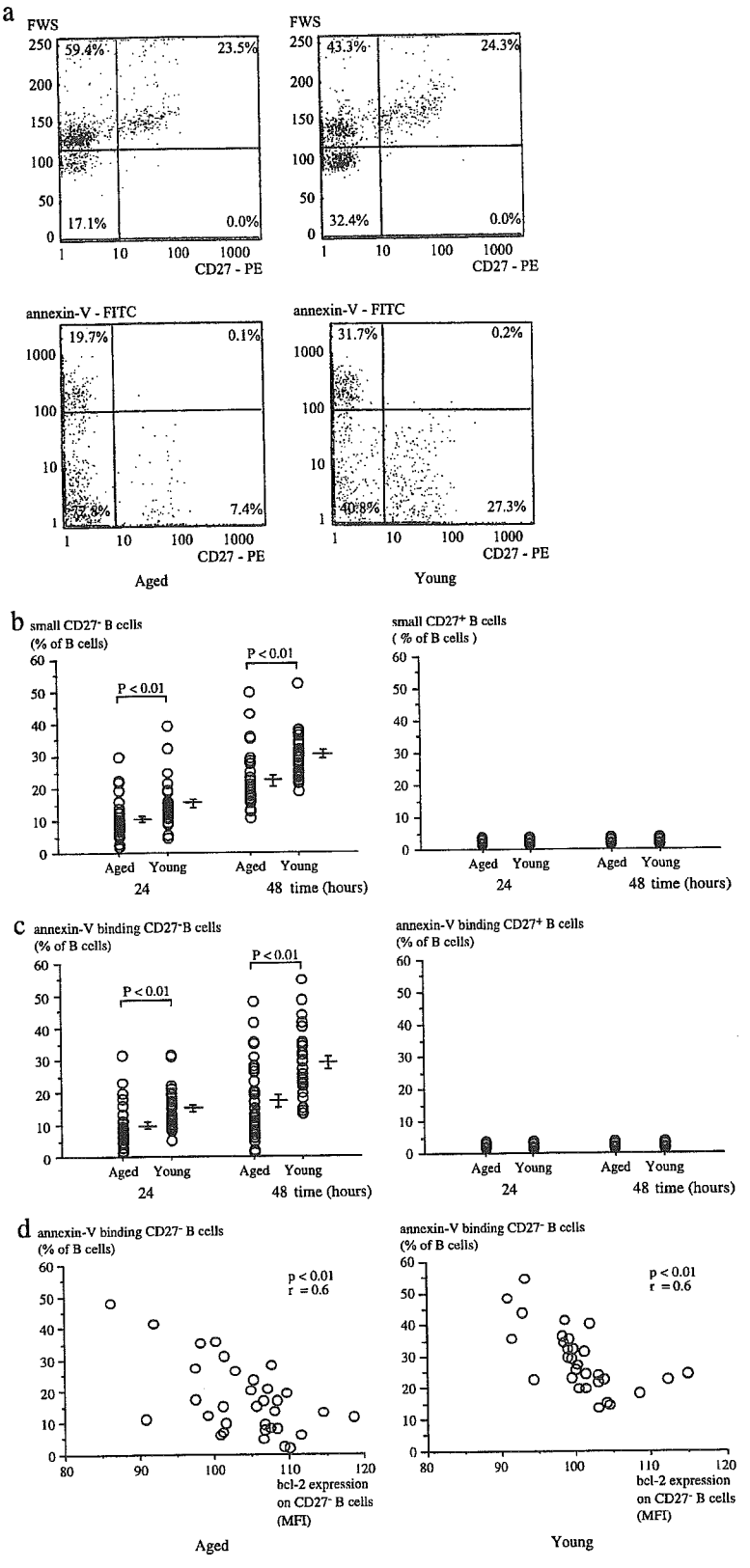
The quantitative and qualitative features of memory B cells in aged humans have not been fully investigated. An extreme depletion of $CD27^+$ (memory) B cells from aged individuals was indicated in the present study (Fig. 1d). Although the absolute number of $CD27^-$ (naive) B cells from aged individuals was also reduced, the rate of reduction of the $CD27^+$ B cells was much higher than that of the $CD27^-$ B cells (Fig. 1e). Thus, depletion of memory B cells is a characteristic phenomenon in aged humans.

Germinal center B cells display high CD95 expression. Thereafter, most of these B cells are induced into apoptosis (17), which indicates that CD95 antigen expression on B cells might be induced through antigen-specific immune responses. In the analysis of peripheral B cells from healthy individuals, $CD38^-IgD^-$ (memory) B cells have been shown to have a significantly higher fraction of CD95 expression than that of $CD38^+IgD^+$ (naive) B cells (19). Thus, the induction of CD95-related apoptosis is possibly important as a mechanism of cell death of peripheral memory B cells (post-germinal center B cells). Similar results were obtained in the present study. In addition, aged individuals showed an increased percentage of $CD95^+$ $CD27^+$ B cells compared with that of young individuals (Table 2), suggesting that an apoptotic pathway induced by CD95 signaling is important for memory B cell survival in both aged and young humans.

Bcl-2 intensity, a critical anti-apoptotic factor, was significantly higher in $CD27^+$ B cells than in $CD27^-$ B cells (Table 2). In both young and aged individuals, the percentages of $CD27^+$ B cells were stable during culture, and few small or annexin-V-binding $CD27^+$ B cells were detected (Fig. 2b and c). These findings suggest that memory B cells are less susceptible to apoptosis than are naive B cells. The present study does not clarify the mechanism of memory B cell depletion caused by aging. To examine whether an age-related increase of $CD95^+$ $CD27^+$ B cell fractions is related to this loss of memory B cells, further investigation, such as a functional analysis, will be necessary.

Quantitative and qualitative alterations were found in naive B cells as well as in memory B cells. $CD27^-$ (naive) B cells became more predominant in the peripheral B cells of aged individuals (Fig. 1c). $CD27^-$ B cells of aged individuals showed a decreased susceptibility to apoptosis under the *in vitro* culture condition, as indicated by cell size and annexin-V binding (Fig. 2b and c). Thus, $CD27^-$ B cells seem to become 'apoptosis-resistant' in aged humans, as the *in vitro* condition itself is generally thought to cause cells to become more apoptotic when compared with the *in vivo* condition. Under the *ex vivo* condition before culture, $CD27^-$ B cells showed a significantly higher bcl-2 intensity in aged individuals than in young individuals (Table 2). Bcl-2 plays a critical role in controlling the apoptotic pathway, and over-expression of bcl-2 is known to increase the resistance of lymphocytes to apoptosis (18). It is possible that the bcl-2-related anti-apoptotic pathway plays an important role in 'apoptosis resistance' in the naive B cells of aged humans.

Activated and proliferated B cells augment CD38 expression under various stimulations including T-cell-independent and -dependent responses (14, 20). In general, lymphocyte activation is closely related to increased susceptibility to apoptosis (21). In HIV-infected patients, $CD27^-$ B cells with high CD38 intensity have shown a low level of bcl-2 intensity and a high susceptibility to apoptosis (22), suggesting that persistent HIV infection can induce naive B cells to change into an activated and apoptosis-susceptible phenotype. In the present study, $CD27^-$ B cells from aged individuals showed an increased percentage of CD38 expression compared with that of young individuals (Table 2), but the intensity was similar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FWS
59.4%
23.5%
17.1%
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CD27 - PE
FWS
43.3%
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annexin-V - FITC
19.7%
0.1%
72.8%
7.4%
CD27 - PE
Aged
annexin-V - FITC
31.7%
0.2%
40.8%
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bcl-2 expression
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Aged
annexin-V binding CD27⁻ B cells
(% of B cells)
p < 0.01
r = 0.6
bcl-2 expression
on CD27⁻ B cells
(MFI)
Young

between the young and aged individuals. In addition, the $CD27^-$ B cells were less susceptible to apoptosis in aged individuals. Although CD38 expression on $CD27^-$ B cells increases in frequency with age, it is probable that naive B cells from aged humans are not an activated phenotype.

Some investigators have reported that serum Ig levels of aged humans are significantly higher than those of young humans (23, 24). In the present study, the serum Ig levels of young and aged individuals were comparable (Table 1), suggesting that the production of Ig in aged humans is maintained at a level equivalent to that of young humans. The mechanism of serum Ig maintenance is unknown. Antibody-secreting cells are thought to be derived from peripheral B cells. The age-related B cell alterations found in the present study, as characterized by memory B cell depletion and apoptosis resistance in naive B cells, might affect the differentiation of peripheral B cells into antibody-secreting cells. Further investigation will be necessary to clarify this issue.

Human aging induces both quantitative and qualitative changes in peripheral B cells. During aging, memory B cells decrease and apoptosis-resistant naive B cells increase. We have also reported a higher accumulation of somatic mutations in Ig variable region genes of peripheral IgG B cells among aged humans (4). Thus, it is possible that human aging affects the developmental system of peripheral B cells. This behavior is possibly associated with presumed immunological dysfunctions and hematological disorders in aged humans. The detailed mechanisms and pathological significance of the age-related B cell alterations found in the present study should be addressed.

Abbreviation

PC5 PE–cyanin 5.1

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Fig. 2. Susceptibility to apoptosis of peripheral $CD27^-$ and $CD27^+$ B cells. Apoptotic B cells were measured using cultured PBMC stained with anti-CD19, anti-CD27 and annexin-V at 24 and 48 h. The level of apoptosis was evaluated by small cell size and annexin-V binding. (a) Representative FACS analysis of B cells with small cell size (small B cells) in one young (right) and one aged (left) subject (top). PBMC were cultured for 24 and 48 h, followed by a dual staining with anti-CD19 and anti-CD27, after which, among gated B cells, the cell sizes of the $CD27^-$ and $CD27^+$ B cells were measured by the level of forward light scatter. The small B cells were clearly separate from the B cells with normal cell size. Representative FACS analysis of annexin-V-binding B cells in one young (right) and one aged (left) subject (bottom). PBMC were cultured for 24 and 48 h, followed by a staining with anti-CD19, anti-CD27 and annexin-V, after which annexin-V binding the $CD27^-$ and $CD27^+$ B cells were measured among the gated B cells. Culture data at 48 h is shown. Each number in the quadrant is the percentage of all B cells. (b) Percentage of small $CD27^-$ B cells of all B cells (left) and percentage of small $CD27^+$ B cells of all B cells (right) in the young and aged subjects. The horizontal and vertical bars represent the mean levels and SE, respectively. (c) Percentage of annexin-V-binding $CD27^-$ B cells of all B cells (left) and percentage of annexin-V-binding $CD27^+$ B cells of all B cells (right) in the young and aged subjects. The horizontal and vertical bars represent the mean levels and SE, respectively. (d) Correlation between the percentage of annexin-V-binding $CD27^-$ B cells and bcl-2 intensity on $CD27^-$ B cells in young (right) and aged (left) subjects.

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Atherosclerosis 178 (2005) 303–309

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Association between chronic *Helicobacter pylori* infection and acute ischemic stroke: Fukuoka Harasanshin Atherosclerosis Trial (FHAT)

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Received 16 February 2004; received in revised form 18 August 2004; accepted 26 August 2004

Abstract

Helicobacter pylori (*H. pylori*) have been associated both epidemiologically and pathogenetically with coronary atherosclerosis, but data on the relationship between chronic *H. pylori* infection and stroke are lacking. Therefore, we investigated the relationship between *H. pylori* infection and acute ischemic stroke in 62 patients with their first stroke and 143 controls. The stroke patients were all admitted to Harasanshin General Hospital (Fukuoka, Japan) and the controls were asymptomatic age-matched outpatients with hyperlipidemia who did not have cardiac disease or infections. All patients underwent cranial CT scanning and/or brain magnetic resonance imaging, duplex ultrasonography of the extracranial carotid arteries, and transthoracic echocardiography. *H. pylori* infection was diagnosed by detection of anti-*H. pylori* IgG antibodies, the ^{13}C-urea breath test, and histology. Conditional logistic regression analysis was performed to analyze the data. The 62 stroke patients and 143 controls were aged from 41 to 92 years. Chronic *H. pylori* infection was associated with a higher risk of stroke due to small artery occlusion (odds ratio: 9.68; 95% CI: 3.56–33.08, $P < 0.001$) and a lower risk of cardioembolic stroke (odds ratio: 0.27; 95% CI: 0.03–1.53). Chronic *H. pylori* infection still showed an overall association with ischemic stroke (odds ratio for all subtypes combined: 2.57; 95% CI: 1.09–6.08) after adjusting for major cardiovascular risk factors. These results suggest that chronic *H. pylori* infection may be a triggering factor that increases the risk of acute ischemic stroke.

Keywords: *Helicobacter pylori*; Ischemic stroke; Stroke subtypes; Carotid atherosclerosis

1. Introduction

Atherosclerosis is a highly prevalent disease, and is currently the greatest cause of morbidity and mortality in developed societies. Many risk factors are involved in the development of atherosclerosis, which manifests as coronary artery disease (CAD) and myocardial infarction (MI), including hyperlipidemia, hypertension, smoking, and diabetes mellitus [1], but much of the risk remains unexplained. The pathogenesis of atherosclerosis involves the processes of vascular injury, inflammation, degeneration, and thrombosis, but the stimulus that triggers the inflammatory response is largely unclear.

The pulse wave velocity can be used as an indicator of arterial stiffness [2,3], and it is regarded as a marker of vascular damage [4,5]. An instrument was recently developed that can measure the branchial-ankle pulse wave velocity (baPWV) by the volume rendering method. Yamashina et al. have reported a high validity and reproducibility of baPWV measurements, suggesting that this parameter may be an acceptable indicator of vascular damage and may be suitable

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0021-9150/$ – see front matter © 2004 Published by Elsevier Ireland Ltd.
doi:10.1016/j.atherosclerosis.2004.08.025

for screening large populations to detect vascular disease [6].

Chronic infection with *Helicobacter pylori* (*H. pylori*) has been seroepidemiologically linked to CAD and atherosclerosis [1,7]. Ischemic stroke comprises a heterogeneous mixture of different stroke subtypes caused by atherosclerotic as well as non-atherosclerotic mechanisms [9]. Although stroke is pathogenetically related to coronary atherosclerosis, data about the association between chronic *H. pylori* infection and cerebrovascular disease are limited [9–14]. It appears that the first paper regarding ischemic cerebrovascular disease and *H. pylori* was published in 1998 [14], and it was only a small study. To make a valid and reliable assessment of the role of chronic *H. pylori* infection in cerebrovascular disease, the underlying mechanism of ischemic stroke must be taken into consideration by stratifying the subjects into different etiologic subtypes. Because direct detection of *H. pylori* in the cerebral vasculature would require samples of cerebral vessels, which are not clinically available, surrogate markers such as the anti-*H. pylori* antibody titer must be used to assess the association between stroke and infection with this organism.

The present case-control study was performed to investigate whether *H. pylori* infection was an independent risk factor for various etiologic subtypes of ischemic stroke.

2. Methods

2.1. Subjects and methods

All of the patients with acute cerebrovascular disease admitted to the Division of General Medicine at Harasanshin General Hospital (Fukuoka, Japan) during the year 2002 were considered eligible for the present study. Between July 1 and December 31, 2002, a total of 62 patients who suffered their first ischemic stroke were registered for this study.

2.2. Selection of stroke patients

Patients with their first stroke were enrolled in the study if they met the following criteria: (a) first ischemic stroke, (b) admission to hospital for treatment, and (c) admission within 72 h of the onset. Stroke was defined according to World Health Organization criteria [15]. Cerebral infarction was diagnosed on the basis of the initial CT and MRI data. All patients underwent ultrasonography of the neck and intracranial arteries. The carotid arteries were assessed by color flow B-mode Doppler ultrasound (SONOS 5500, PHILIP) according to the standard method [16,17]. The vertebrobasilar system was evaluated as described by Bartels [18]. In some cases, the ultrasound images were unsatisfactory, so MRI angiography was performed to determine the presence/absence of atherosclerotic lesions.

Patients without clinical or imaging evidence of atherosclerosis who had atrial fibrillation and/or echocardiographic findings suggestive of possible cardiogenic embolism were classified as having thromboembolic stroke. The other patients were diagnosed as having large artery stroke if there was >50% stenosis of the extracranial carotid or an intracranial artery, small artery occlusion if they had a clinical lacunar syndrome associated with appropriate CT changes or a typical clinical syndrome despite normal CT scans, or undefined stroke if their condition was not due to either of these mechanisms [8]. The present study only included patients with large vessel stroke and cardioembolic stroke, while the other subtypes were excluded because both atherosclerotic and non-atherosclerotic mechanisms might be involved.

During hospitalization, neurological evaluation was always done by one neurologist who applied the specified study criteria for classification of the patients. All evaluations were performed at the Department of Neuroradiology.

2.3. Selection of controls

The control subjects were chosen from among asymptomatic age-matched outpatients with hyperlipidemia who did not have cardiac disease or infections. The absence of atherosclerosis in the control subjects was assessed as follows: normal 12-lead ECG, normal echocardiography findings, <25% stenosis of the carotid arteries on Doppler ultrasonography, and normal lower limb arteries on physical examination. A history of cardiac disease meant exclusion from the control group.

2.4. Baseline evaluation

Data were collected by interview, physical examination, and neurological examination performed by trained health professionals, detailed review of all available medical records, and laboratory tests of fasting blood samples. Stroke patients were evaluated on day 7 after the onset of symptoms, while blood samples were taken within 24 h of admission (86% of the blood samples were obtained within 48 h after stroke onset). Control subjects were evaluated in the same manner as the stroke patients at the Stroke Prevention Clinic of the Department of Neurology between July 2002 and January 2003. Patients and control subjects were defined as hypertensive if they had a diastolic blood pressure >90 mmHg and a systolic blood pressure >140 mmHg or if they had been treated with antihypertensive therapy for at least 1 year. Patients were classified as diabetic if they had a fasting glucose level >126 mg/dL on two occasions or if they had been treated with antidiabetic drugs for at least 1 year. Patients were defined as smokers if they reported daily smoking of >10 cigarettes for at least 1 year during the last 10 years, and they were considered to be hyperlipidemic if the total cholesterol level was >220 mg/dL or if they had been treated with lipid-lowering drugs for at least 1 year. The BMI (kg/m^2) was calculated as a measure of obesity. All subjects reporting previous *H. pylori* eradication therapy were excluded from the study. The stroke patients

and controls lived in the same geographic area (Fukuoka City).

The type of ischemic stroke was classified according to Trial of Org 10172 in Acute Stroke Treatment (TOAST) criteria into large artery atherosclerosis, cardiogenic embolism, small artery occlusion, other etiologies, and undefined. Classification was performed by one neurologist on the basis of clinical findings and the results of standardized diagnostic tests, including CT or MRI, vascular imaging, and ECG or echocardiography.

2.5. Laboratory tests

All samples were stored at −80 °C and were analyzed simultaneously by technicians who were unaware of whether each sample belonged to a patient or a control.

2.6. IgG antibody to H. pylori

Serum IgG antibody to *H. pylori* was detected by ELISA [19] according to the manufacturer's instructions. Samples were tested in duplicate and results were expressed in arbitrary units. The reference limits were previously determined in our laboratory using serum samples from persons with or without *H. pylori* infections. A value >7.5 U was defined as positive, whereas values <3.0 U were negative (sensitivity and specificity >95%). Values between 3.0 and 7.5 U were defined as borderline and were excluded from the study.

2.7. ^{13}C-urea breath test

Each patient underwent a ^{13}C-urea breath test (UBT) by drinking 100 mg of ^{13}C-urea in water after an overnight fast. Breath samples were collected before and 20 min after the administration of ^{13}C-urea. The $^{13}CO_2/^{12}CO_2$ ratio ($\Delta^{13}CO_2$) in the breath bag was analyzed by a small infrared spectrometer (UBiT-200; Otsuka Electronics Co., Hirakata, Japan) and the results were expressed as the percent excess (parts per thousand) of $^{13}CO_2$ ($\Delta^{13}CO_2$). This method has been shown to have an excellent correlation (r = 0.996) with mass spectrometric measurement of $^{13}CO_2$ [20,21]. ^{13}C-UBT values below 3.5% were considered negative for *H. pylori*.

2.8. Brachial-ankle pulse wave velocity (baPWV)

The baPWV was measured using a volume plethymograph (PWV/ABI; Colin, Co., Ltd., Komaki, Japan), which simultaneously recorded the PWV, blood pressure, electrocardiogram, and heart sounds [6]. Each subject was examined in the supine position, with the electrocardiographic leads on both wrists, a microphone for detecting heart sounds taped at the left sternal edge, and cuffs on both arms and ankles. The cuffs were connected to a plethymographic sensor that determined the pulse volume waveform and to an oscillometric pressure sensor that measured the blood pressure. Pulse volume waveforms were recorded using a semiconductor pressure sensor with the acquisition frequency set at 1200 Hz. Waveforms for the arm and ankle were stored in 10 s batches with automatic gain analysis and quality adjustment. In this study, baPWV data were obtained after at least 5 min of rest. The coefficient of variation for reproducibility of baPWV values in healthy subjects was reported to be 2.4% for the interobserver coefficient of variation (n = 15) and 5.8% for the intraobserver coefficient of variation (n = 17) [22].

2.9. Detection of H. pylori infection

H. pylori infection was identified by histologic examination, the ^{13}C-UBT, and serologic evaluation. Patients in whom at least one of these three tests was positive were classified as *H. pylori*-positive and those in whom all three tests were negative were considered to be *H. pylori*-negative.

2.10. Statistical analysis

Odds ratios (ORs) and 95% confidence intervals (CIs) for the risk of ischemic stroke associated with *H. pylori* infection were estimated by univariate analysis, as well as by multiple logistic regression analysis with adjustment for age, sex, smoking history, diabetes, and hypertension.

Because C-reactive protein (CRP) values do not show a normal distribution, a non-parametric test (the Mann–Whitney U-test) was used to compare this variable between groups. All analyses were performed with SPSS software (ver. 8). When not otherwise stated, data are presented as the mean ± S.D. and P = 0.05 (two-tailed) was considered to indicate statistical significance.

2.11. Ethics

The design of this study was approved by the Ethics Committee and the Data Protection Committee of Harasanshin General Hospital (Fukuoka, Japan). Informed consent for the collection of blood was obtained from all patients (or their closest relatives).

3. Results

One hundred and three patients with stroke and 281 control subjects were considered for the study, but 51 patients and 138 control subjects were excluded for the following reasons: unclassified stroke subtype (13 patients), refusal to participate in the study (19 patients and 38 control subjects), previous *H. pylori* eradication therapy (9 patients and 21 control subjects), abnormal ECG (27 control subjects), abnormal echocardiography findings (31 control subjects), and asymptomatic carotid stenosis (21 control subjects). Therefore, 62 patients and 143 control subjects were investigated further.

Table 1
Characteristics of the subjects

	Stroke patients ($n = 62$)	Controls ($n = 143$)	P value
Age [years, mean ± S.D.]	71.5 ± 11.3	69 ± 9.3	N.S.
Male sex [%]	40 (65%)	48 (34%)	$P < 0.001$
Blood pressure [mean ± S.D., mmHg]			
Systolic	161.4 ± 31.4	132 ± 20.4	$P < 0.001$
Diastolic	84.2 ± 13.5	78.1 ± 11.1	$P < 0.01$
Smoking [%]			
Ever	31 (50%)	65 (46%)	N.S.
Never	31 (50%)	78 (55%)	N.S.
History [%]			
Hypertension	55 (89%)	47 (33%)	$P < 0.001$
Diabetes mellitus	26 (42%)	16 (11%)	$P < 0.001$
IMT [mean ± S.D., mm]	1.22 ± 0.35	1.13 ± 0.41	N.S.
baPWV [mean ± S.D., cm/s]	2,292.8 ± 665.1	1,527.4 ± 314.8	$P < 0.001$

3.1. Characteristics of the subjects

The clinical characteristics of the stroke patients and control subjects are displayed in Table 1. The mean age of the 62 stroke patients was 71.5 years (S.D. 11.3), and 40 of them (65%) were men. Their average systolic blood pressure and diastolic blood pressure was 161.4 and 84.2 mmHg, respectively. Among the 62 patients, 50% were recent or former smokers, 89% had a history of hypertension, and 42% had diabetes. The mean carotid intima/media thickness (IMT) was 1.22 mm and baPWV was 2292.8 cm/s.

3.2. Prevalence of chronic H. pylori infection

Table 2 shows the prevalence of seropositivity to *H. pylori* and *H. pylori* infection. Infection was detected in 48/62 stroke patients (77%) compared with 64/143 controls (45%) and the difference was significant ($P < 0.0001$). When analyzed for sex, 34/40 male stroke patients (85%) were infected by *H. pylori* compared with 18/48 controls (38%) ($P < 0.0001$). Among the women, 14 out of 22 stroke patients (64%) were infected compared with 46 out of 95 controls (48%) ($P < 0.05$). Chronic *H. pylori* infection was found in 40 patients (89%) with small artery occlusion, 6 patients (67%) with large artery atherosclerosis, and 2 patients (25%) with cardiogenic embolism.

Table 2
Crude prevalence of chronic *H. pylori* infection in each group

	Stroke patients ($n = 62$)	Controls ($n = 143$)	P value
All	48 (77%)	64(45%)	$P < 0.0001$
Age (years)			
<70	19 (74%)	31(40%)	$P < 0.01$
≥70	29 (81%)	33(50%)	$P < 0.01$
Sex			
Male	34 (85%)	18(38%)	$P < 0.0001$
Female	14 (64%)	46(48%)	$P < 0.05$
Stroke subtype			
Small artery occlusion	40 (89%)		
Large artery atherosclerosis	6 (67%)		
Cardioembolic	2 (25%)		

3.3. ORs for H. pylori infection in stroke patients and controls after adjustment for possible confounding factors (Table 3)

Chronic *H. pylori* infection showed an overall association with ischemic stroke when all of the stroke subtypes were combined. There was no significant difference in the prevalence of chronic *H. pylori* infection between patients with cardiogenic embolism and the control subjects, whereas the prevalence of infection was significantly higher in the patients with small artery occlusion than in the controls (univariate analysis showed OR: 9.68 and CI: 3.56–33.08 ($P < 0.0001$)). However, there was no significance difference in the prevalence of chronic *H. pylori* infection between the patients with large artery atherosclerosis and the control subjects.

3.4. ORs for association between chronic H. pylori infection and stroke subtypes

Conditional logistic regression analysis (Table 4) showed that chronic *H. pylori* infection was significantly associated with a higher risk of stroke due to small artery occlusion and large artery atherosclerosis. In contrast, there was a significant inverse correlation between chronic *H. pylori* infection and cardiogenic embolism (adjusted OR: 0.137; 95% CI: 0.0236–0.796). Despite these differential associations with the stroke subtypes, chronic *H. pylori* infection also showed an overall association with ischemic stroke (all subtypes combined).

3.5. C-reactive protein (CRP)

Measurement of CRP was performed in all stroke patients and control subjects. The CRP level was 1.13 ± 2.03 mg/dL in the stroke patients (all subtype combined) and 0.32 ± 0.87 mg/dL in the control subjects, showing a significant difference ($P < 0.001$). There was no significant difference of CRP between the patients with cardiogenic embolism and the control subjects, whereas the CRP level was significantly higher in patients with small artery occlusion and large

Table 3
Odds ratios for *H. pylori* infection after adjustment for possible confounding factors

	Ischemic stroke				Stroke subtype									
	(all subtypes)				Small artery occulusion				Large artery atherosclerosis				Cardioembolic	
	OR	RR	95%	CI	OR	RR	95%	CI	OR	RR	95%	CI	OR	RR	95%	CI
Age (years)																
<70	0.62	0.72	0.32	1.18	0.74	0.79	0.36	1.52	0.27	0.28	0.03	1.47	0.99	0.99	0.18	5.47
≥70	0.61	1.40	0.85	3.09	1.35	1.26	0.66	2.78	4.10	3.90	0.76	41.41	1.01	0.18	5.59	6.18
Male sex	3.57	2.42	1.84	7.09***	3.49	2.66	1.66	7.61**	2.77	2.66	0.57	17.59	1.34	1.33	0.24	7.43
Smoking	1.20	1.14	0.63	2.28	0.89	0.91	0.43	1.81	1.44	1.42	0.30	7.48	3.55	3.41	0.62	36.72
Hypertension	15.8	7.93	6.53	44.4***	9.90	6.56	3.86	30.31***	8.61	8.08	1.12	388.17*				
Diabetes mellitus	5.67	2.80	2.62	12.67***	2.45	1.94	1.07	5.48*	15.78	13.58	2.85	161.89**	4.15	3.88	0.74	23.33
large IMT	1.68	1.43	0.88	3.20	1.31	1.24	0.64	2.69	4.36	4.13	0.80	44.01	1.19	1.18	0.22	6.57
high baPWV	5.84	2.77	2.56	13.82***	6.07	3.43	2.62	14.34***	1.36	1.34	0.13	7.57	1.59	1.57	1.15	9.42
high CRP	21.39	4.88	8.60	59.67***	11.31	5.16	4.94	26.90***	5.14	4.71	1.05	27.21*	4.02	3.77	0.72	22.57
H. pylori infection	4.20	2.85	2.06	9.03***	9.68	6.64	3.56	33.08***	1.69	1.66	0.35	10.77	0.27	0.28	0.03	1.53

* $P < 0.05$.
** $P < 0.001$.
*** $P < 0.0001$.

artery atherosclerosis than in the controls (univariate analysis showed OR: 11.31 and CI: 4.94–26.90 ($P < 0.0001$); OR: 5.14 and CI: 1.05–27.21 ($P < 0.05$), respectively) (Table 3).

4. Discussion

4.1. Main findings

In the present case-control study, *H. pylori* infection was associated with an increased risk of ischemic stroke due to small artery occlusion or large artery atherosclerosis versus a decreased risk of stroke caused by cardiogenic embolism. There was also a strong association between *H. pylori* infection and the overall risk of stroke. Moreover, we found no difference in the prevalence of *H. pylori* infection among patients with two different stroke subtypes (large artery and small artery stroke) and controls. Our study showed that the presence of *H. pylori* infection might be increased in patients with stroke that is due to large artery and small artery disease but not in patients with cardiogenic embolism.

Although CRP (a sensitive marker of systemic inflammation) was increased in both groups of stroke patients compared with control subjects, the *H. pylori*-positive patients showed significantly higher CRP levels than the *H. pylori*-negative patients.

4.2. Chronic H. pylori infection and stroke subtype

Our findings were consistent with the results of some previous studies that have addressed the relationship between chronic *H. pylori* infection and ischemic stroke.

Markus and Mendall [14] reported elevated levels of IgG antibody for *H. pylori* in patients with lacunar stroke, which is comparable to small artery occlusion [8]. The adjusted OR was 2.51 (95% CI: 1.19–5.28), which was compatible with our findings (OR: 9.68; 95% CI: 3.56–33.08). The classic lacunar hypothesis is that lipohyalinosis of small arteries caused by diabetes mellitus and hypertension represents the underlying pathogenesis of this stroke subtype [21]. In recent years, however, evidence has been obtained that lacunar infarcts also share the mechanisms involved in atherosclerotic disease [23]. Similar trends were demonstrated for stroke caused by large artery atherosclerosis in the study performed by Markus and Mendall [14] and the present study (adjusted OR: 2.17; 95% CI: 1.11–4.21 and adjusted OR: 1.69; 95% CI: 0.35–10.77, respectively), although the association did not reach statistical significance in our study, possibly because of the small number of patients with this stroke subtype.

Unfortunately, comparison of the role of *H. pylori* in cardioembolic stroke could not be performed between the two studies because Markus and Mendall [14] combined their data for stroke due to cardiogenic embolism and stroke due to undefined causes. Since cardioembolic stroke is mainly caused by disorders such as atrial fibrillation that lead to thromboembolic occlusion of the cerebral arteries [8], our