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*J Immunol* 2005; 175:3262-3267; doi: 10.4049/jimmunol.175.5.3262
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Regulation of Aged Humoral Immune Defense against Pneumococcal Bacteria by IgM Memory B Cell

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Elderly persons have a high incidence of lethal infections by encapsulated bacteria. However, mechanisms involved in their poor defense and maintenance of immunological memory have been poorly understood. The present study characterized the population of B cells known as IgM memory B cell compartment and their response by pneumococcal vaccine in elderly people. CD27+ memory B cells, particularly IgD−IgM+CD27+ IgM memory B cells, had dramatically declined in the aged. Their Ig syntheses by B cells and the differentiation into plasma cells were diminished in vitro compared with those in adults. A rise of anti-pneumococcal IgM in sera of elderly persons was found with lower levels compared with those in adults after pneumococcal vaccination. Although diminished function itself of aged B cells surely exist, decline of the IgM memory B cell pool is expected to result in a poor humoral immunity against pneumococcal infection in elderly people. The Journal of Immunology, 2005, 175: 3262–3267.

The secondary immune response is crucial for disease prevention and depends entirely on the immunological memory that is carried by memory B cells and memory T cells (1). The mechanisms with which immunological memory is maintained after infections or vaccination are complex and a matter of debate (2). The most dramatic health problem of the aged immune system is the increasing rates of morbidity and mortality from recurrent and invasive infections of the respiratory tract caused by encapsulated bacteria such as Streptococcus pneumoniae (2, 3). Related effects include diminished protective immunity after prophylactic vaccines, blunted reactivity to tuberculin skin tests, and re-emergence of latent infections (2). The impaired response of the elderly to most vaccines and the greater susceptibility of the elderly to lethal infections by encapsulated bacteria have fostered a view that immune senescence is the chief cause of pneumatic infections (8, 9). S. pneumoniae is the chief cause of pneumonia in older adults. The effectiveness of the pneumococcal polysaccharide vaccine, which was associated with a significant reduction in the risk of pneumococcal bacteremia as reported previously (10), on IgM memory B cells, the origins and characteristics of which were highlighted recently.

Materials and Methods

Study populations

One hundred thirty healthy subjects were enrolled in this study. Adults were 65 healthy blood donors (21–64 years of age, 32 women and 33 men). In the group of elderly persons, 65 elderly persons (65–99 years of age, 37 women and 28 men) living in their homes or in an institution for elderly self-sufficient people were recruited. The volunteers were regarded as eligible if they had no clinically significant diseases and conditions such as diabetes mellitus, cancer, collagen diseases, various kinds of infections, and any treatment with immunosuppressive drugs. Also, none of them suffered from ongoing bacterial infections at the time of analysis.

Blood sampling and cell preparation

Peripheral blood (PB)2 samples were obtained from healthy volunteers after the informed consent of the study was given. Mononuclear cells (MNCs) were isolated from PB by Ficoll-Hypaque (Pharmacia) density gradient centrifugation. CD19+ B cells were enriched to 95–98% by magnetic cell separation using the MiniMACS system (Miltenyi Biotec).

Flow cytometric analysis

PB MNCs were stained with a combination of anti-IgD-FITC (DAKO Japan) or anti-IgM-FITC (DAKO Japan), anti-CD20-PerCP (Becton Dickinson) or anti-CD19-PerCP (BD Biosciences), and anti-CD27-biotin (8H5; IgG1) (14), followed by streptavidin-PE (Sigma-Aldrich). Conjugation of biotin to anti-CD27 mAb was performed by the standard technique using N-hydroxysuccinimido-biotin (Sigma-Aldrich) in our laboratory. Purified B cells after activation were stained with a combination of CD38-FTTC (Immunotech) and CD20-PE (DAKO Japan). Staphylococcus aureus Cowan strain (SAC) and propidium iodide were obtained from Sigma-Aldrich, and human rIL-2 was obtained from Genzyme. Dead cells were

2 Abbreviations used in this paper: PB, peripheral blood; MNC, mononuclear cell; SAC, Staphylococcus aureus Cowan strain.
removed by staining with propidium iodide (Sigma-Aldrich). Flow cytometric analysis was then performed by FACScan (BD Biosciences).

**Ig assay**

The amount of serum IgG from adults and elderly persons was measured by a latex agglutination test. For the IgG, IgM, and IgA syntheses, MNCs or purified B cells were cultured with SAC plus IL-2. The cells were cultured for 8 days at 37°C in a humidified atmosphere with 5% CO2. The final cell density was 2.5–5/10⁵/ml in a volume of 200 μl/well. The plates were coated with goat anti-human Igs (Southern Biotechnology Associates) for the detection of IgG, IgM, and IgA. The cultured supernatants were harvested and added to 96-well flat ELISA plates (Nunc). The standard human IgG, IgA, or IgM (Sigma-Aldrich) was also added to the plates. After an overnight incubation, supernatants were discarded, and the plates were washed with 0.05% Tween 20 in PBS. Alkaline phosphatase-labeled goat anti-human IgG, IgA, and IgM at a dilution of 1/2500 was added for the detection of IgG, IgA, and IgM, respectively. After 2 h of incubation at room temperature, color detection was performed by 3-[cy clohexylamino]-1-propanesulfonic acid buffer containing p-nitrophenyl phosphate (Sigma-Aldrich). Calibration was performed with PBS at standard zero levels. In this ELISA system, no cross-reaction between IgG, IgA, and IgM occurred.

**Polysaccharides and sera**

Pneumococcal Ags, pneumovax (1 vial of valium is 0.5 ml containing 25 mg of each Ag), which include 23 types of polysaccharides 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, and 33F, were obtained from Banyu.

**Vaccination**

Healthy elderly persons who hoped for pneumococcal vaccination were immunized s.c. in the arm with 1 vial of pneumovax (a 0.5-ml volume containing 25 μg of each of the 23 types of polysaccharides) only one time. Before vaccination and 4 wk after vaccination, sera and PB heparinized samples from the vaccinated persons were obtained and used to measure specific Abs by ELISA and investigate B cell populations by flow cytometric analysis.

**FIGURE 1.** Marked decline of IgM memory B cells in elderly persons. A. MNCs from cord blood and PB of adults (30 years old) or elderly persons (60, 70, 80, and 90 years old) were stained with anti-IgD-FITC, anti-CD20-PerCP, and anti-CD27-biotin, followed by streptavidin-PE. Three-color analysis was conducted by gating CD20-positive B cells. Data were displayed as density plots with green (FITC) fluorescence, IgD, on the x-axis and orange (PE) fluorescence, CD27, on the y-axis by the log scale. The percentages of positive cells are indicated. B. MNCs from 65 adults (65 years old) and 65 elderly (≥65 years old) persons are stained as described above. Data are displayed as dot plots with age on the x-axis and percentage of positive cells on the y-axis. The percentages of IgD−CD27− naive B cells and CD27+ memory B cells, including IgD−CD27+ (IgM memory) and IgD−CD27+ memory B cells, within the CD20+ cell population are shown. The correlation between age and the percentages of IgD−CD27− naive B cells, CD27− memory B cells, IgD−CD27+ B cells, and IgD−CD27+ B cells within the CD20+ cell population was r = 0.548 (p < 0.001), r = 0.651 (p < 0.001), r = 0.700 (p < 0.001), and r = 0.521 (p < 0.001), respectively. C. MNCs from adults (n = 7; □) or elderly persons (n = 7; □) were stained with anti-IgG-FITC, anti-IgM-FITC, or anti-IgA-FITC, followed by streptavidin-PE. Surface IgG, IgM, or IgA expression on IgD−CD20+ B cells was evaluated.
IgM Abs against pneumococcal polysaccharides

Specific IgM Abs against pneumococcal polysaccharides were determined by ELISA designed according to the standardized ELISA protocol with few modifications. The 96-well flat ELISA plates (Nunc) were coated with 0.5 μg/ml pneumovax, which is composed of 23 polysaccharides (Banyu) and were blocked by 0.25% BSA in PBS for 4 h. Serum samples were diluted 20 times with PBS including 0.6% BSA. The diluted standard sera ranging from 1/10 to 1/160 and samples were added to each well. After overnight incubation at 4°C, the wells were washed with 0.05% Tween 20 in PBS. Alkaline phosphatase-labeled goat anti-human IgM (Sigma-Aldrich), at a dilution of 1/2500, was added for the detection of specific IgM Ab. After 2 h of incubation at room temperature, color detection was performed by 3[cyclohexylamino]-1-propanesulfonic acid buffer containing p-nitrophenyl phosphate (Sigma-Aldrich). Absorbance was read at 405 nm.

Statistical analysis

Data from individual experiments were expressed as mean ± SD. Data were analyzed with StatMatelII software (ATMS), and correlation coefficients were obtained using SOKAN in the software. Statistical significance was determined using paired or unpaired Student’s t-test, and p < 0.05 was considered to be statistically significant.

Results

Analyses of PB B cell subsets

Circulating PB B cells obtained from 65 healthy adults (<65 years old) and 65 healthy elderly persons (≥65 years old) were examined. As reported previously (15–17), adult B cells could be separated into three subpopulations on the basis of CD27 and IgD expression: IgD⁺CD27⁻ naïve B cells, IgD⁺CD27⁺ (IgM memory) B cells, and IgD⁻CD27⁺ B cells. In this study, we included IgM-only memory B cells (16 ± 7% in adult IgD⁻CD27⁺ B cells), which are unclass-switched memory B cells, into IgD⁻CD27⁺ B cells, a majority of which are composed of switched memory B cells (78 ± 13% in adult IgD⁻CD27⁺ B cells). In contrast, cord blood B cells consist predominantly of naïve B cells (Fig. 1A). The percentages of IgM memory B cells and IgD⁻CD27⁺ memory B cells in PB B cells increased during childhood and adulthood and peaked at ~30 years of age. An especially notable finding in this study revealed that IgM memory B cells markedly reduced with age (r = −0.700), and IgD⁻CD27⁺ memory B cells also reduced to a slight degree (r = −0.521), especially in individuals ≥50 years of age (Fig. 1B).

We also evaluated B cell populations by the expression of CD19 or IgM. In our experiments, as shown in Fig. 2A, when we gated on CD19, the percentages among each B cell population was almost identical to those gated on CD20 (10 elderly persons, CD20 or CD19 gated naïve B cells, IgM memory B cells, and...
IgG−CD27− memory B cells were 77.01 ± 5.50% and 76.37 ± 5.63, 4.33 ± 1.02, and 5.23 ± 1.15%, 16.33 ± 3.86% and 16.64 ± 3.35%, respectively. Also, when we used IgM instead of IgD, the difference in percentages among each B cell population was barely recognized (Fig. 2A). Due to the known reduction in the total number of PB B cells in the aged, the increased levels in naive B cell percentages had no impact on the absolute number of naive B cells in the aged, whereas the absolute number of memory B cells, especially IgM memory B cells, significantly decreased (Fig. 2B).

These results show that circulating IgM memory B cells are strongly reduced in elderly people compared with those in normal adults.

**B cell functions in elderly people**

The declines of memory B cells in elderly persons led us to examine whether Ig productions were different from adults to elderly persons. We found similar levels of serum IgG in the elderly persons in the study compared with those of adults (1468 ± 35.5 mg/dl in elderly persons (n = 60) vs 1407 ± 3.8 mg/dl in adults (n = 60); not significant by Student’s t test). PB MNCs isolated from the elderly produced only moderate levels of IgA, IgM, and IgG in response to stimulation with SAC and IL-2 compared with those taken from adults (Fig. 3A). To exclude effects by T cells and monocytes, we purified B cells by positive selection using CD19 microbeads. The highly purified B cells in adults produced substantial amounts of IgG, IgM, and IgA after being stimulated with SAC plus IL-2, whereas Ig syntheses in elderly persons were remarkably diminished (Fig. 3B).

We also investigated the generation of plasma cells from highly purified B cells in elderly persons and adults. We detected plasma cells by the expression of high levels of CD38 and low levels of CD20, which were identified morphologically as plasma cells (i.e., basophilic cytoplasms with pale Golgi zone and eccentric nuclei) (data not shown). After stimuli, the induction of the differentiation into the plasma cell was decreased in elderly persons compared with that in adults (Fig. 3, C and D). Statistical significance was found between the plasma-cell inductions in elderly persons and adults (p < 0.05) (Fig. 3D). These findings show that Ig syntheses and plasma cell differentiation in B cells of elderly persons fall and their B cells have similar characteristics of naive B cells.

**Effect of pneumococcal polysaccharide vaccine**

The marked reduction with aging of IgM memory B cells, which may predominantly produce anti-polysaccharide IgM, raised the possibility of the change of IgM memory B cell compartment in circulating B cells after pneumococcal polysaccharide vaccination. To investigate this idea, we examined the population of memory B cells known as IgG−CD27+ and IgM memory B cells before and after vaccination. Specific IgM Abs to pneumococcal polysaccharides were detected both in young adult and aged sera. OD levels of the pneumococcus-specific serum IgM Abs in elderly persons were two-thirds of that in young adults before vaccination, and an ~2-fold increase in OD was found in sera of both groups after vaccination (Fig. 4A). Interestingly, marginal levels of increase in IgM memory B cells, but not in IgG−CD27+ memory B cells, were recognized after the vaccination. Significance was found in the percentages of IgM memory B cells before and after vaccination (Fig. 4B). Thus, pneumococcal vaccination, at least some part, has an effect on IgM memory B cells, probably resulting in an elevation of anti-polysaccharide IgM.

**Discussion**

In this study, we investigated the effects of IgM memory B cells, which are proposed to provide the splenic marginal zone and be

![FIGURE 3](http://www.jimmunol.org/) Ig synthesis and plasma cell differentiation by B cells. A, MNCs (10⁵/well) from adults (n = 20) or elderly persons (n = 20) were stimulated with SAC and IL-2. Eight days after being stimulated, the supernatants were harvested, and their IgA, IgM, and IgG concentrations were measured by ELISA. Final concentrations of SAC and IL-2 were 0.01% and 50 ng/ml, respectively. □, <65 years old; ▪, ≥65 years old. B, The amounts of IgG, IgA, and IgM secreted in supernatants of highly purified B cells cultured under the condition described in A were measured (▲, <65 years old, n = 9; ○, ≥65 years old, n = 10). Results are shown as means ± SD. *p < 0.05. C, B cells from an adult and an elderly person were incubated with SAC plus IL-2 for 8 days, and two-color analysis using anti-CD38-FITC and anti-CD20-PE was performed by flow cytometry. CD38 and CD20 were used as a surface marker of plasma cells and B cells, respectively. D, Percentages of the induced plasma cells. B cells in adults (▲) and elderly persons (○) were incubated with SAC plus IL-2, and CD38-expressing cells were detected by flow cytometry. Values are the mean ± SD. *p < 0.05.
Surely, in our experiments, some elderly persons had a little degree of IgM memory B cells. However, the percentages of IgD and CD27, it is conceivable that their findings -axis. Results are shown as means ± SD. *p < 0.05. B, Percentages of IgM memory B cells (IgD CD27- B cells) and IgD CD27- B cells in CD20- B cells in elderly persons before (□) and 4 wk after (□) vaccination. Values are shown as means ± SD (n = 10); *p < 0.05.

responsible for the protection against encapsulated bacteria, on aged humoral immune defense. Memory B cells, in particular IgM memory, diminished markedly in the aged accompanied with reduction of plasma cell differentiation potency and Ig syntheses. In addition, levels of anti-pneumococcal IgM in sera of elderly persons were lower than those of young adults after pneumococcal vaccination.

In the aged immune system, the number of T cells and their response to Ag are declined (18). One well documented significant T cell change is the gradual shift from CD45RA+ CD4+ naive toward an increase in CD45RO+ CD4+ T cells that represent activated or memory phenotype (19). However, the memory T cell population in aged persons is reported to be hyporesponsive against pathogens, and the loss of optimal IL-2 production may participate in the aging process and may represent the main Ag-independent defect in the CD4+ T cells (20). In contrast to our knowledge of memory T cell functions in the elderly, it is not known whether there is an age-associated change in the naive and memory distribution in B cells. In regard to the change in total B cell number, a reduction in the proportion of B cells in the PB has been found in elderly persons (21), and a decline in CD19+ B cells during aging has been noticed in tonsillar lymphocytes (22). In contrast with our findings, Colonna-Romano et al. (23) reported that the percentage of CD27+ B cells increase in the elderly. Surely, in our experiments, some elderly persons had the considerable percentages of IgD CD27- memory B cells, but extremely a little degree of IgM memory B cells. However, the percentages of CD27+ B cells in total B cells range from 0 to 90 in 80- to 100-year-olds in this study. When B cells are separated by the expression of IgD and CD27, in conceivable that their findings will become reliable. Surface expression levels of each Ig class on IgD-CD27- memory B cells in young and elderly persons resembled (Fig. 1C), implying that IgM-only and each switched memory B cells diminished equally during aging. A statistically significant age-related increase in the serum levels of Ig isotypes (IgG and IgA but not IgM) and IgG subclasses (IgG1, IgG2, and IgG3, but not IgG4) was reported by Ginaldi et al. (18). Although the number of circulating B cells in elderly persons was barely reduced in our study (data not shown), their serum IgG was the same level. The discrepancy between the same serum IgG levels and decreased differentiation potency in plasma cells may be due to other factors such as circulating PB plasma cells and long-lived plasma cells. It is also reported that aging is associated with a decreased diversity in the Ab response reflected in the high-affinity Abs after immunization with the foreign Ag (4, 21). These findings are content with our data of the decrease in IgD CD27+ and IgM memory B cells in elderly persons.

The most definitive marker of memory B cells is somatic hypermutation in the Ig V-region genes; through this process, the generation of highly diverse Abs with high affinity can be achieved (24). To be effective, memory B cells must be able to differentiate rapidly into plasma cells and to produce high-affinity Ag-specific Abs efficiently during the secondary immune response. Triggering via B cell Ig receptors by Ags, cytokines such as IL-2 and IL-10, and direct cell-to-cell contact between T and B cells play an important role in the differentiation into plasma cells. In regard to B cell Ig synthesis, although the possibility remains that functions itself of aged B cells are declined, a decrease in both of two CD27+ memory B cell pools may result in the impaired differentiation into plasma cells and the diminished production of high-affinity Abs against pathogens in the elderly.

Bacteria such as S. aureus and Homophone influenza are known to cause post-influenza pneumonia, but S. pneumoniae is the most prominent pathogen causing secondary bacteria pneumonia, especially in elderly people (25). However, S. pneumoniae is a common cause of pneumonia but not always the most common cause, because agents vary in different clinical populations. Regarding defense from S. pneumoniae infections, many components of the immune system are likely involved in the defense. B cells and Abs may be especially important for the defense from blood-borne infections. Recently, it was reported that increased susceptibility to secondary pneumococcal pneumonia is at least in part caused by excessive IL-10 production and reduced neutrophil function in the lung (26). In young children, the response to T-independent Ag has been reported to be defective with a high incidence of infections caused by encapsulated bacteria. The splenic marginal zone is especially well equipped for rapid humoral responses and is unique in its ability to initiate an immune response to encapsulated bacteria (27), suggesting the importance of the spleen for host immune defense toward encapsulated bacteria infections. Our data suggest that the remarkable age-related decline in IgM memory B cell compartment in elderly persons may have an influence on their humoral immunity. So, it is expected that strategies targeting the maintenance of the IgM memory B cell pool is valuable in the treatment of this condition.

IgD CD27+ memory B cells can produce IgG, IgM, and IgA, whereas IgM memory B cells predominantly produce IgM (7, 28). A complete absence of the IgD CD27+ memory B cell population, which results in severe susceptibility to infections, is found in some diseases involving primary immunodeficiency such as X-linked hyperIgM syndrome (29) and common variable immunodeficiency (30). Some patients of X-linked hyperIgM syndrome and common variable immunodeficiency have IgM memory B cell phenotypes with/without somatic hypermutation, and the presence of IgM memory B cells somewhat correlates with their clinical aspects (31). B cell proliferation can be triggered by polyclonal stimuli derived from microbial products, such as lipopolysaccharides or unmethylated single-stranded DNA motifs (CpG oligonucleotides), which stimulate B cells via TLR4 and TLR9, respectively (32). The sensitivity to polyclonal stimuli represents a key feature of human memory B cells (33). Although the exquisite sensitivity of switched memory B cells to bystander help may be instrumental in maintaining systemic IgG Ab levels, the capacity of IgM memory B cells to respond to CpG in the absence of cytokines could be instrumental in maintaining levels of natural Abs to bacterial Ags (34, 35). Given the findings that functions in the

FIGURE 4. Effects of pneumococcal polysaccharide vaccine on production of IgM Ab specific for pneumococcal polysaccharide and IgM memory B cells. A, The levels of the concentration of IgM specific for pneumococcal polysaccharide in 20x dilution of sera were measured by ELISA in three young adults (□) and five old adults (□) before and after vaccination. Polysaccharide-specific IgM is indicated as scale in OD on the left y-axis. Results are shown as means ± SD. *p < 0.05. B, Percentages of IgM memory B cells (IgD CD27- B cells) and IgD CD27- B cells in CD20- B cells in elderly persons before (□) and 4 wk after (□) vaccination. Values are shown as means ± SD (n = 10); *p < 0.05.
aged spleen are declined during aging (12, 13) and the incidence of lethal infections by pneumococcal infections increases during aging; the spleen may be the central organ in defense against encapsulated bacteria infections.

In conclusion, besides that circulating IgM memory B cells correspond to splenic marginal zone B cells and they are provided from the spleen as reported recently (9), decline of splenic functions may reflect diminished numbers of aged IgM memory B cells. Effectiveness of pneumococcal polysaccharide vaccine in older adults on protection against pneumococcal infections may be associated with the increase and activation of circulating IgM memory B cells, resulting in rapid synthesis of anti-polysaccharide IgM Abs.

Disclosures
The authors have no financial conflict of interest.

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