Immune profile of asplenic patients following single or double vaccine administration: A longitudinal cross-sectional study

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ABSTRACT

Objective: Splenectomy poses a lifelong threat for the development of uncontrolled sepsis despite vaccination. As it is impractical to measure the levels of each antibody against 23 most frequent bacterial serotypes, different surrogate markers of immune response should be identified.

Material and Methods: Forty-eight patients with benign disorders were vaccinated with Pneumo-23 and Act-HIB before or at the day of surgery. The immunological response and opsonization capacity of the patients after splenectomy was analyzed through the quantitative measurement of IgG, IgM, C3, and C4 titers; flow-cytometric analysis of (CD3+) T-lymphocytes and (CD19+) B-lymphocytes; and isolation of CD27+ B cells by immunomagnetic positive selection. Blood samples were drawn at the sixth month and 5 and 7 years after surgery.

Results: The mean follow-up period was 98.4 months. All the patients in this series had normal IgG, C3, C4 levels and a normal distribution of CD19+ B-cells and CD8+ T-cells in three follow-up periods. Moreover, C3 levels markedly improved to 133.5 ± 37.3 mg/dL at 5 years and remained stable thereafter. CD19+ B-lymphocyte values have progressively improved to the normal range in 98% patients at 7 years. Further, low levels of CD27+ B-cell population (memory cells) was observed in only 12.5% patients at the last follow-up. Adequate seroconversion of IgG, IgM with normal C3, C4, and CD19+ B-cell levels were accomplished in almost all patients. Early postoperative death and late overwhelming infections did not occur.

Conclusion: Our results are indicative of the resumption of the immune function following Pneumo-23 and Act-HIB administrations, instigated by the probable activation of B cells and adequate production of C3, C4, IgG, and IgM antibodies in remote lymphoid tissues.

Keywords: Splenectomy, opsonins, CD3, CD19, pneumococcal prophylaxis, haemophilus influenza prophylaxis

INTRODUCTION

Despite being an important therapeutic method for some hemolytic diseases [hereditary spherocytosis, idiopathic thrombocytopenic purpura (ITP), thalassemia, and idiopathic autoimmune hemolytic anemia] splenectomy is still a potentially morbid procedure and renders the patient susceptible to the development of overwhelming sepsis which, despite vaccination, poses a life long threat (1-4). The altered clearance of encapsulated microorganisms (i.e., *Streptococcus pneumoniae*, *Meningococcus* and *Haemophilus influenzae type B*) in asplenic patients may lead to fatal infections, especially in elderly and in patients whose primary disease involves the reticuloendothelial system (2, 5). Immunogenicity of pneumococcal polysaccharide vaccines is definitely demonstrated in healthy adults as well as in patients with diabetes mellitus, chronic pulmonary disease and asplenia (6). However, there is still no consensus on the protective antibody threshold following vaccination against *S. pneumoniae* (7), and it is impractical to measure the levels of each antibody against 23 most frequent bacterial serotypes in current practice; thus, different surrogate markers of immune response should be identified. Close correlation exists between vaccine-induced immunogenic activity and the concentration of serum IgG, IgM, and IgA antibodies (8, 9), opsonophagocytic activity (10), T-cell activation, and level of antibody secreting cells (11). In light of the previous literature, antibody titers of IgG, IgM, C3, and C4 were quantitatively measured and flow-cytometric analysis of (CD3+) T-lymphocytes, (CD19+) B-lymphocytes were performed in this group to demonstrate seroconversion, the adequacy of immunological response, and the opsonization capacity. In addition, the percentage of CD3+/CD16+CD56 Natural Killer (NK) lymphocytes and CD4+/CD8+ (helper/suppressor) lymphocyte ratio were investigated to bring out NK-mediated cytotoxicity and to evaluate the immune status of these asplenic patients being susceptible to immune deficiency in the long-term.

Finally, immunoglobulin IgM+ peripheral blood B cells expressing the CD27 cell surface antigen were examined to detect the so-called "memory marker" for *Streptococcus pneumoniae* (12).

MATERIAL AND METHODS

After receiving informed consent, 48 adult patients who had undergone elective or urgent splenectomy for diagnostic and therapeutic indications between May 1999 and May 2013, were enrolled in this longitudinal cross-sectional single-center study.

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Routine preoperative vaccination was a standard policy for all patients scheduled for elective splenectomy, and these cases were vaccinated on average eight days (range: 3–27 days) before surgery. Pneumo-23 (Aventis-Pasteur), a 23-valent polysaccharide vaccine against *Streptococcus pneumoniae*, was administered to all patients and Act-HIB (Aventis-Pasteur), a vaccine against *Haemophilus influenza type B* was added to the immunization practice after January 2002 and was concurrently applied to 23 patients. For all urgent cases, antibiotic prophylaxis was mandated for a period of 2 weeks and vaccination was strictly conducted on the day of splenectomy. The perioperative prophylactic antibiotics used were amoklavin+clavulanic acid or piperacillin+tazobactam. Early and delayed infections and their causative agents were recorded and medical measures were promptly undertaken. Infections that required hospitalization were classified as serious. In-hospital death or death within 30 days of discharge was accepted as early postoperative death.

An accessory spleen was not demonstrated in any patient, either at the images of preoperative computed tomography of the abdomen or during surgical exploration.

To demonstrate seroconversion of immunoglobulins after vaccination and to reveal the ongoing opsonizing capacity of the immune system, hemogram and white blood cell distribution with antibody titers of IgG and IgM, plasma C3 and C4 levels, and flow-cytometric analysis of (CD3+) and (CD19+) lymphocytes were quantitatively measured in all patients at the sixth month postsurgery and the aforementioned parameters with CD4+/CD8+ T-cells and the percentage of CD3/CD16+CD56 NK-cells were evaluated at the targeted mean follow-up of 5 and 7 years after surgery for the whole group, which had been completed at May 2009 and September 2012. Additionally, Human Memory B Cells (CD27+/B-cells) were isolated with a two-step method from frozen peripheral blood nucleated cells at 7 years after surgery. A blind laboratory analysis was performed by the specialist not being aware of the primary disease, surgical treatment, and vaccination status of the patients at the time of sampling.

Becton-Dickinson Simultest CD3/CD19, CD4/CD8, and CD3/CD16+CD56 (BD Biosciences 2350 Quine Drive San Jose, CA 95131 USA) was used for enumerating percentages of mature human T and B lymphocytes, helper/inducer and suppressor/cytotoxic lymphocytes, and NK-cells in erythrocyte-lysed whole blood. White blood cell counts were maintained in the range of 3500–9400/mm³ in all samples. The fluorochrome-labeled monoclonal antibodies were used to identify lymphocyte subpopulations. The stained cells were subsequently introduced into the flow cytometer and the emitted light was collected and processed.

IMMAGE Immunochemistry Systems (Beckman Coulter, Inc. 4300 N. Harbor Blvd., Fullerton, CA 92835 USA) was used for IgG and IgM measurements with near infrared particle immunoassay technology. C3 and C4 reagent was used for the quantitative determination of complement C4 and C3 in human serum by rate nephelometry.

The EasySep Human Memory B Cell Isolation Kit is used to isolate CD27+ B cells from frozen peripheral blood nucleated cells

by immunomagnetic positive selection method. CD2, CD3, CD14, CD16, CD36, CD43, and CD56 are separated using a magnet without the use of columns. Following pre-enrichment, CD27+ B cells are selected using human CD27 + selection cocktail. Cells are targeted with tetrameric antibody complexes recognizing CD27+ B cells and dextran-coated magnetic particles. Labeled cells are separated by magnet, kept in the tube, and the unwanted cells are separated. Normal value for CD27+ B cells were 32%–56% of all peripheral blood B cells.

Normal values for opsonins and immunoglobulins and lymphocyte subset reference ranges were as follows: C3 (79.0–152.0 mg/dL), C4 (16.0–38.0 mg/dL), IgG antibody level (751–1560 mg/dL), IgM antibody level (46.0–304.0 mg/dL), distribution of CD3 (61%–85%), CD19 (7%–23%), NK (6%–29%), CD4 (28%–58%), and CD8 (19%–48%).

Statistical Analysis

All statistical analyses were performed using the Statistical Package for Social Sciences (SPSS 11.0 for Windows, Chicago, IL, USA). Results were expressed as mean values $\pm$ SD and a p value less than 0.05 was considered to be significant. Because the laboratory assessments were complete in all patients, Student's t-test was merely used to examine differences between these parametric data.

RESULTS

There were 21 male and 27 female patients. The age of the patients ranged from 17 to 63 years, with a mean of 42.3 ± 5.45 years. The follow-up was complete in all but three patients and ranged from 10 to 174 months, with a mean of 98.4 months. The demographic characteristics of the study group is shown in Table 1.

Of the 48 patients with benign disorders, there were 14 cases of immune thrombocytopenic purpura (ITP) and six autoimmune hemolytic anemia (AIHA). In addition, four patients presented with isolated hydatid disease, 22 patients had traumatic rupture of the spleen (including two iatrogenic trauma during operation), and two patients had splenic hamartoma.

The operative morbidity was 4%. Postoperative hemorrhage to the left subphrenic space occurred in one female patient with AIHA and one female patient with splenic trauma. These patients required laparotomy and recovered uneventfully. The hospitalization period ranged from 5 to 53 days, with a mean of 12.7 days. Three patients with benign hematologic disorders (two ITP and one AIHA) died because of myocardial infarction (two patients) and intracerebral hemorrhage (one patient) during the follow-up. One patient with splenic trauma died due to uncontrolled diabetes and ketoacidotic coma 4 years after surgery. There was no early postoperative death in this series. In our clinical survey, death from overwhelming postsplenectomy infection was nil. Overall mortality was 8%.

The immune profile data of 48 patients collected at the sixth month after splenectomy and at the mean follow-up of 5 years is shown in Table 2.

All of the patients in this series had normal IgG levels in the first two blood samplings (Table 2). The mean values were 1417.4 ± 353.4 mg/dL and 1360.5 ± 387.8 mg/dL, respectively.

Table 1. Demographic data of the study group

Splenectomy Indication	ITP and AIHA	Trauma and Splenic Mass
Number of patients	20	28
Sex		
(Male/Female)	6/14	15/13
Pneumo-23 vaccine		
Before splenectomy/After splenectomy	20/0	6/22
ACT-HIB vaccine		
Before splenectomy/After splenectomy	12/0	4/7
Comorbid disease	11*	7 [†]
Morbidity	1	0
Mortality	3/20	1/28
ITP: idiopathic thrombocytopenic purpura; AIHA: autoimmune hemolytic anemia Pneumo-23 vaccine: a 23-valent polysaccharide vaccine against <i>Streptococcus pneumonia</i> ACT-HIB vaccine: a vaccine against <i>Haemophilus influenza type B</i> *3 diabetes mellitus, 6 hypertensive atherosclerotic cardiac disease, 1 kidney transplant, 1 psoriasis [†] 2 chronic obstructive pulmonary disease, 2 diabetes mellitus, 2 coronary artery disease, 1 hydatid disease of liver		

Subnormal IgG levels (<751 mg/dL) were not observed in any patient at any sampling. Mean IgM levels were 103.9±61.0 mg/dL at 6 months after operation and 95.3±71.6 mg/dL at 5 years of splenectomy. One patient with ITP had subnormal levels (38.0 mg/dL) at sixth month that did not improve during the follow-up (38.1 mg/dL at 5 years). Further, two patients (one with ITP and the other with AIHA) had equivocal and subnormal IgM levels (45.0 and 38.0 mg/dL) at 5 years. Despite these subnormal levels of IgM, these three patients had no evidence or history of any serious infection. C3 and C4 levels were almost within normal range in all subjects. Moreover, C3 (major opsonin) levels significantly improved from a mean of 108.1±23.7 mg/ml to 133.5±37.3 mg/dL during the follow-up (p=0.002).

The mean distribution of CD3 decreased from 63.3%±11.4% to 57.5%±12.3%. In a separate analysis, the percentages of CD3+ T-lymphocytes ranged 45%-55% in five patients (4 ITP, 1 AIHA) at the sixth month. The percentage was normalized in AIHA patient but reduced further in all ITP patients. Finally, ten patients (9 ITP, 1 splenic trauma) demonstrated subnormal CD3+ T-cell distribution at five years. Contrary to the progressive reduction in CD3+ T-cells, CD19+ B-lymphocyte values have progressively improved to normal range in 12 of 13 patients (10 from ITP & AIHA and 3 from trauma groups) who had flow cytometric distribution of less than 7% at the sixth month. Depressed CD19 level persisted only in a patient with ITP and was 5% in both measurements.

The distribution of lymphocyte subpopulations at 5 years were as follows: CD4 (range: 16%-56%; mean, 30.0±2.34%), CD8 (range: 19%-47%; mean, 35.8%±2.7%) and NK cells (range, 3%-57%; mean: 21.2%±3.1%). CD8 was normal in every individual in both periods. At 5 years, 5 patients with ITP had reduced distribution of CD4 and three of them had concomitant de-

Table 2. Immunological variables at the postoperative sixth month and five years (mean±standard deviation)

	Normal values	6 th month	5 years	p
IgG (mg/dL)	751-1560	1417.4±353.4	1360.5±387.8	0.618
IgM (mg/dL)	46.0-304.0	103.9±61.0	95.3±71.6	0.439
CD3 (mg/dL)	79.0-152.0	63.3±11.4	57.5±12.3	0.118
CD19 (%)	7 to 23	8.8±7.8	12.7±6.1	0.096
C3 (mg/dL)	79.0-152.0	108.1±23.7	133.5±37.3	0.002
C4 (mg/dL)	16.0-38.0	22.7±5.2	23.7±6.7	0.523
WBC count		17649±6834	10996±5321	0.000
Neutrophil (%)		77±10	52.5±11	0.000
CD4 (%)	28 to 58	-	30.0±2.34	
CD8 (%)	19 to 48	-	35.8±2.7	
CD3-CD16+/ CD56+ (NK cells)		-	21.2±3.1	

Ig: immunoglobulin; WBC: white blood cells; NK: natural killer
*Normal values for opsonins, immunoglobulins, T and B lymphocytes with natural Killer-cells were as follows: C3 (79.0-152.0 mg/dL), C4 (16.0-38.0 mg/dL), IgG antibody level (751-1560 mg/dL, IgM antibody level (46.0-304.0 mg/dL), distribution of: CD3 (61%-85%), CD19 (7%-23%), CD4 (28%-58%), CD8 (19%-48%), NK cells (6%-29%)

pressed NK cell levels. However, all these patients have demonstrated normal IgG, C3, C4 and CD19+ B-cell distributions.

The comparison of immunological variables at the postoperative five and seven years is shown in Table 3. In the last period, three patients refused to give a blood sample, and the other 3 patients could not be reached. Therefore, sampling for immune monitorization at the 7th year could have been made from 42 patients. Further, CD27+ B-cell distribution could be applied for the first time.

All patients had normal IgG, IgM, CD3, and CD19 levels at the 7th year sampling. Except three patients in ITP and AIHA group, C3 levels were almost within normal range. Three patients had 51, 71, and 73 mg/dL C3 levels at the final analysis. No statistically significant difference was observed in the fifth and seventh year immunological evaluation. We demonstrated low levels of CD27+ B-cell population in only 2 patients in ITP and AIHA group and in 4 of 28 patients in the trauma and splenic mass group.

The mean CD27+ B-cell population was 21.6%±7.8% (95% CI, 13.4-29.8) in these six patients who are assumed to be immunologically suppressed. The most conspicuous findings in our study group are the absence of the history of treatment because of an infection by any encapsulated microorganisms and patient loss because of any septic complication after splenectomy.

DISCUSSION

In this study, vaccination preoperatively or at the day of operation has proved to be efficient against post-splenectomy sepsis in patients with benign hematological disorders, benign splenic mass, and splenic trauma. This confirms the prior report of Jockovich et al. (13) who had reported no OPSI among

Table 3. Immunological variables at the postoperative five and seven years (mean±standard deviation)

	5 years		7 years		p
IgG	95% CI	1360.5±387.8 1248-1473	95% CI	1157.8±224.5 1090-1225	NS
IgM	95% CI	95.3±71.6 74.5-116.1	95% CI	107.3±55.2 90.7-123.9	NS
CD3	95% CI	57.5±12.3 53.9-61.1	95% CI	66.6±11.2 63.2-69.9	NS
CD19	95% CI	12.7±6.1 10.9-14.5	95% CI	14.1±5.9 12.3-15.9	NS
C3	95% CI	133.5±37.3 122.7-144.3	95% CI	128±49.6 113.1-142.9	NS
C4	95% CI	23.7±6.7 21.8-25.6	95% CI	21.9±3.3 20.8-22.7	NS
WBC count	95% CI	10996±5321 9451-12541	95% CI	8970±3280 7985-9955	0.02
Neutrophil (%)	95% CI	52.5±11 49.3-55.7	95% CI	66.3±14.4 62-70.6	NS
CD4	95% CI	30.0±2.34 29.3-30.7	95% CI	37.6±2.5 36.9-38.4	NS
CD8	95% CI	35.8±2.7 35.0-36.6	95% CI	40.2±5.5 38.6-41.9	NS
CD3-CD16+/CD56+ (NK cells)	95% CI	21.2±3.1 20.3-22.1	95% CI	18.7±3.6 17.6-19.8	NS
CD27+ B cells			95% CI	39.6±21.8 32.8-46.4	

WBC: white blood cells; NK: natural killer; NS: not significant

*Normal values for opsonins, immunoglobulins, T and B lymphocytes with NK-cells were as follows: C3 (79.0-152.0 mg/dL), C4 (16.0-38.0 mg/dL), IgG antibody level (751-1560 mg/dL), IgM antibody level (46.0-304.0 mg/dL), distribution of: CD3 (61 to 85%), CD19 (7 to 23%), CD4 (28 to 58%), CD8 (19%-48%), NK cells (6%-29%). Normal value for CD27+ B cells were 32%-56% of all peripheral blood B cells

patients vaccinated before splenectomy. The spleen and peripheral lymphoid tissue have some common immunological properties. First, they are the major sites of IgM production in the body, a special feature allowing the clearance of encapsulated microorganisms and production of IgM against previously unrecognized antigens. Second, T-cells in the splenic white pulp in particular are composed of CD4+ (helper-inducer) cells and possess the same phenotype as those that reside in the paracortical region of the peripheral lymph nodes. In this way, splenectomy-induced blunted production of IgM and CD4+ T-cells could be partially or totally compensated by the peripheral lymphoid tissue.

In contrast, the primary B-cell follicles in the white pulp was asserted as being different from the follicles in the lymph nodes in terms of populating memory B-cells that express CD19 and CD20 surface antigens. When exposed to an antigen, these memory B-cells proliferate into antibody-secreting mature plasma cells (14). Increased susceptibility to encapsulated bacterial infections has been attributed to the lack of immunoglobulin M memory B cells in splenectomized patients. The denominator in the infected patients is the lack of CD27+, IgM+, and IgD+ memory B cells (15, 16). However, it is not clear whether the spleen is the unique site for the generation of IgM memory B cells; there are some studies reporting that IgM memory B cells can be generated in the absence of germinal centers (17). In our study, we have demonstrated the distribution of CD27+ mem-

ory B-cell population only once in September 2012 and found normal levels in 36 of 42 patients. However, as our technique does not discriminate IgM⁺IgD⁺ B cells from IgM only B cells, it is premature to conclude that the vast majority of our patients have immunologically recovered after splenectomy.

The spleen was postulated to be the only source of opsonins and C3, C4, C3b, and C4b are the major generated opsonins (1). The clearance of encapsulated microorganisms is accomplished with the induction of opsonization by anticapsular polysaccharide IgG antibodies. These antibodies facilitate the deposition of complement opsonins on the capsule of the pathogen (18, 19). Bacteria coated by opsonins are optimally destroyed by phagocytic cells, such as macrophages and neutrophils. Thus, one can speculate that splenectomized patients are theoretically vulnerable to sepsis indefinitely because of the loss of opsonizing capacity and lack of memory B-cell proliferation during antigen exposure. However, the early and long-term immunological profile of patients in our study did not support the existing literature. The percentage of CD19+ mature B lymphocytes progressed toward normal range in all patients but not in our series. The recovery of this lymphocyte subset aroused suspicion on the concept that CD19+ and CD20+ memory B cells uniquely reside in the follicles of splenic white pulp. Along with the improvement of CD19+ lymphocytes, all patients had normal IgG levels in both blood samples drawn at the sixth month and five and

seven years after splenectomy. Moreover, the normal C3 and C4 levels attained in all patients with benign disorders in our previous (20) and present study led to skepticism about the absolute loss of opsonizing capacity of the immune system following splenectomy. Except three patients with benign hematological diseases who have demonstrated equivocal or subnormal IgM levels in the first two examination periods, the remaining have had normal and stable values throughout the study. A similar outcome was reported by Ruben et al. (1) who had demonstrated adequate antibody response to a single-dose meningococcal polysaccharide vaccine in asplenic subjects with non-lymphoid tumors or splenic trauma. The rate of seroconversion in IgG, IgM, and IgA class antibodies was almost similar to those obtained in vaccinated control subjects except the IgM category in patients with non-lymphoid tumors, which remained stable without any increase.

Splenectomized patients with different etiologies run 7% risk of sepsis over a 10-year period, and the frequency is highest within the first three years. The Advisory Committee on Immunization Practice recommends the administration of pneumococcal vaccine (23-valent polysaccharide type) to all patients two weeks before elective splenectomy: a protocol conducted in all elective patients in this study (21).

The evaluation of our results revealed an adequate seroconversion of IgG in all and IgM in more than 85% of patients and normal C3 and C4 levels in all patients with benign hematological disorders and splenic trauma. Further, CD19+ mature B lymphocyte titers were almost within normal limits for the whole group over the long term, indicating the ongoing humoral immunological responsiveness. Although no patient had pneumococcal or *H. influenza* infection or died because of overwhelming sepsis, a booster dose of vaccine was not required. Our results are indicative of the resumption of the immune function following Pneumo-23 and Act-HIB administration, instigated by the probable activation of T and B cells and the adequate production of C3, C4, IgG, IgM antibodies in remote lymphoid tissues. This assumption was previously substantiated by Slifka et al. (22) who have demonstrated long-lasting B-cell immunity induced by conjugate vaccines, and this persistent immunity with no additional boosting was contributed to long-lived plasma cells of the bone marrow. As no patient experienced a serious infection or died because of one within 7 years of follow-up, it is possible to consider these responses as prognostically important.

CONCLUSION

Longer follow-up is required to provide a prognostic immune profile index capable of predicting which patients are at a risk of postsplenectomy sepsis and to specifically conclude the prognostic capacity of the immunological parameters investigated in this study.

Ethics Committee Approval: Ethical approval is not required because this study is principally based on systematic evaluation and analysis of retrospective screening of patient files and surveillance records.

Informed Consent: Written informed consent was obtained from patients who participated in this study.

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