Immune Response Capacity After Human Splenic Autotransplantation

Restoration of Response to Individual Pneumococcal Vaccine Subtypes

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Objective

To evaluate features of general immune function, in particular the restoration of the humoral immune response to pneumococcal capsular polysaccharides, in humans undergoing a spleen autotransplantation after splenectomy because of trauma.

Summary Background Data

After splenectomy, patients have an increased risk of overwhelming infection or sepsis involving encapsulated bacteria such as pneumococci. The value of human spleen autotransplantation after splenectomy because of trauma has long been questioned. Mononuclear phagocyte system function appeared to be similar to that in splenectomized persons. The presence of specific antipneumococcal antibodies would allow other parts of the mononuclear phagocyte system, such as those in the liver, to phagocytose opsonized bacteria.

Methods

Ten consecutive patients undergoing splenectomy followed by autotransplantation were compared with the next 14 consecutive patients undergoing splenectomy alone. After a minimum of 6 months, the patients were vaccinated with 23-valent pneumococcal vaccine. Blood samples were taken at the time of vaccination and after 3 and 6 weeks for antipneumococcal capsular polysaccharides IgM and IgG enzyme-linked immunosorbent assay against types 3, 4, 6, 9, 14, and 23. Splenic regrowth was evaluated by scintigraphy.

Results

Surprisingly, several of the nonautotransplanted patients showed scintigraphic activity, indicating the presence of either accessory spleens or traumatic seeding (splenosis). Significant antibody titer increases (more than twofold) were found for both IgM and IgG in the autotransplanted patients. Splenectomized-only patients showed no significant increase in Ig levels in patients without splenic regrowth and partial improvement in patients with splenosis/accessory spleens.

Conclusions

Considering this significant antipneumococcal antibody increase, spleen autotransplants can be expected to permit an adequate humoral response to pneumococcal infections and presumably also to other TI-2 antigens, and to protect against overwhelming postsplenectomy infection or sepsis.

The spleen has an important function in the host's response to infections by clearing polysaccharide encapsulated bacteria. This response involves the clearing from the bloodstream as well as the rapid production of specific antibodies against the polysaccharide antigens. After splenectomy, there is an increased risk of septic complications with a high mortality rate, especially in children. This is known as the "overwhelming postsplenectomy infection syndrome."[1,2]

The spleen is the major organ for the phagocytosis of low or nonopsonized particles; hence, after splenectomy there is

a decrease in the capacity of the mononuclear phagocyte system. Another important function of the spleen is production of tuftsin, a stimulator of phagocytic activity. Therefore, we can expect that after splenectomy the phagocytic activity of the polymorphonuclear granulocytes is decreased.[3,4] Finally, the spleen provides a rapid primary immunologic reaction by the production of immunoglobulins after initial contact with an antigen, leading to the elimination of the antigen. It has been proposed that the initiation of the primary immune response to polysaccharide antigens, including pneumococcal polysaccharides, is specifically related to the spleen, in particular to the marginal zone.[5] This implies that after splenectomy there will be an impaired antibody response in relation to pneumococcal vaccination.[6]

Animal experiments have suggested that autotransplantation of splenic tissue (AST) after splenectomy might restore defects in the immune system.[7-9] Most human studies to confirm or disprove this have so far been confined to nonspecific splenic function tests.[10,11] In view of the highly specific functions of the spleen, this is unsatisfactory, and for further progress we must find new parameters testing real spleen-related immune functions.[12] An example of detailed, adequate testing of specific splenic functions was given in a case report describing a patient with extensive posttraumatic splenosis.[13]

As an initial evaluation of immune capability after splenectomy with or without autotransplantation, we have determined the general humoral response capability. The function of the nonspecific immune system was evaluated using tests for phagocytosing and opsonization capacities.[14]

The most important question to be answered in this study is whether the specific antibody response after pneumococcal vaccination in splenectomized patients will be better if AST is performed. We evaluated specific antibody responses after vaccination with polyvalent pneumococcal vaccine with respect to five pneumococcal subtypes in splenectomized patients with and without AST.

PATIENTS AND METHODS

Patients

During a period of 4 years, we studied 24 male patients with a mean age of 27.3 years (range 15 to 55 years) who had undergone splenectomy after traumatic rupture of the spleen or during partial gastrectomy for peptic ulceration. Splenectomy was carried out only when splenorrhaphy or other conservative therapy was not possible (University Hospital protocol). During the operation, a thorough examination for ectopic splenic tissue was performed. When ectopic tissue was found, the patient was excluded from the study.

To exclude the immediate effects of trauma or surgery, the period of time between the operation and the investigations related to the present study was 0.5 to 2.5 years. The

subsequent clinical follow-up period varied from 5 to 8 years (mean 6.5). The study was organized prospectively, with the patients entered at the time of splenectomy. In the first 10 consecutive splenectomy patients, autotransplantation was performed; the next 14 consecutive patients, including some splenectomized patients from other hospitals, did not undergo the autotransplantation procedure. No selection was applied whatsoever. There were no differences in indications for splenectomy or otherwise between the groups in the different hospitals.

This project was approved by the hospital's medical ethics committee, and informed consent was obtained from all patients.

Spleen Autotransplantation

In 10 patients, autotransplantation was performed after splenectomy with 20 to 30 g splenic tissue. A slice measuring $5 \times 5 \times 1$ cm, including part of the capsule, was taken from the spleen and divided with a scalpel into cubes of in general about 3 mm, with a maximum of 5 mm diameter. This autotransplant was then fixed in the greater omentum with a pursestring suture, and the site was marked with a silver clip. In the remaining 14 patients, no autotransplantation was performed. During their hospital stay, all patients received prophylactic antibiotics (cefuroxime).

Fc-Receptor Scintigraphy

In all patients, Fc-receptor scintigraphy with IgG-coated and ^{99m}Tc-labeled red cells was performed to check the mononuclear phagocyte system function and consequent presence of autotransplants. Because the red blood cells are coated with low-density immunoglobulin, this provides a low level of opsonization that causes a reversible pool of the labeled red cells in splenic tissue, when present, and not in the liver. In this way, a selective visualization of splenic tissue (red pulp mononuclear phagocyte system) is obtained. We described this method in more detail previously.[15] In short, the test procedure for the scintigram is as follows. Red cells from a single donor had previously been incubated with a suspension of 1:2 antirhesus (D) antiserum. Small numbers of these cells were stored in 10-ml ampules at $-80°C$. On the day of the test, the contents of one ampule were labeled with ^{99m}Tc. The abdominal region was scanned with a dual-headed gamma camera and computer.

Phagocytosis and Opsonizing Capacity Test

Granulocytes were isolated, washed, and resuspended in Ca^{2+}-containing HEPES buffer at a final concentration of 15×10^6 cells/ml.[14] Staphylococcus aureus was cultured in BHI broth and washed in phosphate-buffered saline. The bacteria were spectrophotometrically brought to a final concentration of 10^9 bacteria/ml in Ca^{2+}- and Mg^{2+}-containing

HEPES buffer. A suspension was made of 1 ml HEPES with bacteria and 60 ml (3%) serum. Opsonization took place in the presence of Mg^{2+} and Ca^{2+} for proper complement activation both via the classical and alternative pathways. After the opsonization of the bacteria and after warming the granulocytes to 37°C, they were put together in a ratio of 2:1 to determine the phagocytosis in a spectrophotometer. Phagocytosis was measured during 30 minutes as an increase in light transmittance given as a percentage, compared with reference values.

Nitro Blue Tetrazolium Test

The nitro blue tetrazolium test is a standard test for evaluating the phagocytic function of granulocytes.[16,17] A drop of blood is put on a slide prepared with endotoxin and incubated. After washing with saline, the granulocytes adhere to the glass. Subsequently, the slide is incubated with nitro blue tetrazolium and pooled serum of healthy donors. The active granulocytes reduce nitro blue tetrazolium to blue formazan after phagocytosis and can be counted. The result is given as the percentage of positive cells, counting 100 cells.

Phagocytosis Killing Test

Granulocytes were purified, washed in HEPES with saline, and put into a bacteriologic-grade 96-well incubation plate together with serum.[18] Cultures of *S. aureus* were put in phosphate-buffered saline, and this suspension was added to the plate in a two-step dilution. After incubation at 37°C, 10 μl from each well was put into nutrient agar. After incubation, the well with <5 bacterial colony-forming units was considered the bactericidal titer and was used to measure the largest bacterial population that was eliminated by phagocytosis. The result is given as a percentage of the dilution, compared with granulocytes of healthy donors.

Helix pomatia Hemocyanin Test

This test is used to test general humoral immune capacity.[19,20] A vaccination of 1 mg *Helix pomatia* hemocyanin (HPH) is given subcutaneously and blood is taken before and 3 and 6 weeks after vaccination to determine specific antibodies IgG, IgA, and IgM. An indirect enzyme-linked immunosorbent assay (ELISA) technique is used. The results are given in arbitrary units. Patients with increased levels of specific anti-HPH levels on the day of immunization or abnormally high levels after 3 or 6 weeks were excluded from the test.

Immunoglobulin and Complement Levels

The levels of immunoglobulins and of C3, protein factor B (PFB: C3 activator), and C4 were measured in an auto-

matic nephelometer. The radial immunodiffusion technique was used for C1q, and C3d was measured by an ELISA with rabbit antihuman C1q and C3d antibodies. The reference values were obtained from healthy volunteers of the laboratory and checked with standard values. The immunoglobulin and complement levels were compared to the standards used by the laboratories in the University Hospital. All determinations of one test were performed at the same time.

Immunization

All patients were immunized after the splenectomy, with a lag time of ≥6 months to allow full regrowth of the transplanted splenic tissue. Immunization was performed with 23-valent pneumococcal vaccine (Pneumovax, Merck, West Point, PA). Blood was taken on the day of immunization and after 3 and 6 weeks to measure IgM and IgG against pneumococcal polysaccharide subtypes 1, 3, 4, 6, and 14 (Danish nomenclature) using an ELISA technique, as previously described.[21] Purified pneumococcal polysaccharides of different subtypes were obtained from the American Type Culture Collection (Bethesda, MD).

ELISA Procedure

The ELISA was performed similar to previously described methods.[21] Wells of microtiter plates were coated with a 150-μl solution of each of the five pneumococcal polysaccharide types (1, 3, 4, 6, and 14) and in addition with Pneumovax; polysaccharides were used in a concentration of 5 μg/ml antigen dissolved in 0.1 M $NaHCO_3$ (pH 9.6) and incubated overnight at 4°C. After washing four times with 150 μl 0.9% NaCl containing 0.05 Tween 20 (Saline T), plates were freeze-dried and kept at 4°C until further use.

Measurements of IgG and IgM against the pneumococcal antigens were performed as follows. Patient and control sera (150 μl) were diluted 1:640 to 1:5120 for the IgG antibodies and 1:160 to 1:1280 for the IgM antibodies in 5 mM Tris-HCl buffer (pH 7.2) containing 0.05% Tween 20 (Tris T) and incubated for 90 minutes at 37°C. Subsequently the plates were washed five times with Saline T and incubated for 90 minutes with 150 μl peroxidase conjugated antihuman IgM (Nordic Immunology, Tilburg, The Netherlands). After incubation, the plates were washed five times with Saline T and were incubated for 45 minutes at 20°C with substrate (150 μl 0-phenylene diamine diluted in 0.025 phosphatase buffer [3 mg/ml] containing 0.02% H_2O_2, pH 6.3). The reaction was stopped by the addition of 50 μl 1N H_2SO_4. The absorbance was measured at 492 nm. The negative standard was a mixture of serum obtained from 3-month-old healthy babies, in whom pneumococcal infections were unlikely and residual maternal antibody was low.

The results are given in relative units, allowing determination of antibody increases after vaccination. The positive control was a mixture of sera of vaccinated volunteers.

Table 1. RESULTS OF IMMUNOLOGIC TEST PARAMETERS IN PATIENTS AFTER SPLENECTOMY (MEAN VALUES AND ONE STANDARD DEVIATION)

Parameters	Reference	AST	EST	NST
Phagocytosis and opsonizing capacity test (%)				
Opsonization	100 ± 10	121 ± 23	122 ± 24	139 ± 25
Phagocytosis	100 ± 10	106 ± 18	99 ± 13	94 ± 7
Nitro blue tetrazolium test (%)	>80	89.4 ± 5.3	90.1 ± 5.7	87.8 ± 5.4
Phagocytosis killing test (%)	>96	96.3 ± 4.0	97.7 ± 3.9	99.0 ± 0.4
Helix pomatia hemocyanin test (units)				
IgG 0 weeks	<1	0.5 ± 1.2	1.0 ± 1.0	0.2 ± 0.4
3 weeks	>6	16 ± 10	18 ± 18	33 ± 34
6 weeks	>6	21 ± 20	15 ± 16	22 ± 21
IgA 0 weeks	<1	0.2 ± 0.4	0.3 ± 0.7	0.4 ± 0.5
3 weeks	>6	26 ± 17	25 ± 20	64 ± 35
6 weeks	>6	25 ± 28	14 ± 11	39 ± 30
IgM 0 weeks	<1	4 ± 3	3 ± 2	4 ± 6
3 weeks	>6	17 ± 7	21 ± 21	18 ± 9
6 weeks	>6	16 ± 8	18 ± 14	18 ± 1
Immunoglobulin (g/l)				
IgG	8.5–15	13.0 ± 2.2	11.7 ± 2.3	12.2 ± 2.9
IgA	0.9–4.5	2.6 ± 0.8	3.3 ± 1.8	3.1 ± 0.8
IgM	0.6–2.6	1.4 ± 1.0	0.9 ± 0.5	1.3 ± 0.7
Complement				
C1q (mg/l)	100–250	103 ± 4.6	100 ± 3.9	101 ± 5.3
C3 (g/l)	0.64–1.20	1.07 ± 0.2	0.93 ± 0.33	0.90 ± 0.16
C3d (mg/l)	0.8–5.2	3.5 ± 0.8	3.0 ± 0.6	3.0 ± 0.6
PFB (g/l)	0.19–0.40	0.32 ± 0.05	0.30 ± 0.03	0.35 ± 0.03
C4 (g/l)	0.11–0.40	0.30 ± 0.07	0.28 ± 0.05	0.29 ± 0.12

AST = patients with autotransplantation (n = 10); EST = ectopic splenic tissue (n = 8); NST = no splenic tissue (n = 6).

Statistics

The incidence of patients with increases of antibody levels against pneumococcal polysaccharides of twofold or more were analyzed using the Fisher's exact test. The levels of antibodies in the different groups were compared using the Mann–Whitney/Wilcoxon test. The Kruskal–Wallis and Wilcoxon's nonparametrical tests were used for statistical analysis. $P < 0.05$ was accepted as significant.

RESULTS

Patients

No patient developed an overwhelming postsplenectomy infection syndrome during the period of investigation, nor was there any evidence of immune deprivation such as an increased general infection rate. There were no complications attributable to the presence of the autotransplants.

Based on the scintigraphic results, three groups of patients could be distinguished. The Fc-receptor scintigram showed a hot spot at the sites of autotransplantation grafts in the 10 patients (AST) in which this procedure had been performed. In 8 of the 14 patients who did not undergo AST, a hot spot on the scintigram suggested splenosis or ectopic splenic tissue (EST). These hot spots showed random localization, not similar to the autotransplanted tissue,

at the site of the silver clip marking. Ectopic tissue present as accessory spleens was less likely because of the inspection during laparotomy, excluding patients in whom accessory splenic tissue could be identified. In six cases no activity was found on the abdominal scintigram except for the liver; this group was considered to have no splenic tissue (NST). The patients are comparable in terms of age, sex, and the time between splenectomy and immunization with the pneumococcal vaccine.

Immune Tests

All the phagocytosis activity tests showed normal results in relation to the reference values (Table 1), and no significant differences were found between the patient groups. Complement and immunoglobulins also showed normal values, without significant differences between the groups.

Vaccination

The results of the HPH test showed values in the normal range for all three patient groups, without significant differences (see Table 1). One NST patient had a strong reaction on immunization; because the value (after 3 and 6 weeks, IgG 26 and 832, IgA 51 and 104, IgM 20 and 254 units) by

Table 2. HUMAN SPLEEN AUTOTRANSPLANTATION: ELISA RESULTS OF SPECIFIC ANTI PNEUMOCOCCAL CAPSULAR POLYSACCHARIDE (PPS) ANTIBODY AFTER PNEUMOVAX 3 AND 6 WEEKS AFTER VACCINATION

Subtype:		AST			EST			NST		
Weeks:		0	3	6	0	3	6	0	3	6
PPS1	IgG	39	65	130	54	58	56	45	52	57
	IgM	35	196	150	36	61	78	57	69	68
PPS3	IgG	16	16	16	25	18	18	15	15	15
	IgM	73	158	185	57	133	151	108	105	114
PPS4	IgG	72	250	300	81	139	111	84	112	119
	IgM	55	289	296	43	76	87	78	120	134
PPS6	IgG	97	97	121	109	127	116	108	116	150
	IgM	45	57	78	43	62	62	64	69	80
PPS14	IgG	223	343	351	181	230	280	196	226	276
	IgM	49	82	110	52	69	60	88	90	112

AST = patients with autotransplantation (n = 9); EST = ectopic splenic tissue (n = 7); NST = no splenic tissue (n = 6)
Two–fold or more rises are underlined.

far exceeded the mean plus two times the standard deviation, this was excluded from the mean values. Such incidental abnormal values have been reported previously.[22] The patients in the three groups showed no differences in the levels of specific IgG and IgM in the serum before vaccination for each of the tested serotypes. Six weeks after vaccination, most patients in the three subgroups showed a twofold increase of IgM antibodies against the whole vaccine (AST, 8/9; EST, 7/7; NST, 4/6).

The specific antibody responses against the different subtypes are listed in Table 2. There is a remarkable difference in the three groups with respect to the antibody reactions against the several serogroups. The AST patients showed a twofold rise of antibody titer against serogroups 1, 3, 4, and 14 for IgM antibodies, and 1 and 4 for IgG antibodies. The EST group showed a twofold increase in the IgM titer to types 3 and 4 and no rise in the IgG titers. The NST group showed neither an IgM nor an IgG rise to the tested antigens. Increase in antibody titers was the highest in the AST group, particularly with respect to IgM antibodies. After 3 weeks there was a significant increase in the IgM antibody response compared to the NST group to serotype 1, type 3, type 4, and to the total vaccine (p < 0.05). Comparing the AST group with the EST group, there was a reduced response in the latter patients for type 4 (p < 0.02). The EST group compared with the NST group showed an enhanced IgM response to type 3 (p < 0.05)

Six weeks after vaccination, the differences in the IgM antibody response showed the same pattern. At this time there was a significant difference in the IgG antibody response for type 6 and type 14, AST versus NST; type 14, AST versus EST; and type 6, EST versus NST (all p < 0.05).

DISCUSSION

In a nontrauma situation, it is clear that pneumococcal vaccination should be performed before splenectomy. The usefulness of vaccination after splenectomy in case of trauma is often disputed. Previous animal studies reported that splenectomized subjects had a better antipneumococcal capsular polysaccharides antibody response to pneumococcal vaccination after spleen autotransplantation.[23] Another animal study demonstrated that autotransplantation improved the immunoglobulin levels after reimmunization.[24] Although the positive effect of spleen autotransplantation on restoration of immune functions in humans has been subject to doubt, the present study demonstrated that a better antibody response to pneumococcal vaccine was found in patients after splenectomy if an autotransplantation was performed, in agreement with an earlier case report on a patient with extensive splenosis.[13]

In humans, deliberate spleen autotransplantation is performed in the highly vascularized greater omentum. Some reviews[9,11,25] have found that in humans the total volume of the autotransplanted spleen, not the individual fragment size, is critical in relation to the extent of necrosis, which is known to occur in the initial phase after autotransplantation. In fact, the reticular structure remains intact in a rim inside the preserved outer area of the autotransplant. We believe this reticular structure is important in the specific anatomy of the spleen, especially with respect to the sinusoidal structure of the marginal zone; therefore, we maintain this structure by avoiding the use of very small fragments or even putting the tissue through a mesh. Because we tend to agree that limiting the total volume is critical, using fragments of sufficient size, again theoretically, could serve as a "homing" structure for cellular outgrowth and repopulation of the spleen fragments.

In our study, we did not find a decrease in polymorphonuclear granulocyte function after splenectomy because of the lack of the normally present stimulating effect of tuftsin[3,4]: we found no difference in polymorphonuclear granulocyte function after splenectomy with or without AST or EST. This situation has also been described by others.[26,27] This difference may be related to the difference in time intervals between splenectomy and the functional studies, which in most reports were quite short. In our study this lag time was deliberately increased to avoid the immediate effects of trauma and surgery and to allow any compensatory mechanisms to develop.

Testing the primary immunologic response with HPH did not show any differences between the patient groups: all results were within the normal range. However, this test is not specifically spleen-dependent, and it seems very likely that the extrasplenic B-cell compartments compensate for the loss of the splenic B cells.

In 8 of the 14 patients who did not have autotransplants, there was activity on the scintigram suggesting the presence of EST. This activity, observed at different sites in the abdomen, may result from the presence of accessory spleens or may be more likely due to splenosis from seeded (autotransplanted) splenic cells after splenic rupture.[13,28–30] There is a strong possibility that such "born-again" spleens will provide immune protection after splenectomy; this was extensively discussed by Hathaway et al.[13] However, failure of EST to prevent fatal pneumococcal septicemia after splenectomy has been described.[31] None of the patients in this limited survey showed any clinical evidence of immune deficiency in the follow-up period. The importance of the presence of EST is not yet clear and deserves more study.

It is likely that pneumococcal infections occur only in persons who lack antibodies to the capsular polysaccharide of the colonizing Streptococcus pneumoniae. In fact, studies conducted with cell wall polysaccharide antigens to measure an antibody response do not distinguish between antibodies directed to capsular polysaccharides and to cell wall polysaccharide antigens. Musher et al[32] suggested using a specific ELISA with an absorption step to remove antibodies to cell wall polysaccharide antigens to specify the antibody response to the specific polysaccharides.[32] Nevertheless, in our ELISA, we used capsular polysaccharide antigens and were able to demonstrate an antibody response to the polysaccharides in healthy vaccinated individuals[21] and in some patients (AST group to subtype 1, 3, 4, and 14).

From our results, it appears that AST patients had a significantly better IgM response to serotypes 1 and 14 in comparison with EST patients, whereas for serotype 3 and 4 there was no substantial difference in the IgM response. It has been proposed previously that EST in humans does not normalize the altered antibody responses after splenectomy[33]; the limiting factor in these cases is not yet clear. One possibility is that either the reticular structure present in these accidentally implanted fragments or the blood supply is insufficient to allow restoration of adequate lymphoid

tissue.[34] In the NST group there was no significantly improved reaction at all.

The fact that the most important increase in immunoglobulin was of the IgM class indicates that the respective pneumococcal capsular polysaccharide subtypes were encountered for the first time and that the spleen probably is essential in eliciting an adequate primary humoral response.

Because with splenectomy the restoration of clearance by autotransplantation is insufficient,[15] restoration of the humoral response becomes even more important, allowing good opsonization of pneumococcal capsular polysaccharides, which enables clearance by the liver's mononuclear phagocyte system. When small amounts of regenerated autotransplanted spleen are present, with adequate response capacities to T-cell independent antigen type 2 (e.g., pneumococcalpolysaccharide), specific antibodies will be formed. These antibodies will cause opsonization of the antigen (encapsulated bacteria) and, although the phagocytosing capacity of the splenic fragments will not play an important role, the cells of the mononuclear phagocyte system in the liver are then perfectly capable of removing these well-opsonized particles.

In conclusion, this study shows a surprising effect of AST after splenectomy on specific antibody responses after pneumococcal vaccination. Although it is not yet clear whether complete protection against all pneumococcal subtypes can be obtained, spleen autotransplantation may be expected to help limit the risk of overwhelming postsplenectomy infection syndrome. This study demonstrates that spleen autotransplantation can play a role in the management of severe splenic injury in which splenectomy is inevitable, particularly when followed by vaccination with a polyvalent pneumococcal vaccine. Our results may provide a foundation for more extensive clinical studies with longer-term follow-up to put our findings in a useful clinical context. Further, the use of a panel of specific spleen-dependent antigens, such as different pneumococcal polysaccharide subtypes, can provide a procedure for testing functional splenic immune response capacity (e.g., to test the success of an autotransplantation procedure).

Acknowledgments

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