Spleen autotransplantation provides restoration of functional splenic lymphoid compartments and improves the humoral immune response to pneumococcal polysaccharide vaccine

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After splenectomy, patients have an increased risk of overwhelming post-splenectomy infection or sepsis involving encapsulated bacteria such as pneumococci. The value of spleen autotransplantation after splenectomy because of trauma has long been questioned. Much attention has been given to the restoration of mononuclear phagocyte system (MPS) function, which appeared to be similar to that of splenectomized individuals. The presence of specific anti-pneumococcal antibodies may enhance phagocytosis of opsonized bacteria by other parts of the MPS, as present in the liver. Therefore, in the present study we have evaluated the restoration of the humoral immune response after spleen autotransplantation, especially to pneumococcal capsular polysaccharides (PPS).

Wistar rats were divided into three groups that were operated as follows: splenectomy, splenectomy followed by autotransplantation, and sham operation. After 12 weeks the rats were vaccinated with 23-valent pneumococcal vaccine. Blood samples were taken after 3 days, 3 and 6 weeks for anti-PPS IgM and IgG ELISA against types 3, 4, 6, 9, 14 and 23. In addition, immunohistological studies were performed on the autotransplants. Significant antibody titre rises were found in a main proportion of the autotransplanted rats, comparable with sham-operated rats. Splenectomized rats showed as well significantly lower increase in Ig-levels, as significant differences in proportion of rats showing a minimum 2-fold increase of antibody level, considered to represent an adequate response. The titres were highest 3 days after vaccination. Immunohistochemical studies demonstrated structurally functional autotransplants including an intact marginal zone.

Considering this significant anti-pneumococcal antibody response, spleen autotransplants can be expected to enable an improved humoral response to PPS, and to contribute to protection against OPSI after splenectomy.

Introduction

After splenectomy, patients have an increased risk of overwhelming post-splenectomy infection (OPSI) or sepsis (OPSS). Such an infection has a high risk of mortality, especially in children. Fatal OPSI is in most cases caused by pneumococci, the capsular polysaccharides of which are considered to be T-cell-independent type 2 (TI-2) antigens. It is known that the initiation of the antibody response to such antigens depends on the presence of splenic tissue, in particular a functional marginal zone B-cell compartment. Although spleen autotransplantation has been performed in an attempt to restore normal spleen immune function, until now it has not been known whether autotransplants of splenic tissue are capable of initiating a primary humoral reaction to TI-2 antigens. In animal experiments, focusing on the influence of the marginal zone of splenic transplants on the antibody response to TI-2 antigens, the restoration of the antibody titres correlates with the return of B-cells, finally reaching titres indistinguishable from those of normal mice. In a recent study, it was shown that autotransplanted tissue in newborn rats leads to a well-developed marginal zone and that this regenerated tissue provides
significant protection against pneumococcal infections introduced via the respiratory tract. In most human studies the function of autotransplants was only tested by functional evaluation of the mononuclear phagocyte system (MPS). An example of detailed, adequate testing of specific splenic functions was given in a case report describing a patient with extensive post-traumatic splenosis. In most animal experiments using pneumococci or Pneumovax® only general antibody titres were evaluated, and not subtype specific antibody titres. The drawback of such an approach is that highly immunogenic PPS-types may elicit a considerable response, thereby masking potential non-responsiveness to other, less immunogenic types. Subsequently, the risk of OPSI in this situation may still be present for the latter PPS-types.

In the present study we investigated the effect of splenic autotransplants on the antibody responses to individual pneumococcal capsular polysaccharides of types 3, 4, 6, 9, 14 and 23. Antibody responses after vaccination with a pneumococcal capsular polysaccharide vaccine were compared in sham-operated, splenectomized and spleen-autotransplanted animals, respectively. To ensure full regeneration of white pulp in the splenic transplants, vaccination was performed 12 weeks after operation.

Materials and methods

Animals and operation

Male Wistar rats were housed under standard laboratory conditions on a 12-h light and dark cycle. They were fed with standard laboratory rat food (Hope Farms, Inc, Woerden, The Netherlands) and tap water ad libitum. Rats with a body weight of approximately 200 gr. of about three months of age were used for all experiments.

Sham operation (n=10), splenectomy (n=10) and splenic autotransplantation (n=10) were performed as described by Pabst et al. In short, all operations were performed via an upper midline incision, under ether anaesthesia and clean but not sterile conditions. In splenic autotransplantation the spleen was removed and half of the spleen tissue was cut into pieces of approximately 1 mm³, which were sutured into an omental pouch. The abdomen was closed in two layers.

Vaccination

For vaccination we used a 23-valent pneumococcal vaccine (Pneumovax®, Merck, Sharp, and Dohme, West Point, PA, USA) which contains the PPS types 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F, and 33F. The sham operated, splenectomized, and spleen-autotransplanted rats, (for all n=10) were vaccinated 12 weeks after operation. Vaccination was performed by intramuscular injection in the left hind leg with one dose (0.5 Fl) of Pneumovax®.
Before vaccination (day 0) and 3 days, 3 weeks and 6 weeks after vaccination 500 µl blood was taken from the retro-orbital plexus under mild anaesthesia. Six weeks after vaccination the animals were sacrificed and spleen or spleen autotransplants, were obtained at autopsy. The spleens and the autotransplants were weighed and tissue blocks were immediately frozen by immersion in liquid isopentane (cooled in a freezer to -80°C) and stored in a freezer at -80°C until sectioning.

Anti-PPS ELISA

To detect the anti-PPS 3, 4, 6, 9, 14 and 23 IgM and IgG antibody titres in serum, a sandwich enzyme-linked immunosorbent assay was used as described previously. In short, ELISA plates were coated overnight at 37°C with 10 µg of PPS subtypes per ml in 0.9% NaCl. Pooled serum from all sham-operated animals (n=10), immunised with Pneumovax® (day 21) was used as an internal reference and assigned 100 U/ml antibody for all serotypes. To determine the anti-PPS concentrations in a given sample, serial dilutions were titrated into the plate. Adsorption with pneumococcal cell wall polysaccharide (CPS) was carried out by incubating serum samples overnight at 4°C with 50 µg of CPS (State Seruminstitute, Copenhagen, Denmark) per ml. The ELISA was processed by adding peroxidase-conjugated goat anti-rat IgM or IgG (Tago, Burlingame, Calif.) and incubated for 3 h at 37°C. Subsequently, the wells were allowed to react with 1.0 ml of 5.5 mM O-phenylenediamine-0.015% H2O2 in citrate phosphate buffer (pH 5). The yellow-brown reaction product was measured after 10 to 15 min at 450 nm with an ELISA reader.

Immunohistochemistry

To detect the different injected PPS-types, we used group or type specific rabbit anti-pneumococcal polysaccharide (anti-PPS) antibodies (State Seruminstitute, Copenhagen, Denmark). As second step antibody we used a peroxidase conjugated Swine anti-rabbit immunoglobulin (SAR®; Dakopatts (Glostrup, Denmark). Kupffer cells in the liver and red pulp macrophages in the spleen were stained using the mAb ED2, marginal zone macrophages and metallophils in the spleen using mAb ED3 and as a B-lymphocyte marker the anti-IgM mAb His40.

Cryostat sections (4 µm) were air dried for 20 min, fixed for 10 min in acetone, air dried and washed in phosphate buffered saline (PBS) pH 7.4, for 5 min. Next, sections were incubated for 30 min with an appropriately diluted type or group specific anti-PPS antibody directed against PPS-type 3,4,6,9 or 14 with 5% normal rat serum (NRS). Subsequently, sections were incubated with SAR® diluted 1:20 in PBS with 5% NRS for 15 min. The peroxidase activity was visualised using 3-amino-9-ethylcarbazole (AEC) and H2O2 as a reagent. Finally, sections were counterstained in Mayer’s hematoxylin and embedded in Kaiser’s glycerin-gelatin.
Statistical analysis

The incidence of rats with ≥ twofold rises of the antibody levels against pneumococcal polysaccharides were analysed with the Fisher exact test. The increase of levels of the antibodies in the different groups was compared using the Kruskal-Wallis and Mann-Whitney test. P< 0.05 was taken as significant.

Results

Immunohistology of autotransplanted splenic tissue

Eighteen weeks after autotransplantation the splenic transplants showed a reduced weight. The weight of the transplants was about 18% of the spleen weight in sham operated age matched rats. As about half of the spleen was transplanted, this equals approximately 36%

Fig 1. Microphotographs (all immunoperoxidase staining) demonstrating restoration of spleen lymphoid compartments including the functionally most relevant area, the marginal zone (12 weeks after autotransplantation procedure). A. all B-cell areas stained with IgM antibody His40: overview (x40) and B. magnification (x 100); C. marginal zone macrophages stained with ED3 (x 100). D. Staining for PPS1: localisation in a dendritic pattern in the germinal centre; presumably on follicular dendritic cells. (X 100).

C = lymphocyte corona; GC = germinal centre; F = follicle; MZ = marginal zone; P = periarteriolar lymphocyte sheath
of the originally implanted tissue weight. A clear demarcation of red and white pulp was seen and the latter was positioned directly beneath the capsule. Central parts of the transplants still contained fibrotic tissue. A clear compartmentalisation in marginal zone, mantle zone and germinal centre was detected with the anti-IgM mAb staining all B lymphocytes, and ED3 positive macrophages were present in the marginal zone. PPS-type 3, 4 and 9 but not PPS-type 6 and 14 were detected in the germinal centres in most of the splenic transplants. The PPS were localised in a pattern consistent with localisation on follicular dendritic cells (FDC). None of the PPS types localised in the marginal zone of the transplants, consistent with earlier kinetic studies, demonstrating that PPS localise in vivo in the marginal zone at an early time point\(^a\), with a subsequent shift in localisation to the follicle/germinal centre showing (from 3 days after immunisation) a dendritic pattern. The above findings are illustrated in figure 1 (page 89).

**Antibody responses**

The IgM and IgG anti-PPS antibody response was studied in groups of animals that were sham operated, splenectomized, or splenectomized and autotransplanted. IgM and IgG anti-PPS antibody titres of the experimental groups in serum withdrawn before immunisation did not differ significantly from antibody titres in naive, non-immunised rats. Day 0 was considered baseline for each group, and for the other time points (3, 21, and 42 days after vaccination) the increase of PPS-type specific Ig level was determined in comparison to this baseline level.

In figure 2 the mean values for both IgM and IgG are given for each time point relative to baseline levels including standard deviations.

In sham-operated rats, immunisation with Pneumovax\(^b\) induced a more than two-fold increase in both IgM and IgG antibody titres against all pneumococcal serotypes tested. The highest antibody titres were observed at day 3 after immunisation. At day 21, IgM antibody titres against most serotypes had dropped to almost pre-immunisation levels while IgG titres against serotypes 3, 4, and 9 still were higher than before immunisation (compared with baseline: 2.1-, 3.3- and 3.7-fold increase, respectively).

**Fig 2.** (page 91-92-93)

Graphical representation of increase in PPS type-specific IgM and IgG between day 0 (day of vaccination) and A. day 3, B. day 21 and C. day 42, respectively. A. \(^*\) increase in immunoglobulin level significantly lower than sham-operated rats (\(^*\) p<0.05; \(^**\) p<0.01). In B. and C. IgM levels are all below a mean of 2-fold increase (minimum of 2-fold increase is considered clinically relevant). For IgG at 21 days only for PPS9 is a relevant mean increase observed for the autotransplant group, not different from the sham operated group, whereas the splenectomy group has significant lower increase (p<0.05). For the other PPS no relevant increase was observed for either the splenectomy or the autotransplant group (the seemingly high increase for PPS23 was due to very low zero values for this PPS).
Figure 2A: Increase in PPS-specific IgM levels between day 3 and day 0

- **sham**
- splenectomy
- autotransplantation

Figure 2B: Increase in PPS-specific IgG levels between day 3 and day 0
Increase in PPS-specific IgM levels between day 21 and day 0

- sham
- splenectomy
- autotransplantation

Increase in PPS-specific IgG levels between day 21 and day 0

Fig. 2B
**Fig. 2C**

**Increase in PPS-specific IgM levels between day 42 and day 0**

- **sham**
- **splenectomy**
- **autotransplantation**

**Increase in PPS-specific IgG levels between day 42 and day 0**

PPS types:

- PPS 3
- PPS 4
- PPS 6
- PPS 9
- PPS 14
- PPS 23

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In accordance with previous publications we found that splenectomy had a clearly negative effect on the anti-polysaccharide antibody response. At day 3 after immunisation in splenectomized rats compared with sham operated rats, a significant lower antibody titre was observed for all PPS types except PPS type 23 for both IgM and IgG (fig. 2). Splenectomy, followed by autotransplantation can partly rescue the antibody response to Pneumovax® vaccination: at day 3 IgM response to PPS 3, 14 and 23, and IgG antibody responses to pneumococcal serotypes 4, 6, 9, and 23 were similar in the autotransplantation group as compared with the sham-operated group.

When evaluating the number of rats in each group reaching ≥ two-fold increase in immunoglobulin titres, compared with the sham-operated group (fig. 3), both at day 3 and day 21, the splenectomy group showed a significant lower relative number of adequate responders for all PPS types except PPS14 for IgM and for all but PPS3 for IgG (p< 0.05).

**Fig 3.** Graphical representation of number of rats per group with minimum of 2-fold increase in PPS type-specific IgM and IgG between day 0 (day of vaccination) and day 3. (Significant lower numbers compared to sham-operated rats: * p<0.05; # p= 0.05)
In contrast, for the autotransplanted group for IgM only for PPS4 and 6 and for IgG only for PPS3 and 4 was a lower number of responders found, both at day 3 and day 21. No other immunoglobulin titres were significantly different from the sham operated group. At day 42 only individual rats in the splenectomy and autotransplantation group showed more than two-fold increase in immunoglobulin titre, whereas the sham operated rats showed >2-fold responses for IgG levels for PPS4 (three rats), PPS9 (five rats) and PPS14 (two rats).

Discussion

Reimplantation of splenic tissue has been postulated to account for a lower incidence of OPSI among patients splenectomized for trauma\(^8,19\). However, the efficacy of splenic autotransplantation in restoring the immune response after splenectomy has not yet been established firmly. Some studies support the notion that immunisation combined with autotransplantation leads to higher antibody levels and may therefore offer a survival benefit\(^9\). In this study it is demonstrated that autotransplantation followed by vaccination confers the ability to mount an improved IgM and IgG antibody response to pneumococcal capsular polysaccharide antigens. Restoration of the early IgM response after vaccination is not the most surprising, as TI-2 antigens that enter the bloodstream will normally localise in the spleen followed by a rapid IgM response\(^21\). The IgG results are concurrent with earlier reports\(^22\) and with the fact that in humans, a single vaccination with Pneumovax\(^8\) induces significant levels of IgG antibodies. Although the early IgG response might be the consequence of a classic secondary immune response, the non- or low-responsiveness for most PPS subtypes after splenectomy does indicate that this is not likely. In previous studies we have demonstrated that localisation of PPS on follicular dendritic cells in follicle and germinal centre is dependent on a serum factor, most likely a complement-fragment (probably C3d), but not on the presence of specific antibody\(^16,23,24\). This implies that PPS after entering the marginal zone, may induce PPS-specific B-cells migrating to the follicle/germinal centre\(^16,25,26\), where PPS-complement complexes at the same moment are already localised on FDC\(^16,23,24\). The germinal centre is considered to be the area where the isotype-switch takes place, enabled by immune complexes on local follicular dendritic cells. Tentatively, it can be suggested that specific for this type of antigen an early isotype switch to IgG can take place independently of the presence of substantial levels of specific IgM.

It should be noted that the clinical presentation of a pneumococcal infection in splenectomized patients (i.e. pneumococcal sepsis) is unlike that of other patients with increased susceptibility to pneumococcal infections due to insufficient anti-polysaccharide antibodies. In the latter category of patients, pneumococcal infections may cause pneumonia, meningitis or otitis, but seldom results in sepsis. This can be explained, because when a functional spleen is present, even badly opsonized pneumococci can be cleared by the spleen MPS, due to the specific low flow spleen red pulp architecture\(^21\) thus preventing bacteremia and sepsis. As in the case of splenectomy the restoration of
clearance by autotransplantation is insufficient\textsuperscript{11}, and restoration of the humoral response becomes even more important, allowing good opsonization of PPS. Despite the observation of normal total IgM or IgG antibody titres against TI-2 antigens, a low or absent response to individual subtypes may be present. The capacity for an early, adequate humoral response is of utmost importance, as in case of pneumococcal bacteremia with risk of sepsis, only protection can be expected when a rapid humoral response can be elicited. Only in this case the bacteria may be opsonized. When small amounts of regenerated autotransplanted spleen are present, with adequate response capacities to TI-2 antigen, specific antibodies will be formed. These antibodies are able to cause opsonization of the antigen (encapsulated bacteria) and, because phagocytosing capacity of the splenic fragments will not play an important role, the cells of the mononuclear phagocyte system as present in the liver are then capable of removing these opsonized particles\textsuperscript{21}.

The architecture of the splenic transplants demonstrated that we were dealing with regenerated splenic fragments with a largely restored white pulp. A functional white pulp is characterised by follicles with a functional germinal centre and a distinct marginal zone that is occupied with B lymphocytes, capable of inducing a primary immune response against TI-2 antigens in an appropriate vascular micro-environment. From the extensive studies by Pabst and co-workers, it was concluded that the younger the recipient and the donor, the better the regeneration and perfusion of the regenerated splenic tissue\textsuperscript{27,28}. In addition to these results which showed mainly adequate regeneration when using fetal/newborn spleens, we obtained similar results using rats of 3 months old ("adolescent"). This may be due to strain differences. Although for the human situation an estimation of age range, probably limiting the success of spleen autotransplantation, is hard to give, it seems likely that there is a much better potential for splenic regeneration in children and possibly young adults, than in older subjects.

The question how much spleen tissue should be transplanted has been a matter of controversy with respect to both total amount of transplanted tissue and size of the individual spleen fragments\textsuperscript{9,19}. In different animal models the implanted amount of spleen tissue did not affect the resulting amount of regenerated tissue, whereas a particle size does seem to play a role\textsuperscript{27}. Others and we think that particle size should at least allow the reticular/stromal structures of individual white pulp nodules to remain intact\textsuperscript{27} and should allow ingrowth of proliferating vessels at the implantation site. Although a minimum amount of transplanted spleen tissue seems necessary, a too large mass will result in increase of necrosis, resulting in fever and other complications.

In our study the response capacity was evaluated 12 weeks after transplantation, as for rats it was reported\textsuperscript{27} that regeneration of spleen transplants was in general completed. For other species it may take more time to complete this regeneration; 3-6 months is supposed to be the maximum time frame in which regeneration is completed. At present it is not known whether at this time point also functional maturation is reached, although from our results it appears that at least an initial capability to respond to TI-2 antigens is present. Future studies should reveal whether improvement of immune responses could be
obtained when postponing the antigen challenge.
The conclusion of this study is that autotransplanted splenic tissue in the omentum major after splenectomy has the possibility to grow out to immunological functioning spleens with a functional marginal zone. Consequent on this we demonstrated the capacity of early specific antibody formation in the autotransplanted rats comparable to normal, whereas after splenectomy a significantly decreased response was observed for most PPS-types. Autotransplantation followed by immunisation after a time interval will lead to significant increase of antibody levels for most PPS types in most subjects comparable to sham operated rats, whereas after splenectomy a significantly lower number of subjects show a relevant increase in antibody levels. In particular because high immunoglobulin levels are already found at an early time point (3 days) this may give protection against OPSI and offer a survival benefit. Although this has also to be proven in humans, these findings are a positive argument that autotransplantation of splenic tissue is of value and may provide protection against OPSI.

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