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# Lack of effect of splenic regrowth on the reduced antibody responses to pneumococcal polysaccharides in splenectomized patients

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## SUMMARY

The experiments were to determine if ectopic splenic tissue in humans would restore to normal those antibody responses which are reduced in patients who have been splenectomized. The IgM and IgG antibody response to subcutaneous injection of polyvalent pneumococcal polysaccharide vaccine (PNEUMOVAX) was determined in 34 patients who had been splenectomized for trauma and 14 controls, by measuring the concentration of antibody specific for five of the serotypes in the vaccine in serum samples taken before and 1 month after the immunization. The patients had significantly lower post-immunization concentrations of IgM antibody for three of the five serotypes measured, and IgG for two of the five. The antibody response to the immunization was assessed by comparing the post- to the pre-immunization concentration of antibody by analysis of covariance. The patients had a significantly lower IgM response to three of the five serotypes measured and IgG response to four of the five. It is concluded that in adult humans the spleen is important in the maintenance of normal humoral immune responses.

The presence and degree of ectopic splenic regrowth (splenosis) in the splenectomized patients was assessed by a spleen-specific radio-isotopic scan. There was no difference between patients with splenosis and those without, or between those with different degrees of splenosis, in any of the parameters of the antibody response measured. This is *in vivo* evidence indicating that ectopic splenic tissue in humans does not normalize the altered antibody responses observed following splenectomy.

**Keywords** splenosis splenectomy septicaemia polyvalent pneumococcal polysaccharide vaccine

## INTRODUCTION

Patients who have been splenectomized have an increased risk of developing overwhelming septicaemia, due mainly to infection by fast-growing encapsulated bacteria (Singer, 1973), particularly *Streptococcus pneumoniae* (Francke & Nell, 1981). Therefore, there has been great interest in determining what alterations in immunological function occur in humans following splenectomy, particularly those which predispose to septicaemia, and what treatments will protect patients from the consequent infections. One treatment currently advocated is the autotransplan-

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tation of slices of spleen into omental pouches at the time of splenectomy. Despite the lack of clear evidence that ectopic splenic tissue has a protective effect, autotransplantation is currently practised by some surgeons (Kusminsky *et al.*, 1982), in the hope that this will decrease the incidence and/or the severity of post-splenectomy infections.

Spontaneous regeneration of splenic tissue (splenosis) frequently occurs following splenectomy for traumatic rupture of the spleen (Kiroff *et al.*, 1983). We have studied patients with splenosis, a 'natural' form of splenic autotransplantation, to investigate if ectopic splenic tissue has the ability to normalize antibody responses. Using a polyvalent pneumococcal polysaccharide vaccine, we have investigated three aspects of the humoral immune response *in vivo*. The first is whether the antibody response of splenectomized patients differs from that of intact controls. The second is whether the antibody concentration achieved by the patients is sufficient to protect against future infection, while the third is whether the presence of splenosis tissue affects the in-vivo antibody response. This latter question is of particular importance, given the feasability of auto-transplantation of splenic tissue at the time of splenectomy.

## MATERIALS AND METHODS

*Patients.* The study population consisted of 34 otherwise healthy individuals who, following trauma to the spleen, had been splenectomized between three and 13 years previously (median interval 6 years). The group consisted of 25 males and nine females, age range 17 to 70 years (median age 29 years). Fourteen healthy volunteers (10 males, four females; median age 32 years, range 19 to 43) acted as controls. Informed consent was obtained from all participants. The study conformed to the guidelines of, and was approved by, the Research Review Committee of the Royal Adelaide Hospital.

*Vaccine.* Fourteen valent pneumococcal vaccine (PNEUMOVAX: Merck, Sharp and Dohme; batch number 1961E) was administered subcutaneously to all patients and controls. This vaccine contains 50 μg of each of the following serotypes: 1, 2, 3, 4, 6A, 7F, 8, 9N, 12F, 14, 18C, 19F, 23F and 25 (Danish nomenclature).

*Pneumococcal Antibody Assay.* Concentrations of IgG and IgM antibody to serotypes 2, 7F, 9N, 14, and 23F were determined by ELISA (Voller, Bidwell & Bartlett, 1979). Blood was taken immediately before and 4 weeks after immunization from all controls and patients. The sera were stored at −70°C until all samples were assayed simultaneously.

The capsular polysaccharides of the pneumococcal serotypes 2, 7F, 9N, 14 and 23F (Health Research Inc., New York) were dissolved in distilled water at a final concentration of 1·0 mg/ml, and the working polysaccharide solutions (WPS) were made by diluting each stock solution with bicarbonate coating buffer (pH 9·6) to achieve the following final concentrations for each serotype: 2, 50 μg/ml; 7F, 50 μg/ml; 9N, 100 μg/ml; 14, 5 ug/ml and 23F, 50 μg/ml. To coat the plates (Nunc) 100 μl of each WPS was added to the appropriate wells of micro-ELISA plates and incubated overnight in a moist chamber at room temperature. The plates were then washed twice with a solution of 0·05% Tween 20 in normal saline. Sample and control sera were tested at a dilution of 1:2,100, prepared in phosphate buffered saline, pH 7·6, containing 0·05% Tween 20 and 100 mg/ml bovine serum albumin (BSA). The IgG concentrations were quantified by using the following dilutions of a standard serum prepared in the BSA diluent: 1:500, 1:1,000, 1:2,100, 1:4,200, 1:8,400, 1:16,800, 1:33,600, 1:67,200. These dilutions correspond to antibody nitrogen concentrations of: 5,880; 2,940; 1,400; 700; 350; 175; 87·5 and 43·75 ng/ml. The standard serum was provided by Dr F. Sheffield of the National Institute for Biological Standardization, London, and had been standardised by ELISA against a radio-immuno-assay evaluated standard serum from Dr G. Schiffman, State University of New York. Because a standard serum for quantifying IgM in terms of antibody nitrogen concentration was not available, the IgM results are expressed in arbitrary units.

For the test, 100 μl of the dilution of the serum being tested, or the dilution of the standard, were added to wells of an ELISA plate. Pre- and post-immunization serum samples were always tested on the same plate. Also, 100 μl of BSA diluent was added to the wells in one row to serve as a blank.

Plates were incubated at room temperature in a moist chamber for 2 h before being washed three times with 0·05% Tween 20 in normal saline. Anti-human IgG (or IgM) alkaline phosphatase conjugate (Behring) was used as the labelled indicator antibody. A 100 μl aliquot of a 1 : 5 dilution of the conjugate in BSA diluent was added to all wells. Plates were incubated at room temperature for 3 h and then washed 3 times with 0·05% Tween 20 in normal saline. One tablet of *p*-nitrophenol phosphate (Sigma) was dissolved in 5 ml of diethanolamine buffer, pH 9·8, to form the substrate solution. Then 150 μl of substrate solution was added to each well of the prepared plate and incubated at room temperature for exactly 20 min. The reaction was stopped by the additon of 50 μl of 2 M sodium hydroxide. The optical density at 405 nm was then determined automatically on a multiscan reader coupled on line to a micro-computer. The absorbance for each sample was determined and the concentration of serotype-specific IgG or IgM antibody was automatically calculated from the standard curve derived from the reference serum.

*Spleen Scans.* Splenic scanning was performed following the i.v. reinjection of heat damaged, radio-labelled autologous erythrocytes (Kiroff *et al.*, 1983). Anterior, lateral and posterior scans of the entire abdomen and pelvis were taken. Each scan was assessed independently by two observers, and the patients were assigned to one of four groups based on the area of splenic uptake in the posterior view. Group 1 consisted of those patients with no evidence of splenosis, Group 2 with minimal, Group 3 with moderate and Group 4 with large splenosis (Kiroff *et al.*, 1983). The allocations of each observer were identical for all but two patients. In each of these two instances the observers initially disagreed on whether the degree of regrowth was minimal or moderate.

*Analysis of Results.* The results were analysed to determine the most appropriate method of statistically testing the data. It was found that the antibody concentrations were not normally distributed about the mean (increased positive coefficient of skewness) and that the variance of the results increased as the mean increased (assessed by F-test), i.e., the variance was found to be heterogeneous. Logarithmic transformation ($\log_{10}$) of the data resulted in homogeneous variance for the groups (F-test), enabling comparisons to be made between the groups.

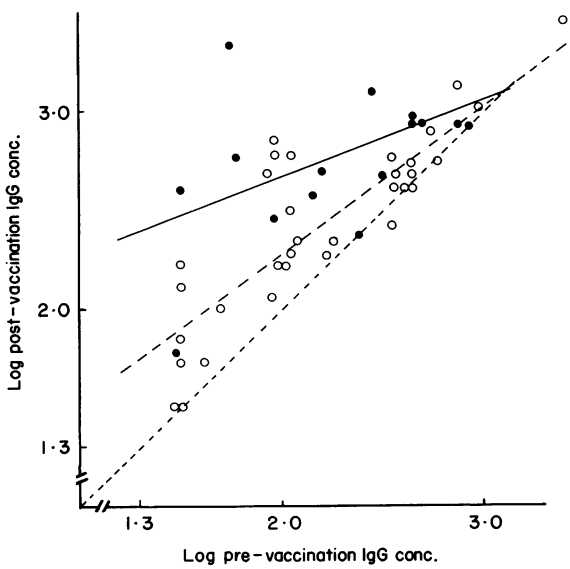


**Fig. 1.** An illustration of the statistical analysis employed, using data from this study. The line y = x conforms to the regression that would exist if there were no response. The regression line y = 0·78 + 0·75x describes the IgG response to serotype 2 by the patients (○). The regression line y = 1·88 + 0·38x describes the IgG response to serotype 2 by the controls (●). The variances for the two populations were calculated and shown to be homogeneous, which allows the groups to be compared. F for the slope = 3·827 ($0·05 < P < 0·01$) while F for elevation = 8·83 ($P < 0·1$). Therefore the control and the splenectomized populations were significantly different in their response to serotype 2, with the controls having the greater response.

We have assumed that the post-immunization concentration of antibody in an individual results from three components. The first is the pre-immunization concentration of antibody. The second is the ability of the population or group (i.e., patient or control, adult or child, etc.) to respond to the antigen, and this component is measured by the regression coefficient of the response of the group. The third influence is the variation specific to the individual, this component being related to the variance of the group. The appropriate statistical test to compare the responses of different groups, a test which takes into account these factors and which also allows for the differences between individuals in pre-immunization antibody concentrations, is the analysis of convariance.

The results from one representative experiment, plotted in Fig. 1, help explain the basic approach used in the analysis of covariance. When the logarithms of the pre-vaccination and post-vaccination serum antibody concentrations are plotted as the abscissa and ordinate respectively for each individual in a population studied, then by linear regression analysis, a line can be drawn through the obtained group of points, with the general formula $y = a + bx$ (Fig. 1). When the slope of this line (b) is equal to 1, and the y intercept (a) is equal to 0, then, as a group, the population under study has failed to respond to the vaccine. If the results from two populations are so graphed then the regression lines may differ from each other with respect to one or two parameters, *viz.*, the slope or the elevation (the distance between the lines) (Fig. 1). We were able to independently compare either the slopes or the elevations, by calculating deviations from the regression lines for each group by the F-test.

The antibody responses of the subgroups, defined by the degree of splenosis measured, were compared using a modification of the analysis of covariance enabling multiple comparisons. Fisher's exact probability test was used to compare the percentage of the patients and the controls who had a concentration of specific antibody $>300$ ng/ml following the immunization. The absolute serum antibody concentration after immunization was compared between the groups using the unpaired Wilcoxon test.

For the statistical analysis, IgG concentrations less than 38 ng/ml were considered to be equal to 38 ng/ml and IgM concentrations less than 2 units were considered to be equal to 2 units. The results are given as the medians of the patient or the control group, with the 95% confidence limits in parentheses.

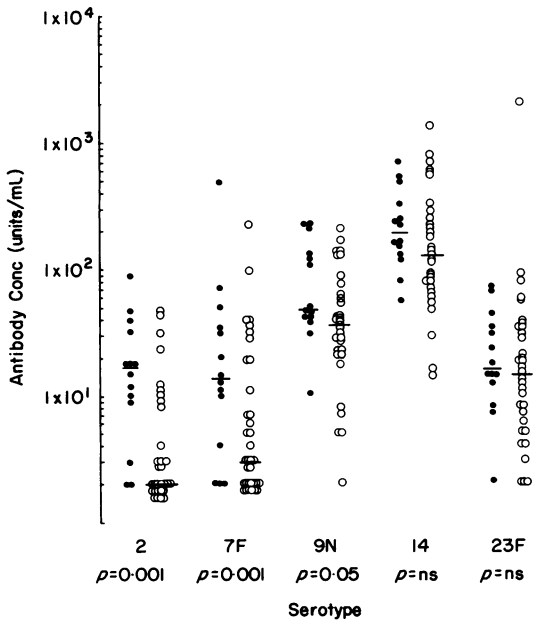


**Fig. 2.** The post-immunization concentration of IgM antibody, specific for 5 of the serotypes injected, in the serum of splenectomized (Spx, ○) and control (Con, ●) subjects.

## RESULTS

*Specific IgM antibody response*

There was no difference between patients and the controls in the concentration of specific IgM antibody in the serum collected before the immunization, for each of the five serotypes tested. The serotype specific antibody concentrations measured in the serum collected one month after the immunization are illustrated in Fig. 2. The concentrations of IgM antibody specific for the serotypes 2, 7F and 9N were higher in the controls than the patients. Thus, the median IgM concentration for serotype 2 was 16·5 units/ml (2–40) in the controls and 2 (2–4) in the patients. For serotype 7F the median concentration was 12 (2–49) in the controls and 3 (2–7) in the patients, and for 9N, 45·5 (30–207) and 38·5 (22–52) respectively (Wilcoxon, $P<0·05$). There was no significant difference between the controls and the patients in the median concentration of IgM antibody specific for serotype 14 and 23F: 183 (77–461) vs. 121·5 (76–200) and 16 (7–41) vs. 14 (6–28) respectively.

The regression equations, F-values and significance levels for the IgM antibody response are detailed in Table 1. The IgM response to the serotypes 2, 7F and 9N was greater in the controls than the patients.

*IgG Antibody Response*

There was no difference between the patients and the controls in the concentration of specific IgG antibody in the serum collected before the immunizaton, for each of the serotypes assayed. The serotype specific antibody concentrations measured in the sera collected one month after the immunization are shown in Fig. 3. The median concentration of IgG specific antibody in the patients for serotype 2 (355 ng/ml; 163–548) and serotype 7F (453; 218–592) was significantly less than in the controls: 543 (248–928) and 662 (204–1227) respectively (Wilcoxon, $P<0·05$). Although the median post-immunization concentration of antibody specific for 9N in the patients (519; 233–673) was lower than in the controls (747; 294–1109), the difference was not significant ($P=0·08$). There was no difference between the patients and the controls in the median concentration of antibody specific for the serotypes 14 and 23F; 854 (439–1277) vs. 867 (619–1197) and 753 (237–1116) vs. 765 (407–1014) respectively.

The regression equations, F-values and significance levels for the IgG antibody response are detailed in Table 2. The IgG response to the serotypes 2, 7F, 9N and 14 was higher in the controls than in the patients, but there was no significant difference for the serotype 23F.

**Table 1.** Statistics for the regression lines for the IgM antibody response by the control (Con) and splenectomized (Spx) subjects. The controls had a higher IgM response than the patients for three of the five serotypes (2, 7F & 9N) (analysis of covariance).

| Serotype | | y-intercept | Slope | F for Slope | F for Elevation |
|---|---|---|---|---|---|
| 2 | Con | 1·167 | −0·105 | 1·804 ($P$=NS) | 18·24 ($P<0·005$) |
| | Spx | 0·211 | 0·977 | | |
| 7F | Con | 0·633 | 1·432 | 0·023 ($P$=NS) | 4·35 ($P<0·05$) |
| | Spx | 0·325 | 1·293 | | |
| 9N | Con | 0·454 | 0·885 | 0·017 ($P$=NS) | 4·1 ($P<0·05$) |
| | Spx | 0·252 | 0·928 | | |
| 14 | Con | 0·276 | 0·912 | 1·39 ($P$=NS) | 1·67 ($P$=NS) |
| | Spx | 0·017 | 1·01 | | |
| 23F | Con | 0·197 | 0·911 | 2·955 ($P$=NS) | 2·036 ($P$=NS) |
| | Spx | −0·053 | 1·09 | | |

NS not significant.

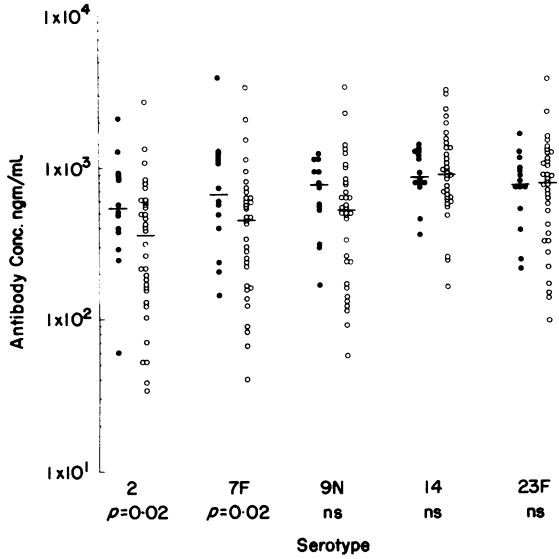


**Fig. 3.** The post-immunization concentration of IgG antibody, specific for five of the serotypes injected, in the serum of splenectomized (Spx, ○) and control (Con, ●) subjects.

*The effect of splenosis on the antibody responses*

Splenosis tissue was detected in 21 (66%) of the 32 patients studied. The patients were allocated into groups according to the presence or not of splenosis, and those patients with splenosis were further allocated into three subgroups according to an estimate of the degree of splenosis. Thus 11 patients had no splenosis, 10 had minimal, seven moderate and four large splenosis (who were estimated to have an almost normal volume of spleen tissue).

There was no difference in the specific IgG or IgM antibody response to any of the serotypes tested between patients who had splenosis, whether minimal, moderate or large, and those in whom it was not demonstrated. In particular, splenosis did not restore to normal the low final antibody concentrations or the deficient response to serotypes 2, 7F, 9N and 14.

**Table 2.** Statistics for the regression lines for the IgG response by the control (Con) and the splenectomized (Spx) subjects. The controls had a higher IgG response to four of the five serotypes (2, 7F, 9N & 14) (analysis of covariance).

| Serotype | | y-intercept | Slope | F for Slope | F for Elevation |
|---|---|---|---|---|---|
| 2 | Con | 1·878 | 0·38 | 3·83 ($P$=NS) | 8·83 ($P$<0·005) |
| | Spx | 0·777 | 0·754 | | |
| 7F | Con | 1·187 | 0·691 | 0·076 ($P$=NS) | 5·95 ($P$<0·025) |
| | Spx | 0·853 | 0·749 | | |
| 9N | Con | 1·679 | 0·454 | 5·82 ($P$<0·025) | 5·42 ($P$<0·025) |
| | Spx | 0·659 | 0·811 | | |
| 14 | Con | 2·052 | 0·324 | 4·49 ($P$<0·05) | 0·1 ($P$=NS) |
| | Spx | 0·717 | 0·804 | | |
| 22F | Con | 1·473 | 0·522 | 2·22 ($P$=NS) | 1·32 ($P$=NS) |
| | Spx | 0·809 | 0·753 | | |

NS not significant.

**Table 3.** The proportion (%) of control (Con) and splenectomized (Spx) subjects who had a pre- or post-immunization IgG antibody concentration in excess of 300 ng/ml for each serotype. No significant difference between the patients or the controls was noted (Fisher's exact probability test).

| | Pre-immunization | | | Post-immunization | | |
|---|---|---|---|---|---|---|
| Serotype | Spx ($n=34$) | Con ($n=14$) | $P$ | Spx ($n=34$) | Con ($n=14$) | $P$ |
| 2 | 41 | 36 | 0·24 | 53 | 79 | 0·07 |
| 7F | 38 | 43 | 0·24 | 59 | 79 | 0·12 |
| 9N | 47 | 50 | 0·24 | 68 | 79 | 0·22 |
| 14 | 91 | 79 | 0·18 | 91 | 100 | 0·35 |
| 23F | 71 | 64 | 0·24 | 82 | 86 | 0·32 |

*Protective response to immunization*

A total IgM and IgG antibody concentration in the range of 250–300 ng/ml, as determined by radio-immuno-assay (RIA), is considered to protect against pneumococcal infection (Smit *et al.*, 1977). Because we were not able to measure the IgM antibody concentration except in arbitrary units, we have compared the proportions of patients and controls who had a post-immunization IgG antibody concentration in excess of 300 ng/ml. The results in Table 3 show that, in this respect, there was no difference between the patients and the controls, for each of the serotypes.

*The effect of the interval since operation*

No significant correlation was found between the concentration of specific IgG or IgM antibody, either before or after the immunization, and the interval between the splenectomy operation and the time of immunization.

## DISCUSSION

Since the initial report by King and Schumacker (1952) of overwhelming septicaemia in splenectomized infants, considerable data has accumulated which underlines the importance of the spleen in defence against infection (Singer, 1973; Gopal & Bisno, 1977). Patients splenectomized as part of treatment of an underlying disease such as thalassaemia, idiopathic thrombocytopenic purpura, autoimmune haemolytic anaemia or malignant haematologic disease are at greatest risk of septicaemia, but even in patients splenectomized for trauma the incidence is around 1%, with a mortality of about 50% (Singer, 1973). The deliberate auto-implantation of splenic tissue at the time of operation has therefore been advocated as a means of maintaining normal spleen function, but there is little information in humans on its effectiveness.

In this study we have measured the concentration of IgM and IgG antibody specific for five serotypes of pneumococcus. There was no statistically significant difference between the patients and the controls in the concentrations of antibody in the sera taken before the immunization, but the patients had significantly lower post-immunization concentrations of IgM antibody for three of the five serotypes measured, and IgG for two of the five.

Reports on the antibody response to pneumococcal or meningococcal polysaccharide antigens in splenectomized patients differ, with some indicating that splenectomy has no effect (Sullivan *et al.*, 1978; Ammann *et al.*, 1977) and others suggesting that it leads to an impairment of the response (Ahonkhai *et al.*, 1979; Hosea *et al.*, 1981; Aaberge *et al.*, 1984). In part the differences noted may result from differing underlying pathological conditions, and/or the use of immunosuppressive drugs in some of the patient groups. In addition, the methods of assessing the antibody response and

analysing the results have differed between the studies. Frequently a 'fold increase' (i.e. the ratio of the post- to the pre-immunization antibody concentration) is used to measure the antibody response to antigen challenge. Following preliminary testing of our data, we concluded that a more appropriate method was an analysis of covariance on the logarithmically transformed data. With this technique we found that the IgM antibody response to the serotypes 2, 7F and 9N and the IgG response to 2, 7F, 9N and 14 were significantly less in the patients than in the controls.

It is not certain what concentration of serum antibody is protective against pneumococcal infection, nor what constitutes an adequate response to pneumococcal vaccine. Based on the antibody concentrations found to be present before proven infections, it has been suggested that 300 ng/ml of antibody nitrogen is adequate to protect against serious pneumococcal infections (Landesman & Schiffman, 1981). We found that, although the overall response of the patients was lower, there was no difference between the percentage of patients or controls who achieved serum concentrations of antibody greater than 300 ng/ml following immunization.

Many patients in this study had intra-abdominal splenosis detected by scanning. Splenosis is thought to result from the growth of unintentionally autotransplanted tissue or cells, dissociated from the spleen either during its traumatic rupture or at surgery. The number of pitted red blood cells, which are normally culled from the circulation by the spleen (Koyama *et al.*, 1962), increases in the peripheral blood of splenectomized patients, but the number of these cells in this group of patients was inversely proportional to the degree of splenosis, indicating that splenosis tissue retains this function (Kiroff *et al.*, 1983). The fact that splenosis tissue was visualised in the spleen scans, which measure the uptake of damaged autologous erythrocytes by the liver and spleen, also indicates that it has retained some splenic function.

Evidence from animal experiments regarding the ability of splenosis tissue to confer protection against infection is inconclusive (Likhite, 1978; Cooney *et al.*, 1979; Brown, Hosea & Frank, 1981), although it is reported the autotransplants in splenectomized rats partially restore the antibody response to an intravenously injected particulate antigen, sheep red blood cells (Schwartz *et al.*, 1978; Church, Mahour & Lipsey, 1981). There is no evidence from human studies that splenosis tissue is beneficial. Rice and James (1980) reported on two cases of fatal pneumococcal septicaemia that occurred 8 and 9 years after splenectomy for trauma. At autopsy, one patient had over 100 nodules of splenic tissue, the largest of which weighed 3 g, while splenic tissue in the other patient weighed 92 g. Moore *et al.* (1983) reported that a patient with 2 g of splenic tissue implanted into the omentum after splenectomy for trauma died 5 months later from pneumococcal septicaemia. However, the amount of spleen transplanted was very small, and the patient was an alcoholic with cirrhosis.

Previous studies have shown that many *in vitro* immunological functions, which are altered in splenectomized patients, are not normalized in those patients with splenosis. These include immunoglobulin synthesis *in vitro* (Drew *et al.*, 1984), serum immunoglobulin concentrations (Drew *et al.*, 1984), natural killer cell activity and lymphocyte responses to mitogens (Ferrante *et al.*, 1985). Our study has demonstrated that spontaneously occurring splenosis does not restore the defective *in vivo* antibody responses to polysaccharide vaccine.

Our results show that, following immunization, the median concentration of IgG antibody for each of the five serotypes tested exceeded the concentrations generally considered to be protective (Landesman & Schiffman, 1981), suggesting that vaccination of all splenectomized patients is warranted. That the spleen is important for the maintenance of a normal humoral immune response is shown by the lower antibody responses in splenectomized patients. However, while the culling function for red cells was at least partially restored in the patients with splenosis, splenosis tissue could not completely compensate for the immunological function of the intact spleen.

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