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The role of the spleen in the immune response following naturally acquired exposure to encapsulated bacteria

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Summary. Female New Zealand White Rabbits following splenectomy ($n=9$), splenectomy with 50% splenic autotransplantation ($n=8$) and sham laparotomy ($n=9$) have been serially exposed to type 2 *Streptococcus pneumoniae* and *Haemophilus influenzae* by aerosol inhalation. Animals were sampled for 3 weeks after exposure and the IgG and IgM type-specific antibody response measured by enzyme-linked immunosorbent assay. *Haemophilus influenzae* initiated a substantial anti-haemophilus IgG response which was not diminished by splenectomy. The anti-haemophilus IgM response was present in sham-operated animals, absent following splenectomy, and partially restored by splenic autotransplantation. Type 2 *Streptococcus pneumoniae* induced a minimal IgG and IgM antibody response in all animals irrespective of the presence or absence of a spleen. The results support the role of the spleen in mediating IgM production against polysaccharide encapsulated bacteria. The differential degree of immune response produced by the two organisms may explain in part the differential frequency with which these two organisms infect man following splenectomy.

There is a greater susceptibility to infection with polysaccharide-encapsulated bacteria following splenectomy. *Streptococcus pneumoniae* is the commonest responsible organism although an enhanced risk of infection with *Haemophilus influenzae* and *Neisseria meningitidis* has also been reported (Singer 1973; Spirer 1980; Pearson 1980; West & Grosfeld 1985; Bohnsack & Brown 1986; Di Cataldo *et al.* 1987).

The spleen plays a complex part in the antibody response to infection. This includes the processes of antigen recognition and subsequent antibody synthesis. A poor humoral antibody response to infection may therefore be a factor in the increased susceptibility to infection after splenectomy (Cohn & Schiffman 1987).

The majority of the reports of the effect of splenectomy on the humoral immune response have documented the changes in circulating antibody following subcutaneous immunization with soluble polyvalent pneumococcal vaccine. The reported experimental work on the protective effect of the spleen against pneumococcal infection has been based on mortality data following intraperitoneal or intravenous innoculation with various types of *Streptococcus pneumoniae.* However, these methods are not truly repre-

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sentative of the circumstances of pneumococcal infection in man because they do not reproduce the route of acquisition of encapsulated bacteria which are predominantly acquired via the respiratory tract.

This paper concerns the antibody response following exposure of animals to live bacteria by aerosol inhalation. The effects of sham laparotomy, total splenectomy and splenic autotransplantation on the IgG and IgM immune response have been measured.

Materials and methods

Animals

Female New Zealand White Rabbits approximately 3 months of age were used (Ranch Rabbits, Crawley Down, UK). Group A ($n=8$) underwent total splenectomy with 50% by weight of the spleen autotransplanted into omental pouches, group B ($n=9$) total splenectomy and group C ($n=9$) sham laparotomy. All animals were housed under identical conditions and fed a standard diet (B Rabbit Standard diet, SDS (UK) Ltd) with free access to water. Animals were given a 12-week recovery period to allow splenic regeneration.

Bacteria

Type 2 *Streptococcus pneumoniae* was obtained from laboratory stocks at Ninewells Hospital. The organism was maintained on blood agar plates (5% whole horse blood in Columbia Agar base (Oxoid Ltd, UK)) and grown at 37°C in 5–10% CO_2. Type b *Haemophilus influenzae* was obtained from a patient with Haemophilus meningitis. The organism was grown on chocolate blood agar (blood agar heated to 70°C) at 37°C in 5–10% CO_2. The organisms were subcultured every 2–3 days.

Exposure of animals to bacteria

Organisms were grown overnight and harvested into 0.15 M NaCl, washed three times in sterile 0.15 M NaCl and resuspended to an optical density of 1.50 at 540 nm. This suspension was shown by quantitative plating to contain between 10^7 and 10^8 organisms per ml. Rabbits were immobilized and the face was placed within 5 cm of a Hudson nebulizer (Hanley's Medical Supplies, UK) containing the bacterial suspension. The head of the animal was enclosed in a $30 \times 30 \times 30$ cm Perspex box to increase the inspired concentration of bacteria. Laboratory air (British Oxygen Company, UK) was fed to the nebulizer at a flow rate of 6 l/min for 30 min.

All animals were first exposed to *Streptococcus pneumoniae* type 2 and sampled for 3 weeks. Following a recovery period of 2 weeks the animals were subsequently exposed to *Haemophilus influenzae* by an identical method.

Blood cultures

Samples for qualitative blood culture were obtained from the marginal vein of the ear immediately after exposure, 2, 4 and 24 h post-exposure. One millilitre of blood was drawn into a sterile syringe and added to 20 ml of Robertson's Cooked Meat medium. The tube was subcultured after overnight incubation at 37°C.

Antibody assays

Antibody titres were measured using an enzyme-linked immunosorbent assay. Bacteria were harvested after overnight culture, washed three times in sterile 0.15 M NaCl and heat killed (100°C for 15 min). The purity of the suspension was tested by subculture and repeat culture after killing confirmed the absence of growth. Bacteria were sonicated at 20 kHz in ten 30-s cycles with 1 min between cycles, then diluted so that a 1:10 dilution solution had an optical density of 0.25 at 540 nm. The prepared antigen was stored at 4°C until used.

The solid phase of the assay was formed by coating the antigen onto 96-well microtitre plates (Nunc type F, Gibco, UK). Full strength

antigen was added to 0.05 M carbonate/bicarbonate coating buffer (pH 9.65; 0.167 g Na_2CO_3 and 0.286 g $NaHCO_3$ in 10 ml sterile water) in a ratio of 9:1. 100 μl of the antigen/buffer mixture was dispensed into each well and the plates were incubated at 37°C for 3–4 h and then a 4°C overnight. Plates were stored at 4°C until used.

Goat anti-rabbit IgG (Fc specific, Nordic Immunological Laboratories, UK) was reconstituted with PBS (phosphate buffered saline (pH 7.6; 5.12 g Na_2HPO_4, 0.62 g NaH_2PO_4. $2H_2O$, 34 g NaCl, 0.4 g NaN_3 in 4 l of purified water), precipitated in 18% Na_2SO_4 at room temperature for 40 min, reconstituted with 2.5 ml of PBS and dialysed for 48 h with PBS at 4°C with three changes of buffer. The protein content was checked by measuring the optical density at 280 nm. The purified dialysed antibody, 0.7 mg, was mixed with 1000 units of alkaline phosphatase (Bovine intestinal alkaline phosphatase type VII-T, Sigma), 10 μl 25% aqueous glutaraldehyde (Sigma) and made up to 1.25 ml with PBS, incubated at room temperature for 4 h and dialysed against PBS for 48 h with three changes of buffer. The alkaline-phosphatase-linked goat anti-rabbit IgG conjugate was stored at 4°C until used.

Alkaline-phosphatase-linked goat anti-rabbit IgM conjugate was purchased ready prepared (Southern Biotechnology Associates, Inc., UK distributor AR Horwell (Reagents) Ltd, London).

Blood for antibody assays was taken from the marginal vein of the ear prior to exposure and on days 1–7, 10, 14 and 21 after exposure. This was allowed to clot at room temperature, and the serum stored at −20°C until assayed.

Serum samples were diluted and 100 μl dispensed into the wells of antigen-coated microtitre plates. The plates were incubated for 45 min at 37°C and washed three times with PBST (PBS with Triton; pH 7.6 1 l of PBS with 1 ml 10% Triton × 100). The goat anti-rabbit conjugate (100 μl), either IgG or IgM, was dispensed into each well. IgG conjugate was used at a dilution of 1:100 and IgM conjugate at 1:1000 dilution. The plate was incubated at 37°C for 45 min, washed three times with PBST and 100 μl of *p*-nitrophenyl phosphate in glycine buffer substrate dispensed into each well (one 5 mg tablet of disodium *p*-nitrophenyl phosphate hexahydrate (Sigma 104–105 phosphatase substrate) added to 5 ml of 1:10 diluted substrate buffer, pH 10.2, made by dissolving 7.5 g glycine, 3.088 g NaOH, 0.40 g $ZnCl_2$ and 0.27 g $MgCl_2$ in 200 ml of sterile water).

The plates were left at room temperature to allow colour development and absorptions were read at 405 nm (MR 600 plate reader, Dynatech, USA) when control 'positive' readings had reached a predetermined optical density. Controls included 'negative' wells which were treated with PBS and 'positives' which were dilutions of a serum known to contain high levels of the antibody being assayed.

For each assay a standard curve from the control serum was drawn. Each of the dilutions of the control serum was assigned an antibody level (arbitrary units) and the antibody level per ml of serum under test was read from the curve for the control line.

Expression of results

Antibody levels are expressed as units per ml serum. The units of antibody are constant for any one assay and were assigned arbitrarily. The units are not directly comparable between the different assays. Changes in antibody levels are expressed as the multiple or fold increase (FI) of the pre-exposure value. A fold increase of 1.0 represents the pre-exposure value, a value of less than 1.0 represents a fall in antibody and a value of greater than 1.0 represents an increase. With this type of data a fold increase of greater than 2.0 is usually taken as a significant antibody increase (Pedersen *et al.* 1982; Aaberge *et al.* 1984).

Antibody levels for any one group of animals are expressed as the geometric mean together with the 95% confidence interval. Changes in antibody titres on any one day for

any one group of animals are expressed as the geometric mean fold increase (GMFI) compared to the pre-exposure value.

Comparisons of groups are made with non-parametric methods using Wilcoxon's Signed Rank Test for paired data and the Mann–Whitney *U*-test (two-tailed) for unpaired data. Correlations are made using Spearman's Rank Correlation Coefficient.

Results

No animals died during the studies from the effects of infection. Six of the autotransplanted animals had histological evidence of regenerated spleen in the omentum; the other two animals showed areas of granulation tissue, although small areas of regenerated spleen could not be excluded. Blood cultures were consistently negative.

Pneumococcal antibody response

Table 1 gives the pre-exposure pneumococcal IgG and IgM antibody levels for each group of animals in units/ml. The geometric mean fold increase and 95% confidence interval for each group of animals at 21 days after exposure and the number of animals attaining a greater than twofold increase is also indicated. The pre-exposure geometric mean pneumococcal IgG antibody level for the sham-operated animals (group C) was slightly lower than the other two groups ($P<0.02$).

In all three groups the IgG geometric mean

Table 1. Anti-type 2 pneumococcus antibody response. Values give the geometric mean and 95% confidence interval for pre-exposure IgG and IgM antibody levels in units/ml, the geometric mean fold increase (FI) and 95% confidence interval in type-specific IgG and IgM antibody 21 days after exposure for each group of animals and for each organism. The number of animals attaining a fold increase of greater than 2.0 by day 21 after exposure is also indicated

	Group A Autotransplant ($n=8$)	Group B Splenectomy ($n=9$)	Group C Sham laparotomy ($n=9$)
Anti-type 2 pneumococcal IgG			
Pre-exposure antibody level (units/ml)			
Geometric mean	3222	1746	451*
Confidence interval	(1316–7888)	(1049–2905)	(229–888)
21 Days after exposure			
Geometric mean FI	1.29	1.20	1.65
Confidence interval	(0.81–2.03)	(0.87–1.66)	(1.07–2.59)
Number with FI > 2.0	3/8	2/9	3/9
Anti-type 2 pneumococcal IgM			
Pre-exposure antibody level (units/ml)			
Geometric mean	220	130†	75‡
Confidence interval	(142–340)	(106–158)	(51–112)
21 Days after exposure			
Geometric mean FI	0.88	0.95	1.57
Confidence interval	(0.67–1.16)	(0.69–1.32)	(1.26–1.95)
Number with FI > 2.0	0/8	0/9	1/9

Statistical difference by Mann–Whitney *U*-test (two-tailed): * Group C lower than both groups A and B ($P<0.02$); † Group B lower than group A ($P<0.05$); ‡ Group C lower than both groups A and B ($P<0.02$)

FI 21 days after exposure to the pneumococcus remained below 2.0. Only three animals in group A, two in group B and three in group C individually attained a level above this. Initially, in the first few days after exposure there appeared to be a small decrease in pneumococcal IgG titre. This was observed both in the individual animals and in the overall geometric mean for the group. After day 5 there was a small increase in all groups. The rise in Groups A and B was not statistically significant; however, the rise in the sham-operated group just reached statistical significance by day 21 compared to the pre-exposure level ($P<0.05$) although the geometric mean FI (1.65) remained below 2.0. The maximum IgG fold increase for any individual animal in each group was 2.23 for group A, 2.35 for group B and 5.35 for group C.

The pre-exposure level of pneumococcal IgM antibody was lower in the sham-laparotomy group (group C) than in the other two groups ($P<0.02$).

The splenectomy group (group B) was also lower than the autotransplanted group ($P<0.05$). There was very little, if any, IgM response to the type 2 pneumococcus. Only one animal in group C reached a greater than twofold increase. There was a slight antibody increase in the sham-operated group and this rise, although small (geometric mean FI = 1.57), did reach statistical significance for the group by the 7th post-infection day ($P<0.01$). The maximum IgM fold increase for any individual animal in each group was 1.44 for group A, 1.74 for group B and 2.49 for group C.

Haemophilus influenzae antibody response

The overall responses of the same animals 21

Table 2. Anti-haemophilus influenzae IgG antibody response. Values give the geometric mean and 95% confidence interval for pre-exposure IgG antibody levels in units/ml, the geometric mean fold increase (GMFI) and 95% confidence interval (CI) in type-specific IgG antibody 21 days after exposure for each group of animals and for each organism. The number of animals attaining a fold increase of greater than 2.0 by day 21 after exposure is also indicated

		Group A Autotransplant	Group B Splenectomy	Group C Sham laparotomy
Pre-exposure antibody level (units/ml)				
Geometric mean		1633	972	566
Confidence interval		(1164–2290)	(121–7791)	(76–4182)
Day after exposure				
2	GMFI	0.92	0.86	0.95
	95% CI	(0.70–1.19)	(0.69–1.07)	(0.78–1.16)
5	GMFI	2.11	1.75	1.95
	95% CI	(1.48–3.00)	(0.94–3.26)	(0.96–3.95)
7	GMFI	2.93	4.08	6.91
	95% CI	(1.73–4.97)	(2.05–8.12)	(2.10–22.74)
10	GMFI	4.94	10.08	9.69
	95% CI	(2.59–9.41)	(2.56–39.94)	(2.44–38.40)
14	GMFI	7.33	11.75	15.08
	95% CI	(3.69–14.58)	(2.86–48.30)	(3.36–67.61)
21	GMFI	9.40	9.22	13.02
	95% CI	(5.14–17.21)	(2.13–39.81)	(2.78–60.97)
Number with FI > 2.0		8/8	7/9	7/9

days after exposure to *Haemophilus influenzae* are summarized in Table 2 for haemophilus IgG and Table 3 for haemophilus IgM antibodies.

The pre-exposure haemophilus IgG antibody titres were all similar (Table 2). All groups had a geometric mean FI of at least 9.0 by day 21 after exposure. Only two animals in group B and two animals in group C failed to respond with a fold increase of greater than 2.0. There was an initial small decrease in antibody before the levels rose by the 5th post-exposure day. All groups demonstrated a statistically significant increase by the fifth day. The sham laparotomy group (group C) appeared to peak at day 14 (GMFI = 15.08) but this was not statistically significant from the peaks seen for the other two groups. The maximum FI for any individual animal in anti-haemophilus IgG at day 21 was 18.33 for group A, 75.15 for group B and 172.0 for group C. These maximal responses occurred in different animals from those giving maximal IgG responses following type 2 pneumococcus exposure.

The pre-exposure level of anti-haemophilus IgM was lower in the autotransplanted than in the other two groups (Table 3). In contrast to the response for anti-type 2 pneumococcus IgM, there was a response in some of the animals following the *Haemophilus* exposure. Three animals in group A, two in group B, and five in group C reached a greater than twofold increase. Both the sham-laparotomy and the autotransplanted groups reached a geometric mean fold increase of at least 2.0 by the 21st day after exposure. It was noted that in the autotransplanted group and in the sham-operated

Table 3. Anti-haemophilus influenzae antibody response. Values give the geometric mean and 95% confidence interval for pre-exposure IgM antibody levels in units/ml, the geometric mean fold increase (GMFI) and 95% confidence interval (CI) in type-specific IgM antibody 21 days after exposure for each group of animals and for each organism. The number of animals attaining a fold increase of greater than 2.0 by the 21st day after exposure is also indicated

		Group A autotransplant	Group B Splenectomy	Group C Sham laparotomy
Pre-exposure antibody level (units/ml)				
Geometric mean		36*	53	62
Confidence interval		(28–44)	(43–65)	(47–83)
Day after exposure				
5	GMFI	1.38	1.03	1.50
	95% CI	(1.03–1.85)	(0.90–1.17)	(0.96–2.34)
7	GMFI	1.80	0.94	1.75
	95% CI	(1.50–2.16)	(0.80–1.12)	(1.16–2.65)
10	GMFI	2.22	0.94	1.74
	95% CI	(1.43–3.45)	(0.78–1.14)	(1.01–2.99)
14	GMFI	2.35	1.04	2.10
	95% CI	(1.53–3.62)	(0.80–1.34)	(1.23–3.58)
21	GMFI	1.84	1.43	2.13
	95% CI	(1.24–2.74)	(1.06–1.91)	(1.29–3.52)
Number with FI > 2.0		3/8	2/9	5/9

Statistical difference by Mann–Whitney *U*-test (two-tailed): *group A lower than both groups B and C ($P < 0.02$).

group the levels of anti-haemophilus IgM rose quite quickly and were statistically significant within 7 days and at their highest levels by day 14. In contrast, in the total splenectomy group any increase occurred considerably later and was apparent only by day 21. The splenectomy group (group B) showed a poor response, geometric mean FI = 1.43 by the 21st day. The maximal FI for any individual animal in each group was 5.38 for group A, 2.52 for group B and 7.41 for group C.

The changes in anti-haemophilus IgG titre appeared to be greatest in animals with the lowest pre-exposure antibody titre. Thus, an inverse correlation (Spearman's Rank) was observed in both the splenectomy and sham-laparotomy groups between pre-exposure anti-haemophilus IgG titre and the fold increase at 21 days (group A $R_s = -0.19$, n.s.; group B $R_s = -0.83$, $P < 0.001$; group C $R_s = -0.85$, $P < 0.001$). There was no correlation between the pre-exposure anti-type 2 pneumococcal IgG and anti-haemophilus IgG titres for the individual animals ($R_s = 0.01$, n.s.).

Discussion

These results demonstrate that inhalation of *Streptococcus pneumoniae* type 2 induced a minimal antibody response in both the IgG and IgM classes of immunoglobulin. In contrast, *Haemophilus influenzae* given to the same animals under identical conditions produced a vigorous IgG antibody response which was not influenced by splenectomy. The anti-haemophilus IgM response, which was greatest in the sham-operated animals, appeared to be abolished by splenectomy and was partly restored by splenic reimplantation.

The crucial role of the spleen in the synthesis of IgM is supported by the findings that serum IgM levels are reduced after splenectomy (Spirer 1980; Gavrillis *et al.* 1974; Andersen *et al.* 1976; Sullivan *et al.* 1978; Drew *et al.* 1984; Linne *et al.* 1984), and that IgM production correlates with splenic size (Gavrillis *et al.* 1974; Van Wyck *et al.* 1978). The data from the present study would support the view that the spleen is required to initiate an adequate IgM response. The increase seen in anti-pneumococcal IgM in sham-operated animals (GMFI = 1.57) was almost as great as the anti-pneumococcal IgG response (GMFI = 1.65), whereas the anti-pneumococcal IgM response was absent in animals after total splenectomy and after splenic reimplantation. Similarly, the anti-haemophilus IgM response was greatest after sham laparotomy and minimal after total splenectomy. Furthermore, the restoration of the IgM response after splenic reimplantation, suggests that reimplanted spleen may be immunocompetent.

The principal response to *Haemophilus influenzae* in the present study occurred in the IgG class and was not modified by splenectomy. The assay method used in the present experiments measured only total IgG antibody and it was therefore not possible to establish whether responses were confined to the IgG_2 sub-class which is thought to regulate thymus-independent antibody responses against polysaccharide antigens (Mosier *et al.* 1977; Mosier & Subbarao 1982; Morrell *et al.* 1972; Amlot & Hayes 1985; Oldfield *et al.* 1985). The vigorous anti-haemophilus IgG response would suggest that anti-haemophilus responses are not completely T-independent or that the capacity to produce T-independent responses can develop outside the spleen. In support of this view there is evidence that suggests that haemophilus antibody responses are not completely T-independent (Siber *et al.* 1980), that T-independent responses can occur in extra-splenic sites (MacLennan *et al.* 1982; Gray *et al.* 1985) and that T-independent responses can recover following splenectomy (Amlot & Hayes 1985).

The lack of an IgM response to the pneumococcus could simply represent insufficient synthesis. However, IgM is reported to be a better opsonin than IgG against pneumococci (Brown *et al.* 1982) and this could

result in preferential consumption of IgM with a lack of detectable serum response, as seen in the present studies.

The most striking observation of this study was the different IgG antibody response to the two encapsulated organisms, *Streptococcus pneumoniae* type 2 and *Haemophilus influenzae*. The considerably marked response to the latter organism suggests that *Haemophilus* is more antigenic. Certainly, there were no differences in the infecting dose, the method or duration of exposure to the two organisms, and each animal served as its own control for comparisons. It is possible that pre-exposure of the animals with the pneumococcus resulted in an exaggerated response to subsequent *Haemophilus* exposure. However, there was no correlation between the degree of response in the individual animals to the two organisms and the order of exposure was probably not relevant.

The aerosol route of administration was adopted in this study because it is more representative of the normal method of acquisition of pneumococcal infection. The poor response to pneumococci and the excellent IgG response to *Haemophilus influenzae* may explain why the latter organism is a less frequent cause of infection after splenectomy than the pneumococcus.

In conclusion, the results of this study provide evidence that the spleen has a role in the regulation of IgM antibody responses to encapsulated bacteria. This humoral immunity which is lost following splenectomy can be partially restored by splenic autotransplantation. Furthermore, the poor antibody responses to pneumococcal exposure compared to the excessive response to *Haemophilus influenzae* may, in part, explain the disproportionate frequency with which these organisms infect man following splenectomy.

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