# Specific and natural antibody production during *Salmonella typhimurium* infection in genetically susceptible and resistant mice

P. MATSIOTA-BERNARD,*† W. MAHANA,* S. AVRAMEAS† & C. NAUCIEL* *Laboratoire de Microbiologie, Faculté de Médecine de Paris-Ouest, Garches and †Unité d'Immunocytochimie, CNRS URA 359, Institut Pasteur, Paris, France*

## SUMMARY

Genetically susceptible (C57BL/6) and resistant (CBA) mice were infected with an avirulent strain of *Salmonella typhimurium* and studied over a 35-day period for the production of antibodies directed against bacterial antigens including lipopolysaccharide (LPS) (specific antibodies) and antibodies directed against self antigens [natural antibodies (NAb)]. Antibodies directed against LPS and self antigens were detected by enzyme immunoassay (EIA) and those directed against other bacterial antigens by immunoblotting. We found that serum natural antibody titres in C57BL/6 and CBA mice were similar and correlated with the bacterial load in the spleen and liver. In C57BL/6 mice, anti-LPS antibodies remained polyreactive and of the IgM isotype. In contrast, CBA mice, after an early increase in polyreactive IgM anti-LPS antibodies, mounted a specific anti-LPS IgG antibody response. The immunoblotting results demonstrated that the IgM polyreactive antibodies in the resistant and susceptible mice recognized bacterial antigens of different molecular weights and that CBA, but not C57BL/6 mice, were able to produce IgG antibodies recognizing bacterial components. Our results suggest that the synthesis of antibodies directed against bacterial antigens and natural antibodies follow, at least partially, distinct pathways, but they do not allow us to determine whether these two antibody populations are produced by the same or distinct B-cell subpopulations.

## INTRODUCTION

*Salmonella typhimurium* is a facultative intracellular pathogen which induces a disease in mice which is similar to human typhoid fever. Natural resistance of mice is controlled by several genes;[1] in particular, *Ity* regulates the growth rate of virulent strains in macrophages during the early phase of infection.[2] A sublethal inoculum of *S. typhimurium* persists for several weeks in the spleen and liver of mice, its rate of clearance being regulated by the H-2 complex.[3] *Salmonella typhimurium* infection may induce suppression of several immune responses, including depression of the proliferative response of spleen lymphocytes to B and T mitogens,[4] a decrease in interleukin-2 (IL-2) production by spleen lymphocytes after stimulation with concanavalin A (Con A),[4,5] a depressed ability to mount delayed-type hypersensitivity reactions[6] and a depressed antibody response to sheep red blood cells.[5,7] Depression of immune responses is generally more pronounced in mice genetically

susceptible to *S. typhimurium* infection.[4,6] Immunosuppression seems to be mediated by activated macrophages.[7,8]

The aim of this study was to examine whether dysregulation of the antibody response during infection involves the production of both antibodies directed against the infecting organism, and the polyreactive antibodies usually recognizing self antigens[9,10] [natural antibodies (NAb)]. NAb present similarities to the autoantibodies that appear at relatively high titres in certain autoimmune conditions.[11,12] The IgM NAb in both normal and autoimmune mice are considered to be produced by Ly-1+ B cells,[13,14] at present referred to as B-1 cells. Several studies have demonstrated that NAb levels are increased after viral and parasitic infections, as well as after injection of bacterial compounds such as tetanus toxoid and lipopolysaccharide (LPS).[15,16] It has been postulated that NAb participate in non-specific host defences.[17]

In the present study we compared the production of natural antibodies and antibodies directed against bacterial antigens in genetically susceptible (C57BL/6) and resistant (CBA) mice during *S. typhimurium* infection.

## MATERIALS AND METHODS

### Mice and S. typhimurium *infection*

Female C57BL/6 (*Ity*ˢ) and CBA (*Ity*ʳ) mice were obtained from Iffa Credo (L'Arbresle, France) and used at between 6 and 8 weeks of age.

Abbreviations: EIA, enzyme immunoassay; LPS, lipopolysaccharide; NAb, natural antibodies; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulphate; TNP/BSA, trinitrophenyl/bovine serum albumin.

Correspondence: Dr P. Matsiota-Bernard, Laboratoire de Microbiologie, Faculté de Médecine de Paris-Ouest, Hôpital Raymond Poincaré, 104, bd Raymond Poincaré, 92380 Garches, France.

*Salmonella typhimurium*, strain C5TS,[18] a temperature-sensitive mutant derived from the virulent strain C5 (kindly provided by C. E. Hormaeche, Cambridge, U.K.), was grown for 18 hr at 30° in tryptic soy broth. Mice were inoculated intravenously with $10^6$ bacteria in 0·2 ml of saline. Viable bacteria were enumerated in the spleen, at various intervals, by plating 10-fold serial dilutions in saline of spleen homogenates onto tryptic soy agar. Colonies were counted after overnight incubation at 30°.

### Antigens

BALB/c mouse brain tubulin, muscle actin and myosin were prepared according to published methods.[9,10] Double-stranded type I deoxyribonucleic acid (dsDNA) from calf thymus (Sigma, St Quentin-Fallavier, France) was solubilized in phosphate-buffered saline (PBS) and then sonicated. Trinitrophenyl groups were coupled to bovine serum albumin (TNP/BSA), using 2,4,6-trinitrobenzene sulphonic acid according to a procedure previously described by Little and Eisen.[19] LPS from *S. typhimurium* was purchased from Difco (Detroit, MI).

### Antibodies

Sheep anti-mouse Ig antibodies were isolated using an Ultrogel–Ig immunoadsorbent[20] and coupled to *Escherichia coli* β-galactosidase, using the one-step glutaraldehyde procedure.[21] Sheep anti-mouse IgM and sheep anti-mouse IgG were purchased from Biosys (Compiègne, France), passed through Ultrogel immunoadsorbents in order to eliminate possible cross-reactions between Ig isotypes and coupled to *E. coli* β-galactosidase or to horseradish peroxidase as described previously.[21]

The fluorescein isothiocyanate (FITC)-labelled rat anti-mouse IgM and the biotin-labelled rat anti-Ly-1 used for the double labelling of Ly-1+ B cells in the fluorescence-activated cell sorter analysis were a kind gift of Dr D. Portnoi (Institut Pasteur, Paris).[22]

### Enzyme immunoassays (EIA)

*Direct assay.* Groups of five mice were bled on days 7, 14, 23 and 35 post-infection and their sera were individually tested for antibody activity against tubulin, actin, myosin, dsDNA, TNP/BSA and LPS. Ninety-six-well polystyrene flat-bottomed microtitre plates (CML, Nemours, France) were coated with the various antigens (1 hr at 37° and overnight at 4°) as described previously.[9] The plates were washed extensively with PBS containing 0·1% Tween-20 and dilutions (1/100) of the serum samples were tested in duplicate (1 hr incubation at 37°). After extensive washing, β-galactosidase-labelled sheep anti-mouse Ig (1 μg/ml) was added. After another 1 hr of incubation at 37°, the plates were washed and the enzyme activity revealed using the enzyme substrate (*o*-nitrophenyl-β-D-galactopyranoside). The optical density was measured at 414 nm with a Multiskan Titertek apparatus (Flow Laboratories, Puteaux, France).

In addition, serum IgM and IgG anti-TNP/BSA titres and IgM and IgG anti-LPS titres were determined using a β-galactosidase-labelled sheep anti-mouse IgM or IgG antibody as the second antibody (both at 1 μg/ml concentrations).

*Competitive assay.* The dilution of sera (at post-infection days 7 and 23) which gave 50% of the maximum optical density in EIA using LPS-coated plates was determined by direct assay, as described above. Mice sera were then incubated at this
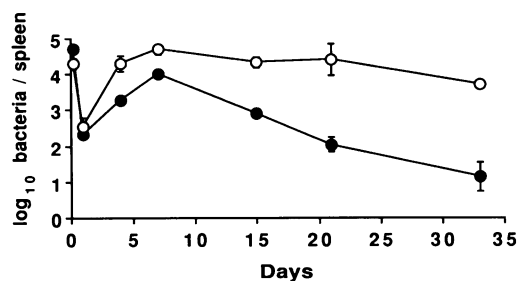


**Figure 1.** Time-course of infection in CBA (●) and C57BL/6 (○) mice after intravenous inoculation of $10^6$ *S. typhimurium* C5TS. Data are the mean number of bacteria recovered from the spleens of three mice per group ±SD.

dilution with decreasing concentrations of either LPS or TNP/BSA for 1 hr at 37°. The mixture was then allowed to react for 1 hr at 37° with LPS-coated plates. After washing, antibodies bound to the plates were measured as described above.

### Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

*Salmonella typhimurium* was cultured overnight in tryptic soy broth, centrifuged at 5000 *g* for 15 min and resuspended in PBS. The bacterial suspension was sonicated and centrifuged for 15 min at 5000 *g*. The supernatant was then centrifuged for 30 min at 100,000 *g* and the pellet separated in a 12% SDS gel with a 3% stacking gel. The protein markers used ranged from 70,000 to 14,000 MW. Gels were then placed for 5 min in a transfer buffer (Tris 48 mM, glycine 39 mM, SDS 0·0375%, methanol 20%) and blotted onto nitrocellulose paper for 1 hr, after which the nitrocellulose was washed first in PBS with 0·3% Nonidet P-40 (NP-40) for 30 min, then with PBS-0·5% gelatin-0·1% Tween-20 for 1 hr. After five additional washings with PBS-0·1% Tween-20, the nitrocellulose was incubated with the mouse sera diluted 1/200 in the PBS-0·5% gelatin-0·1% Tween 20 buffer for 1 hr. After washing, the nitrocellulose was incubated for 1 hr at 37° with horseradish peroxidase-labelled goat antiglobulin to mouse IgM or IgG (1/500 diluted). After extensive washing, the substrate solution was added for 10 min (Tris 0·1 M pH 7·6, 4-chloro-1-naphtol 2 mM and 3% $H_2O_2$) before washing in water and air drying.

### Flow cytometry

For flow cytometric analysis, groups of three infected mice were killed at different post-infection times and individually tested. Spleen cells were dissociated and washed twice in Hanks' balanced saline solution. Aliquots of $5–10 \times 10^5$ lymphocytes were suspended in 100 μl of ice-cold PBS (pH 7·2) containing 0·1% sodium azide and 1% foetal calf serum (FCS). For two-colour staining, 20 μl of FITC goat anti-mouse IgM and biotin-labelled rat anti-Ly-1 were added to each tube and allowed to incubate in ice for 20 min. After washing, the cells were resuspended in PBS containing sodium azide and FCS and phycoerythrin-avidin was added. After 20 min the cells were washed again and fixed with paraformaldehyde (1%) for later analysis. For two-colour analysis, 5000–10,000 events were analysed using a flow cytometer (FACScan, Becton Dickinson,

**Table 1.** Kinetics of antibody titres during *S. typhimurium* infection in resistant (CBA) and susceptible (C57BL/6) mice

| | Days after infection | | | | |
|---|---|---|---|---|---|
| | 0 | 7 | 15 | 23 | 35 |
| *CBA mice* | | | | | |
| Actin | 270 ± 34 | 311 ± 52 | 414 ± 49 | 368 ± 41 | 310 ± 34 |
| Myosin | 154 ± 19 | 320 ± 33 | 503 ± 58 | 415 ± 51 | 380 ± 39 |
| Tubulin | 158 ± 24 | 250 ± 23 | 396 ± 37 | 326 ± 44 | 290 ± 25 |
| dsDNA | 353 ± 33 | 558 ± 72 | 467 ± 59 | 624 ± 71 | 389 ± 42 |
| TNP/BSA | 403 ± 51 | 537 ± 52 | 1077 ± 99 | 953 ± 81 | 711 ± 74 |
| LPS | 28 ± 4 | 83 ± 12 | 420 ± 38 | 1133 ± 93 | 1291 ± 117 |
| *C57BL/6 mice* | | | | | |
| Actin | 150 ± 21 | 270 ± 23 | 414 ± 39 | 487 ± 45 | 450 ± 42 |
| Myosin | 80 ± 9 | 190 ± 33 | 498 ± 58 | 488 ± 51 | 415 ± 39 |
| Tubulin | 130 ± 29 | 258 ± 23 | 455 ± 37 | 557 ± 54 | 561 ± 55 |
| dsDNA | 304 ± 39 | 440 ± 74 | 613 ± 69 | 909 ± 91 | 1100 ± 102 |
| TNP/BSA | 344 ± 51 | 660 ± 52 | 1309 ± 99 | 1722 ± 171 | 1415 ± 124 |
| LPS | 31 ± 7 | 89 ± 17 | 135 ± 21 | 621 ± 79 | 752 ± 104 |

\* Antibody titres were determined by an EIA method and expressed as mean OD ($\times 10^3$) ± SD.



**Figure 2.** Time–course of anti-TNP/BSA (a) and anti-LPS (b) titres during *S. typhimurium* infection in C57BL/6 and CBA mice. (□) IgM and (○) IgG antibodies in C57BL/6 mice, (■) IgM and (●) IgG antibodies in CBA mice (mean of five animals).

Mountain View, CA). The FACScan software program was used to analyse data. In order to exclude granulocytes and macrophages as well as dead cells from the analysis, the lymphocyte population was gated according to 90° scatter and forward scatter.
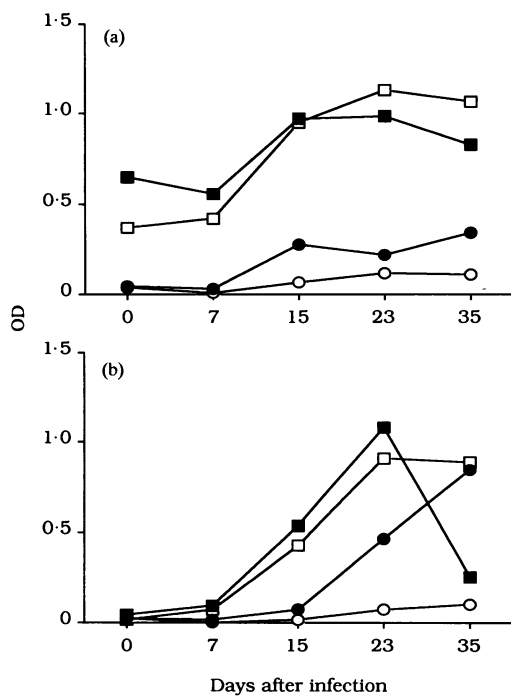
## RESULTS

**NAb in the sera of CBA and C57BL/6 mice during *S. typhimurium* infection**

Mice were infected with C5TS, an avirulent mutant of *S. typhimurium* which induces protracted infection and allows the survival of genetically susceptible mice.[3] As previously reported the clearance rate of *S. typhimurium* C5TS from the spleen was lower in C57BL/6 (H-2b) than in CBA (H-2k) mice[3] (Fig. 1). NAb titres in the sera of susceptible and resistant mice were studied using a panel of antigens: actin, myosin, tubulin, dsDNA, TNP/BSA. The mice sera (five in each group) were individually tested before and at different times after infection.
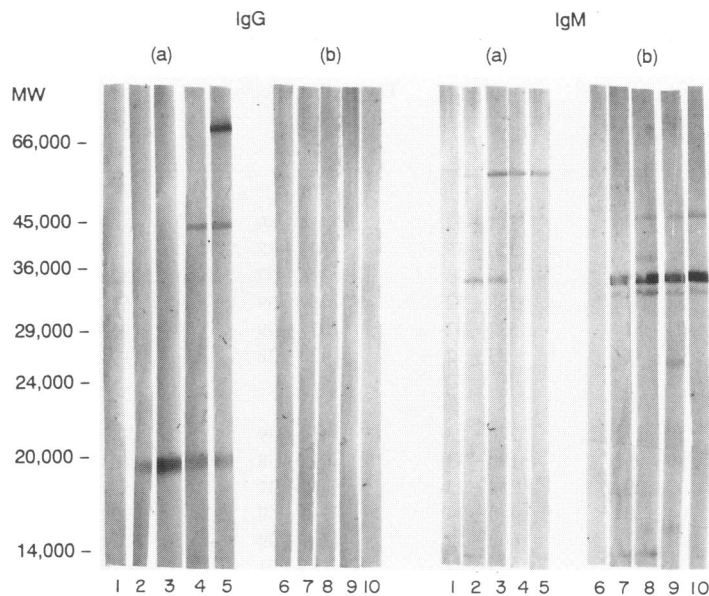
As shown in Table 1, CBA mice presented a slight increase in total titres of NAb against all antigens tested as early as day 7 post-infection. The highest of NAb titres (a twofold increase) were detected 15 days post-infection, after which they declined to almost initial values on day 35. No given autoantibody specificity was produced preferentially. However, as the anti-TNP/BSA antibody titres were higher than those against the other antigens and as it has been reported that almost all NAb carry anti-TNP activity[23] isotype analysis of this response was undertaken. We found that resistant CBA mice presented a moderate (twofold) elevation of IgM anti-TNP/BSA antibody titres on days 15, 23 and 35, post-infection, whereas IgG anti-TNP/BSA antibodies were raised on day 15 and continued to increase slightly until day 35 (Fig. 2a).

Susceptible C57BL/6 mice presented a slight increase in total NAb titres begining on day 7 after the infection, but in contrast to CBA mice, total NAb titres continued to increase on days 15 and 23 (a threefold overall increase) and remained elevated even on day 35 (Table 1). As with CBA mice, anti-TNP/BSA activity was more pronounced than that directed against the autoantigens. Isotype analysis of the anti-TNP/BSA response demonstrated that IgM anti-TNP/BSA antibody levels in the sera of the C57BL/6 mice were elevated starting on day 15 after

**Figure 3.** Immunoblot analysis of sera of infected CBA and C57BL/6 mice. The mouse-specific anti-bacterium response was examined using an extract of the bacterium. The IgG and IgM anti-bacterium activities at days 0 (lanes 1 and 6), 7 (lanes 2 and 7), 15 (lanes 3 and 8), 23 (lanes 4 and 9) and 35 (lanes 5 and 10) post-infection in the sera of CBA mice (a) and C57BL/6 mice (b) are compared.

infection, remaining increased until day 35. C57BL/6 mice presented only a slight increase in serum IgG anti-TNP/BSA antibody levels that remained inferior to those of CBA infected mice (Fig. 2a).

As the IgM NAb in both normal and autoimmune mice are considered to be produced by Ly-1$^+$ B cells, we examined the evolution of this cell population in the spleens of the infected mice, finding the proportion of splenic cells bearing both IgM and Ly-1 markers to have remained stable (approximately 2·5%) before and during the infection in both susceptible and resistant mice.

### Anti-LPS antibody activity

In CBA mice, anti-LPS antibodies detected by an EIA method using anti-mouse Ig antibodies were raised as early as 15 days after infection. High anti-LPS antibody titres were found on days 23 and 35. In contrast, anti-LPS-specific antibodies were found in low titres in the sera of C57BL/6 mice until day 23. While C57BL/6 mice mounted a significant anti-LPS response (days 23 and 35), this response always remained lower than that of the CBA mice (Table 1).

When we undertook isotype analysis of the anti-LPS response, we found that CBA mice mounted an elevated IgM anti-LPS response on day 15. This IgM response reached its maximum value on day 23 and then declined. The IgG anti-LPS response increased on day 23 after infection and continued to increase on day 35. In the sera of C57BL/6 mice, the IgM anti-LPS antibody levels were elevated from day 15 after infection and remained increased until day 35. C57BL/6 mice did not present an IgG anti-LPS response during the period studied (Fig. 2b).

In order to examine further the specificity of the anti-LPS response during infection, the eventual polyreactivity of the anti-LPS antibodies on days 7 and 23 post-infection was analysed. We found that the anti-LPS response of the CBA mice

on day 7 post-infection was at least partially polyreactive, since it could be inhibited by TNP/BSA (27% inhibition of the anti-LPS response at TNP concentrations of 1 and 0·5 mg/ml), but was strictly specific on day 23. In contrast, the anti-LPS antibodies in C57BL/6 mice remained at least partially polyreactive even on day 23. Indeed, no complete inhibition of the anti-LPS activity even at high LPS concentrations (1 mg/ml) was obtained and partial inhibition by the TNP/BSA (35% inhibition at 1 and 0·5 mg/ml TNP concentrations, 25% inhibition at 0·25 mg/ml) was observed.

### Antibody activity directed against other bacterial antigens

The mouse response to bacterial antigens was further examined by SDS–PAGE and immunoblotting using an extract of the bacterium as the antigen source (Fig. 3). We found that CBA resistant mice developed IgM antibodies which recognized two different proteins at days 7 and 10 post-infection, and another protein of 60,000 MW on days 23 and 35 post-infection. CBA mice also developed IgG antibodies which recognized proteins different from those recognized by the IgM antibodies during the post-infection period in which they were tested. C57BL/6 mice mounted anti-bacterium responses which were different from those of the CBA mice. They produced IgM antibodies which recognized proteins of molecular weights different from those recognized by the CBA IgM antibodies. In addition, C57BL/6 mice were unable to raise IgG antibodies which recognized components present in the bacterial extract.

### DISCUSSION

The clearance rate of *S typhimurium* C5TS from the organs is lower in C57BL/6 (H-2$^b$) than in CBA (H-2$^k$) mice. In this study we found that serum NAb titres in both CBA and C57BL/6 mice were similar and parallel to the bacterial load in spleen and liver. Susceptible mice, however, mounted a different specific anti-

body response from that found in resistant mice. Serum anti-LPS antibodies were not only raised much later after infection in susceptible C57BL/6 mice, but their titres also remained relatively low even on day 35 post-infection. In C57BL/6 mice, these anti-LPS antibodies remained polyreactive and of the IgM isotype, whereas CBA mice, after an increase of polyreactive IgM anti-LPS antibodies, mounted an IgG-specific anti-LPS response. The immunoblotting results demonstrated that IgM antibodies of resistant and susceptible mice recognized proteins of different molecular weights, and confirmed that C57BL/6 mice were unable, during a 35-day period, to raise IgG antibodies which recognized antigens present in the bacterial extract.

NAb were found to be increased in the sera of both susceptible and resistant mice during infection. The rise of NAb titres may reflect a state of polyclonal activation related to the presence of LPS on the infecting bacteria. The impairment of specific antibody responses in C57BL/6 mice during *S. typhimurium* infection agrees well with previous studies which showed that C57BL/6 mice infected with C5TS presented marked depression to B and T mitogens at 2–3 weeks after the infection,[4] a defect of IL-2 production after Con A stimulation[4] and a decrease in anti-sheep red blood cell antibody production.[7] A depressed antibody response has also been described during *S. typhimurium* infection in C3H mice.[5,8] *In vitro* antibody response could be restored by the addition of IL-4.[5] Immunosuppression in mice during *S. typhimurium* infection was found to be more pronounced in genetically susceptible mice.[4,6]

In contrast to the impairment of specific antibody responses in C57BL/6 mice during infection, the NAb response did not seem to be affected. Two hypotheses can be advanced to explain our results. According to the first hypothesis, bacterial antigens may stimulate cells which bear NAb as polyspecific receptors. Following this antigenic stimulation, these cells may undergo a series of divisions and mutations leading to the production of specific anti-bacterial antibodies.[12,15] In our experiments, resistant mice mounted IgM polyreactive NAb responses soon after infection, and specific IgG anti-LPS and anti-bacterium antibodies gradually replaced them. Susceptible mice, however, seemed unable to undergo this process. It must be noted that CBA IgM polyreactive antibodies were found by immunoblotting to recognize proteins of molecular weights different from those recognized by the C57BL/6 IgM polyreactive antibodies. This suggests that B cells carrying polyreactive receptors in CBA mice recognize bacterial antigens different from those of similar B cells present in C57BL/6 mice. Recognition of these antigens may provide sufficient antigenic stimulation to the polyspecific receptors of CBA mice for the immunoglobulin switch and the production of more specific antibodies. In contrast, the recognition of other epitopes may not give sufficient antigenic stimulation to the polyspecific receptors of the C57BL/6 mice that remain in the early phase of the NAb response and continue to produce polyreactive unmutated antibodies.

According to the second hypothesis NAb and antibodies directed against bacterial antigens may be produced by different B-cell subpopulations. In C57BL/6 mice only the cell population producing the antibodies directed against the bacterial antigens is affected during the infection. The mechanism of this preferential disregulation of cells producing specific antibodies during the infection in susceptible mice remains unclear.

C57BL/6 mice seem to remain in the early stage of non-specific defence by producing only IgM NAb. Since NAb are polyreactive and recognize different antigens, they facilitate opsonization and subsequent phagocytosis of micro-organisms,[12, 15, 24] and may serve as a first line of defence prior to specific antibody production. However, when bacteria reach high growth, NAb production may not be sufficient and successful defence may be related to the production of specific IgG antibodies, as well as to the T-cell-mediated response.[25]

Ly-1[+] B lymphocytes are supposed to produce polyreactive IgM antibodies which react with self constituents. In our study, the Ly-1^{+} B-cell proportion in the spleen was not found to be affected during the infection in either susceptible or resistant mice. There are two possible explanations for these results. The first is that while the proportion of splenic Ly-1^{+} B cells is not modified during infection, their proportion is enhanced at other sites (the peritoneal cavity). The second possibility is that NAb are not produced exclusively by Ly-1^{+} B cells. In fact, we and others have previously reported that CD5^{+} B cells of normal human neonates, the corresponding Ly-1^{+} B cells in mice, are not the only lymphocyte subpopulation that produce NAb in culture.[26,27]

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