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Lyme carditis in immunodeficient mice during experimental infection of *Borrelia burgdorferi*

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Summary. Recently, we described the severe combined immunodeficency (scid) mouse as a laboratory model for *B. burgdorferi* infection. Scid mice inoculated with the virulent low-passage tick isolate *Borrelia burgdorferi* ZS7 developed a severe pancarditis involving endocardium, myocardium and epicardium in the absence of functional *B*- or *T*-cells. Soon after inoculation perivascular infiltration was observed, later diffuse infiltration of the interstitium of the subendocardial and subepicardial areas was seen. The infiltrate was mainly mononuclear and predominantly composed of Mac-1$^+$ cells. Concomitantly, fibroblast proliferation and augmented collagen deposition occurred in the interstitium. This was associated with the presence of *B. burgdorferi* organisms. The histopathological and ultrastructural findings observed in scid mice resemble those observed in human Lyme carditis. The data emphasize the suitability of the scid mouse as a model in which to study the role of the immune system in the pathogenesis of Lyme carditis.

Key words: *Borrelia burgdorferi* – Experimental infection – Mouse model – Immunodeficiency (scid) – Carditis

Introduction

Lyme borreliosis is caused by the spirochaete *Borrelia burgdorferi*, which is transmitted to human hosts primarily by ticks of the genus *Ixodes* (Steere et al. 1983). It appears as a multi-system illness with dermatological, rheumatic, central and peripheral nervous and cardiac features (Goldings and Jericho 1986). The primary manifestation of *B. burgdorferi* infection in humans is characterized by an expanding skin lesion: erythrema chronicum migrans (ECM). Subsequent haematogenous spread of the *B. burgdorferi* organisms may affect other organ systems such as liver, heart and joints. About 8% of Lyme disease patients develop cardiac symptoms, the most common of which are fluctuating degrees of atrioventricular block including complete heart block (Steere et al. 1980, 1986). There is increasing evidence that patients suffering from Lyme disease may also develop acute myopericarditis, left ventricular dysfunction and/or frank cardiomegaly. Cardiac involvement lasts usually only weeks but may be recurrent. Fatal cases have been described (Steere et al. 1980; de Koning et al. 1989).

As yet little is known about the pathogenesis of Lyme disease due in part to the lack of an appropriate animal model. We have recently shown that severe combined immunodeficiency (scid) mice inoculated with viable low passage tick isolates of *B. burgdorferi* experience a persistent spirochaetaemia and a progressive disease in the absence of any immune response (Schaible et al. 1989a). They develop a multi-systemic illness with extensive pathological alterations in different organs, such as joints, liver, kidney and heart.

In the studies reported here we used histological, immunohistochemical and electron microscopical methods to explore the histopathological evolution of carditis in *B. burgdorferi* infected C.B-17 scid and in normal C.B-17 mice.

The presence of *B. burgdorferi* organisms in the myocardium of infected animals and the simultaneous development of a chronic progressive inflammation in the heart tissue of scid mice, but not of normal C.B-17 mice, suggests the involvement of the spirochaetes themselves and/or inflammatory reactions rather than specific immune reactions unique to the pathogenesis of Lyme carditis. These findings are discussed in the context of the features observed in human Lyme carditis.

Materials and methods

Adult mice of strains C.B-17 scid (homozygous for the scid mutation, Bosma et al. 1983) and normal C.B-17 were bred at the Max-Planck-Institut für Immunbiologie, Freiburg, FRG, under specific

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pathogen-free conditions. Female and male animals between 6 and 10 weeks of age (8 animals of each strain) were used in this study. Scid mice were kept in sterile tents during the study.

The *B. burgdorferi* strains B31 (ATCC 35210) and ZS7 (isolated from a *Ixodes rizinus* tick of the Freiburg area) and the isolation procedure of *B. burgdorferi* from mice and ticks were described elsewere (Schaible et al. 1989a, b).

To detect *B. burgdorferi* directly in blood samples haematocrit centrifugation for enrichment and immunofluorescence staining for visualization was performed as described elsewhere (Schaible et al. 1989a).

Organs were removed from mice at different time intervals post-inoculation and were stored either in liquid nitrogen for preparation of cryostat sections or in 5% formaldehyde for embedding in paraffin. Sections of 4–7 µm were prepared and stained with haematoxylin-eosin for conventional histological examination.

Immunohistological staining was performed using the streptavidin-biotin-peroxidase detection system (Kramer et al. 1989) and the following monoclonal antibodies (mAb): rat anti-Ly 2 (culture supernatant of clone 53–6.7, kindly provided by Dr. J. Ledbetter, Genetic Biosystems Inc, Seattle, Wash.), rat anti-L3T4 mAb (culture supernatant of clone H-129–19.6, kindly provided by Dr. M. Pierres, Centre d'Immunologie, Luminy, France), rat anti-Mac-1, rat anti-Mouse IgG, rat anti-mouse IgM (all used at 10 µg/ml; commercially obtained from Boehringer Mannheim, Mannheim, FRG) and mouse anti-Ia^d mAb (purified IgG; used at 10 µg/ml, kindly provided by Dr. G. Hämmerling, German Cancer Research Centre, Heidelberg, FRG). *B. burgdorferi* was visualized by using a peroxidase labelled anti-flagellin (41 kDa antigen) mAb (Wilske et al. 1988) kindly prepared by Prof. Vernes, apiBioMérieux, Lyon, France.

Ultra-thin sections for electron microscopy were prepared from myocardial samples of 3 scid mice after immersion fixation with 1.5% glutaraldehyde/1.5% paraformaldehyde in 0.2 mol phosphate buffer. The specimens were post-fixed in osmium tetroxide and embedded in Epon-Araldite. Thin sections (0.5 µm) were prepared and stained with paraphenylene-diamine and toluidine blue for light microscopy or stained with uranyl acetate and lead citrate for electron microscopy.

Scid mice and normal C.B-17 mice were inoculated with spirochaetal organisms and their organs were studied by immunhistochemistry and electron microscopy 23, 29, 56 and 87 days after inoculation. For inoculation 1×10^8 viable organisms of either the viable low-passage tick isolates *B. burgdorferi* ZS7 and ZQ1 low-virulent long-passage *B. burgdorferi* strain B31 organisms or ultraviolet (UV) inactivated organisms of the strain ZS7 were used.

Results

Starting on day 7 (beginning of observation period) inoculated scid mice developed clinical signs of arthritis in the tibiotarsal joints, which gradually increased with time after inoculation. However, there was no evidence of heart failure and none of the scid mice died during the observation period. No arthritis was seen in scid mice inoculated either with UV irradiated *B. burgdorferi* ZS7 organisms or with low-virulent *B. burgdorferi* B31 organisms. In contrast to scid mice, normal C.B-17 mice that were inoculated with viable *B. burgdorferi* ZS7 organisms failed to demonstrate symptoms of arthritis.

Scid mice inoculated with viable *B. burgdorferi* ZS7 showed histological alterations in the heart as early as day 7 post-infection; these gradually increased in extent up to day 87 (end of observation time: Schaible et al. 1989a). In contrast, acute inflammation occurred much later and was much less pronounced in similarly infected

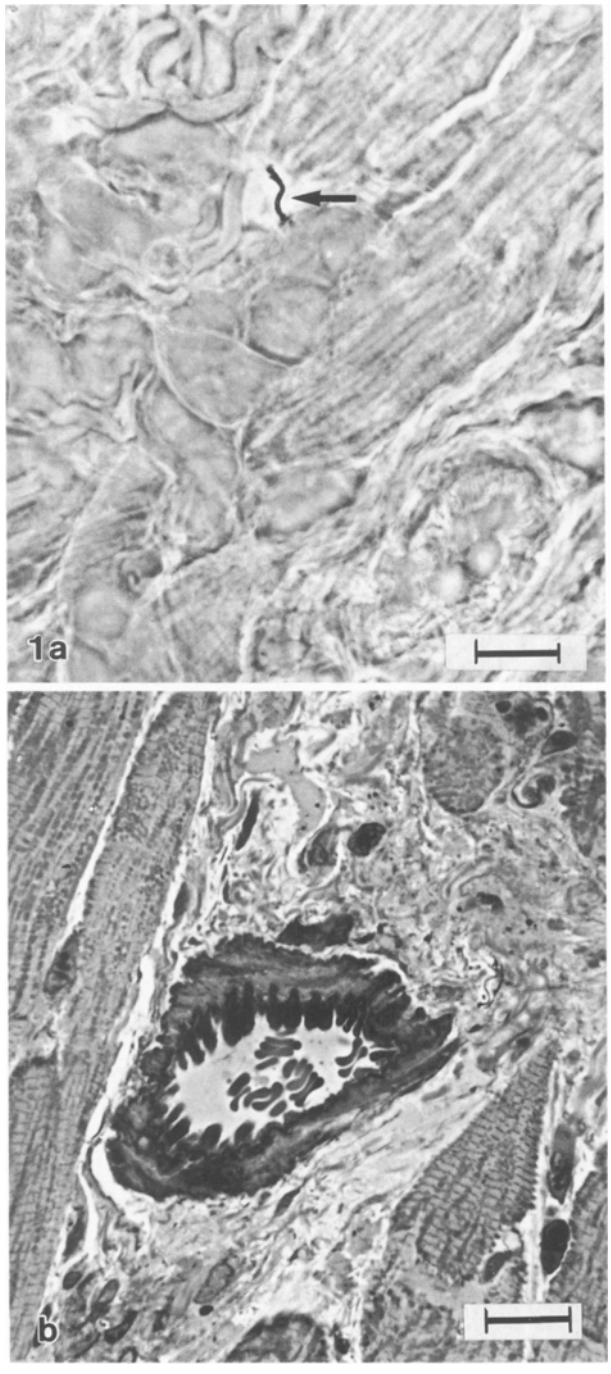

Fig. 1. A Immunoperoxidase staining for *B. burgdorferi*. Light micrograph of the myocardium of *B. burgdorferi* infected C.B-17 scid mice demonstrating a loosely coiled spirochaete (*arrow*) present in the interstitium laying on a large macrophage. The interstitium is focally infiltrated with numerous large cells. Myocytes are not affected and show normal content of contractile material. Scale bar = 9.2 µm **B** Light micrograph (semi-thin section) of the myocardium of *B. burgdorferi* ZS7 infected C.B-17 scid mice at day 22. The increased interstitial space around a small artery containing collagen, fibroblasts and monocytes is shown. Myocardial cells are not affected and show normal content of contractile material. *Scale bar = 8 µm*

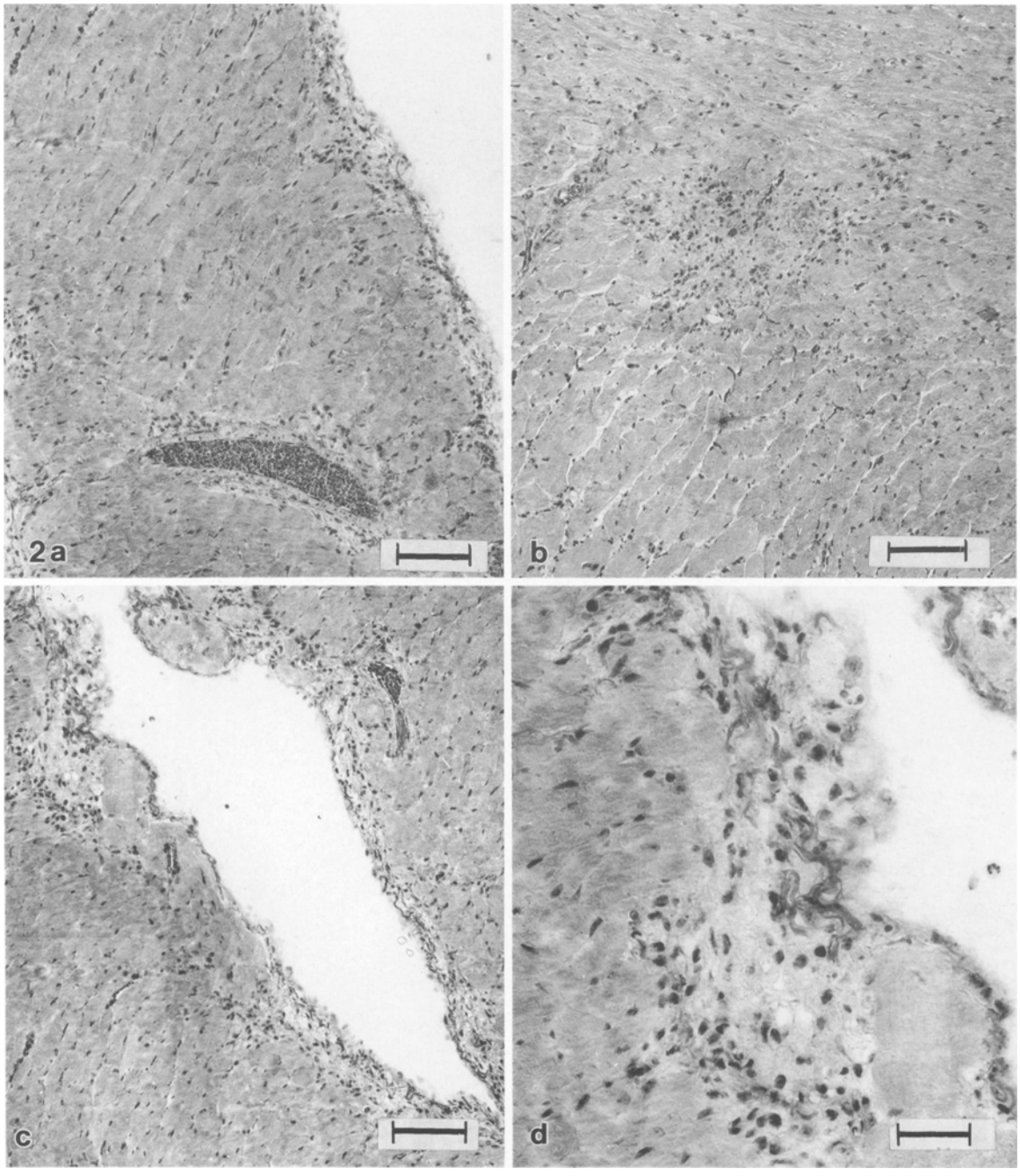

Fig. 2A–D. Light micrographs of haematoxilin-eosin stained sections of the myocardium of *B. burgdorferi*-infected C.B. scid mice at day 21 post-infection. **A** Severe inflammatory infiltration of the perivascular area and the interstitium of the epicardium. Scale bar: 20 μm. **B** Inflammatory infiltration by mononuclear leucocytes of the interstitial area of the myocardium. Scale bar: 20 μm. **C** Inflammatory infiltration of the subendocardial area. Scale bar: 20 μm. **D** Higher power micrograph of the inflammatory infiltration of the subendocardial area. Scale bar: 10 μm

normal C.B-17 mice. No histopathological changes were observed in scid mice infected with either UV irradiated *B. burgdorferi* ZS7 organisms or with the attenuated strain B31 organisms.

Spirochaetes were detected in the perivascular space and the interstitial areas of the myocardium from infected scid mice (Fig. 1a). Organisms and/or sliced parts thereof were observed 2–3 per high-power field ($\times 400$) and could be followed through 2–3 serial 5 μm sections.

The first histological alterations were observed in the myocardium (from day 7 on) and were characterized by an inflammatory infiltration of perivascular space (Fig. 1b) followed by an increasing involvement of the interstitial myocardium. In addition to the myocarditis, endocarditis and pericarditis developed in later stages (day 21) of the *B. burgdorferi* infection with subendocardial and subepicardial infiltrations, predominantly by mononuclear leucocytes (Fig. 2a–d).

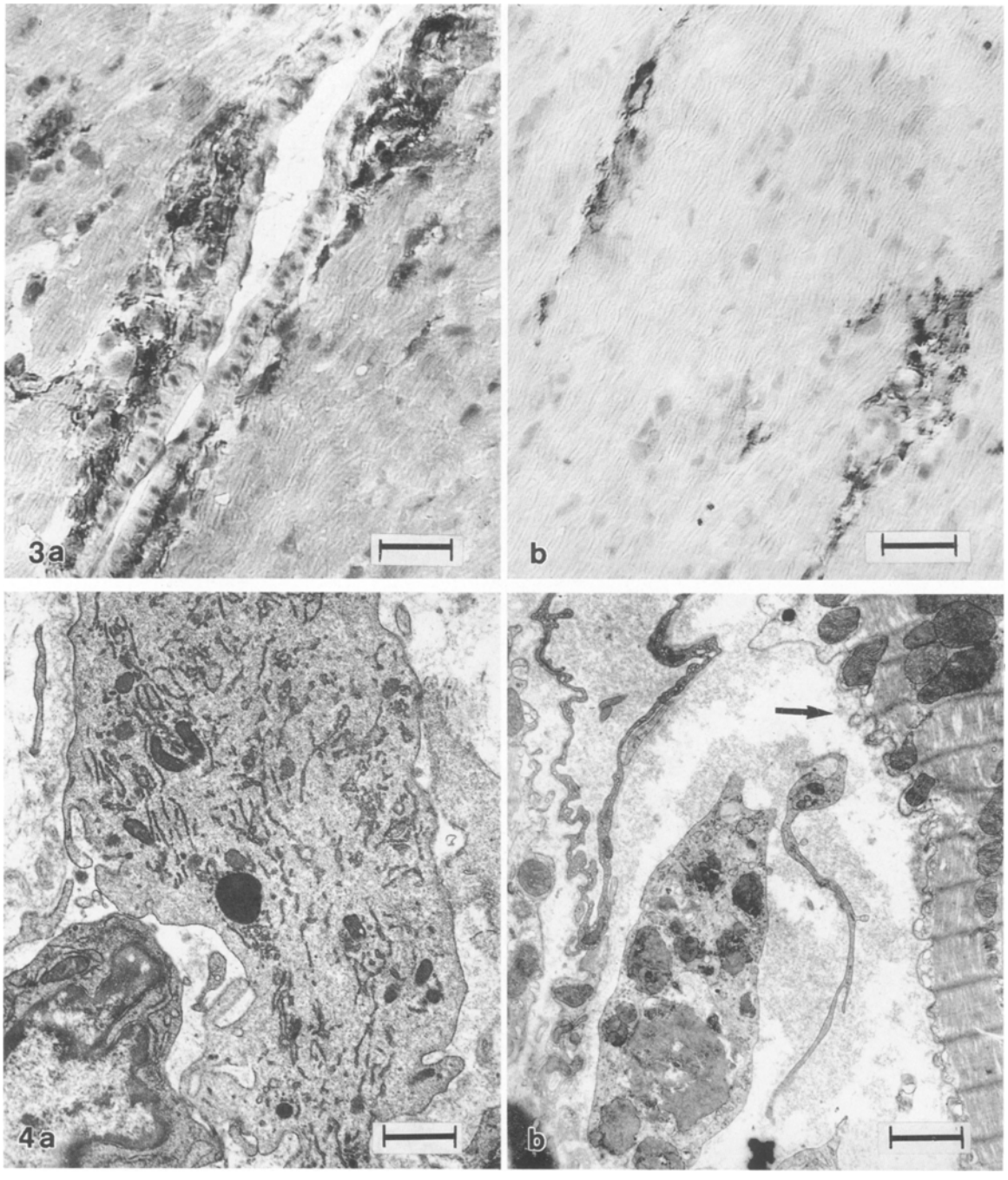

Fig. 3A, B. Immunoperoxidase staining for Mac1 of frozen sections of the heart obtained from *B. burgdorferi* infected C.B-17 scid mice. **A** Infiltration of the perivascular area of a small myocardial artery at day 22. Scale bar = 16 μm. **B** Progressive infiltration of the myocardial interstitial space at day 29. Myocytes are not affected, no focal myocyte necrosis. Scale bar = 16 μm

Fig. 4A, B. Electron micrograph of ultra-thin sections prepared from the myocardium (day 87 post-infection) of *B. burgdorferi* infected C.B-17 scid mice. **A** Large cell with dense cytoplasm, long strands of endoplasmatic reticulum, and membrane bound electron dense granules of uniform density can be seen. Scale bar: 1.4 μm. **B** An activated macrophage like cell with inclusions of intermediate size and variable density (which are likely to represent ingested material) and with numerous profiles of endoplasmatic reticulum is depicted. The golgi region is more expanded than in quiescent cells. *Arrow:* Myocytes are not affected: the contractile material and the mitochondria appear normal. Scale bar: 2.6 μm

The major histological findings in the heart of scid mice were interstitial activation (comprising proliferation of fibroblasts and collagen deposition) and cellular infiltrations of the myocardium. Inflammatory cells in the perivascular (Fig. 3a) and interstitial space (Fig. 3b) of the myocardium predominantly consisted of Mac-1$^+$ monocytes. As expected for immunodeficient scid mice, no cells bearing either immunoglobulin (Ig) or T-cell surface markers (L3T4) were observed (Table 1). At day 16 Mac-1$^+$ cells were localized mostly around blood

Table 1. Immunohistochemical evaluation of the myocardium of C.B-17 scid and C.B-17 mice

Day	16[a]/16[b]	22[a]/23[b]	29[a]/n.d.	87[a]/87[b]
Mac 1				
Percent involved arteries	60%/0%	92%/0%	89%/n.d.	97%/30%
$Mac1^+$ cells/mm^2 in the interstitium	4,3/5.4	24,3/5.4	18,9/n.d.	59,4/9.3
$Mac1^+$ cells/artery	8/0	8/0	8/n.d.	6/3
Infiltration of the endocardium by $Mac1^+$ cells	+/−	+++/−	++/n.d.	+/+
L3T4				
$L3T4^+$ cells/mm^2	0/1.2	0/2.3	0/n.d.	0/3.0
IgM/IgG				
Expression of IgM/IgG	−/+	−/+	−/n.d.	−/+

C.B-17 scid ([a]) and C.B-17 ([b]) mice were inoculated with viable *B. burgdorferi* ZS7 organisms. Hearts were prepared at different time points (C.B-17 scid mice: day 16, 22, 29, and 87; C.B-17 mice: day 16, 23, and 87) post-infection and frozen sections were stained for the respective lymphoid cell surface antigens by using monoclonal antibodies as described. Positive staining cells per mm^2 were counted at a magnification of 250 using an appropriate Zeiss ocular. Furthermore, the percentage of involved arteries was calculated from evaluation of the whole sections. Positive staining cells per artery were calculated from counting at least 25 involved arteries. Infiltration of the myocard by $Mac1^+$ cells, as well as expression of IgM/IgG, is scored semi-quantitatively using a scale between − (no infiltration/no expression) and +++ (strong infiltration).

vessels affecting up to 60% of the small arteries. Perivascular infiltration increased further to more than 95% of arteries affected on day 87 at which time a prominent interstitial infiltration was seen (Table 1). It now consisted of fibroblasts and collagen deposits in addition to Mac-1^+ cells (59.4 cells/mm^2). There was no development of focal scars or granulomata. From day 22 on Mac-1^+ cells were also found in the interstitium, mainly of the subendocardium (Table 1).

In contrast to scid mice, the myocardium of normal C.B-17 mice previously inoculated with viable ZS7 *B. burgdorferi* organisms was normal when examined on day 16 and 23 and showed only 3–4 Mac-1^+ cells/mm^2 (Table 1) a cell number usually found under non-pathological conditions (Chow et al. 1988). However, occasionally significant perivascular infiltrations consisting of Mac-1^+, $L3T4^+$ and Ig^+ cells were also observed in the myocardium at later stages (day 87). The valves and the endocardium of infected C.B-17 mice were also affected (Table 1).

The myocardium from *B. burgdorferi*-inoculated scid mice was examined ultrastructurally. Analysis revealed the presence of numerous mononuclear cells of different sizes in the interstitial space of the myocardium. In general, these cells had abundant cytoplasm; some exhibited the classical features of monocytes. Other large cells showed characteristics of both monocytes and large granular lymphocytes: they expressed long strands of endoplasmatic reticulum, membrane-bound dense granules and tubular inclusions (Fig. 4a). Although a definite classification of these cells by morphological criteria was not possible, they are reminiscent of natural killer cells (Grossi and Ferrarini 1982).

In addition, numerous fibroblasts were observed in the perivascular space of small vessels and in the interstitium of the myocardium from *B. burgdorferi* ZS7 infected scid mice. Deposition of collagen bundles was increased (data not shown). Using morphological criteria myocytes were not affected in the tissue samples studied: neither mitochondrial alterations nor myofibrillar changes or damage of the sarcolemma were seen (Fig. 4b). No spirochaetes could be detected in the electron microscopic sections studied, which was probably due to the limited areas examined.

Discussion

Lyme disease is a multi-systemic disease and is often associated with significant cardiac abnormalities (Steere 1989). Although the pathogenesis of *B. burgdorferi* infection is far from being understood, at least two possibilities could be envisaged to explain the development of clinical expression of this chronic infectious disease. Spirochaetes may be able to induce the disease (1) by direct interaction with host tissues, (2) via non-specific inflammatory reactions, or (3) via specific immune responses, that is to say via specific T-cells and/or antibodies generated during the infection. The findings reported here, that *B. burgdorferi* infection of scid mice deficient in functional B- and T-cells results in a persistent spirochaetosis with the development of a chronic progressive disease associated with a severe pancarditis, are in accordance with the first two hypotheses. We assume that the inflammatory reactions observed in the heart of infected scid mice are either the consequence of direct interactions of *B. burgdorferi* with structures of the extracellular matrix and/or of the activation of host cells such as neutrophils, macrophages and granulocytes by the spirochaetes. In particular, the finding of Mac-1^+ monocytes as the major constituent of interstitial infiltrations in the myocardium of *B. burgdorferi* infected scid mice emphasizes the role of this cell population in the pathogenesis of Lyme carditis.

Alterations in the heart with similar histopathological appearance have been reported in humans. Autopsy examination of one fatal case (Steere et al. 1980) and two non-fatal cases (de Koning et al. 1989) with cardiac involvement during Lyme disease revealed heavy infiltrations of lymphocytes and plasma cells in the interstitium of the myocardium. In the fatal case transmural myocarditis was seen. Focal aggregates involved myocardial fibres. Neither myocardial fibre necrosis nor obliterative microangiopathy was observed (Duray and

Steere 1986). The presence of spirochaetes in the myocardium of these patients is suggestive of *B. burgdorferi* as the causal pathogenic agent of pancarditis.

The development of similar cardiac alterations in the absence of functional T- and B-lymphocytes in mice does not exclude the involvement of immune responses in the pathogenesis of the disease in man. In fact, T-lymphocytes have been found in inflammatory infiltrates in the heart of *B. burgdorferi* infected C.B-17 mice, as well as in biopsies of the heart of patients with Lyme carditis (Steere et al. 1980; de Koning et al. 1989). In addition, a possible role for activated T-lymphocytes in bacterial as well as in virus-induced carditis has been proposed in previous reports (Guthrie et al. 1984; Steinmüller et al. 1988). The scid model of experimental Lyme borreliosis described here now allows us to study the role of T-lymphocytes in Lyme carditis in passive cell-transfer experiments.

The fact that *B. burgdorferi* infected normal C.B-17 mice did not develop a spirochaetosis and showed a later onset of moderate pathological changes in the heart is also indicative of the involvement of the immune system in the elimination of the spirochaetes and protection against the disease. At present, it is not known which element of the immune system is responsible for the mitigation of clinical symptoms in the heart of *B. burgdorferi*-infected normal mice and whether similar mechanisms control the disease in humans. Preliminary results suggest a qualitative difference in the humoral immune response to *B. burgdorferi* generated in mice when compared with man. It has been reported that in mice inoculated with pathogenic *B. burgdorferi*, the first antibodies to appear in serum are those reacting with the spirochaetal outer surface antigens OspA and OspB (Coleman and Benach 1987). In humans the primary antibody response is directed mainly to periplasmic flagellar antigens (Dattwyler et al. 1988), whereas antibodies to OspA and/or OspB are only generated later during the disease. The differential appearance of putative protective antibodies specific for surface structures of *B. burgdorferi* may therefore influence both the generation of the disease and the pattern of organ involvement during spirochaetal infection. This assumption is supported by our finding that passively transferred monoclonal antibodies to outer surface protein A prevent Lyme borreliosis in infected scid mice (Schaible et al. 1990).

To date a correlation between the different clinical expressions of Lyme disease and *B. burgdorferi*-specific immune response (antibodies and T-cells) has not been established. Moreover, it has been shown that certain stages of Lyme disease can develop in the absence of measurable antibody responses (Dattwyler et al. 1988). Although the documentation of B- and T-cell responses specific for *B. burgdorferi* may provide important information as to the pathophysiology of this infectious disease, the inability to document any *B. burgdorferi* specific immune response may not necessarily indicate the absence of an ongoing Lyme borreliosis. The data provided here strongly support the notion that even in, or because of, the state of immunological non-responsiveness (tolerance) severe progressive Lyme carditis could develop. These considerations should be taken into account in the clinical evaluation of Lyme carditis.

Future studies will focus on the involvement of the host's immune response in the clinical course and in particular the pathogenesis of Lyme disease. The scid mice mouse is a suitable laboratory animal model for this purpose.

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