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Depletion of lymphocytes, but not neutrophils, via apoptosis in a murine model of *Vibrio vulnificus* infection

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Vibrio vulnificus causes severe sepsis in humans. There are several reports about the relationship between host immunity and bacterial growth in *V. vulnificus* infection. However, the effect on leukocytes of *V. vulnificus* infection *in vivo* has not been elucidated. A murine model of *V. vulnificus* infection was used to investigate its effects on leukocytes in this study. Bacteria were recovered from the blood of mice 3 h after subcutaneous injection in the right lower flank. They were detected in 87·5 % ($n = 7/8$) of mice at 6 h, but this value decreased to 12·5 % ($n = 1/8$) at 12 h. In contrast, the number of lymphocytes in peripheral blood had already started to decrease at 3 h, and reached a minimum at 6–9 h post-inoculation. Typical DNA laddering, a hallmark of apoptosis, was also detected in thymocytes and splenocytes at 6 and 9 h, and showed a tendency to disappear by 12 h. Although the number of lymphocytes decreased in the model, the numbers of neutrophils did not. These results suggested that *V. vulnificus* has selective cytotoxicity for lymphocytes in peripheral blood *in vivo*, and the lymphocyte depletion was probably associated with apoptosis *in vivo*.

INTRODUCTION

Vibrio vulnificus, a Gram-negative estuarine bacterium, causes septicaemia in humans who suffer from liver cirrhosis, alcoholism, haemochromatosis, immunocompromised conditions or diabetes (Hlady & Klontz, 1996; Linkous & Oliver, 1999; Strom & Paranjpye, 2000). Primary septicaemia is caused by the ingestion of seafood contaminated with *V. vulnificus* or through wound infections involving exposure to contaminated sea water or marine products. Mortality from this infection exceeds 50 %, and increases to more than 90 % in patients who go into shock shortly after admission to hospital (Koenig *et al.*, 1991). Infected individuals with primary septicaemia usually present with fever and chills. Within 24 h of these first signs of illness, secondary cutaneous lesions appear, including ecchymoses, bullae and progressive cellulitis (Starks *et al.*, 2000).

Several putative virulence factors of *V. vulnificus*, including a capsular polysaccharide, a metalloprotease and iron-sequestering systems, have been studied *in vitro* and *in vivo* (Fan *et al.*, 2001; Johnson *et al.*, 1984; Miyoshi *et al.*, 1998; Morris *et al.*, 1987). Of these, the capsular polysaccharide confers resistance to phagocytosis by human polymorphonuclear leukocytes or murine peritoneal macrophages *in vitro* (Kreger *et al.*, 1981; Tamplin *et al.*, 1985). Indeed, encapsulated phenotypes of *V. vulnificus* have higher lethality towards mice than unencapsulated phenotypes (Wright *et al.*, 1981; Yoshida *et al.*, 1985). This is the one of the reasons why encapsulated strains are predisposed to cause sepsis in humans.

In a recent study, it was reported that extensive loss of lymphocytes via apoptosis was observed in patients who died by polymicrobial sepsis (Hotchkiss *et al.*, 2001). In a mouse model of polymicrobial sepsis, general-caspase inhibitor and caspase-3 inhibitor prevented lymphocyte apoptosis and improved survival (Hotchkiss *et al.*, 2000). Therefore, lymphocyte apoptosis is involved in lethality in polymicrobial sepsis. However, the relationship between lethality and lymphocyte apoptosis has yet to be clarified.

There are few reports concerning the relationship between apoptosis of host cells and growth of *V. vulnificus in vivo*. We reported that clinical isolates of *V. vulnificus* can induce apoptosis in the macrophage-like cell line J774, and in mouse peritoneal macrophages both *in vitro* and *in vivo* (Kashimoto

Abbreviations: H&E, haematoxylin and eosin; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP–biotin nick end labelling.

et al., 2003), indicating that *V. vulnificus* might induce immunodysfunction by interacting with leukocytes. However, the effects of *V. vulnificus* infection on leukocytes *in vivo* are not fully understood. This study aimed to clarify the effect on leukocytes by using the murine model of *V. vulnificus* infection.

METHODS

Growth conditions of *V. vulnificus* and *Escherichia coli* strains. *V. vulnificus* strain K44 was isolated from the blood of a septicaemic patient at Kurashiki Central Hospital in Japan. K44 was routinely grown in Marine broth (Difco) at 37 °C for 12 h. Injected bacteria were prepared as follows. The bacterial concentration was adjusted to an OD_{600} of 0·5, and then 100 µl adjusted bacterial culture was added to 5 ml fresh Marine broth. This was then grown for a further 2 h at 37 °C with agitation to obtain exponential-growth-phase bacteria. Bacteria were harvested by centrifugation at 2500 ***g*** and 4 °C for 10 min, and were suspended in PBS (pH 7·4) at a concentration of about 10^7 c.f.u. ml^{-1}. The solution was adjusted to the desired bacterial concentration by measuring the OD_{600}. Concentrations were verified by plating serial dilutions of the samples on thiosulfate/citrate/bile salt/sucrose (TCBS) agar (Nissui Pharmaceutical) and counting the c.f.u. after incubation. *E. coli* DH5α was used as a control; it was prepared by the same method as above except that Luria–Bertani (LB) broth and agar were used.

Animals and treatment. Male, 5-week-old ddY, C.B-17 +/+, C.B-17 scid/scid, C3H/HeJ and C3H/HeN mice were used in this study. Mice were housed in plastic cages in groups and were maintained on a standard laboratory diet (Rat Chow MF; Oriental Yeast) and tap water, and exposed to a 12 : 12 h light and dark cycle. The ambient temperature during the study was maintained at about 21 °C. The Kitasato University guidelines for animal treatment, which meet the principles of laboratory animal care, were followed. Injection of the bacteria into the mice was carried out as described previously (Kashimoto *et al.*, 2003). Mice were given subcutaneous injections of 5×10^6 *V. vulnificus* K44 or *E. coli* DH5α suspended in 0·5 ml PBS, in the right lower flank, and then killed at the indicated times by an overdose of anaesthetic. Infection of mice was verified by culture of blood obtained by heart puncture under sterile conditions. Aliquots (100 µl) of the blood were incubated for 24 h at 37 °C. The number of bacteria was determined on TCBS agar for *V. vulnificus* and on LB agar for *E. coli*.

Analysis of DNA fragmentation. DNA fragmentation was analysed as described previously with some modification (Szondy *et al.*, 1998). Briefly, thymus and spleen tissues from the mice were gently glass-ground to dissociate the cells, and then suspended in cold Hanks' balanced salt solution (HBSS), which was then gently passed through a stainless steel mesh to obtain single-cell suspensions (thymocytes and splenocytes). Cells (5×10^6) in HBSS were centrifuged, and the pellet was suspended for 10 min at 4 °C in lysis buffer (10 mM Tris/HCl, pH 7·4; 10 mM EDTA; 0·5 % Triton X-100). Samples were then centrifuged at 14 000 ***g*** for 5 min, and the supernatant containing fragmented DNA was digested with 200 µg RNase ml^{-1} (Sigma Aldrich) for 1 h at 37 °C. The sample was then treated with 200 µg proteinase K ml^{-1} (Merck) for 30 min at 50 °C. The supernatant was precipitated overnight using isopropyl alcohol. DNA fragments were separated on 2·0 % agarose gels, visualized with ethidium bromide, and photographed.

Histology. Thymus and spleen were fixed in 10 % (v/v) buffered formalin (pH 7·2), dehydrated in a graded alcohol series, and embedded in paraffin wax. Three-micrometre sections were stained with haematoxylin and eosin (H&E). Terminal deoxynucleotidyl transferase-mediated dUTP–biotin nick end labelling (TUNEL) staining was performed to detect apoptotic cells. Briefly, paraffin-embedded sections were dewaxed in xylene and rehydrated by passage through a graded ethanol series, ending with PBS. Sections were permeabilized using proteinase K. After washing, the 3′-OH ends of DNA fragments were stained by the method described by the manufacturer (*In situ* Apoptosis Detection kit POD; Roche Diagnostics). The sections were visualized with 3,3′-diaminobenzidine tetrahydrochloride solution and counter-stained with methylene green.

Percentage of apoptotic lymphocytes. The apoptotic splenocytes were detected by TUNEL staining on sections of spleen from C3H/HeJ (LPS non-responder) and C3H/HeN (LPS responder) mice 9 h after inoculation with the bacteria ($n = 5$). The percentages of apoptotic splenocytes were calculated from a sample of at least 300 cells in the white pulp of the spleen.

Flow cytometry. The single-cell suspensions of thymocytes and splenocytes, prepared as described above, were washed twice in cold HBSS. After washing, 1×10^6 cells were resuspended in 50 µl PBS with 1 % bovine serum albumin (BSA) and 0·01 % sodium azide. To detect each cell surface marker, the antibodies anti-mouse CD4-PE, anti-mouse CD8-FITC, anti-mouse CD3-FITC and anti-mouse CD45R (B220)-PE (Immunotech) were added to the cell suspension at appropriate dilutions, and incubated for 30 min at 4 °C. Cell suspensions were washed twice with PBS containing 1 % BSA and 0·01 % sodium azide, and then the numbers of stained cells were determined by counting about 10 000 cells on a fluorescence-activated cell sorter (FACS 440; Becton Dickinson).

Statistical analysis. All results are shown as means ± SDs. For multiple comparisons of the data, we used one-way ANOVA followed by Tukey's test; $P < 0.05$ was considered statistically significant. Data from studies with only two groups were analysed by the Student's *t*-test for equal variance or Welch's *t*-test for unequal variance after Bartlett's test.

RESULTS AND DISCUSSION

Depletion of lymphocytes, but not neutrophils, in *V. vulnificus*-infected mice

Chronic liver disease, alcoholism, haemochromatosis or other iron-overload disorders increase susceptibility to *V. vulnificus* infection in humans (Linkous & Oliver, 1999; Strom & Paranjpye, 2000). In particular, chronic liver diseases such as cirrhosis or viral hepatitis are associated with the highest risk of fatal outcomes for *V. vulnificus* infections because of the decreased synthesis of iron-binding proteins in the liver: 80 % of people suffering from chronic liver diseases die when infected with *V. vulnificus* (Strom & Paranjpye, 2000). In fact, it had been experimentally demonstrated that iron overload in mice enhanced susceptibility to *V. vulnificus* administered by intraperitoneal or intravenous injection (Stelma *et al.*, 1992; Wright *et al.*, 1981). We also confirmed that the 50 % lethal dose (LD_{50}) of the K44 strain used in this study by intraperitoneal challenge was decreased from $5\cdot0\times10^7$ to less than 1·0 through iron-overloading (data not shown). However, lymphocyte apoptosis was induced in iron dextran injected control mice without the injection with bacteria, indicating that the iron-treated model was not appropriate for our purpose. Therefore, we observed the effects of *V. vulnificus* on leukocytes in the mice model without iron overload in this study.

The number of mice in which *V. vulnificus* could be detected

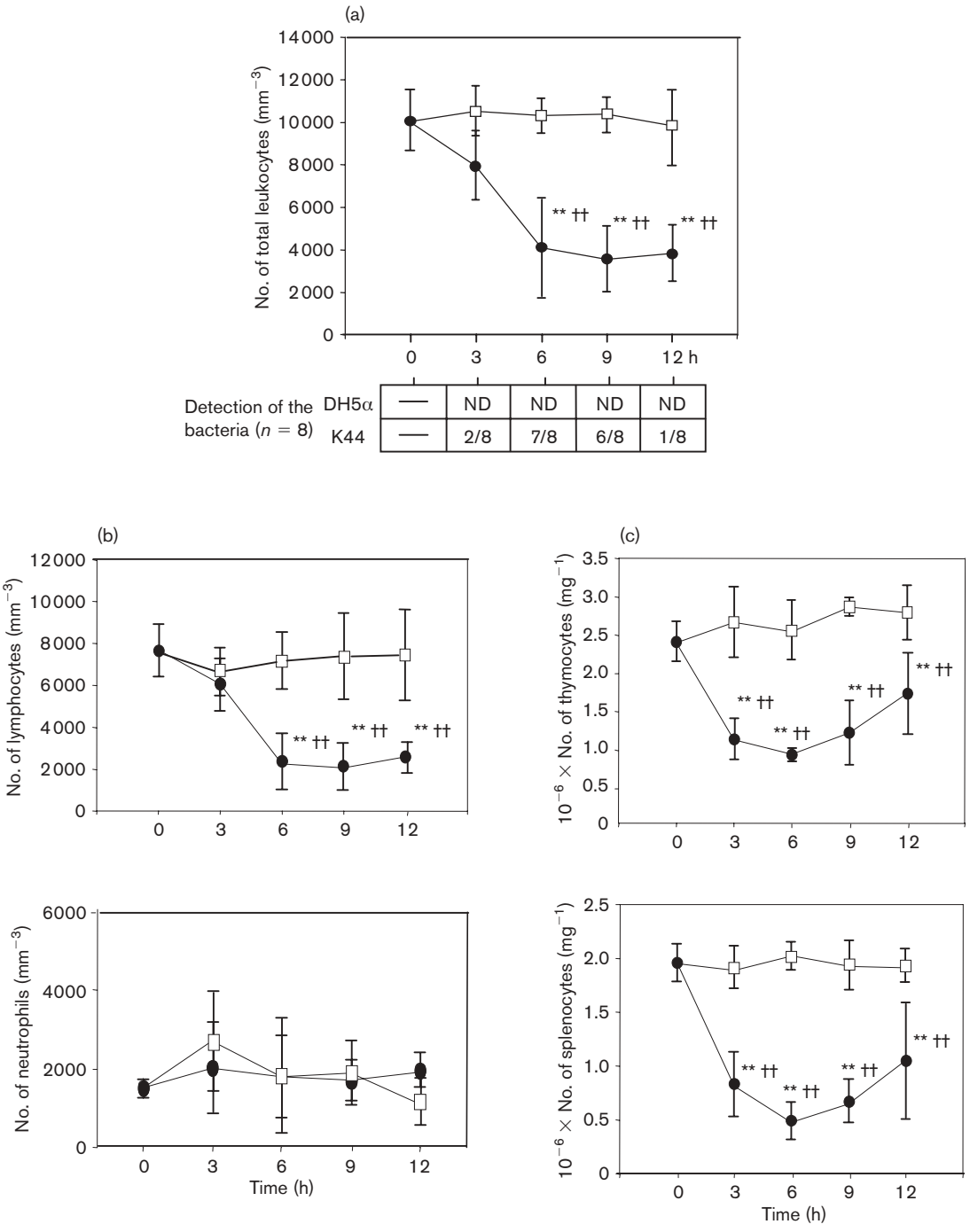


Fig. 1. Lymphocyte depletion is associated with bacterial growth. (a) Leukocyte depletion and numbers of mice in which bacteria were detected in the blood at various time intervals ($n = 8$). 'Detection of the bacteria' indicates the number of *V. vulnificus*-positive mice detected from 100 μl blood (detected/total). ND, Not detected. (b) Depletion of lymphocytes in mice injected with *V. vulnificus*. Numbers of lymphocytes and neutrophils were determined from a total leukocyte count and differential cell count. (c) Depletion of thymocytes and splenocytes in *V. vulnificus*-injected mice. The thymocytes and splenocytes were isolated as described in Methods. ●, K44-injected mice; □, control mice injected with *E. coli* DH5α. Data represent means ± SDs. Data were analysed by ANOVA. **Significant decrease compared with the mice before treatment ($P < 0{\cdot}01$). ††Significant decrease compared with the mice injected with *E. coli* DH5α at the same time point ($P < 0{\cdot}01$).

from blood samples started to increase by 3 h (2/8 mice), and reached a maximum at 6 h (7/8) after injection (Fig. 1a). The number of bacteria isolated from the blood increased from 10^2 to 10^4 c.f.u. ml^{-1} (data not shown) in a time-dependent manner until 9 h after injection. However, the number of leukocytes showed a tendency to decrease at 3 h, and reached a minimum value 6–9 h after injection (Fig. 1a). The number of leukocytes was still depressed at 12 h (Fig. 1a). No bacteria were detected throughout the experimental period in the mice that were injected with *E. coli* DH5α and served as controls (Fig. 1a). The types of depleted leukocytes in peripheral blood were determined. As shown in Fig. 1(b), the number of lymphocytes tended to decrease at 3 h, and decreased to about 25 % of the control value by 6 h post-inoculation. The numbers of lymphocytes were significantly decreased in the K44-injected mice in comparison with those of the control groups injected with *E. coli* strain DH5α ($P < 0{\cdot}01$) (Fig. 1b). Despite the severe decrease in lymphocytes in the K44-injected mice, the numbers of neutrophils showed no significant differences from the control groups at any time

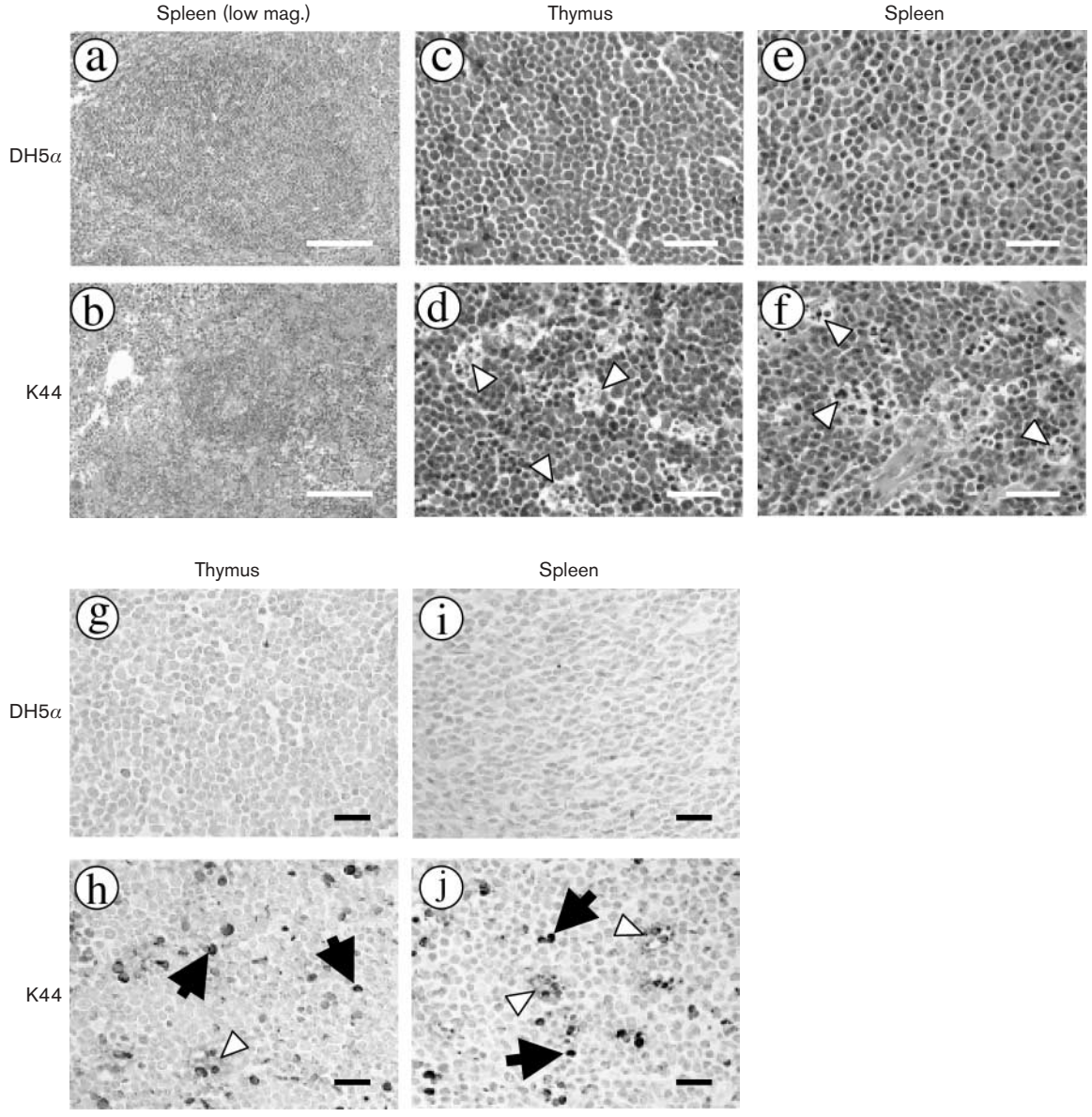


Fig. 2. Histology of the thymus and spleen in mice inoculated with *V. vulnificus* or *E. coli*. The sections of thymus and spleen were stained with H&E (a–f) and TUNEL (g–j). Atrophy of the white pulp appeared in the *V. vulnificus*-injected mice (b). Dead cells were observed in the macrophages as a 'starry sky' appearance in both thymus and spleen in the *V. vulnificus*-injected mice (d and f; arrowheads). Some lymphocytes (arrows) and dead cells phagocytosed by macrophages (arrowheads) appeared positive for TUNEL staining (h and j). Little pathological change could be observed in mice injected with *E. coli* DH5α (a, c, e, g and i). Bars, 100 μm (a and b) and 40 μm (c–j).

points (Fig. 1b). It was clear that lymphocyte numbers declined in the peripheral blood of K44-injected mice; therefore, we examined the number of lymphocytes in the thymus and spleen of these mice. The numbers of both thymocytes and splenocytes were markedly decreased at 3 h after injection, reaching a minimum at 6 h (Fig. 1c). The numbers of thymocytes and splenocytes tended to recover at 12 h (Fig. 1c). It is interesting to note that the decreases in thymocytes and splenocytes were caused earlier than the loss of peripheral blood lymphocytes (Fig. 1b, c), suggesting that the loss of peripheral lymphocytes was a result of the decrease in lymphocytes in the lymphoid tissues. Thus the experimental time points at which the depletion or recovery of lymphocytes was observed corresponded with bacterial growth *in vivo*.

Depletion of lymphocytes was associated with apoptosis

Histological analysis of the thymus and spleen at 9 h after injection of K44 showed phagocytosis of dead cells by macrophages as evidenced by a 'starry sky' appearance in H&E-stained sections (Fig. 2d, f; arrowheads). Atrophy of lymphoid follicles (white pulp) in the spleen was observed in the K44-injected mice (Fig. 2b). These results suggested that the rapid depletion of lymphocytes occurred by cell death. In the thymus and spleen sections stained using the TUNEL method, the dead cells were phagocytosed by macrophages (identical to the 'starry sky' appearance; arrowheads) and several lymphocytes showed positive reactions (Fig. 2h, j; arrows). These changes were not observed in the control groups injected with *E. coli* DH5α (Fig. 2a, c, e, g and i). In a recent study, it was reported that extensive loss of lymphocytes via apoptosis was observed in patients who died by polymicrobial sepsis (Hotchkiss *et al.*, 2001). A decrease in the area of lymphoid follicles could also be demonstrated by microscopic examination (Hotchkiss *et al.*, 2001). In our experiment with *V. vulnificus*-infected mice, we detected atrophy of lymphoid follicles in the spleen (Fig. 2b). These results suggested that a similar effect on lymphocyte apoptosis occurred in our *V. vulnificus*-infected mouse model as well as polymicrobial sepsis in human. Hotchkiss *et al.* (2001) reported that general-caspase inhibitor and caspase-3 inhibitor prevented lymphocyte apoptosis and improved survival in polymicrobial sepsis in a mouse model. To investigate the role of lymphocyte apoptosis in lethality, we injected *V. vulnificus* into the mice which had been administered the pan-caspase inhibitor (z-VAD-fmk; 6 mg kg^{-1}) 2 h before injection of the bacteria. However, the survival rates and time were not significantly affected by the treatment with z-VAD-fmk compared with z-FA-fmk administered mice (data not shown). Because we cannot exclude the possibility that the various different points (bacterial species, mouse strains, challenge dose and concentration of inhibitor) between our mouse model and the model of polymicrobial sepsis have an influence on this result, we could not decide whether the prevention of lymphocyte apoptosis will become an effective therapy for *V. vulnificus* infection at the present time.

Lymphocyte apoptosis was associated with bacterial growth *in vivo*

To confirm that positive reactions of TUNEL staining on histological analysis were associated with lymphocyte apoptosis and that the degree of apoptosis was related to bacterial growth *in vivo*, we measured DNA laddering, a hallmark of apoptosis, in the thymocytes and splenocytes obtained from the *V. vulnificus*-injected mice at all experimental time points. As shown in Fig. 3, K44 induced DNA fragmentation in the thymus and spleen at 3, 6 and 9 h. Only slight fragmentation of DNA was detected at 12 h (Fig. 3). However, no fragmentation of DNA in the thymus or spleen of control mice was observed (Fig. 3). In addition, we showed that the number of lymphocytes in peripheral blood reached a minimum 6–9 h post-inoculation, and that the number of thymocytes and splenocytes clearly decreased by 3 h and then recovered by 12 h after injection (Fig. 1a, c). Typical DNA laddering observed at 6 and 9 h tended to disappear by 12 h

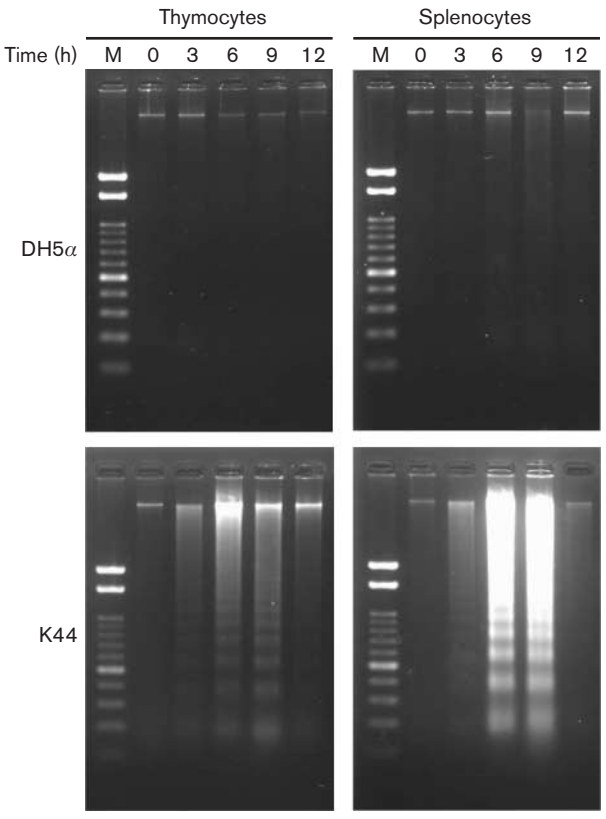


Fig. 3. *V. vulnificus* infection induced DNA fragmentation in thymocytes and splenocytes. Low-molecular-mass DNA was extracted from the cells of mice inoculated with *V. vulnificus* or *E. coli*, and electrophoresed on 2·0 % agarose gels, as described in Methods. Fragmentation of DNA was detected in both thymocytes and splenocytes of *V. vulnificus*-injected mice as early as 3 h post-inoculation (lower panels). However, only slight fragmentation could be detected at 12 h post-inoculation. No such changes were seen in mice injected with *E. coli* DH5α. One representative experiment of three replicates is shown. M, 100 bp DNA ladder marker.

in these tissues (Fig. 3). Thus the experimental time points at which the depletion or recovery of lymphocytes was observed corresponded to a distinct presence or absence of DNA fragmentation in the lymphocytes. These results indicate that lymphocyte apoptosis was associated with bacterial growth *in vivo*.

Phenotypic characterization of lymphocyte subsets following *V. vulnificus* infection

We determined the subsets of thymocytes and splenocytes that were affected by apoptosis. The cell surface phenotypes of lymphocytes were detected using flow cytometry at 6 h after inoculation of bacteria. In both the thymocytes and splenocytes, there were no differences in the percentages of analysed subsets of the K44-injected group compared with the group injected with *E. coli* DH5α (Fig. 4). We calculated the absolute numbers of all analysed subsets of lymphocytes from the total numbers of lymphocytes (Fig. 1b) and the percentage of each subset (Fig. 4a). The absolute numbers of all subsets in the K44-injected group were decreased in comparison with the DH5α-injected group (Fig. 4b).

Participation of LPS in lymphocyte apoptosis

It is well known that LPS, a cell-wall component of Gram-negative bacteria, is an important mediator of the inflammatory response and is an important factor of lymphocyte apoptosis in infection with Gram-negative bacteria. LPS activates the cells through Toll-like receptors (TLRs). Among TLR family members, both TLR2 and TLR4 have been shown to recognize LPSs (Erridge *et al.*, 2004). C3H/HeJ mice have selectively impeded LPS signal transduction, caused by the mutation in the third exon of the TLR4 gene (Poltorak *et al.*,

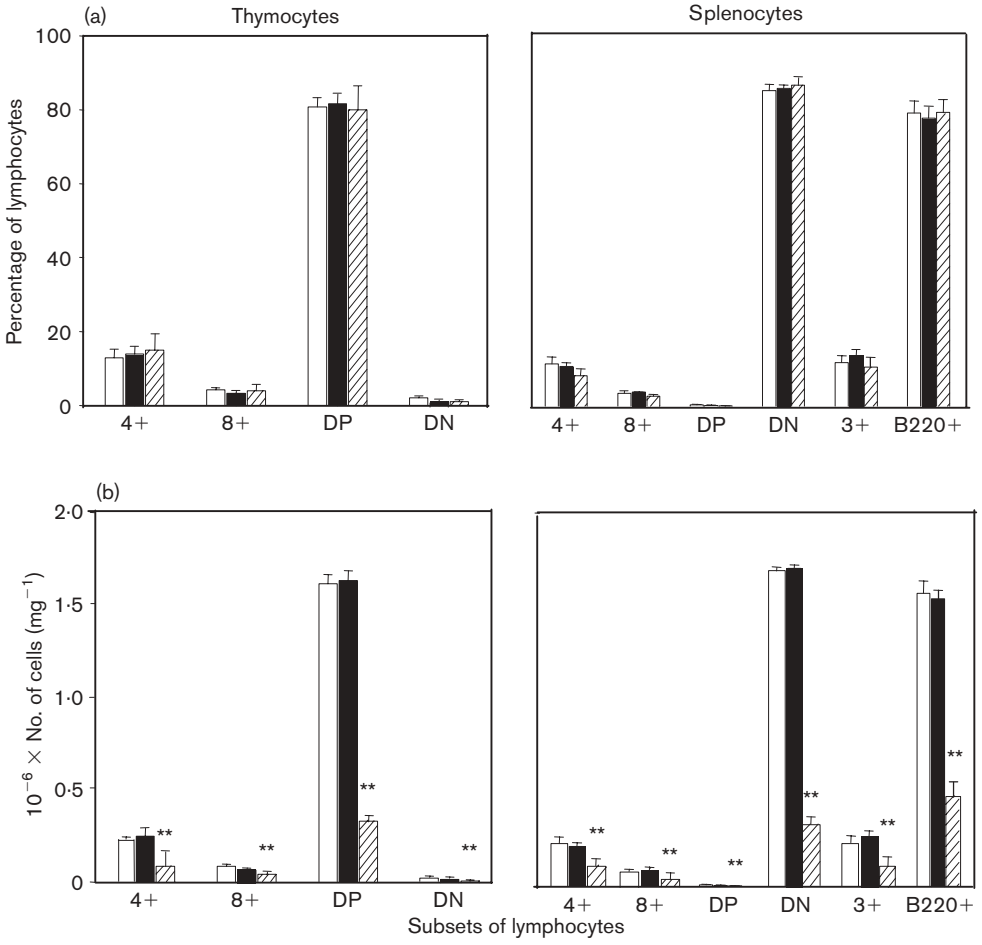


Fig. 4. Subtyping and absolute numbers of lymphocytes. Thymus and spleen were dissociated from *V. vulnificus*- or *E. coli*-injected mice at 6 h post-inoculation. Thymocytes and splenocytes were isolated as described in Methods. There were no changes in lymphocyte subsets in mice injected with either *V. vulnificus* or *E. coli* DH5α (a). The absolute numbers of thymocytes and splenocytes were calculated from the total numbers of lymphocytes and the percentage of each subset (b). Open bars, non-injected mice; filled bars, mice injected with *E. coli* DH5α; hatched bars, K44-injected mice. **Significant decrease compared with the mice injected with *E. coli* DH5α ($P < 0{\cdot}01$). 4+, $CD4^+$ T-subset; 8+, $CD8^+$ T-subset; DP, $CD4^+$ $CD8^+$ lymphocytes; DN, $CD4^-$ $CD8^-$ lymphocytes; 3+, $CD3^+$ T cells; B220+, CD45R $(B220)^+$ B cells.

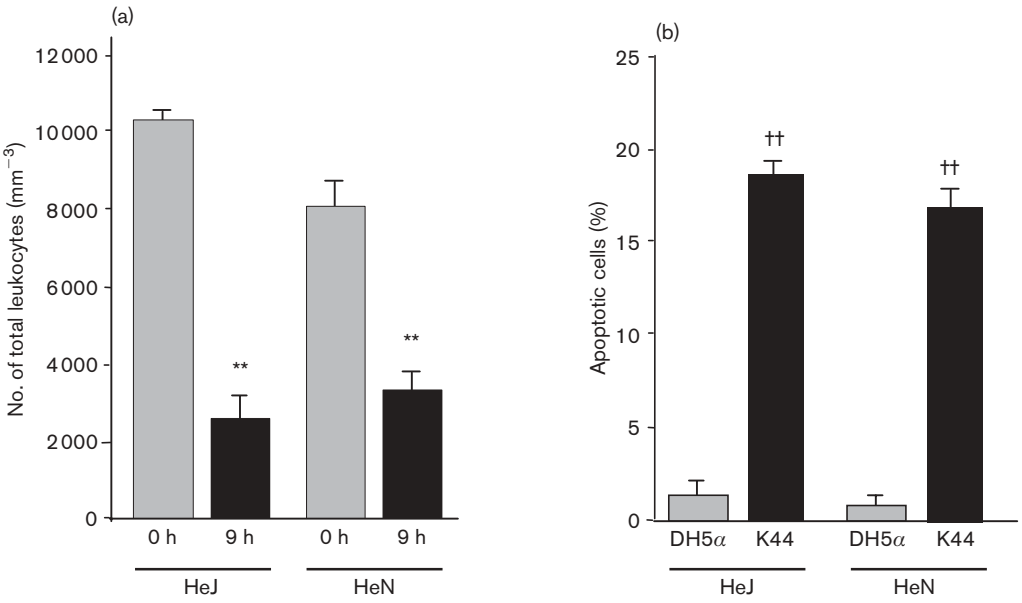
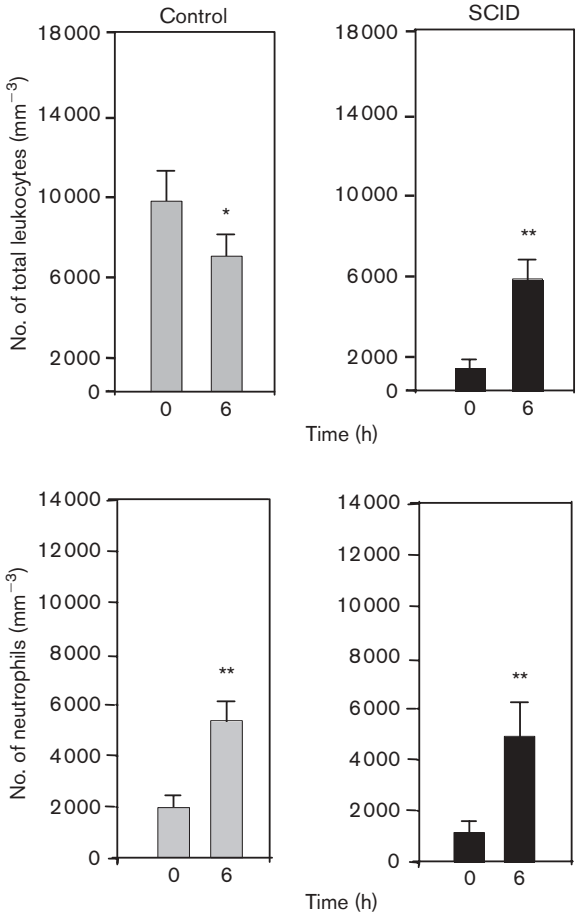


Fig. 5. Influence of *V. vulnificus* infection on the number of leukocytes and percentage of apoptotic splenocytes in C3H/HeJ mice and C3H/HeN mice ($n = 5$). The numbers of total leukocytes in these mice before inoculation were compared with the values at 9 h post-inoculation of K44 strain (a). The apoptotic splenocytes were detected by TUNEL staining of a section of the spleen, and the percentages of apoptotic splenocytes were calculated from at least 300 cells in the white pulp of the spleen (b). Data represent means ± SDs. Data were analysed using Student's *t*-test. **Significant decrease compared with the mice before the treatment ($P < 0{\cdot}01$). ††Significant increase compared with the mice injected with *E. coli* DH5α ($P < 0{\cdot}01$).



1998). However, C3H/HeN mice maintain normal LPS signal transduction by TLR4. To investigate the role of TLR4 in lymphocyte apoptosis in our experimental model, we compared the number of lymphocytes and percentage of apoptotic splenocytes between C3H/HeJ and C3H/HeN mice 9 h after inoculation with *V. vulnificus*. As shown in Fig. 5, there was no significant difference in the number of lymphocytes or percentage of apoptotic lymphocytes between these mice groups, indicating that TLR4 does not participate in *V. vulnificus*-induced lymphocyte apoptosis. Recent studies have demonstrated that TLR2 is largely required for signalling by LPSs from many Gram-negative bacteria (Erridge *et al.*, 2004). Therefore, it is possible that the lymphocyte apoptosis in our model was induced through TLR2 signalling. The other possibility involves the cytolysin of *V. vulnificus*, which is one of the major secreted toxins and induces apoptosis in various types of cells *in vitro* (Fan *et al.*, 2001; Kim *et al.*, 1998; Kwon *et al.*, 2001). Since the effect of cytolysin on lymphocytes has not been clearly demonstrated *in vivo* at the present time, further investigation is necessary to determine the contribution of this toxin to lymphocyte apoptosis in our mouse model.

Fig. 6. Influence of *V. vulnificus* infection on the numbers of neutrophils in control and SCID mice ($n = 5$). Control strain, C.B17 +/+; SCID strain, C.B17 scid/scid. Data represent means ± SDs. Data were analysed using Student's *t*-test. *,**Significant difference compared with the mice before treatment ($P < 0{\cdot}05$ or $0{\cdot}01$, respectively).

Effect on the number of neutrophils in *V. vulnificus*-injected severe combined immunodeficient (SCID) mice

The total number of leukocytes reached a minimum 6–9 h after injection of K44 into ddY mice (Fig. 1a). However, the number of neutrophils in these mice was not decreased by *V. vulnificus* injection (Fig. 1b). To gain further clarity on the question of whether *V. vulnificus* induces neutrophil apoptosis *in vivo*, we injected the K44 strain into the SCID mouse, which has a high percentage of neutrophils (about 70 %) and very few lymphocytes in peripheral blood. The total number of leukocytes and neutrophils in the K44-injected SCID mice increased more than threefold compared with the pre-injection value (Fig. 6). However, in the K44-injected control mice (+/+), the total number of leukocytes decreased to 70 % of pre-injection numbers within 6 h (Fig. 6). These results indicated that growth of *V. vulnificus* did not clearly induce apoptosis in neutrophils *in vivo*. From the results shown in Figs 1(c) and 6, we concluded that *V. vulnificus* appeared to induce apoptosis specifically on lymphocytes in peripheral blood *in vivo*, which led to a loss in overall leukocyte numbers in the peripheral blood, and the lymphocyte apoptosis must be closely related to the growth of *V. vulnificus in vivo*.

In conclusion, we report here that *V. vulnificus* induces lymphocyte, but not neutrophil, depletion in association with apoptosis *in vivo*.

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