HOST RESPONSE TO INFECTION

Induction of thymocyte apoptosis in mice by *Yersinia enterocolitica* products

Y.-S. LIN, K.-H. CHEN, C.-F. KUO, K.-J. HUANG and J.-J. WU*

Department of Microbiology and Immunology and *Department of Medical Technology, National Cheng Kung University Medical College, Tainan, Taiwan, Republic of China

In-vivo administration of the culture supernates from *Yersinia enterocolitica* resulted in thymus atrophy in C3H/HeJ mice, known to be lipopolysaccharide (LPS)-non-responders. The thymocytes underwent apoptosis as characterised by fragmented DNA ladders on agarose gel electrophoresis, a cell death detection ELISA and a morphological study by the TUNEL reaction. As a control, LPS treatment did not induce thymocyte apoptosis in C3H/HeJ mice. Flow cytometric analysis indicated that thymus atrophy was due predominantly to the deletion of CD4+CD8+ T cells. When cells were undergoing apoptosis, an elevation in the percentage of T-cell receptor (TCR)-**a**β**high** cells was observed at 24 h, which was correlated with the increase in the percentages of cells expressing high levels of the Vp6 and Vp8 TCR. Gel electrophoretic analysis demonstrated the presence of protein bands with mol.wts ranging from 17 to 65 kDa in *Y. enterocolitica* culture supernates.

Introduction

*Yersinia enterocolitica*, a gram-negative coccobacillus belonging to the Enterobacteriaceae, has been associated with human and animal diseases [1, 2]. These pathogens cause gastro-enteritis; one complication of infection with *Y. enterocolitica* in man has been the subsequent development of autoimmune disease [3–5]. Reactive arthritis stimulated by *Y. enterocolitica* provides an excellent model for the study of pathogenesis of inflammatory arthritis. Recent studies showed that this pathogen activated a Th1-like T-cell subset in patients with *Yersinia*-triggered reactive arthritis [6–8]. A 19-kDa antigen, which was identical to the urease β-subunit [9, 10], has been shown to act as a target for human synovial T lymphocytes in reactive arthritis [10]. Nevertheless, the role that bacterial superantigens may play in the development of autoimmune diseases should also be taken into consideration. A number of bacterial T-cell superantigens have been identified, including staphylococcal enterotoxins, streptococcal pyogenic exotoxins and *Mycoplasma arthritidis* mitogen [11–13]. Furthermore, *Y. pseudotuberculosis*-derived superantigen has been identified and cloned [14–16]. *Y. enterocolitica* was reported to produce antigenic material that had properties consistent with those of T-cell superantigens [17, 18]; however, the superantigen produced by *Y. enterocolitica* has not been characterised.

Induction of apoptosis by bacteria and bacterial products has been reported in macrophages and neutrophils [19–23]. Bacterial superantigen-induced apoptosis in lymphocytes and thymocytes has also been described [24, 25]. In this study, the effect of *Y. enterocolitica* products on mouse thymus was examined *in vivo*. The effects of *Y. enterocolitica* products on various thymocyte subpopulations with respect to CD4/CD8 and specific T-cell receptor (TCR) V**β**-bearing cells were investigated.

Materials and methods

Mice

Breeding stock of C3H/HeJ mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and maintained on standard laboratory chow and water *ad libitum* in our animal centre. Their progeny, aged 4–6 weeks, were used for the experiments.

Bacterial culture supernate

The antigen preparation method was modified from the procedure described by Abe et al. [14]. *Y. enterocolitica* serotype O8 (ATCC 23715) was grown in 2 L of CYG medium (casamino acid and yeast extract broth supplemented with glucose, pH 8.5) at 25°C for 5 days...
with vigorous shaking. The culture supernate (SN) was collected by centrifugation at 7000 g for 30 min, filtered through a 0.22-µm filter to remove the residual bacteria, then lyophilised and resuspended in 200 ml of phosphate-buffered saline (PBS). The concentrated SN was precipitated by gradual addition of ammonium sulphate up to 80%, and centrifuged at 10 000 g for 30 min. The precipitates were resuspended in 5 ml of PBS and dialysed against PBS with a 6000–8000-mol wt membrane, then concentrated with a Negative Pressure Micro Protein Dialysis Concentrator (Spectrum Medical Industries, Los Angeles, CA, USA) to 3–5 ml. After filtration, the protein concentration was determined by BioRad protein assay (BioRad, Hercules, CA, USA). The CYG medium control was also prepared by the procedure described above but without any bacterial inoculum. The components present in the SN were analysed by SDS-PAGE with a 12.5% gel, which was loaded with 40 µg of proteins (in 20 µl) followed by Coomassie Blue staining.

Measurement of thymus weights and thymocyte numbers

Mice were inoculated with *Y. enterocolitica* SN preparation or LPS from *Escherichia coli* serotype O55:B5 (Sigma). Control groups received an equivalent volume of PBS or CYG medium alone. After 24 h, mice were killed and the thymus was removed and weighed accurately. Single cell suspensions were then prepared and the cell number was determined with a haemocytometer.

**DNA extraction and agarose gel electrophoresis**

Single cell suspensions prepared from thymus glands (1 × 10⁸ in a 1.5-ml microcentrifuge tube) were pelleted and resuspended in cold lysis buffer (500 µl) containing 20 mM Tris-HCl (pH 7.4), 10 mM EDTA and Triton X-100 0.2% for 10 min, and the lysate was centrifuged for 10 min at 10 000 rpm. Then, proteinase K 100 µg/ml was added and incubated at 50°C for 6 h and the extract was digested with RNase at 50 µg/ml for a further 2 h at 37°C. The DNA in the viscous solution was extracted twice with phenol and once with chloroform:isoamyl alcohol (24:1) and then precipitated with 2-propanol 50% and 1 µl of glycogen for 3 h at −20°C. Electrophoresis was performed on agarose 1% gel in 90 mM Tris-borate buffer (pH 8.0) containing 2 mM EDTA. The gel was stained with ethidium bromide 1 µg/ml and visualised with UV light.

**Cell death detection ELISA**

Isolated single cell suspensions from thymus were prepared and the fragmented DNA was determined quantitatively with the cell death detection ELISA kit (Boehringer Mannheim GmbH, Mannheim, Germany). The procedure used followed the manufacturer's instructions.

**TUNEL reaction**

Mice were treated with *Y. enterocolitica* SN or CYG medium alone for 24 h, and the presence of apoptotic cells in the thymus was analysed by the terminal deoxytransferase-mediated dUTP nick end-labelling (TUNEL reaction). The ApopTag *In Situ* Apoptosis Detection Kit for Peroxidase (Oncor, Gaithersburg, MD, USA) was used; tissue fixation and staining procedures used followed the manufacturer's instructions.

**Immunofluorescence analysis**

Mouse thymocytes (50 µl) at concentration of 2 × 10⁷/ml were suspended in Minimum Essential Medium (Life Technologies, Inc., Grand Island, NY, USA) containing sodium azide 0.1% and fetal calf serum (HyClone) 2%. Cells were then incubated with various fluorescent-labelled monoclonal antibodies (MAbs) for flow cytometry. The antibodies used included PE-conjugated anti-CD4 (PharMingen, San Diego, CA, USA) and FITC-conjugated anti-CD8 MAb (Boehringer Mannheim GmbH), FITC-conjugated anti-TCR-αβ and FITC-conjugated anti-TCR Vβ3, 5, 6, 7, 8.1, 8.2, 9, 11 or 13 MAb (PharMingen). After incubation for 30–45 min on ice, the mixture was washed twice with ice-cold medium and the cells were resuspended in cold medium to approximately 1 × 10⁶ cells/ml. The stained cells were analysed by flow cytometry (FACScan, Becton Dickinson, Mountain View, CA, USA) with excitation set at 488 nm.

**Statistical analysis**

Comparison of TCR Vβ subpopulations among the three groups was done by analysis of variance (ANOVA) followed by the Duncan multiple range test. Differences were considered significant at p < 0.05.

**Results**

**Induction of thymocyte apoptosis by *Y. enterocolitica* SN preparation**

Intraperitoneal administration of *Y. enterocolitica* SN for 24 h caused a reduction in thymus weight (Fig. 1a) and thymocyte number (Fig. 1b) as compared to the PBS and CYG medium controls. A similar effect was observed with intravenous injection of the SN (data not shown). The reduction in thymus size and cell number was sustained at 48 h (data not shown). Furthermore, administration of LPS did not cause thymus atrophy in C3H/HeJ mice (Fig. 1). DNA from mouse thymocytes was extracted and analysed by agarose gel electrophoresis. The appearance of fragmented bands of DNA was detected following *Y. enterocolitica* SN treatment (Fig. 2, lane 3), but not in PBS- (lane 2) and LPS-treated (lane 4) groups. The fragmented DNA was further quantified by the cell death detection ELISA. As thymocytes would undergo apoptosis spontaneously, there was a basal level of DNA fragmentation in both
Y. enterocolitica-induced thymocyte apoptosis

PBS- and CYG medium-treated controls. Treatment with *Y. enterocolitica* SN caused an enhancement of DNA fragmentation (Fig. 3). Morphological studies by the TUNEL reaction revealed a higher degree of thymocyte apoptosis in *Y. enterocolitica* SN-treated group than in those mice given control medium (Fig. 4).

The changes in thymocyte subpopulations were examined by flow cytometry. Results indicated a predominant loss of CD4+CD8+ cells in the thymus of *Y. enterocolitica* SN-treated mice. Both the percentage (Fig. 5) and yield (Table 1) of CD4+CD8+ cells were reduced after treatment. However, LPS-treated mice showed a pattern similar to that of the PBS-treated group (Fig. 5, Table 1).

**Effects of Y. enterocolitica SN on TCR-αβ and various subsets of Vβ-bearing cells**

In addition to the CD4/CD8 subpopulations, changes in other surface phenotypes during apoptosis were examined. There was an increase in the percentage of TCR-αβ<sup>high</sup> cells (Fig. 6, centre panel) as compared with the PBS-treated control (Fig. 6, upper panel). The changes in thymocytes bearing specific Vβ elements were investigated further. Fig. 7a shows the increase in the percentages of Vβ6- and Vβ8-bearing cells 24 h after administration of *Y. enterocolitica* SN. Data obtained from averages of three experiments are shown.
Fig. 4. Morphological study of *Y. enterocolitica* SN-induced thymocyte apoptosis. Mice were treated with CYG medium (A, B) or YE (400 µg) (C, D) for 24 h, and the apoptotic cells were analysed by the TUNEL reaction. Magnification, ×50 (A, C); ×190 (B, D).

in Fig. 7b. The cells bearing Vβ3, 5, 7, 9, 11 and 13 remained unchanged relative to the PBS-treated control.

As shown in Fig. 6 (lower panel), C3H/HeJ mice inoculated with LPS showed a similar percentage of TCR-αβ<sup>high</sup> thymocytes to that of the PBS control. Analysis of the percentages of cells bearing other Vβ markers also showed levels similar to those of the PBS-treated control (Fig. 7).

Protein patterns in *Y. enterocolitica* SN

The protein compositions in *Y. enterocolitica* SN and in the CYG medium were analysed by SDS-PAGE and Coomassie Blue staining. Results showed the presence of protein bands corresponding to mol. wts of 65, 52, 48, 45, 40, 30, 27, 26, 23 and 17 kDa in *Y. enterocolitica* SN, but not in the medium control (Fig. 8).

Discussion

The present study demonstrated that *Y. enterocolitica* produced factor(s) that caused depletion of thymocytes. During the process of apoptosis, there was an increase in the percentage of TCR-αβ<sup>high</sup> cells, which was correlated with an increase in the percentages of Vβ6- and Vβ8-bearing cells. A recent report showed upregulation of TCRαβ/CD3, CD69 and CD25 expression on dying thymocytes, which led to the suggestion that apoptosis caused a radical alteration in the expression of cell surface molecules [26]. An increase in the expression of TCRαβ/CD3 surface markers on thymocytes during apoptosis induced by staphylococcal enterotoxin B, a bacterial superantigen, has also been found (unpublished data). In this study, the increase in the percentages of TCRαβ<sup>high</sup> cells, especially in Vβ6<sup>high</sup> and Vβ8<sup>high</sup> cells, after *Y. enterocolitica* SN administration was consistent with those previous findings showing upregulation of the expression of cell surface markers.

*Y. enterocolitica*-derived antigens might possess superantigenic activity [17]. The stimulatory activity was found in culture supernates, as well as in membrane and cytoplasmic fractions. With *Y. enterocolitica* cytoplasmic fractions, it was demonstrated that *Y. enterocolitica* stimulated murine T cells bearing Vβ3, 6 and 11 and possibly 7 and 9, as well as T-cell hybridomas bearing Vβ3, 7, 8.1, 9 and 11, but not 2, 8.2, 8.3 and 13. As a result of the broad Vβ repertoire stimulated by *Y. enterocolitica* antigens, it was suggested that this micro-organism might possess multiple superantigens, each of which interacts with
Y. ENTEROCOLITICA-INDUCED THYMOCYTE APOPTOSIS

Fig. 5. Changes in CD4/CD8 thymocyte subpopulations after Y. enterocolitica SN administration to C3H/HeJ mice. Groups of three or four C3H/HeJ mice were inoculated with PBS, YE (80 µg) or LPS (100 µg). After 24 h, the thymocytes were stained with PE-conjugated anti-CD4 and FITC-conjugated anti-CD8 MAb, and analysed by FACScan.

Table 1. Changes in numbers of CD4/CD8 thymocyte subpopulations after Y. enterocolitica SN administration to C3H/HeJ mice*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CD4⁺CD8⁺</th>
<th>CD4⁺CD8⁻</th>
<th>CD4⁻CD8⁺</th>
<th>CD4⁻CD8⁻</th>
</tr>
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<tbody>
<tr>
<td>PBS</td>
<td>62.2(3.5)</td>
<td>9.2(0.5)</td>
<td>1.9(0.1)</td>
<td>2.4(0.1)</td>
</tr>
<tr>
<td>YE</td>
<td>20.2(9.5)</td>
<td>6.1(2.9)</td>
<td>2.3(1.1)</td>
<td>1.5(0.7)</td>
</tr>
<tr>
<td>LPS</td>
<td>66.7(14.7)</td>
<td>10.3(2.3)</td>
<td>3.6(0.8)</td>
<td>4.5(1.0)</td>
</tr>
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*Mice were treated as described in Fig. 2. The number of thymocyte subpopulations was determined by multiplying the number of thymocytes per mouse by the percentage of each thymocyte subpopulation.

Fig. 6. Increase in TCR-αβhigh thymocytes after Y. enterocolitica SN administration to mice. Groups of three or four C3H/HeJ mice were inoculated with PBS, YE (80 µg) or LPS (100 µg). The thymocytes were stained with FITC-conjugated anti-TCR-αβ MAb and analysed by FACScan. The percentages of TCR-αβhigh and TCR-αβlow cells are indicated.

a distinct spectrum of Vβ-bearing T cells, or alternatively, might have varying binding affinities for different Vβ [17]. In this study, only Vβ6- and Vβ8-bearing thymocytes appeared to be affected by Y. enterocolitica SN preparation. A positive correlation between the presence of Y. enterocolitica superantigen...
in the SN and the upregulation of these specific Vβ-bearing cells during apoptosis remains to be elucidated. As antibodies against both Vβ8.1 and 8.2 were used in this study, at present it is not possible to differentiate the reactivities between these two subsets.

Induction of thymocyte apoptosis by administration of LPS in vivo has previously been demonstrated in BALB/c [27] and C3H/HeN mice, but not in C3H/HeJ mice [28]. The LPS-non-responder C3H/HeJ mice were used in this study and it was confirmed that LPS did not cause an apoptotic effect on thymocytes of these mice. The presence of LPS in Y. enterocolitica SN preparation was, therefore, unlikely to account for the induction of apoptosis in thymocytes.

Fig. 7. Effect of Y. enterocolitica SN on thymocytes bearing various Vβ segments. C3H/HeJ mice were inoculated with PBS, YE (80 ng) or LPS (100 μg). After 24 h, the thymocytes were stained with FITC-conjugated anti-Vβ3, 5, 6, 7, 8.1, 8.2, 9, 11 or 13 MAb, and analysed by FACScan. a, Cells expressing Vβ6 or Vβ8.1, 8.2; b, the percentages of various Vβ-bearing cells derived from averages of three experiments. Bar indicates the SD; *p < 0.05.
A number of recent reports showed that bacteria induced apoptosis in infected macrophages or neutrophils [19–23]. The present study showed the apoptotic effect of bacteria on thymocytes. The induction of thymocyte apoptosis may result from a direct effect of bacterial products on thymocytes or through the induction of cytokines and steroid hormones. Further studies in vitro should help to delineate whether or not a direct effect occurred. Tumour necrosis factor-α and interleukin 2-driven thymocyte death have previously been reported to be involved [28–30]. Production of cytokines had been demonstrated after Y. enterocolitica infection, although there was differential expression of some cytokines in susceptible and resistant strains of mice [31–33]. Further studies are needed to explore the involvement of cytokine and steroid hormones in thymocyte apoptosis induced by Y. enterocolitica. The protein patterns in Y. enterocolitica SN revealed the presence of many proteins bands with mol.wts between 65 and 17 kDa. The component(s) of Y. enterocolitica products in the SN which is(are) responsible for the induction of thymus atrophy await further characterisation. Whether the superantigen(s) produced by Y. enterocolitica may be involved remains to be seen.

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**Fig. 8.** SDS-PAGE analysis of the Y. enterocolitica products. Y. enterocolitica concentrated SN (lane 1, 40 μg of protein in 20 μl) and CYG medium control (lane 2, 20 μl) were subjected to SDS-PAGE, and the gel was stained with Coomassie Blue.

References