

IMMUNE RESPONSE TO INFECTION

## TH<sub>1</sub> pattern of cytokine secretion by splenic cells from pyelonephritic mice after in-vitro stimulation with hsp-65 of *Escherichia coli*

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**Splenic lymphocytes and peritoneal macrophages from BALB/c mice with *Escherichia coli* pyelonephritis were obtained at various intervals after infection. These cells were stimulated *in vitro* with different antigens and cytokine release was assayed in the supernate of the cultured cells. It was observed that both specific antigens such as outer-membrane proteins (OMPs), porins and heat-shock protein-65 (hsp-65), as well as non-specific mitogens such as phytohaemagglutinin (PHA), were able to induce cytokine production by splenic cells from infected mice. Of all these antigens, hsp-65 was found to be the best inducer of cytokine release. In the acute stage of pyelonephritis, the release of interleukin-2 (IL-2) and interferon- $\gamma$  (IFN- $\gamma$ ) was found to increase with time; both reached their peak values on the seventh day after infection. The TH<sub>1</sub> pattern of cytokine secretion by splenic cells was observed, i.e., IL-2 and IFN- $\gamma$ , whereas there was complete absence of IL-4 secretion. In the chronic stage of pyelonephritis, i.e., 150 days after infection, a decrease in the level of IL-2 and IFN- $\gamma$  was observed. Peritoneal macrophages released IL-1 on stimulation with hsp-65, which increased with the progression of disease. The possible implications of this study for the disease process are discussed.**

### Introduction

Cytokines are known to play an important role in the regulation of host defences against local bacterial infections [1]. They have been shown to aid in the recruitment and chemotaxis of neutrophils, in the proliferation and function of other effector cells and in bacterial clearance [2–4]. Murine helper T-cell clones have been categorised into two distinct subsets by their lymphokine secretion pattern [5, 6]. TH<sub>1</sub> cells produce Interleukin-2 (IL-2) and Interferon- $\gamma$  (IFN- $\gamma$ ), whereas TH<sub>2</sub> cells produce IL-4 and IL-5. In the present study, acute and chronic pyelonephritis were established in female BALB/c mice *via* the ascending route. Cytokine production by splenic lymphocytes and peritoneal macrophages of the pyelonephritic mice was studied after in-vitro stimulation of these cells with specific and non-specific antigens.

### Materials and methods

#### *Mice*

BALB/c mice aged 2 months and weighing 25–30 g were used to establish acute infection *via* the ascending route as described by Sinha *et al.* [7]. For establishment of chronic infection the method of Gupta *et al.* [8] was used.

#### *Antigens*

Hsp-65 was purified from *Escherichia coli* O6:K13:H1 by affinity chromatography. Briefly, bacterial cells were exposed to a temperature of 42°C for 1 h to enhance the expression of hsp-65. This combination of time and temperature has been standardised in our laboratory to obtain expression of hsp-65 without any deleterious effect on the viability of the micro-organisms. These cells were then lysed by sonication and intracellular proteins were separated by ultra-centrifugation at 100 000 *g* for 1 h at 4°C. Proteins were separated by SDS-PAGE and electroblotted on to nitrocellulose paper. Hsp-65 was eluted from the paper strip and was used to raise antiserum in rabbit. This antiserum

was then used to make an affinity column with Sepharose 4B beads which was used to purify hsp-65 antigen as described previously [9]. Outer-membrane proteins (OMPs) were separated by the method of Shand *et al.* [10]. Briefly, cells were lysed by sonication and unsonicated cells and cell debris were removed by centrifugation. Sodium *n*-lauroylsarcosine 1% was added to the supernate and the detergent-insoluble, OMP-enriched fraction was collected by centrifugation at 100 000 *g* for 60 min at 4°C.

Porins were separated by the method of Nurimnen [11]. Briefly, lysozyme was added to the bacterial pellet and then a solution of MgCl<sub>2</sub> with DNAase and RNAase was added. After the cell pellet had been washed, the cell envelope was extracted with Triton X-100 2%. This extract was centrifuged at 10 000 rpm for 30 min at 4°C and the supernate was dialysed extensively, concentrated by lyophilisation and finally purified on a Sepharose 4 B column.

Phytohaemagglutinin and concanavalin A were the other antigens used for non-specific stimulation. These were obtained from Sigma.

### Interleukin assays

*IL-1.* Thymocytes were separated from 1–2-day-old mice by teasing the thymus and the number of thymocytes was adjusted to 10<sup>6</sup> cells/ml. Viability was checked by the trypan blue dye exclusion test. Culture supernates were prepared by culturing peritoneal macrophages from different groups of infected mice after they had been exposed *in vitro* to different antigens. A co-stimulation assay for proliferation of thymocytes was done as described by Mizel [12].

*IL-2.* Assays for IL-2, IL-4 and IFN- $\gamma$  were done with splenic lymphocytes. For the isolation of lymphocytes, spleens were removed at various times after infection and lymphocytes were separated by teasing the spleens. The number of cells was adjusted to 10<sup>6</sup> cells/ml and 100  $\mu$ l of the cell suspension/well were cultured for 24 and 72 h after stimulation with different antigens. Culture supernates were stored at –20°C. Assays were made with the HT-2 cell line, which is IL-2-dependent. Briefly, cells of the HT-2 cell line were washed free from residual IL-2 and resuspended at 10<sup>5</sup> cells/ml in RPMI 1640 medium with fetal calf serum (FCS) 10%. One hundred  $\mu$ l of this suspension were dispensed in individual wells of a microtitration plate and 100- $\mu$ l volumes of test samples were added to these wells. IL-4 receptors were blocked by an anti-IL-4 monoclonal antibody and the plates were incubated at 30°C for 18 h, after which 10  $\mu$ l of a solution of 3-(4,5-dimethylthia-zol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) 5 mg/ml in PBS were added to each well. Plates were incubated for a further 3–4 h and colour was developed by adding 150  $\mu$ l of 0.01 N HCl in isopropanol. Plates were read in an ELISA reader at

a test wavelength of 570 nm and a reference wavelength of 630 nm. A curve of concentration *versus* optical density (equivalent to cell proliferation) was plotted for the standard preparation of IL-2 and this curve was used to determine unknown samples by interpolation.

*IL-4.* To measure IL-4 secretion, IL-2 receptors of HT-2 cells were blocked by anti-IL-2 antibodies instead of anti-IL-4 antibody. A standard curve was plotted with a standard preparation of IL-4 which was then used to measure IL-4 in test supernates by interpolation.

*IFN- $\gamma$ .* To measure IFN- $\gamma$ , a WEHI-279 cell line was used whose proliferation is inhibited by IFN- $\gamma$ . WEHI-279 cells were washed and plated in 96-well microtitration plates at a density of 10<sup>5</sup> cells/ml of RPMI-FCS medium. Different dilutions of culture supernate were added to 100  $\mu$ l of cell suspension in the presence or absence of anti-IFN- $\gamma$  antibody (RG/46 A-2). After incubation at 37°C for 18 h, 10  $\mu$ l of MTT were added to each well and incubation was continued for a further 4 h. One hundred  $\mu$ l of 0.01 N HCl in isopropanol were added to each well after 4 h and the plates were read in an ELISA reader as in the case of IL-2 and IL-4.

## Results

### *IL-1 assay*

Of all the antigens used to stimulate IL-1 secretion, hsp-65 gave maximal stimulation at all times measured (Table 1). OMPs showed better stimulation than porins on the second and seventh days after infection, but on the 14th and 150th day after infection, the responses were similar. The non-specific antigen, concanavalin A, stimulated IL-1 production at all times studied, but significant differences in comparison to hsp ( $p < 0.05$ ) were observed on day 14 and on day 150; the significance was similar to the one observed between OMPs and porins ( $p < 0.01$ ).

### *IL-2 assay*

Table 2 shows an IL-2 assay with three different concentrations of hsp-65. When 10  $\mu$ g of hsp were used for the *in-vitro* assay, IL-2 secretion was not increased on the second day after infection but was increased on days 5, 7 and 14. With 20  $\mu$ g of hsp-65, the amount of IL-2 secreted was significantly increased even 2 days after infection ( $p < 0.05$ ). There was a gradual increase in IL-2 levels from the fifth to the 14th day, but no significant increase was seen during the chronic phase ( $p > 0.05$ ). With 50  $\mu$ g of hsp-65 the level of IL-2 was significantly increased from the second to the 14th day with a slight increase observed on progression of the disease to a chronic state. On the

**Table 1.** IL-1 assay with different antigens

Day	Mean (SEM) 3[H] TdR incorporation (cpm × 10 <sup>3</sup> )				
	Control I	hsp-65 II	OMPs III	Con A IV	Porins V
2	1.2(0.01)	*10.4(0.04)	7.1(0.05)	*8.5(0.04)	6.5(0.038)
5	1.2(0.01)	*12.4(0.06)	7.9(0.04)	*9.2(0.04)	7.1(0.03)
7	1.0(0.01)	**15.5(0.05)	*12.6(0.035)	**13.5(0.04)	*11.9(0.035)
14	1.2(0.01)	***, †20.8(0.05)	**13.9(0.04)	**15.1(0.02)	*12.8(0.04)
150	1.0(0.02)	***, †31.2(0.06)	**13.9(0.04)	**16.9(0.03)	**13.9(0.03)

Students *t* test I versus II or III or IV or V: \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001; II versus III or IV or V: †*p* < 0.05, ††*p* < 0.01, †††*p* < 0.001.

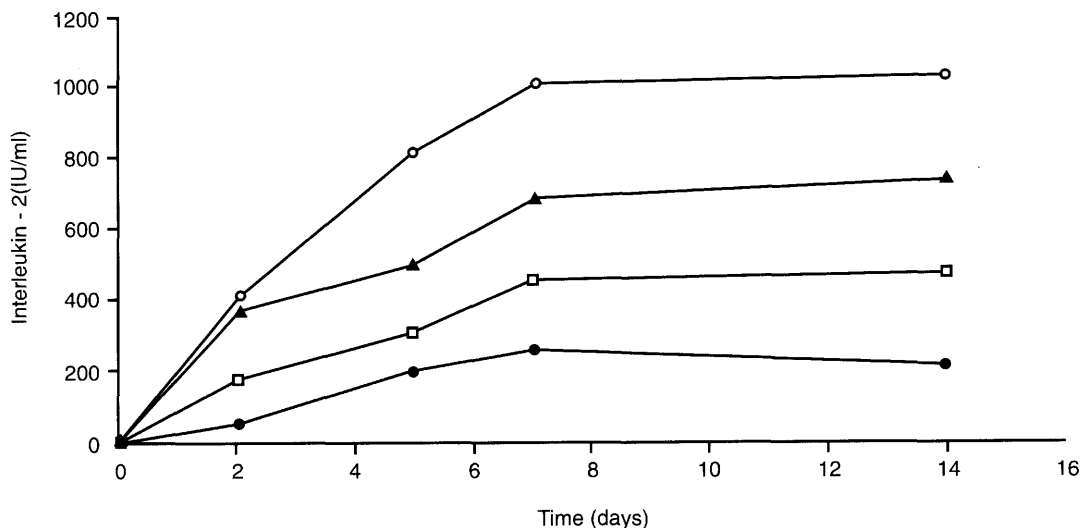
**Table 2.** IL-2 assay with different concentrations of hsp-65

Time (days)	Mean (SEM) IL-2 production (units/ml)		
	10 µg hsp	20 µg hsp	50 µg hsp
2	68.4(2.5)	*280.4(18.4)	*402.6(30.8)
5	*408.2(25.4)	†608.7(30.6)	†808.2(65.5)
7	*442.3(40.2)	†688.5(50.2)	†1026.3(80.8)
14	*468.2(42.4)	†722.4(50.8)	†1048.2(90.4)
150	ND	†760.5(50.2)	†1070.4(90.2)

Data represent mean (SEM) of six different experiments; ND, not done. Control versus 2 or 5 or 7 or 14: \**p* < 0.05, †*p* < 0.01, ††*p* < 0.001.

basis of these findings a 50-µg dose of hsp was selected for comparison with other antigens.

In addition to hsp-65, OMPs and porins also induced IL-2 production (Fig. 1). Production was maximal with hsp at all times in the study and was minimal with porins. However, the IL-2 induction by OMPs was less than that by hsp-65 at all times except on the second day after infection when it was similar. Porins did not mount a significant release of IL-2 on the second day after infection but from the fifth day onwards the level of IL-2 secretion was significantly raised (*p* < 0.05). However, in the later stages of the experiment little difference from the control was observed.

**Fig. 1.** IL-2 secretion with 50 µg of different antigens during acute infection: ○, HSPs; ▲, OMPs; □, PHA; ●, porins.

### IFN-γ assay

Table 3 shows the results of the IFN-γ assay with different concentrations of hsp-65.

With 10 and 20 µg of hsp, IFN-γ secretion was significantly raised only from the fifth day onwards (*p* < 0.05). However, with 50 µg of hsp a significant increase in IFN-γ production was seen even on the second day after infection (*p* < 0.05); production continued to increase significantly (*p* < 0.01) from the fifth day onwards.

Of all the antigens used to stimulate IFN-γ secretion from splenic lymphocytes, hsp showed the maximum potential at all times studied and porins exhibited the

**Table 3.** IFN-γ assay with different concentrations of hsp-65

Time (days)	Mean (SEM) IFN-γ production (units/ml)		
	10 µg hsp	20 µg hsp	50 µg hsp
2	42.3(3.2)	126.3(8.5)	*380.8(25.5)
5	*228.7(25.4)	*366.8(25.5)	†668.4(50.4)
7	*352.5(22.8)	*462.4(30.8)	†802.5(70.2)
14	*388.4(43.3)	*440.5(35.6)	†846.6(75.4)
150	ND	*450.0(28.6)	†860.0(76.2)

Data represent mean (SEM) of six different experiments; ND, not done. Control versus 2 or 5 or 7 or 14: \**p* < 0.05, †*p* < 0.01.

minimum stimulation (Fig. 2). However, when IFN- $\gamma$  secretion induced by OMPs was compared with that by hsp, similar amounts were secreted on the second day after infection but on the fifth day after infection the level of IFN- $\gamma$  secreted after induction by hsp was significantly higher ( $p < 0.05$ ). The response with PHA was similar to the one observed with porins. With PHA, on the second day after infection, low levels of IFN- $\gamma$  secretion were observed ( $p > 0.05$ ), but on the fifth, seventh and 14th days the levels showed a gradual increase that was significantly higher than the controls ( $p < 0.05$ ).

In the chronic stage of pyelonephritis, when animals were killed on the 150th day, the greatest response for IL-2 and IFN- $\gamma$  production by splenic lymphocytes was found with 50  $\mu\text{g}$  of hsp, as was seen in the acute stage of the infection (Fig. 3). At this dose of hsp comparison was made with other antigens of *E. coli*, i.e., OMPs and porins, and PHA. OMPs and hsp were able to stimulate splenic lymphocytes in the chronic stage to induce significant secretion of IL-2 and IFN- $\gamma$ , although secretion of both these interleukins was greater when cells were stimulated with hsp *in vitro*. In the case of IL-2, secretion was significantly greater when splenic lymphocytes were stimulated with hsp than with OMPs. However, the difference was not significant in the case of IFN- $\gamma$  secretion. The other two antigens, i.e., porins of *E. coli* and the non-specific antigen PHA, did not stimulate splenic lymphocytes *in vitro* so as to produce significant amounts of IL-2 or IFN- $\gamma$ .

## Discussion

In the present study the association of hsp-65 and other bacterial antigens with inflammation was studied through their potential to release various cytokines from splenic lymphocytes.

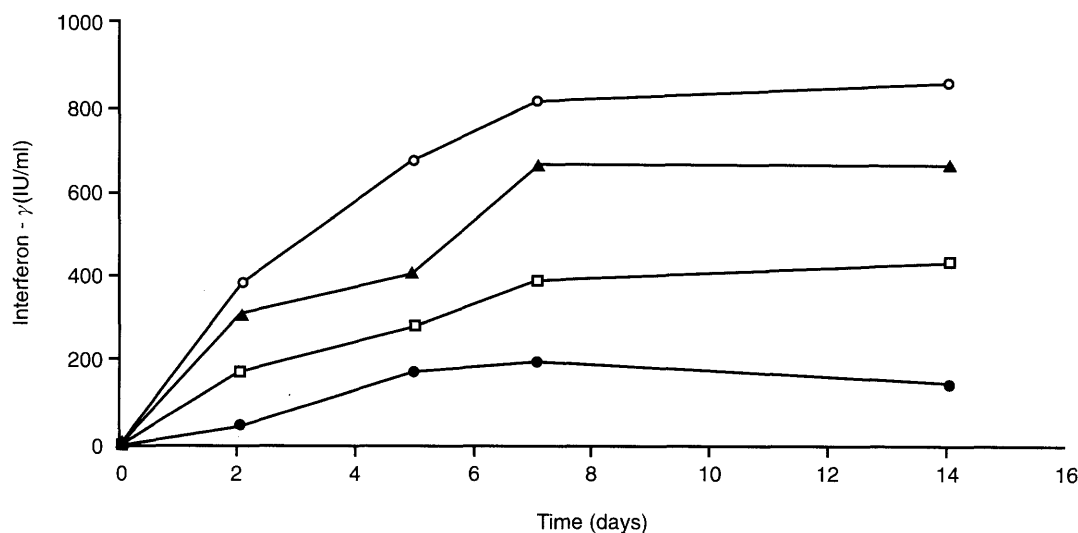


Fig. 2. IFN- $\gamma$  secretion with 50  $\mu\text{g}$  of different antigens during acute infection: ○, HSPs; ▲, OMPs; □, PHA; ●, porins.

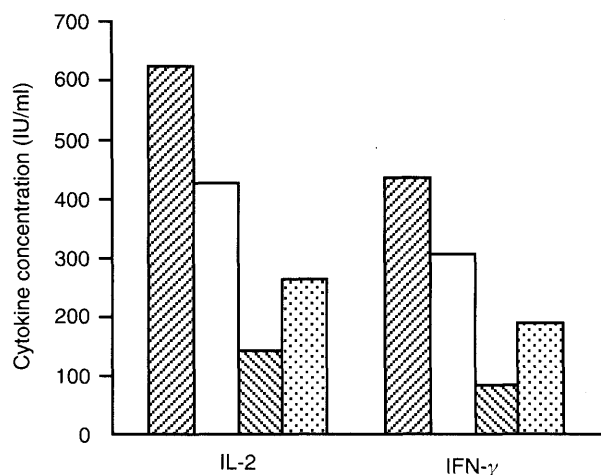


Fig. 3. Interleukin assay with different antigens observed on 150th day: ▨, HSPs; □, OMPs; ▩, porins; ▤, PHA.

Whenever a bacterium enters a mammalian host, it will be exposed to various stress stimuli generated by the host tissues. The ability of the infecting bacteria to survive such an exposure may be a deciding factor in the infection.

Experimental pyelonephritis in rodents is characterised by the presence of numerous neutrophils in the interstitial infiltrate; in the acute phase and in the later stages numerous mononuclear cells are seen [12]; on morphological grounds most of these are lymphocytes. These lymphocytes were found to secrete cytokines when stimulated *in vitro* by different specific and non-specific antigens.

Hightower and White [13] were the first to relate hsp specifically to inflammation. Although the main mediators of the immune response are mononuclear cells, their precise role in a disease process such as pyelonephritis has yet to be fully elucidated [14–17].

Numerous studies have described the presence of cytokines such as IL-1, IL-2, IL-6, IL-4 and IFN- $\gamma$  in overwhelming bacterial systemic and central nervous system infections [18–20]. A recent study has shown antibodies to TNF- $\alpha$  reduce the levels of IL-1 and IL-6 during lethal bacteraemia, suggesting that TNF- $\alpha$  is an essential stimulus for the release of these inflammatory cytokines during septic shock [21]. However, in contrast, complete blocking of the TNF- $\alpha$  response did not alter the markedly elevated levels of IL-6 in the serum and spleen, suggesting that IL-6 production was independent of TNF- $\alpha$  in this infection model [22].

In contrast to bacterial systemic and central nervous system infections, very limited information is available concerning the role of cytokines in localised bacterial infections that are usually not life-threatening and are often cleared spontaneously, including both the acute and chronic stages of pyelonephritis [23, 24].

The role of different cytokines in a BALB/c mouse model of *E. coli* pyelonephritis has been studied [2]. Kishimoto and Harano have studied the presence of mRNA for specific cytokines such as IL-1, IL-6, TNF- $\alpha$ , G-CSF, GM-CSF and CSIF/IL-10. A generalised positive response was observed for 3 days after bacterial challenge, but from the fifth day onwards the pattern of cytokine expression showed that only the localised renal response remained. The source of IL-1 has been shown to be predominantly mesangial cells [25]. Although local cytokine response in renal tissue was not investigated in the present study, it has been observed that both specific antigens such as OMP, porins and hsp-65, as well as the non-specific mitogen PHA, are able to stimulate cytokine production by T-cells of spleens from infected mice. The TH1 pattern was observed in this study, as IL-2 and IFN- $\gamma$  were secreted but IL-4 was not. Furthermore, the level of IL-2 and IFN- $\gamma$  secreted was observed to be time dependent, peak values being reached on the seventh day after infection. These results differ from those of Rugo *et al.* [1] who did not observe IL-2 secretion during acute experimental pyelonephritis in mice. Because these authors did not stimulate the cells *in vitro*, the difference in observation could be due to the basic difference in technique. The results found in the present investigation are in agreement with those of Huygen *et al.* [26], who stimulated spleen cells of BCG-infected mice with different antigens *in vitro*. This test system is comparable to the one employed in the present study. Those authors compared three strains of mice and found that 65-kDa hsp induced higher IL-2 and IFN- $\gamma$  titres in BALB/c mice than in BALB B10 and B6 strains. BALB/c mice were employed in the present study. The data do extend the findings of George *et al.* [27], who observed the production of IL-2 and IFN- $\gamma$  by T-cells from normal healthy individuals stimulated with *Mycobacterium tuberculosis* or group A streptococci.

In the present study splenic lymphocytes and peritoneal macrophages of experimental animals were stimulated with the antigens employed. Hsp-65 was found to be the best stimulant for production of IL-2 and IFN- $\gamma$  when studied *in vitro*. Release of TNF- $\alpha$ , IL-1 and IL-6 as well as IFN- $\gamma$  and IL-4 by human monocytes and lymphocytes, respectively, in response to *Salmonella* serotype Typhimurium antigens (porins) has been reported [28]. The present study corroborates the hypothesis of these workers who proposed that a similar cytokine response would be found with porins of *E. coli in vitro*.

The results of the present study and the previous studies clearly show that hsp expression has the potential to participate in the immune phenomena following infection.

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