Hsp-27, hsp-70 and hsp-90 expression and apoptosis in macrophages during ectromelia (mousepox) virus infection

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Abstract

Viruses remain one of the inducers of the stress response in the infected cells. Heat shock response induced by Moscow strain of ectromelia (mousepox) virus (ECTV-MOS) was studied in vitro in mouse monocyte cell line, RAW 264.7 and in vivo in BALB/c (H-2^d) mice. In our studies we found that ECTV-MOS-infected RAW 264.7 cells up-regulated hsp-70 and hsp-90 expression during the phase of intensive virus replication in vitro. In spleen, lymph nodes (DLN) and liver of BALB/c mice inoculated with ECTV-MOS, virus replication was not accompanied by apoptosis of macrophages identified as CD11b$^+$ cells and high loads of CD11b$^+$/ECTV-MOS$^+$ cells could be detected. Intensive virus replication in the spleen and lymph nodes was accompanied with elevated expression of hsp-27, hsp-70, and hsp-90. Particularly, CD11b$^+$ cells showed up-regulation of hsp-27 (spleen, lymph nodes) and hsp-70 (spleen) at the peak of virus replication and up-regulation of hsp-90 during later stages of infection (15 and 20 day post infection). We conclude that during ECTV-MOS infection in vivo, hsps expressed by CD11b$^+$ cells may play a dual role as anti-apoptotic factors fostering virus replication and as regulators of anti-viral response.

Key words: *mousepox (ectromelia) virus, heat shock proteins, apoptosis.*

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Introduction

Unlike eukaryotic and prokaryotic organisms, viruses are unable to replicate independently and evolved various strategies to force the cells to become virus-producing factories. A basic mechanism used by the cells to escape virus control is apoptosis. Apoptosis is a tightly regulated, irreversible process that results in cell death in absence of inflammation. Cell death limits virus replication and prevents/reduces the infection of neighboring uninfected cells [1]. Viruses have evolved many strategies to modulate apoptosis pathways in order to insure their survival [2].

Heat shock proteins (hsps), are a newly emerging family of apoptosis regulators. They are highly conserved proteins which are involved in protein folding and activation as well as in alteration of protein conformation, or directing damaged proteins to degradation by the proteasome complex [3]. Because hsps interact with proteasome, they can function in antigen presentation and stimulation of the immune response [4, 5]. Hsps bind the antigenic peptides and facilitate presentation on MHC class I and II. In addition, hsps also function as cytokines capable of stimulating antigen--independent immunity [6]. Modulation of apoptosis by hsps is carried out by chaperone activity, inhibiting processing of pro-caspases into active form of proteases [7], preventing caspase recruitment to apoptosome [8, 9] or by inhibition of pro-apoptotic signaling mediators release from mitochondria [10]. Recent data indicate that hsps can also function by chaperone-independent protein-protein interaction. Hsp-70 inhibits JNK and AIF independent of ATPase activity [9, 11].

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By virtue of the ability of hsps to influence cell life and death decision intracellularly and in the context of the immune response, hsps are a logical target for virus modulation.

Studies on HTLV-1 (human T-lymphotropic virus type-1) [12] demonstrated that hsp binds to particular virus proteins and favors clustering, stabilization and facilitates transportation to various compartments of the cytosol. It has been shown that infection of human monocytes with vaccinia virus (VACV), increases the production of viral protein-associated hsp-70, suggesting that hsp may participate in viral clustering [13]. Further evidence is provided by studies showing that during VACV replication in RK13 cells hsp-90 forms complexes with 4a protein of the viral core [14]. The induction of hsps expression is also observed during replication of adenoviruses, cytomegaloviruses, and herpesviruses (HHV-1, human herpesvirus type 1) [15, 16]. Very few viruses have been shown to encode homologues of hsp family members. Herpes simplex virus type 2 (HSV-2) encodes two proteins homologous to hsp family members. One of these, ICP10PK is a homologue to a newly cloned H11 and modulates virus-induced apoptosis, influencing bcl-2, hsp-70 and hsp-27 expressions [17].

Ectromelia virus (ECTV; family *Poxviridae*, genus *Orthopoxvirus*), as a natural pathogen of laboratory mice causing a generalised mousepox ("smallpox of mice") with a high mortality rate and pox skin lesions, has been used as a model for studies of pathogenesis and cellular immune response during generalised viral infection [18-20]. Poxviruses, including ECTV, have been shown to encode many gene products that are able to suppress apoptosis such as inhibitors of cytokine processing and proteolytic activation of caspases, soluble receptors that neutralise various cytokines, factors that inactivate interferon (IFN)-inducible antiviral enzyme activities and analogues of growth factors and hormones [18], and serpins SPI-1, SPI-2 and SPI-3 [18, 21-25]. In our previous paper [26], we have shown that the virulent Moscow strain of *Ectromelia virus* (ECTV-MOS) infection of liver, spleen, conjunctivae and DLN leads to apoptosis and involves activation of caspase-3 and -8 during the effector phase of cell death. We have also shown that ECTV-MOS causes a persistent infection of dendritic cells (DC) and macrophages of spleen, lasting up to 120 day (d.p.i.). Moreover, we investigated the role of hsp-27, -70 and -90 in apoptosis of ECTV-MOS infected monocytes/macrophages in the BALB/c mouse spleen, DLN and liver at the peak of ECTV-MOS infection. The biological implications of these findings are discussed.

Materials and Methods

Virus and cells

Standard procedures were used for the preparation and maintenance of Vero cells (ATTC CCL-81) and RAW 264.7 cells (ATCC TIB-71). The macrophage cell line RAW 264.7 was cultured in RPMI-1640 supplemented with 10% foetal bovine serum (FBS, Invitrogen, Inc.), antibiotics, L-glutamine (Sigma-Aldrich), whereas Vero cells were cultured in MEM supplemented as stated above. The cells were cultured for 24 hours before each experiment. A highly infectious ECTV-MOS strain (ATCC VR-1374) was used throughout all experiments. The virus stock was prepared in BALB/c mouse spleen and titrated ($TCID_{50}$/ml and PFU/ml) in Vero cell line. The virus stock was 5 x 10^7 PFU ml^{-1}. The cells were infected at multiplicity of infection (m.o.i.) = 5. The virus stock prepared in Vero cells was 4 x 10^7 PFU/ml. For control experiments, UV-treated ECTV-MOS stock was used prepared as described [26].

Mice and virus infection

BALB/c mice (H-2^d), genetically susceptible to ECTV--MOS infection, aged 4-6 weeks and of both sexes were purchased from the Centre of Experimental Medicine in Warsaw. All experiments were carried out according to the Warsaw University of Life Sciences-SGGW guidelines on the use and care of laboratory animals.

Groups of 8-9 mice/1 group were infected *via* footpad with ECTV-MOS stock in sterile PBS (0.0005 LD_{50}/mouse, 1 LD_{50} = $10^{3.70}$/ml). Mock infected (only PBS) BALB/c mice were used as the negative control. The BALB/c mice were observed daily during 20 d.p.i. to detect acute form of mousepox or clinical signs of chronic ectromelia (dermal mousepox lesions and conjunctivitis/blepharitis between 10-20 d.p.i. was observed). All experiments were done in triplicates.

Specimen collection

At 5, 10, 15 and 20 d.p.i. the BALB/c mice were sacrificed by cervical dislocation and spleen, DLN and liver were collected. Small fragments of all organs were immediately frozen in liquid nitrogen and further stored at -80°C for sectioning. About 100 mg samples of all organs were quickly homogenized in 1 ml of TRIZOL (Invitrogen, Inc.) and after 5 min. of incubation at room temperature, were stored in -20°C for further RNA isolation. The remaining tissue was used for the preparation of single cell suspensions, as described in our previous papers [19, 27, 28].

Detection of ECTV-MOS antigens by direct immunofluorescence

In short, sections (5 μm thick) were prepared from frozen tissue and dried at room temperature then fixed in cold methanol for 10 min. Following extensive washing in water, FITC-polyclonal rabbit anti-mouse antibody against ECTV-MOS [16, 17, 27] was applied to the sections at a dilution of 1:16 and incubated for 1h at room temperature. Next, sections were washed in water at least 3 times and finally counterstained with Hoechst 33342 (1 μl/ml; Sigma) to expose the nuclei. Stained tissue sections were examined

in a fluorescence microscope (Olympus BX-60 supplied with MicroImage system).

Flow cytometry analysis

Hsp-90, -70 and -27 expression patterns in ECTV-MOS--infected RAW 264.7 cell line as well as in spleen, DLN and liver isolated from ECTV-MOS-infected BALB/c mice were also evaluated by flow cytometry. For these experiments the same set of mAb as for immunohistochemistry was used and the positive cells were detected with a secondary FITC-labeled anti-mouse IgG using BD Cytofix/Cytoperm™ kit. Negative control staining was carried out using mouse IgG1 isotype control antibody. Cells were analyzed in FACScan using CellQuest programme (BD Biosciences) by comparing mean fluorescence intensity (MFI). Phenotyping of hsp-expressing cells was done by staining with PerCP-conjugated, anti-CD11b mAb clone M1/70 (Becton Dickinson) and appropriate anti-hsp mAb described above. Data were evaluated by flow cytometry as described above.

Apoptosis detection

Apoptotic cells were detected using 7-aminoactinomycin D (7-AAD), which is excluded by viable cells but can penetrate cell membranes of dying or dead cells [29]. In short, the cells were stained for single color staining with PerCP--labelled anti-CD11b mAb, as described above. After last washing the cells were resuspended in 1 ml of PBS with 2% FBS and 1 μg of 7-AAD (1 mg/ml in PBS stock) was added to each sample. After 20 min. of incubation in dark, the cells were washed with PBS and resuspended in 1 ml 1% formaldehyde with 2-5 μg/ml of actinomycin D. The cells were next analysed in FACScan as described previously.

Statistical analysis

Data in the text are expressed as mean ± SEM. Differences in mean values were analyzed using Student T-test. All $p \leq 0.05$ values are considered as significant.

Results

Apoptosis in ECTV-MOS-infected RAW 264.7 cells

Cells undergoing apoptosis are characteristic for many morphological and biochemical changes such as decrease in mitochondrial potential, changes in membrane asymmetry, cell shrinkage and membrane blebbing. 7-aminoactinomycin D (7-AAD) staining enables to discriminate live, apoptotic and dead cells by FACS. It intercalates between DNA bases in cells, which lost cell membrane integrity and can be usually defined as necrotic or late apoptotic (7AAD-bright staining), whereas in apoptotic cells 7-AAD interacts with membranes changed during early apoptotic events (7AAD-dim staining) (Schmid et al., 2001). In our study 7-AAD staining correlated well with annexinV/propidium iodide staining and carboxyfluorescein-6/propidium iodide staining (results not shown) and was easily used for multicolor staining.

Flow cytometric analysis showed the significant increase of apoptotic cells in ECTV-MOS infected RAW 264.7 cells at 12 h.p.i. and 18 h.p.i. ($p=0.05$) followed by its decrease at 24 h.p.i. (Table 1A), whereas cells infected with UV-inactivated ECTV-MOS virions showed no apparent induction of apoptosis (Table 1A). These results are consistent with our previous results showing that no apoptosis can be detected until first virus progeny is released from the infected cells [26].

Table 1. Flow cytometry analysis of apoptosis and hsp-27, -70, -90 expression in ECTV-MOS-infected RAW 264.7 cells

A. Apoptosis (%)					
	control	**6 h.p.i.**	**12 h.p.i.**	**18 h.p.i.**	**24 h.p.i.**
Cells infected with UV-inactivated ECTV-MOS	0.43±0.022	11.47±1.2	12.34±1.4	14.23±2.67	13.45±2.12
ECTV-MOS infected cells	0	14.28±2.01	25.46±.3.41	35.78±4.56	10.56±2.34
B. Mean fluorescence intensity (MFI) of hsps staining					
	control	**6 h.p.i.**	**12 h.p.i.**	**18 h.p.i.**	**24 h.p.i.**
hsp 27	103.43±3.22	135.9±4.97	159.04±3.91	132.91±7.27	140±4.69
hsp 70	51.64±4.56	71.56±14.78	61.14±4.52	50.35±7.59	55.73±7
hsp 90	58.42±8.73	34.15±1.48	66.44±3.38	51.26±8.98	46.95±2.49

A. Percentage of apoptosis in ECTV-MOS-infected monocyte RAW 264.7 cells. The cells were infected with 5 p.f.u./cell of ECTV-MOS and UV-inactivated ECTV-MOS and grown in 24-well plates. At the indicated times after infection, cells were collected, stained with 7-AAD and analyzed by flow cytometry. The table represents the percentage of 7-AAD dim staining (apoptotic) population at the indicated times after infection. Data represent means of three experiments ± standard errors of mean (SEM).

B. Hsp 27, 70 and 90 expression in ECTV-MOS-infected RAW 264.7 cells. The cells were infected with ECTV-MOS and grown in 24-well plates. At the indicated times after infection, A – cells were collected, stained with anti-hsp27 or anti-hsp70 or anti-hsp90 monoclonal antibodies and analyzed by flow cytometry. The table represents mean fluorescence intensity (MFI) calculated as the mean of three experiments ± SEM. The data represent means from 3 separate experiments.

Hsp-27, -70 and -90 expression in ECTV-MOS-infected RAW 264.7 cells

Flow cytometric analysis of hsp 27 expression in ECTV--MOS-infected RAW 264.7 cells showed that ECTV-MOS infection significantly increased hsp-70 and hsp-90 expression already at 6 h.p.i. (p=0.05) and expression of hsp-70 (p=0.05) remained significantly elevated at 12 h.p.i., and hsp-90 at 12, 18 and 24 h.p.i. (Table 1B). On the contrary, hsp-27 also increased but it remained very low throughout the tested period (Table 1B). Real-time PCR analysis for hsp-27 and -70 expression at transcription level confirmed results obtained for flow cytometic analysis (Table 1B). Generally, the levels of hsp-70 and -90 expression were higher then the levels observed for hsp 27.

Clinical signs of mousepox

In a rapid (5-7 d.p.i), fatal form of ectromelia BALB/c mice died from acute viral hepatitis within several hours after the onset of disease – dead mice exhibited enlarged inguinal DLN and extensive irregular foci in spleen and liver, similarly as described earlier [26, 27]. In non-rapid form of mousepox BALB/c mice infected by the f.p. inoculation of ECTV-MOS developed between 10–15 d.p.i. ulcerating skin lesions in form of vesicles filled with a serous fluid and crusts on the back skin, tail, tail base, ear lobes, paws, swelling of paws and snout, and well as blepharitis and conjunctivitis with lacrimation. In some serious cases amputation of the tail was observed. No mousepox was observed when PBS or UV-inactivated ECTV-MOS was injected to the BALB/c mice.

Detection of ECTV-MOS antigens and ECTV-MOS-infected CD11b$^+$ cells

ECTV-MOS antigens were present in spleen, DLN and liver isolated from ECTV-MOS infected BALB/c mice. The fluorescence intensity and the number of ECTV-MOS$^+$ cells decreased in the course of infection and reached background staining levels by 20 d.p.i. as shown in previous papers [19, 26]. Localization of ECTV-MOS/CD11b$^+$ cells generally correlated with localization of ECTV-MOS antigen(s) at day 5 and 10, therefore at the beginning of infection, confirming the role of macrophages in virus spread. Later in the course of infection ECTV/MOS/CD11b$^+$ cells were detected in the areas of massive apoptosis and necrosis, but also in neighboring areas. More precisely, ECTV/MOS/CD11b$^+$ cells were detected in lymph nodes at 5 d.p.i. within lymph follicles and consisted about 40% of CD11b$^+$ cells detected in DLN. At 10 and 15 d.p.i., ECTV-MOS/CD11b$^+$ cells were still detected within lymph follicles but their numbers decreased to 10 and 6.25% of CD11b$^+$ cells, respectively. In spleen, ECTV-MOS/CD11b$^+$ cells were detected within germinal centers of lymph follicles at 5 and 10 d.p.i., consisting about 5 and 20% of CD11b$^+$ cells, respectively. ECTV-MOS/CD11b$^+$ cells were also observed in the areas of intensive apoptosis and necrosis, starting at day 10. At day 20, ECTV-MOS/CD11b$^+$ cells were detected at about 8% mainly within the areas surrounding the remaining foci of infection. Similar pattern was observed in liver, where ECTV-MOS/CD11b$^+$ cells were visualized in the perivascular area including the parenchyma, at about 5% of all CD11b$^+$ cells, then in the areas of intensive granulocyte infiltration and necrosis at day 10 and 15 (about 10%). The ECTV-MOS/CD11b$^+$ cells gradually disappeared in liver parenchyma at day 20 d.p.i. (2%).

Apoptosis of CD11b$^+$ cells in the course of ECTV-MOS infection

Dual staining of CD11b$^+$ cells with 7-AAD revealed that in the DLN the percentage of apoptotic cells with CD11b phenotype increased in the course of infection from 11.02±1.89% ($p\leq0.05$) at 5 d.p.i. up to 38.09±5.76% ($p\leq0.005$) and 45±8.1% ($p\leq0.05$) at 15 and 20 d.p.i., respectively (Fig. 1A). On the other hand, the number of CD11b$^+$ cell undergoing apoptosis was significantly increased 12.34±1.26% ($p\leq0.05$) at day 5 after infection and remained at the same level at day 15 and 20 (Fig. 1A). This results show that not only CD11b$^+$ cells but also other type of cells, such as lymphocytes and DC, uderwent apoptosis (data not shown). Total percentage of CD11b$^+$ cells increased at 5 d.p.i. and remained significantly increased ($p\leq0.05$) (which probably accounted for 50% of ECTV-MOS-infected CD11b$^+$ cells). This cells were moving to liver and spleen and possibly to the final site of this poxvirus replication – to the skin [18, 20]. Indeed, the total percentage of CD11b$^+$ cells was increased in the spleen of ECTV-MOS-infected BALB/c mice at 5 and 10 d.p.i. ($p\leq0.05$) to reach the percentage observed in control mice at 15 and 20 d.p.i. (Fig. 1B). The increasing numbers of apoptotic cells reflected the decrease in the number of CD11b$^+$ cells at 10 and 15 d.p.i. – 15±0.90 and 13.85±2.19 ($p\leq0.05$), respectively (Fig. 1B). Again, the CD11b$^+$ cells were not the only type of cells undergoing apoptosis (Fig. 1B). In liver at 15 d.p.i. CD11b$^+$ cells consisted 85.71±7.8% of apoptotic cells, indicating that the CD11b$^+$ cells present at foci of infection and inflammation were eliminated [26] (Fig. 1C). The peak of CD11b$^+$ cells undergoing apoptosis was also observed at 15 d.p.i. (30.76±3.8%, $p\leq0.05$).

Quantitative analysis of hsp-90, -70 and -27 expression

Figure 2 depicts the expression of hsp profiles in DLN, spleen and liver of ECTV-MOS infected BALB/c mice measured by flow cytometry analysis. The percentage of hsp-27$^+$ cells in lymph nodes in the course of ECTV-MOS infection was significantly increased ($p\leq0.005$) already at day 5 (75.44±8.66%) when compared to control lymph nodes (29.84±2.34%) and remained high throughout the whole tested period (Fig. 2A). The percentage of hsp-70$^+$ cells in the lymph nodes was decreasing beginning from 5 d.p.i. (34.33±3.2%, $p\leq0.005$) when compared to control (90±9.6%) and remained decreased at 10 d.p.i. and 15 d.p.i. (46.5±5.6% and 68.38±7.4%, respectively) (Fig. 2A). On the contrary,

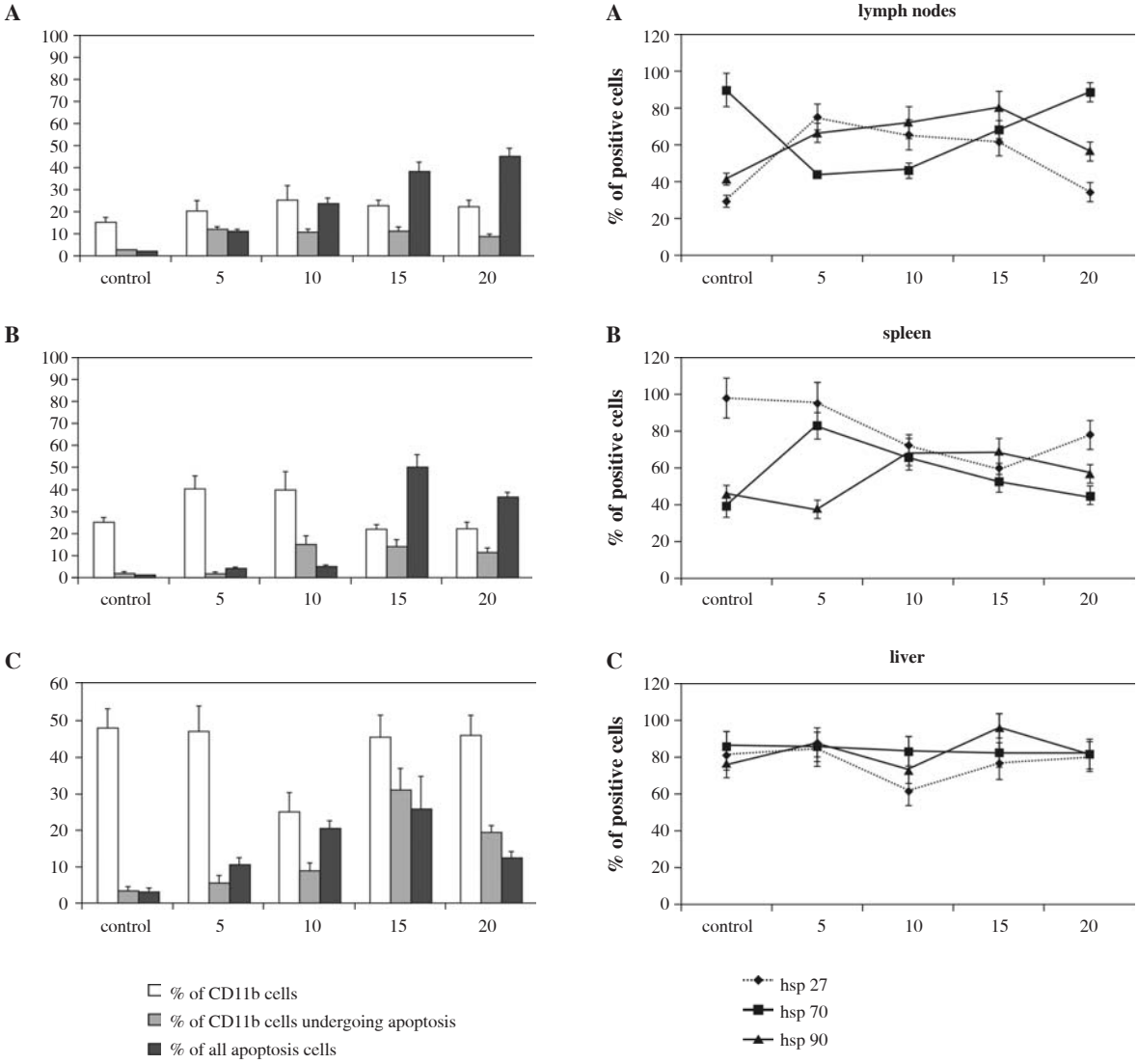

Fig. 1. Percentage of apoptotic CD11b cells detected in ECTV-MOS infected mice by double staining. Cells isolated from DLN [A], spleen [B] and liver [C] of BALB/c infected mice as well as fron non-infected controls were subjected to /-aminoactinomycin staining and anti-CD11b staining. The graph represents total number of $CD11b^+$ cells, the percentage of $CD11b^+$ cells undergoing apoptosis as well as the total number of apoptotic cells in the particular organs. Bars represent mean results obtained from 3 separate experiments ± SEM

Fig. 2. Hsp-27, -70 and -90 expression in DLN [A], spleen [B] and liver [C] of BALB/c mouse infected with ECTV-MOS as detected by flow cytometry. Results are shown as mean results obtained from 3 separate experiments ± SEM

the percentage of hsp-90^+ cells in DLN was significantly increasing during ECTV-MOS infection (Fig. 2A) to reach the peak at 15 d.p.i. (80.39±9.44%), compared to control (42±3.55%).

In the course of ECTV-MOS infection statistically significant ($p \leq 0.05$) increases in the expression of hsp-90 in spleen cells was observed between 5 and 20 dpi compared with control (46.5%) (Fig. 2B). Similarly, hsp-70 expression was 83.66±8.61% ($p \leq 0.05$) at 5 d.p.i., 66.5±7.67% at 10 d.p.i. ($p \leq 0.05$) and 52.89±3.18% ($p \leq 0.005$) at 15 d.p.i. compared with control (39.75±5.38%). At 20 d.p.i., the expression levels for hsp-70 regained numbers observed in uninfected control BALB/c mice (45.67±4.2) (Fig. 2B). The expression pattern of hsp-27 differs from hsp-90 and hsp-70. Initially (5 d.p.i.) there were no differences in expression level of hsp-27 in cells from control and infected animals. Thereafter, expression

levels transiently decreased between 10 d.p.i. (72.73±8.43%, p≤0.05) and 15 d.p.i. (60.43±4.45%; p≤0.05) but increased by 20 d.p.i. (93.77±2.04%) (Fig. 2B).

In liver cells statistically significant increase in hsp 90 expression was observed at 15 d.p.i. (96.26±9.98%; p≤0.05) and 20 d.p.i. (81.11±2.89%; p≤0.05) compared with control (77.49±7.7%). Hsp-70 expression level remained stable throughout the whole tested period (Fig. 2C). Changes of hsp-27 expression were lower but statistically significant. Decrease of its expression was observed at 10 dpi (57.58±1.63%; p≤0.005) and at 15 dpi (59.76±4.71%; p≤0.05). The data suggest that the presence of ECTV-MOS antigens induces the expression of hsp at levels that remain fairly constant during the symptomatic stage of infection. The results also indicate the dependence of hsp expression on the intensity of virus replication in the infected organ, i.e. spleen and liver.

Quantitative flow cytometry analysis of hsp-90, -70 and -27 expression in CD11b$^+$ cells

Since we wanted to test the role of hsps in establishing a persistent infection of macrophages during ECTV-MOS infection of BALB/c mice, expression of hsp-27, -70 and -90 by CD11b cells in DLN, spleen and liver was tested (Fig. 3). About 60% of CD11b cells expressed hsp-27. However, the percentage of CD11b/hsp27$^+$ cell significantly increased at 5 d.p.i. (84.37±3.53%, p≤0.05). On the contrary, the percentage of CD11b/hsp-70$^+$ significantly decreased at 5 and 10 d.p.i. to about 60% (p≤0.05) in comparison to control (96.33±4.45%); 94.34±19.1 at 15 d.p.i. and 98±4.4 at 20 d.p.i. (Fig. 3A). Hsp-90 expression in CD11b cells remained stable during all course of infection (about 80% of CD11b/hsp-70$^+$ cells) except at 15 d.p.i., when the percentage increased significantly to 94.34±19.1% (Fig. 3A). In spleen isolated from ECTV-MOS infected BALB/c mice up-regulation of hsp-27, -70 and -90 in CD11b$^+$ cells was observed at 5, 10 and 15 d.p.i. (Fig. 3B). The up-regulation was almost three fold for CD11b/hsp-27$^+$ cells (83.9±12.32, p≤0.05 at 5 d.p.i., in comparison to 29.76±2.37 in control), two fold for CD11b/hsp-90$^+$ cells (87.92±7.13, p≤0.05 at 5 d.p.i., in comparison to 43.94±6.14 in control) and from 88.94±9.28, p≤0.05 at 5 d.p.i. for CD11b/hsp70$^+$ cells to 71.81±4.43 in control.

In the course of ECTV-MOS infection in liver, the percentage of CD11b/hsp-27$^+$ cells remained at the comparable level (Fig. 3C), whereas the percentage of CD11b/hsp-70$^+$ cells decreased significantly two fold at 5 d.p.i. (from 97.39±2.36 at control to 39.4±8.58 at 5 d.p.i) and remained decreased at 10 d.p.i. (84.69±12.62). For CD11b/hsp-90$^+$ cells we could observe up-regulation at 15 and 20 d.p.i. (97.62±3.01 and 81.23±8, respectively).

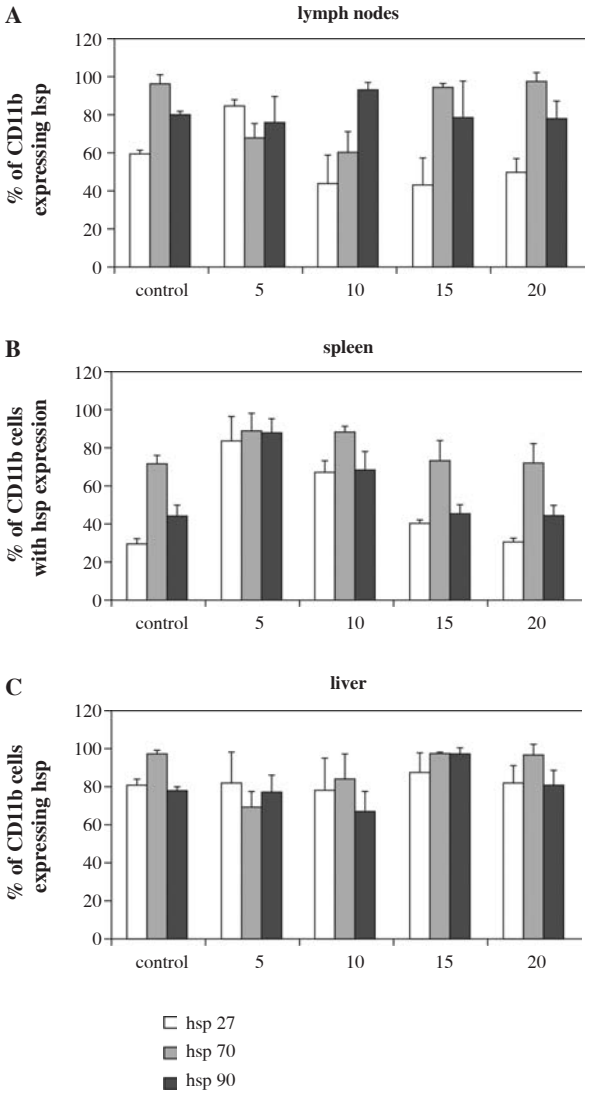

Fig. 3. Percentage of CD11b$^+$ cells expressing hsp 27, 70 and 90 in DLN [A], spleen [B] and liver [C] of BALB/c mice infected with ECTV-MOS. Bars represent mean results obtained from 3 separate experiments ± SEM

Discussion

During in vitro infection of mouse monocyte cell line, RAW 264.7, we observed hsp-70 up-regulation, while hsp-90 expression was fluctuating during the 24-hour infection and hsp 27 expression was detected at very low levels. Therefore, we can conclude that our results were in agreement with previous papers showing up-regulation of hsp-70 expression in VACV-infected monocytes [30, 31]. Up-regulation of hsp-70 was accompanied by low apoptosis levels, as shown by 7-AAD staining and this decrease in hsp-70 expression correlated with an increase in apoptosis levels. Hsp-70 has well-established anti-apoptotic activity. It has been shown to reduce Fas-associated apoptosis in U937 macrophages [32],

and it also conferred protection from other apoptotic stimuli such as UV-irradiation [33] and nitric oxide [10]. The mechanism of apoptosis modulation by hsp-70 has been shown to be related to the inhibition of pro-caspases processing into active form of proteases [7], prevention of caspase recruitment to apoptosome [8, 9] or by inhibition of pro-apoptotic signaling mediators release from mitochondria [10]. Recent data indicate that hsp-70 can also inhibit JNK and AIF independent of ATPase activity [9, 11]. The percentage of apoptotic cells during ECTV-MOS infection increased between 12 and 18 h.p.i., when first mature ECTV virions were released from the infected cells, indicating that hsp-70 expression may be the one of mechanisms by which ECTV-infected cells are rendered apoptosis-resistant. Hsp-90 expression in mouse macrophages in vitro also increased and remained high throughout all tested period, while we showed no significant up-regulation of hsp-27, which may be due to the particular properties of RAW 264.7 cell line. During VACV infection in vitro hsp-90 was shown to change intracellular distribution and transiently associate with virosomes by viral core protein 4a [14]. Therefore, we can conclude that hsp-90 can play a similar role during RAW 264.7 infection with ECTV-MOS in vitro.

CD11b (Mac-1) is a β_2 integrin expressed on macrophages but also on mast cells and natural killer (NK) cells [34] and plays an important role in elimination of infectious agents. Macrophages have been also shown to be sites of ECTV-MOS replication and to be responsible for virus spreading in vivo [18]. Our results showed that in regional lymph nodes, which are the site of primary virus multiplication, before it is released into blood during primary viraemia, about 40% of CD11b cells were ECTV-MOS-infected, while the number of apoptotic CD11b cells was only 10%, which clearly indicated that not all infected macrophages were undergoing apoptosis. Furthermore, this percentage remained constant during the whole tested period, while the percentage of ECTV-MOS-infected CD11b cells was decreasing. After the primary viraemia, ECTV-MOS replicates in spleen and liver between 5 and 10 d.p.i. During this period we detected an increase in CD11b+ cell percentage in spleen (39.6% and 39.21% at day 5 and 10, respectively, in comparison to 24.54% in control) as well as an increase in ECTV-MOS-infected CD11b cells (about 5 and 20%, respectively), while the numbers of apoptotic $CD11b^+$ cells at 5 d.p.i. were low (Fig. 1B) and increased at 10 d.p.i., which is the period of secondary viraemia. In liver, the pattern of infection was similar, with 5% of ECTV-MOS-infected cells at 5 d.p.i. We did not detected apoptotic CD11b cells before 15 d.p.i., when the peak of apoptosis in $CD11b^+$ cells was observed (Fig.1C). Therefore, we can conclude that the replication of ECTV-MOS in the particular organs was not accompanied by the increase in apoptotic CD11b+ cells and their apoptosis was related rather to infection clearance then with the peak of virus replication.

Massive virus replication in these organs may lead to such serious damage that its consequence may even be the death of a host, preventing the virus from closing the whole cycle. There is a question whether increased expression of hsps demonstrating cytoprotective activity facilitates the elimination of infected cells or does it enable the replication of viral DNA and protects it from its elimination by the host organism. It was observed while studying HTLV-1 that hsp may bind to viral proteins and favour its accumulation, also to stabilize and facilitate the transport of virus proteins between different regions of cytosol [12]. As proved, hsp participate in correct polypeptide folding, thus the induction of these proteins as a response to viral infection may be necessary during the synthesis of viral proteins and virions. It is also possible that hsp are necessary during the replicative cycle of viruses. The engagement of hsp during the replication of some viruses like HHV-1, cytomegalovirus and adenovirus was proved [12, 15, 16, 35]. During the adenoviral infection of HeLa cells, a specific viral gene E1A (responsible also for neoplastic transformation) controls the induction of hsp-72 synthesis. However, the lack of hsp induction results in virus being blocked, which suggests that hsp may be necessary to the development of viruses in the cells [12, 35]. Flow cytometry, immunohistochemistry and real-time PCR analysis of hsp-27, -70 and -90 expression allowed to draw a conclusion that in the lymph nodes and spleen, where intensive ECTV-MOS replication took place and inflammatory infiltration occurred, the induction of hsp expression was observed. On the contrary, in liver, where intensive virus replication also occurs, hsps up-regulation was observed late during infection, namely at 10 and 15 d.p.i., although hsp-27, -70 and -90 up-regulation was observed in sites of inflammation at 5, 10 and 20 d.p.i. This may be attributed to the fact that results from RT-PCR and flow cytometry analysis of total expression may differ from the picture observed in immunohistochemistry due to size proportion between the whole organ and inflammation sites. The obtained results point to the dependence of the induction of hsp expression on the increase of virus replication in the infected organ, because that is where the virus replicates most intensively i.e. in spleen and liver the highest levels of hsp-90, hsp-70 and hsp-27 were observed.

In order to elucidate the role of hsps in functioning of macrophages during ECTV-MOS infection, the profile of $CD11b^+$ cells which demonstrate expression of hsp-90, hsp-70 and hsp-27 in spleen and liver was studied. The phenotyping of hsp^+ cells in lymph nodes showed that at 5 d.p.i. an increase in $hsp\text{-}27^+CD11b^+$ cells percentage, together with a decrease in $hsp\text{-}70^+CD11b^+$ cells. Up-regulation of $hsp\text{-}27^+/CD11b^+$ cells was also detected in spleen at 5 d.p.i. (Fig. 3B). Hsp-27 belongs to the small hsp subfamily and does not have ATPase activity. Hsp-27 expression has been shown to suppress apoptosis by inhibiting procaspase-3 processing, and apoptosome formation [36]. It also restores overall structural integrity of the cell by binding and stabilizing F-actin microfilaments [37]. We may therefore conclude that up-regulation of hsp27 in CD11b cells may be related to its

anti-apoptotic role in fostering virus replication. Hsp-70 and hsp-90 up-regulated expression was observed in spleen during the phase of clinical symptoms of the disease (between 5 and 15 d.p.i.) and in liver at the later stages of infection (10 and 15 d.p.i., Fig. 3). This indicates that hsp-70 and -90 may play a different role during replication of ECTV-MOS in liver and spleen, which is dictated by specific environment in both organs. In spleen up-regulation is more related to fostering virus replication, while in liver hsp-70 and -90 may play a role in virus clearance. It is well known that massive necrosis of liver resulting from intensive virus replication leads to early death of ECTV-MOS infected mice [18]. Therefore, an increased expression of hsp-90 and hsp-70 in $CD11b^+$ cells isolated from spleen and liver of BALB/c mice in the phase of clinical symptoms of disease may demonstrate the role of these proteins in ECTV-MOS antigen presentation to the effector cells (i.e. virus-specific $CD8^+$ CTL) of the immune system. Hsp foster the cytotoxic response of T lymphocytes in relation to the proteins and peptides with which they form complexes [3, 4]. Cespedes et al. [19] showed that the increase in $CD11b^+$ cells in spleen correlated with higher percentage of ECTV-MOS-specific $CD8^+$ T cells in spleen and DLN at peak of infection.

Persistence of professional immune cells harboring intracellular pathogen in circulation, spleen and DLN seems detrimental for immunity for two reasons: first, the immune response is deregulated by cytokines and impaired antigen presentation; second the cells were proposed to serve as virus incubators. Cell of monocytic lineage have recently been recognized as a crucial model to study virus-host interactions due to unique capability of these cells to cross-present endocytosed antigens, especially in the context of chaperone proteins. Further understanding of the heat shock response during ECTV infection may help to understand the strategies used by viruses to replicate and spread in their respective hosts.

In conclusion, we would like to stress the role of hsps expressed by $CD11b^+$ cells during ECTV-MOS infection in vivo. We think that hsps play a dual role as anti-apoptotic factors fostering virus replication and as regulators of anti-viral response.

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