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Comparison of Protective Effect of Protein and DNA Vaccines hsp90 in Murine Model of Systemic Candidiasis

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ABSTRACT. Preventive vaccination by a hsp90-expressing DNA vaccine and recombinant hsp90 protein vaccine, both derived from the *Candida albicans* hsp90 using BALB-c mouse model of systemic candidiasis, was performed. Hsp90 mRNA was cloned from a clinical isolate of *C. albicans*, converted to cDNA and cloned into vaccination plasmid pVAX1. Two methods of DNA application were tested: intramuscular (i.m.) and intradermal (i.d.) injection. Recombinant protein was applied by i.d. injection with Freund's adjuvant; the control groups received PBS or Freund's adjuvant only. Mice were vaccinated and after 19 d re-vaccinated. After 3 weeks, the mice were challenged with the live *C. albicans* in a dose of 5×10^6 CFU per mouse. After the challenge, the mice vaccinated i.d. with DNA vaccine survived for 39 and 64 % longer compared to those receiving Freund's adjuvant and/or PBS, respectively. The i.m. application of the DNA vaccine did not provide any significant protectivity. The serum level of anti-candida-hsp90 serum IgG antibodies correlated with the survival rate in both i.d. protein and DNA vaccination approaches. We stressed the importance of specific humoral immunity in the mouse model of systemic candidiasis.

Candida albicans and other *Candida* species are opportunistic yeast that cause both mucosal and systemic candidiasis. Mucosal candidiasis is a frequent manifestation of deficiencies of the phagocytes and T cells (Romani 2000). In contrast, disseminated candidiasis appears in individuals with leukopenia or pancytopenia, often induced by the aggressive chemotherapy for malignant diseases or transplantation (Dorko *et al*. 2002; Tomšíková 2002).

Effective anti-candida host defense is coupled with induction of a specific T_H1 immune response associated with stimulation of the effector phagocytic cells, a mechanism essential in both systemic and disseminated forms of candidiasis (Romani 2000). Lately, the resistance to systemic candidiasis was associated with some *Candida*-specific antibodies. Several candidate antigens were used in experimental vaccination: (*a*) surface mannan polysaccharides, tested either in the form of phospholipomannas (identified 1,2-β-D-mannotriose epitope showing adhesin activity; Poulain *et al.* 2002) or in mannan–protein conjugates (Han *et al.* 1999; Bystricky *et al*. 2003), (*b*) the secretory aspartyl proteinase, one of the virulence factors (De Bernadis *et al*. 2001), (*c*) yeast killer-toxin receptor recognized by the toxin *Pichia anomala* inhibiting in the yeast the synthesis of β-D-glucans (Cassone *et al*. 1997), and (*d*) a 47-kDa fragment of the heat shock protein 90 kDa (hsp90). In the disseminated candidiasis, antibodies against the 47-kDa fragment were protective in the animal. These antibodies were induced by vaccination with the recombinant protein hsp90 or its fragments. Protectivity of anti-hsp90 antibodies was confirmed by the passive immunization of the animals (Matthews *et al*. 1995; Burnie and Matthews 2004).

Here we verified the protective effect of the preventive vaccination by DNA vaccine that expresses the hsp90 protein of *C. albicans* and compared its effect with a protein vaccine containing recombinant hsp90 protein.

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MATERIALS AND METHODS

Isolation of hsp90 DNA and protein vaccines. Chemicals (except where noted) were obtained from *Sigma* (USA). Total RNA (tRNA) was prepared from the clinical isolate of *C. albicans* (*Department of microbiology, Faculty of Medicine, Palacký University*, Olomouc, Czechia). The yeasts were cultivated for 2 d at 32 ºC in Sabouraud-glucose broth (*Imuna*, Slovakia). tRNA was isolated by modification of acid guanidine thiocyanate–phenol–chloroform extraction (Chomczynski and Sacchi 1987). Synthesis of the first strand was driven by SuperScript II reverse transcriptase using oligo(dT)$_{15}$ primer (*Invitrogen*, The Netherlands). The second strand synthesis was performed by DNA polymerase I with the aid of RNAase H according to the manufacturer's protocol (Universal RiboClone cDNA Synthesis System Manual; *Promega*, USA). The hsp90 cDNA was amplified using PCR in two separate reactions allowing cloning of hsp90 cDNA into the prokaryotic expression plasmid pET28b (*Novagen*, USA) and into the mammalian vaccination plasmid pVAX1 (*Invitrogen*). The used primers were synthesized by *Generi-Biotech* (Czechia). The sequences are shown in Table I. Both PCR products were separated on 1 % Tris–EDTA–acetate (TEA) agarose gel, DNA bands with expected length ≈2140 bp were purified (*Qiagen*, Germany), amplified by using the helper cloning plasmid pCRII blunt TOPO (*Invitrogen*) and cloned in plasmid pET28b by restriction sites *Sal*I, *Xho*I (recombinant plasmid pET28–hsp90–CA) and in pVAX1 plasmid by restriction sites *Not*I, *Xho*I (recombinant plasmid pVAX1–hsp90–CA) using T4 DNA ligase (*Invitrogen*) using standard molecular biology approaches (Sambrook *et al*. 1989). PCR reactions were done using Pfu polymerase in a 100 µL volume according to manufacturer's protocol (*Promega*): initial denaturation 94 ºC, 2 min, 35 cycles (denaturation 94 ºC, 30 s; annealing 55 ºC, 30 s; extension 68 ºC, 10 min) and final elongation 72 °C for 10 min. Both DNA inserts were identified by sequencing (*OriGene*, Czechia).

Table I. The sequence of primers for the PCR amplification of inserts *hsp90* for cloning into pET28b and pVAX1

hsp90 cDNA[a]	Primer	Sequence[b]
pET28b	downstream	5′-CGT CGA CAT GGC TGA CGC AAA AGT T-3′
pET28b	upstream	5′-CTC GAG ATC AAC TTC TTC CAT AGC-3′
pVAX1	downstream	5′-GCG GCC GCA CCA TGG CTG ACG CAA AAG TT-3′
pVAX1	upstream	5′-CTC GAG TTA ATC AAC TTC TTC CAT AGC-3′

[a]Primers for cloning of *hsp90* into pET28b and into pVAX1 vectors.
[b]Sites recognized by restriction endonucleases *Sal*I, *Xho*I, *Not*I and *Xho*I were added to 5′ ends of the primers (underlined); the downstream primer for *hsp90*–pVAX1 attaches the Kozak sequence (double underlined) to the 5′ end in the open reading frame of the cDNA and upstream primer attaches stop codon TAA.

For preparation of the protein vaccine and for the specific antibody immunodetection, the recombinant protein hsp90 (r–hsp90–CA) was expressed in *E. coli* BL21(DE3) from recombinant plasmid pET28b carrying *Candida* hsp90 cDNA under the control of the T7 promoter and *lac* operator. The protein was expressed as a fusion protein containing T7 and His tag peptides and purified under native conditions using NiNTA agarose column (*Qiagen*). The eluted fractions of recombinant protein were analyzed using anti-T7-tag-mAb-HRP (*Novagen*), anti-hsp90-mAb-HRP (*Stressgen*, USA), and serum of DNA-vaccinated mice (dilution 1 : 800; secondary antibody: goat anti-mouse-IgG-Fc-specific-Ab-HRP; *Sigma*). The chemiluminescence signal (SuperSignal West Dura; *Pierce*, USA) was recorded using a CCD camera. Molar mass (*M*) of recombinant hsp-90 protein was predicted from cDNA using ProtParamTool software (**http://expasy.cbr.nrc.ca**). The concentration of the recombinant hsp90 protein was determined semiquantitatively by comparison of hsp-90 band densities with bands of standard (fructose-6-phosphate kinase, *M* 85.2 kDa; *Boehringer Mannheim*, Germany) in a serial dilution arrangement on 8 % SDS-PAGE stained by Coomassie Brilliant Blue. Densitometry of scanned gels was done using Scion-Image 4.0.2 software (**http://www.scioncorp.com**).

Recombinant vaccination plasmid pVAX1–hsp90–CA expressing hsp90 cDNA under the control of the CMV promoter was purified for vaccination purposes with the aid of EndoFree Plasmid Mega kit (*Qiagen*). Identity was confirmed by sequencing (*OriGene*). The plasmid was stored at –80 °C.

The vaccination experiment was approved by the *Ethical Committee of the Faculty of Medicine*, *Palacký University* in Olomouc. The challenge *C. albicans* strain, kindly provided by Dr. Turánek (*Research Institute of Veterinary Medicine*, Brno, Czechia), was repeatedly passed through laboratory mice BALB-c to stabilize the LD$_{50}$ value for 10^5 CFU (colony-forming unit) per mouse after intravenous administration.

Vaccination was done in two steps: priming on day zero and booster on day 19. BALB-c mice were divided into 6 groups (D, M_{lo}, M_{hi}, P, S, A; each $n = 12$). An overview of the doses along with the time scheme is presented in Tables II and III. DNA vaccine was administered either i.m. by 5 stabs to m. gluteus in a dose of 1.0 μg or 100 μg per mouse or i.d. by application to front abdomen face of skin. Protein vaccine

Table II. Time schedule of vaccination experiment

Priming	Booster	Challenge	End of experiment
Day 0 –	day 19 sample blood draw	day 41 sample blood draw	day 45 –

Table III. Vaccine composition and doses[a]

Mouse	Day	DNA, μg	Protein, μg	PBS, μL	CFA, μL	ICFA, μL	Route
D1–D12	0	1	–	149	–	–	i.d.
D1–D12	19	1	–	149	–	–	i.d.
$M_{lo}1$–$M_{lo}12$	0	1	–	149	–	–	i.m.
$M_{lo}1$–$M_{lo}12$	19	1	–	149	–	–	i.m.
$M_{hi}1$–$M_{hi}12$	0	100	–	50	–	–	i.m.
$M_{hi}1$–$M_{hi}12$	19	100	–	50	–	–	i.m.
P1–P12	0	–	75	–	80	–	i.d.
P1–P12	19	–	75	–	–	80	i.d.
S1–S12	0	–	–	150	–	–	i.d.
S1–S12	19	–	–	150	–	–	i.d.
A1–A12	0	–	–	70	80	–	i.d.
A1–A12	19	–	–	70	–	80	i.d.

[a]DNA plasmid pVAX1-hsp90-CA was dissolved (concentration of 1 mg/mL) in Tris–EDTA (TE) buffer; recombinant protein r–hsp90–CA was diluted (0.5 mg/mL) in PBS buffer; all chemicals were sterile and endotoxin-free.

(r–hsp90–CA) was applied i.d. to front abdomen face of skin with complete Freund's adjuvant (CFA) and incomplete Freund's adjuvant (ICFA). The first control S group received phosphate buffered saline (PBS) i.m. to m. gluteus. The second control A group received i.d. CFA during priming and ICFA during booster. Forty-one d after initial vaccination, 2 mice from each group were sacrificed in a Ketamine narcosis. The serum was collected to establish the level of specific IgG. Spleen, lungs, heart, kidneys, and liver were then collected. After fixation (10 % glutaraldehyde), the tissues were processed for histological evaluation of tissue changes induced by vaccination. The remaining 10 mice were challenged with a dose of 5×10^6 CFU per mouse by application of *C. albicans* suspension in sterile PBS into the left lateral tail vein. Thirty min after the challenge, candidemia was examined in 5 μL of blood taken from the right lateral tail vein, diluted in 150 μL of sterile PBS and spread on Sabouraud-glucose agar (SGA). *C. albicans* colonies were counted after 1 d.

The mice were continuously monitored and, after death, kidney were aseptically taken, cut and impressed onto SGA for monitoring of residual *C. albicans*. Liver, lung, kidney, heart and spleen were used for histological examination as above. The experiment was terminated 20 d after the challenge.

For the determination of serum anti r–hsp90–CA IgG immunoglobulins, the wells were coated with r–hsp90–CA protein (1 μg per well) in PBS. The sera were diluted 1 : 10 to 1 : 1000 in a blocking buffer (*Pierce*). As a secondary antibody, an anti-mouse-IgG-Fc-specific-Ab HRP conjugated at a dilution of 1 : 15 000 (*Sigma*) in a blocking buffer was used. The reaction was developed using a substrate buffer (*Pierce*), terminated by H_2SO_4 and absorbance A_{492} was measured.

RESULTS

Analysis of plasmids pVAX1–hsp90–CA and pET28b–hsp90–CA. Isolated hsp90 cDNA cloned into the plasmid pVAX1–hsp90–CA and recombinant pET28b were sequenced from the 5′ and 3′ ends in both plasmids. All analyzed bases in the 3′ direction from the START codon and all bases in the 5′ direction from the STOP codon were identical with the hsp90 *C. albicans* sequence (*GenBank*, accession no. X81025)

by using the MACAW program (*National Center for Biotechnology Information*, USA). The sequencing covered approximately 840 bases from a total CDS length of 2124 bases. In the pVAX1–hsp90–CA vaccination plasmid the Kozak consensus region ACC ATG G (START underlined) (Kozak 1991) was also confirmed.

Preparation of the recombinant protein. The translated region of recombinant plasmid pET28–hsp90–CA was composed of 3 parts: (*a*) the first N′ terminal 39 amino acids long sequence containing the T7 tag and His tag, (*b*) its own hsp90–CA protein 707 amino acids long and (*c*) C′ terminal sequence 7 amino acids long containing the His tag. The total length was 753 amino acids and predicted molar mass 85.8 kDa, p*I* 5.04. The identity of recombinant hsp90–CA protein was confirmed on Western blot (Fig. 1). By comparing the densities of r–hsp90–CA with the standard and by establishing the total protein content by the Bradford method, the purity of r–hsp90–CA in the elution fraction used for vaccination was determined 91 % by mass. The concentration 0.5 mg/mL was adjusted before the vaccination.

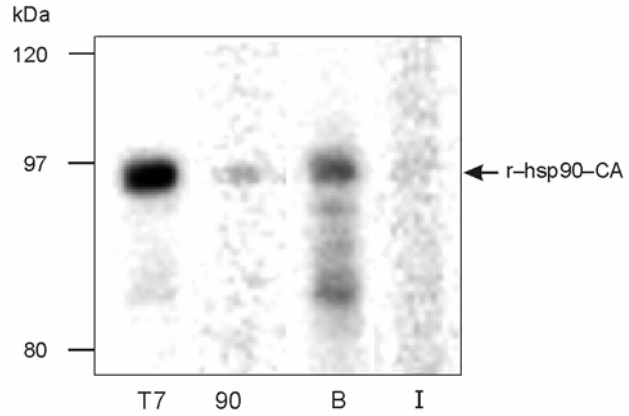

Fig. 1. Western blot analysis of purified recombinant hsp90 protein. Recombinant hsp90–CA was purified using NiNTA agarose column and eluted fractions were analyzed using anti-T-tag-mAb; selected fractions were pooled, separated on 8 % SDS-PAGE, blotted on PVDF membrane and immunodetected by anti-T-tag-mAb (line T7), anti -hsp90-mAb (90) and DNA immunized mouse sera (reactivity of mouse serum: I – 19 d after the first immunization, B – 22 d after booster); for further details *see Material and Methods*.

Immunization of the mice. During the immunization, no signs of clinical complications were observed. No pathological palpation changes were observed in the i.m. or in the i.d. application regions. Only in immunization coupled with application of Freund's adjuvant palpable intradermal granulomas were observed (size ≤10 mm).

Histological examination of organs after immunization. In DNA vaccinated mice, no irreversible pathological changes of the organs were observed. The activation of the spleen, slight hepatocyte swelling and activation of Kupffer cells was observed as compared to the control group. In the group of mice vaccinated with the recombinant hsp90 protein, granulation of hepatocytes, slight edema of the lungs and a slight vacuolar dystrophy of the lining of the kidney canals were observed. In the group vaccinated by the hsp90 protein activation of the spleen reached the highest level in all groups.

Analysis of the serum IgG recognizing recombinant hsp90. The highest level of hsp90 specific IgG was confirmed in sera of mice vaccinated with the recombinant hsp90 protein. The DNA vaccination was associated with weaker increase of serum anti-hsp90 IgG antibodies (Table IV). The i.d. DNA vaccination was the most effective DNA vaccination approach followed by i.m. vaccination (100 μg DNA per mouse).

Table IV. Mouse serum anti-hsp90 IgG and survival after challenge by *C. albicans*

Experimental group	Anti-r–hsp90–CA specific IgG[a]	Survival after challenge, h[b]
Control S (1× PBS)	0.01 ± 0	37 ± 22
Control A (CFA/ICFA)	0.03 ± 0.01	44 ± 27
DNA vaccine i.m. (100 μg)	0.18 ± 0.12	28 ± 14
DNA vaccine i.m. (1 μg)	0.03 ± 0.01	34 ± 23
DNA vaccine i.d. (1 μg)	0.2 ± 0.01	61 ± 22
Protein vaccine i.d. (75 μg)	1.0 ± 0.01	81 ± 18

[a]Levels of anti-r-hsp90-CA antibodies were determined from sera of immunized mice by ELISA on the day of challenge; values were normalized to maximum value (equal to 1 which corresponds to mice immunized with recombinant hsp90–CA protein); confidence interval $p < 0.05$.

[b]Mice were vaccinated according to Tables I and II and challenged by 5×10^6 CFU of live *C. albicans*; confidence interval $p < 0.05$.

The i.m. vaccination by 1 μg DNA per mouse did not induce ELISA-detectable specific antibodies. A corresponding result was confirmed by densitometry on an immunonoblot (*data not shown*).

Clinical results of the challenge experiments. After challenge the number of colonies of *C. albicans* cultivated from individual mice lay in the range 10–160 CFU. In any particular group the candidemia did not correlate significantly with the survival rate. The mice that died within the first 24 h suffered muscle weakness. Later spasms in the upper and lower limbs dominated largely with an activity of the extensors. The behavior of the mice before death indicated a spatial disorientation and inability of goal-directed movement.

Mice vaccinated with the recombinant protein r–hsp90–CA survived on the average for 81 h, which represented an 84- and 118-% increase in survival as compared with the control groups A and S, respectively (Table IV). The most effective DNA vaccination was the i.d. approach. The mice survived an average of 61 h, which represented a 39- and 64-% increase in survival as compared with the control groups A and S, respectively. The i.m. DNA vaccination did not show significant protectivity and rather shortened the survival of the mice after the challenge. Large variance in survival of mice after administration of *C. albicans* was observed in all groups. When testing the LD_{50} of *C. albicans* on unaffected mice, similar differences in survival were observed.

The number of *C. albicans* CFU cultivated from kidney impressions at the time of death increased proportionally with survival rate. In the DNA- and protein-vaccinated mice and in the control group of mice which received Freund's adjuvant, the number varied between 9 and 35 CFU (survival ≤20 h) and 60 and 250 CFU (survival 72–96 h). In the control group of mice that received PBS, the values reached 17–65 CFU (survival ≤20 h) and with a survival rate of 72–96 h, the CFU values did not differ from the other experimental groups.

Three mice (one vaccinated with DNA i.d. and two vaccinated with recombinant protein) survived for over 96 h after the challenge. These mice were sacrificed 20 d after the challenge. The number of *C. albicans* from the kidney impressions was below 25 CFU. In these mice candidemia 30 min after the challenge reached values of 75, 25, and 50 CFU per 5 μL of blood samples.

DISCUSSION

In patients with severe leukocytopenia induced by high doses of chemotherapy and in some patients with terminal stage of AIDS systemic candidiasis reaches up to 50 % mortality despite intensive pharmacotherapy (Dorko *et al*. 2002). Prior induction of candida specific antibodies seems to be promising. An intensive IgG and IgM antibody response to the 47-kDa fragment of hsp90 protein is associated with good prognosis, while in fatal cases, barely any specific antibodies are detected (Matthews *et al*. 1995; Burnie and Matthews 2004).

We showed that i.d. and i.m. hsp90 DNA vaccination approaches did not elicit sufficient protectivity in comparison with recombinant hsp90 protein vaccine. The titers of hsp90-specific IgG induced by i.d. and 100 μg i.m. DNA vaccination reached one-third of the titer after protein vaccination. The i.d. and 100 μg i.m. DNA vaccinations elicit a similar level of specific IgG, nevertheless the i.d. DNA-vaccinated mice survived about twice longer than those vaccinated i.m by 100 μg of DNA vaccine. The low efficiency of i.m. DNA vaccination with a relatively high level of specific IgG is probably associated with improper activation of the immune background, particularly T-cell subpopulations. Both approaches are associated with different activation of specific subclasses of T-lymphocytes (T_H1 and T_H2), which, on the one hand, must stimulate an intense antibody response and, on the other hand, activate the effector macrophage system (Romani 2000). Similarly to previous studies, other factors are probably involved in the protection of mice against *C. albicans* challenge. Differences in protectivity of the tested DNA vaccines (i.m. *vs*. i.d.) can be also associated with differences in MHC I or MHC II, respectively, and restriction of antigens expressed from DNA vaccine inside the host animal cells after i.m. and i.d. administration (Donnelly *et al.* 1997; Bocchia *et al*. 2000). The association of T_H1 response with high i.m. dose of DNA vaccination was confirmed in mice (Donnelly *et al*. 1997). It can be assumed that i.m. hsp90 DNA vaccination did not insure sufficient protectivity in systemic candidiasis especially by unsuitable proportion between elicited T_H1 and T_H2 response but could be efficient in mucous and skin form.

Lower effectiveness of the i.d. DNA vaccination *vs*. protein vaccination is probably associated with the weak expression of the hsp90 antigen from the DNA vaccine after i.d. injection. Increased effectiveness of i.d. DNA vaccination could be achieved by one of the modern application methods, such as Gene-Gun, BioJector, or electroporation (Peachman *et al*. 2003). Higher dose of i.d. applied DNA vaccine can be proposed to be associated with changes in immunomodulation effects of naked DNA resulting paradoxically in diminished protectivity of vaccination intervention (Donnelly *et al.* 1997). Our hsp90 vaccination approa-

ches seems to be effective against the lethal effect of *C. albicans* challenge but not against spreading of candida yeast. Quantitative assessment of *Candida* CFU on SGA suggest that i.d. DNA vaccine as well as protein vaccine still allow multiplication of *Candida* yeast.

hsp90 and other heat shock proteins belong to highly evolutionarily conserved proteins and, therefore, it is necessary to consider the possibility of inducing an autoimmune reaction. It is believed that cross reactivity against conserved determinants of hsp allows to control infection during induction of clonal immunity. This response appears to act much faster and in a higher titer than the induced primary antibody response (Zügel *et al*. 1995). On the other hand, immune response toward evolutionarily conserved hsp antigens is regulates by anti-inflammatory T_H2 cytokines released from low-affinity reacting T-lymphocytes during contact with the conserved hsp epitope. The described anti-inflammatory feedback regulation is permanently induced by the presence of the microorganisms (Gaston 1997). So far there has not been any evidence of induction of autoimmunity by hsp proteins (Weigl *et al.* 1999). This allows us to consider hsp proteins as promising protective antigens appropriate for designing and testing protective vaccines against many types of infection agents.

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