Properties of the recombinant TNF-binding proteins from variola, monkeypox, and cowpox viruses are different

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Abstract

Tumor necrosis factor (TNF), a potent proinflammatory and antiviral cytokine, is a critical extracellular immune regulator targeted by poxviruses through the activity of virus-encoded family of TNF-binding proteins (CrmB, CrmC, CrmD, and CrmE). The only TNF-binding protein from variola virus (VARV), the causative agent of smallpox, infecting exclusively humans, is CrmB. Here we have aligned the amino acid sequences of CrmB proteins from 10 VARV, 14 cowpox virus (CPXV), and 22 monkeypox virus (MPXV) strains. Sequence analyses demonstrated a high homology of these proteins. The regions homologous to cd00185 domain of the TNF receptor family, determining the specificity of ligand–receptor binding, were found in the sequences of CrmB proteins. In addition, a comparative analysis of the C-terminal SECRET domain sequences of CrmB proteins was performed. The differences in the amino acid sequences of these domains characteristic of each particular orthopoxvirus species were detected. It was assumed that the species-specific distinctions between the CrmB proteins might underlie the differences in these physicochemical and biological properties. The individual recombinant proteins VARV-CrmB, MPXV-CrmB, and CPXV-CrmB were synthesized in a baculovirus expression system in insect cells and isolated. Purified VARV-CrmB was detectable as a dimer with a molecular weight of 90 kDa, while MPXV- and CPXV-CrmBs, as monomers when fractioned by non-reducing SDS-PAGE. The CrmB proteins of VARV, MPXV, and CPXV differed in the efficiencies of inhibition of the cytotoxic effects of human, mouse, or rabbit TNFs in L929 mouse fibroblast cell line. Testing of CrmBs in the experimental model of LPS-induced shock using SPF BALB/c mice detected a pronounced protective effect of VARV-CrmB. Thus, our data demonstrated the difference in anti-TNF activities of VARV-, MPXV-, and CPXV-CrmBs and efficiency of VARV-CrmB rather than CPXV- or MPXV-CrmBs against LPS-induced mortality in mice.

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1. Introduction

Viral soluble TNF receptors encoded by poxviruses block the activity of this proinflammatory cytokine; simultaneously, some of them interact with chemokines via an additional C-terminal domain, which is unrelated to any known host proteins [1]. Earlier, it was suggested that TNF-binding proteins contribute to the degree of pathogenicity of the viruses belonging to the family *Poxviridae* [2–4]. The viruses of the genus *Orthopoxvirus*, pathogenic for humans, are of special interest in this respect. They include variola (VARV), monkeypox (MPXV), and cowpox (CPXV) viruses, differing in the level of their pathogenicity for humans and ranges of susceptible hosts [5].

Humans are the only VARV host; hence, this virus is to a maximal degree adapted evolutionarily to overcome the human defense reactions, which develop in response to the infection. Both MPXV and CPXV have wide host range, infecting first and foremost various rodents. Humans are sporadically infected by these viruses. Consequently, MPXV and CPXV are adapted to interactions with the molecular defense reactions of mammals of various species [6].

Tumor necrosis factor (TNF), a potent proinflammatory and antiviral cytokine, is a critical extracellular immune regulator targeted by poxviruses through the activity of virus-encoded family of TNF-binding proteins (CrmB, CrmC, CrmD, and

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CrmE) [7–12]. Various orthopoxvirus species have different sets of TNF-binding proteins [6]. The only gene of this family present in the genomes of three species in question—VARV, MPXV, and CPXV—is that encoding CrmB. Mammalian cytokines and, in particular TNF, display species-specific amino acid variation; hence, this suggests that the TNF-binding proteins of VARV, MPXV, and CPXV named CrmBs may differ in their properties.

Computer analysis of VARV-, MPXV-, and CPXV-CrmB amino acid sequences, which we performed earlier, detected a number of virus species-specific distinctions between these proteins [6,13–16]. Note that the degrees of homology between these protein amounts to 84.8% for VARV and MPXV, 90.8% for VARV and CPXV, and 90.3% for CPXV and MPXV. Presumably, the detected distinctions may determine the differences in their conformation and glycosylation patterns and, consequently, the differences in physicochemical and biological properties.

For a comparative study of the properties of orthopoxvirus TNF-binding proteins, CrmBs, we constructed recombinant baculoviruses carrying the genes of secreted CrmB proteins of VARV (strain India-1967), MPXV (strain Zaire-96-I-16), and CPXV (strain GRI-90) [17] and developed a method for isolation of the recombinant CrmBs from baculovirus-infected Sf21 insect cells [18]. The results of comparative functional analysis of the recombinant CrmB proteins encoded by VARV, MPXV, and CPXV allowed for demonstrating that their properties were different.

2. Materials and methods

2.1. Materials

Grace's medium, antibiotics, and L-glutamine (Gibco/BRL, Invitrogen GmbH, Germany), DMEM (SRC VB Vector, Koltsovo, Novosibirsk oblast, Russia), fetal bovine serum (BioLot Ltd., St. Petersburg, Russia), nitrocellulose membrane (Schleicher@Schuell BioScience Inc., NH, USA), anti-rabbit HRP conjugate and Prestained SDS-PAGE low Range standards (Bio-Rad, Hercules, CA, USA), ECL™ Western blotting detection reagent (Amersham Biosciences Inc., Piscataway, NJ, USA), CP-BU X-ray film (AGFA, Leverkusen, Germany), and LPS (L2880 *E. coli* serotype 055:B5; Sigma-Aldrich, St. Louis, MO, USA) were used in the work. Recombinant human (h, 10^7 U/mg), murine (m, 5×10^6 U/mg), and rabbit (r, 3×10^6 U/mg) TNFs were purified at SRC VB Vector from the corresponding *E. coli* strains [19]; 96-well plates for cell culture and ELISA were produced by Costar (Cambridge, MA, USA).

2.2. Cell lines

Spodoptera frugiperda (Sf21) and L929 murine fibroblast cell lines were obtained from the Cell Culture Collection with SRC VB Vector. A monolayer culture of Sf21 cells was maintained at 27 °C in Grace's insect medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. L929 cells were cultured in Dulbecco's modified Eagle's (SRC VB Vector) medium with the same supplements in 5% CO_2.

2.3. Sequence data analysis

The nucleotide sequences of genes encoding CrmB proteins of VARV, CPXV, and MPXV were obtained from the GenBank database (http://www.ncbi.nlm.nih.gov) using BLAST network server [20]. The predicted amino acid sequences encoded by *crmB* genes were aligned and analyzed for

homology with MUSCLE 3.52 program [21] and BLOSSUM62 matrix [22], respectively.

2.4. Purification of viral recombinant CrmB proteins

Construction of the recombinant baculoviruses expressing viral *crmB* genes was described earlier [16,18]. The corresponding recombinant proteins were purified according to [17]. Protein concentration was determined using Bradford reagent [23] with bovine serum albumin (BSA) as a standard.

2.5. Inhibition of TNF cytotoxicity for L929 cell line

The TNF cytotoxicity for L929 cells was assessed using neutral red staining technique as described in [16,18]. Briefly, 5×10^3 L929 cells per well were incubated in 96-well plates at 37 °C for 24 h in 200 μl of the DMEM containing 5 μg/ml actinomycin D; VARV-, MPXV-, or CPXV-CrmB; and human, mouse, or rat TNF. Cell viability (CV$_\%$; an average of three samples per each variant) was calculated as

$$CV_{(\%)} = 100 \times (OD_{CrmB+TNF} - OD_{TNF})(OD_{cell} - OD_{TNF}),$$

where VARV-, MPXV-, or CPXV-CrmB was the inhibitor.

Optical density was measured at 565 nm in a Microplate Reader EL$_X$808 (Bio-Tek Instruments Inc., Winooski, VT, USA).

2.6. Antibodies and immunoblotting

Antiserum to VARV-CrmB was raised in outbreed rabbits using the emulsified antigen presentation and administration routs as described earlier [18]. Ab$_{VARV-CrmB}$ were precipitated from the immune serum with $(NH_4)_2SO_4$ up to 33% saturation and purified using BrCN-Sepharose 4Â with immobilized recombinant hTNF. Goat anti-rabbit horseradish peroxidase conjugate was used at a dilution of 1:25 000.

Protein samples were analyzed by SDS-PAGE [24]. The gel buffer was with or without 1% 2-mercaptoethanol. Proteins were subsequently transferred to nitrocellulose membrane using Bio-Rad blotting apparatus. The nonspecific binding sites on the membranes were blocked in TBST (140 mM NaCl, 3 mM KCl, 24 mM Tris Base pH 7.4, and 0.2% Tween 20) with 5% fat-free milk for 15 min at 25°C. Membranes were subsequently incubated with the primary Ab$_{VARV-CrmB}$ and appropriate secondary antibodies conjugated with horseradish peroxidase. The peroxidase-mediated luminescence was induced by incubation with ECL™ reagent and detected on X-ray film. Image densitometry was performed using GelPro software (Media Cybernetics Inc., Silver Spring, MD, USA).

2.7. In vivo experiments

All animal experiments were performed according to the Animal Study Protocol approved by the Bioethical Committee, SRC VB Vector. SPF BALB/c male mice (3–4 weeks old; 10–15 g) were obtained from the Experimental Animal Breeding Facility (Pushchino, Moscow oblast, Russia) and kept in a pathogen-free environment during a 2-week adaptation and 72-h experimental periods. Animals were sensitized with a single injection of LPS (300 μg/mouse) and challenged 16 h later with injection of a resolving dose of 350 μg/mouse. The resolving LPS dose was injected in combination with BSA (2 μg/mouse) or recombinant CrmB (0.2 or 2 μg/mouse). The survival of mice was monitored daily for 72 h. VARV-CrmB (at both concentrations) was tested in three separate experiments; the MPXV- and CPXV-CrmBs (at both concentrations), in two separate experiments.

3. Results and discussion

3.1. Computer analysis of amino acid sequences of the VARV-, MPXV-, and CPXV-CrmBs

BLAST network server [20] was used to search the international database GenBank (http://www.ncbi.nlm.nih.gov)

for the nucleotide sequences of VARV, CPXV, and MPXV CrmB genes using CPXV GRI-90 open reading frame (ORF) I4R [13] as a reference for the search. The extracted 46 ORFs (for 10 VARV, 14 CPXV, and 22 MPXV strains) were deduced into the corresponding amino acid sequences, which were aligned by the program MUSCLE 3.52 [21]. Homology analysis of these amino acid sequences [22] detected the following patterns: (i) the homologies of VARV-, CPXV-, and MPXV-CrmB to the extracellular domains of human TNF receptors I and II (TNFRI and TNFRII) amounts approximately to 47 and 54%, respectively; (ii) the intraspecies identities are 98–100%, 85–100%, and 99–100% for VARV, CPXV, and MPXV strains, respectively; and (iii) interspecies homologies equal 82–91% for VARV and CPXV, 84–96% for VARV and MPXV, and 82–92% for CPXV and MPXV.

Note that characteristic of the cellular proteins belonging to the TNF receptor family [25] is a weak homology of amino acid sequences (about 20%) [24]. However, despite the differences in the primary structures, the X-ray analysis of the receptor–ligand complexes demonstrated a similarity of their 3D structures. These data allowed for describing the domain of TNF receptor family (cd00185) containing particular amino acids stretches named 50 s and 90 s loops, involved in the ligand–receptor interaction [26–29]. Using the CDD database [30], we predicted the regions corresponding to the 50 s and 90 s loops of cd00185 domain within the CrmB proteins. Alignment of the CrmB sequences corresponding to cd00185 domain (Fig. 1A) demonstrated that VARV-, CPXV-, and MPXV-CrmBs had virus species-specific nonconservative amino acid substitutions located within presumptive ligand-binding sites; note that MPXV-CrmB contained the highest number of the amino acid distinctions detected in this domain (Fig. 1A). Recently, it was demonstrated by Alejo et al. [1] that CrmB contains a C-terminal domain, named SECRET domain, which binds certain chemokines. Our analysis demonstrated that the amino acid sequence of this domains displayed pronounced distinctions specific of various orthopoxvirus species (Fig. 1B), and the distinctions of VARV were the most pronounced. Revealed distinctions between the analyzed CrmB proteins (Fig. 1A, B) might be responsible for the difference in their biological properties.

3.2. Characterization of the orthopoxviral CrmBs

The recombinant baculoviruses producing TNF-binding proteins of VARV, CPXV, and MPXV were constructed earlier [16,18].

CrmB proteins were isolated from growth medium of Sf21 cells infected with these recombinant baculoviruses using an affinity resin (recombinant hTNF immobilized on acrylamide–agarose matrix) [17]. The purity and electrophoretic mobility of the recombinant CrmBs were determined by SDS-PAGE fractionation followed by Coomassie R250 staining [18], giving 45–47 kDa molecular weight range for the proteins studied. Western blot analysis with $Ab_{VARV-CrmB}$ was then used to characterize the obtained recombinant proteins. Fig. 2A shows an immunoblot pattern of the recombinant proteins (0.01 μg in

each probe) fractionated under reducing conditions (with 1% 2-ME in sample loading buffer). A common fraction detected by $Ab_{VARV-CrmB}$ in all samples corresponds to the proteins with a molecular weight of 45–47 kDa. The intensities of the luminescent signals detected by image densitometry appeared to be different (1:0.2:0.5 for VARV-, MPXV- or CPXV-CrmB, respectively).

Fig. 2B shows the results of immunodetection of the same samples fractionated under reducing and non-reducing conditions. In this experiment, tenfold amount of MPXV- and CPXV-CrmB proteins (0.1 μg) with respect to VARV-CrmB was loaded to compensate for weak signals. Under non-reducing conditions, the molecular weight of VARV-CrmB was detected as 90 kDa. The two other proteins, MPXV- and CPXV-CrmB, were detected as 45–47 kDa bands, although a minor 90 kDa band is also present in the CPXV-CrmB sample.

3.3. In vitro study of the recombinant CrmBs

To study the biological properties of CrmB proteins, we used the test where L929 murine fibroblast cells served as a target for the cytolytic action of human, mouse, or rabbit TNF, while VARV-, CPXV-, or MPXV-CrmB was used as an inhibitor. Initially, ED_{50} values were determined for each of the cytokines listed (data not shown). Then L929 cells were exposed to each cytokine at $6 \times ED_{50}$ dose mixed with twofold dilutions of VARV-, CPXV-, or MPXV-CrmB protein. The viability of L929 cells in each experiment was plotted against CrmB concentration. The resulting curves (Fig. 3 demonstrates such plots for VARV-CrmB; analogous plots for CPXV- and MPXV-CrmBs are not shown) were used to determine the protein concentrations providing a 50% protection of L929 cells (Table 1). The ratio of the concentrations determined demonstrates the distinctions between the neutralizing effects of VARV-, MPXV-, and CPXV-CrmB proteins. VARV-CrmB neutralizes efficiently the cytolytic effects of all the cytokines used. The protective effect of CPXV- and MPXV-CrmBs against hTNF or rTNF was insufficient, but CPXV-CrmB protected L929 cells against mTNF nearly as efficiently as VARV-CrmB. Our data differ from the results of Alejo et al. [1], obtained when studying the TNF-inhibitory activity of recombinant VARV-CrmB using human, mouse, or rat TNFs. These authors discovered that VARV-CrmB inhibited the mouse and rat TNFs with a higher efficiency compared to human TNF. Presumably, these discrepancies stem from the differences in the methods used for assessing the inhibition of TNF cytolytic activity, which were used by Alejo et al. [1] and in this work as well as from different insect cell cultures used for producing VARV-CrmB and differences in their cultivation procedures. Alejo et al. [1] analyzed the TNF-inhibitory activity in supernatants from insect cells infected with a recombinant baculovirus expressing CrmB, whereas in this work we used purified proteins (see Section 3.2). Presumably, certain components of nutrient medium somehow influenced the characteristics of VARV-CrmB TNF-inhibitory activity towards human, mouse, and rat TNFs.

A

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                                                         TNFR domain (cd00185)
                                                  50s loop                        90s loop
HUMAN p55|1TNR|                              TASENHLRHCLSC-SKCR              YW-SENLFQCFNCS
HUMAN p75|P20333|                            TQLWNWVPECLSCGSRSD             LSKQEGCRLCAPLR

CPXV|X94355|GRI-1990|          (27) NGKCKDNEYNRHNLCCLSCPPGTYASRLCDSKTNTNTQCTPCGSGTFTSRNNHLPACLSCNGRCDSNQVETRSCNTTHNRICECAPGYYCLLKGSSGCKACVSQTKCG (134)
CPXV|U90227|MUN-OPV-85|         (28) ......................................................H.....................P.........S..................... (135)
CPXV|U90226|MUN-OPV-89/2|       (29) ..............................................D.......................................D......-........... (133)
CPXV|AF482758|BRI|              (29) ........K..H..............A.D..........................................D......F................ (136)
CPXV|U90235|MUN-OPV90/5|        (29) .....RH.H......................D.......................................D.................I...... (135)
CPXV|U90231|MUN-89/1|           (4)  ........RH.H.....................................................A.......S...........V...... (111)
CPXV|U90234|MUN-OPV90/1|        (4)  ........RH.H.................................................S.......... (111)
CPXV|AY623479|CoPV/DEN/98|      (28) ........RH.H.............E.................................................I...... (135)
CPXV|U90228|MUN-OPV-91/1|       (27) ........RH.H.............................................K.......D...................... (134)
CPXV|U90233|MUN-OPV89/5|        (27) ........RH.H.............................................K.......D...................... (134)
CPXV|U90225|MUN-OPV90/2|        (27) ........KH.H.............S...............................D...................... (134)
CPXV|U90232|MUN-OPV89/4|        (29) ........KH.H.............S.......................................S...................... (133)
CPXV|U90230|MUN-EP2-1975|       (26) ........KH.H.............S.......................................S...................... (133)
CPXV|U90229|CPV58|              (26) ........KH.H.............S.......................................S...................... (133)
VARV|U88146|BUT-1952|           (29) ......T..K...........--...................N...............S...................... (134)
VARV|U18339|GAR-1966|           (29) ......T..K...........--...................N...............S...................... (134)
VARV|U88150|SLN-1968|           (29) ......T..K...........--...................N...............S...................... (134)
VARV|U18341|SOM-1977|           (29) ......T..K...........--...................N...............S...................... (134)
VARV|U88147|CNG-1970|           (29) ......T..K...........--...................N...............S...................... (134)
VARV|U88151|Ch9-2|              (28) ......T..K...........--...................N...............S...................... (133)
VARV|U88145|HAR-1944|           (29) ......T..K...........S.......--...................N...............S...................... (134)
VARV|X67117|IND-1967|Ind3       (28) ......T..K...........--...................N...............S...................... (133)
VARV|L22579|BSH-1975|           (28) ......T..K...........--...................N...............S...................... (133)
VARV|U88152|Ch9-4|              (29) ......T..K...........--...................N...............S...................... (134)
MPXV|U87844|NIG-1971-0082|      (29) ........RSR.........--.......D....H....Q...............S.......A...ST.I.K.... (134)
MPXV|U87846|BEN-1978-3945|      (29) ........RSR.........--.......D....H....Q...............S.......A...RT.I.K.... (134)
MPXV|U87847|ZAI-1979-0005|      (29) ........RSR.........--.......D....H....Q...............S.......RT.I.K.... (134)
MPXV|AF380138|ZAI-1996-I-16|    (29) ........RSR.........--.......D....H....Q...............S.......RT.I.K.... (134)
MPXV|U88543|ZAI-1996-17|        (29) ........RSR.........--.......D....H....Q...............S.......RT.I.K.... (134)
MPXV|U87841|ZAI-1996-16|        (29) ........RSR.........--.......D....H....Q...............S.......RT.I.K.... (134)
MPXV|U87845|ZAI-1977-0666|      (29) ........RSR.........--.......D....H....Q...............S.......RT.I.K.... (134)
MPXV|DQ011155|ZAI-1979-005|     (29) ........RSR.........--.......D....H....Q...............S.......RT.I.K.... (134)
MPXV|U88142|ZAI-1970|           (29) ........RSR.........--.......D....H....Q...............S.......RT.I.K.... (134)
MPXV|DQ011154|CON-2003-358|     (29) ........RSR.........--.......D....H....Q...............S.......RT.I.K.... (134)
MPXV|AY741551||SLN              (29) ........RSR.........--.......D....H....Q...............S.....AL..RT.I.K.... (134)
MPXV|U87843|SLN-1970-0266|      (29) ........RSR.........--.......D....H....Q...............S.....AL..RT.I.K.... (134)
MPXV|DQ011153|USA-2003-044|     (29) ........RSR.........--.......D....H....Q...............S.......A...RT.I.K.... (134)
MPXV|AY603973|WRAIR7-61|        (29) ........RSR.........--.......D....H....Q...............S.......A...RT.I.K.... (134)
MPXV|DQ011156|LIB-1970-184|     (29) ........RSR.........--.......D....H....Q...............S.......A...RT.I.K.... (134)
MPXV|U87995|COP-1958|           (29) ........RSR.........--.......D....H....Q...............S.......A...RT.I.K.... (134)
MPXV|U87842|LIB-1970-0187|      (29) ........RSR.........--.......D....H....Q...............S.......A...RT.I.K.... (134)
MPXV|DQ011157|USA-2003-039|     (29) ........RSR.........--.......D....H....Q...............S.......A...RT.I.K.... (134)
MPXV|U87994|COP-1958|clone:CW-N1 (29) ........RSR.........--.......D....H....Q...............S.......A...RT.I.K.... (134)
MPXV|AY753185|COP-1958|         (29) ........RSR.........--.......D....H....Q...............S.......A...RT.I.K.... (134)
MPXV|U88144|UTC-Rotterdam-1965  (29) ........RSR.........--.......D....H....Q...............S.......A...RT.I.K.... (134)
MPXV|U88143|WMP-1961|           (29) ........RSR.........--.......D....H....Q...............S.......A...RT.I.K.... (134)
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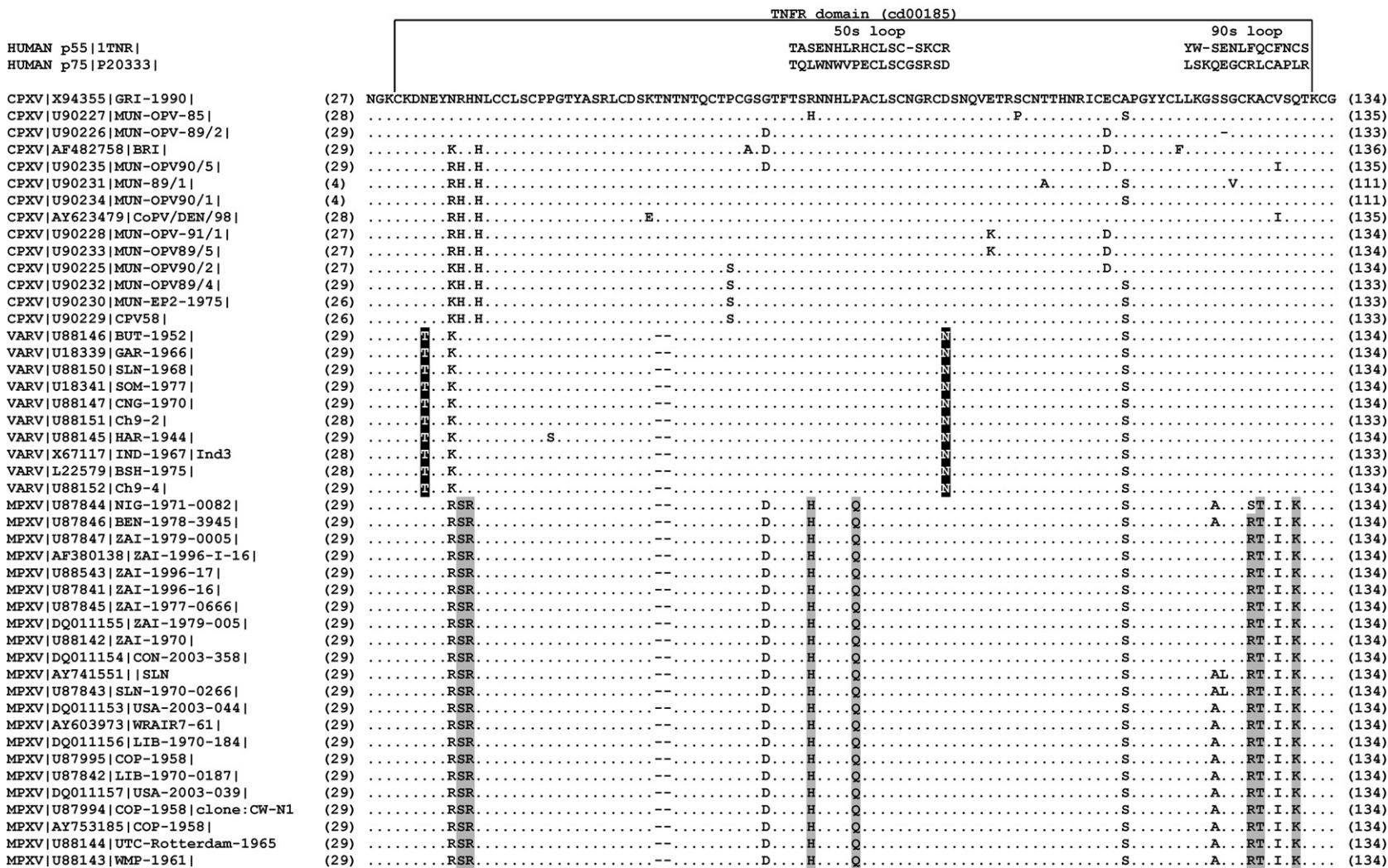
Fig. 1. Amino acid alignment of the functional domains of the orthopoxvirus CrmB proteins. (A) Predicted TNF-binding region. Line on the top indicates cd00185 domain of TNFR family [30]. The 50 s and 90 s loop sequences of human TNFRI and TNFRII proteins are placed above the corresponding sequences of CrmBs. Aligned CrmB sequences are named as "species|GenBank accession number|strain". (B) SECRET domain [1]. Aligned CrmB sequences are named as "species|GenBank accession number". Figures in parenthesis near amino acid sequences indicate the number of amino acid residues at the N- and C-termini of the protein (not shown in the alignment). C-ends of amino acid sequences of proteins are asterisked. The amino acids identical to CPXV-CrmB (GRI-90) are indicated with dots; deletions, with dashes. VARV and MPXV species-specific amino acids are marked with black and gray boxes, respectively.
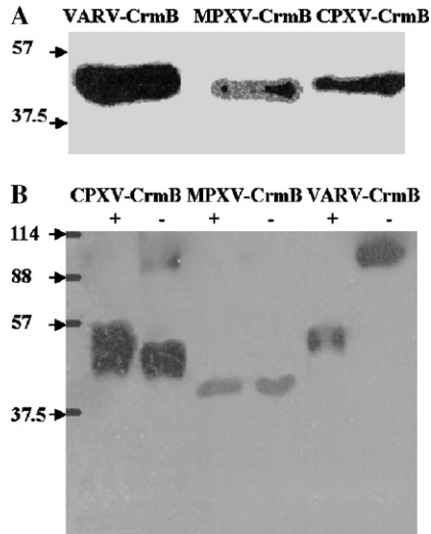
Fig. 1 (*continued*).

Fig. 2. Immunoblotting analysis of purified CrmB proteins. (A) Samples of VARV-, MPXV-, and CPXV-CrmBs (0.01 μg each) were loaded on 10% PAAG in the buffer containing 1% 2-ME, after migration at 150 V for 1.5 h, transferred to nitrocellulose membrane, and incubated with polyclonal AbVARV-CrmB. (B) VARV-CrmB (0.01 mg) and CPXV- and MPXV-CrmBs (0.1 mg each) in the loading buffer with (+) or without (−) 1% 2-ME were separated at 100 V for 4 h and subjected the same procedure. Molecular weight markers (Prestained SDS-PAGE low Range standards) are expressed in kDa.

3.4. In vivo study of the recombinant CrmBs

The recombinant CrmBs were tested in the experimental model of LPS-induced endotoxic shock [31]. SPF BALB/c male mice (10–15 g, 3–4-weeks old) were given LPS intraperitoneally in combination with a CrmB or BSA (see Materials and methods). As is shown in Fig. 4A, only $10 \pm 5\%$ of experimental animals survived over 72 h of observation. Injection of CrmBs only (2 μg/mouse) or BSA (2 μg/mouse) did not cause animal

Table 1
The concentrations of CrmB proteins providing a 50% inhibition of the cytokine-induced lysis of L929 cells

Cytokine	VARV-CrmB (ng/ml)	CPXV-CrmB (ng/ml)	MPXV-CrmB (ng/ml)
hTNF	5.0–8.0	40.0–65.0	70.0–120.0
mTNF	5.5–9.0	5.0–8.0	40.0–60.0
rTNF	6.0–10.0	40.0–60.0	300.0–500.0

death (Fig. 4A). Administration of LPS in combination with VARV-CrmB at doses of 0.2 and 2.0 μg/mouse increased the survival rate to 47 ± 10.9 and $62 \pm 8.6\%$, respectively (Fig. 4A), i.e., VARV-CrmB protein displayed a significant protective effect. MPXV-CrmB and CPXV-CrmB (at both doses used) did not exert any protective effects (Fig. 4B). Note that MPXV-CrmB and CPXV-CrmB even at the doses by one order of magnitude higher (2 μg/mouse) compared with VARV-CrmB (0.2 μg/mouse) displayed no protective effect.

3.5. Concluding remarks

Comparison of amino acid sequences of a great number of various types of human and rodent polypeptides revealed most pronounced interspecies differences in the sequences of the proteins forming the ligand–receptor pairs of the organism's protective systems of these mammals against infectious agents. In addition, the polypeptide ligands and their receptors proved to co-evolve [32].

Pathogenic microorganisms are assumed to be able to cause an accelerated evolution of the defense system proteins (genes) of infected animal species. Such evolutionary changes in the primary structure of the proteins constituting ligand–receptor pairs were suggested to result in alterations of the quaternary structure of the ligand–receptor contact region [32,34]. Consequently, the species-specific mimicry of mammalian
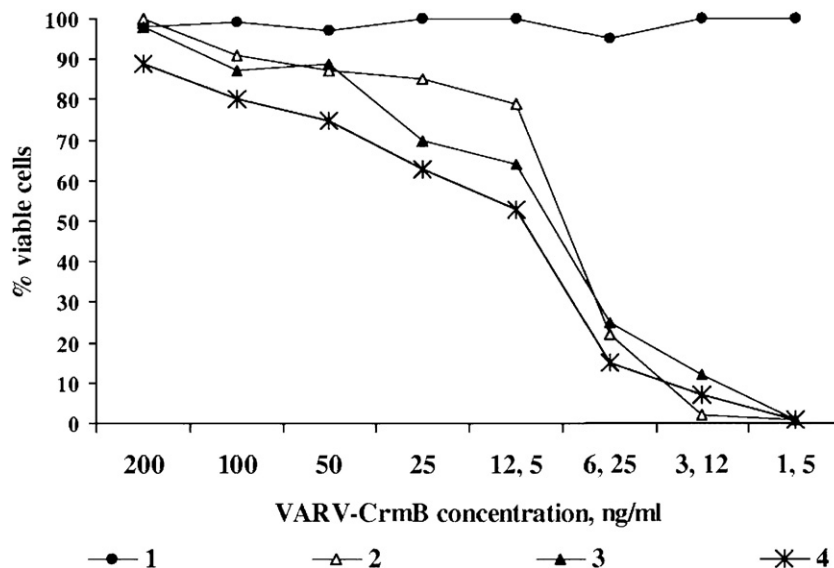

Fig. 3. Inhibition of the cytokine-induced cytolysis of L929 cells by VARV-CrmB proteins. Neutral red staining was used to determine (in triplicate samples) the cell viability after 18 h of exposure to TNFs (at a dose of 6×ED50) alone or in the presence of twofold dilutions of VARVCrmB (1, L929 cells alone; 2, mTNF; 3, hTNF; and 4, rTNF).
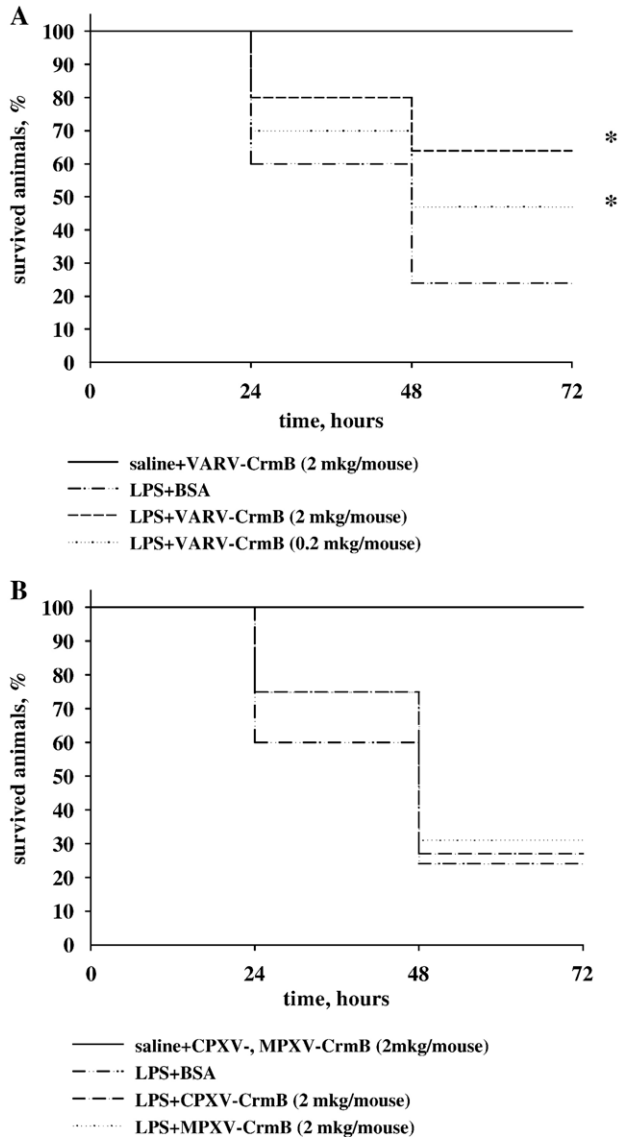
Fig. 4. Biological activity of recombinant CrmB proteins *in vivo*. BALB/c mice were treated intraperitoneally with LPS in combination with BSA and (A) VARV-CrmB (0.2 or 2 μg/mouse) or (B) MPXV- or CPXV-CrmB (2 μg/mouse), as described in Materials and methods (data for MPXV- and CPXV-CrmB injections at a dose of 0.2 μg/mouse are not shown). The control group of animals received saline instead of LPS. The results shown are a compilation of three experiments for (A) and two experiments for (B) using 8–10 mice per treatment group for each experiment. *$P < 0.05$ vs. LPS alone by Fisher's test.

defense system proteins may emerge, providing a narrowed range of hosts sensitive to certain infectious microorganism [32].

Recently accumulated data demonstrate that cytokines and their specific receptors undergo considerable structural alterations during the evolution. Such alterations may occur relatively rapidly on the evolutionary scale. Thus, mouse γ-IFN interacts weakly with human γ-IFN receptor and vice versa. On the other hand, this cytokine binds to its receptor with a high efficiency within the species [33]. It is evident now that any cytokine can function only in a pair with the receptor located on the plasmatic membrane of the cell. That is why the question of co-evolution

of cytokines and their receptors and, on a more global scale, of various receptor–ligand pairs, is of great interest.

Protein families similar to TNF [34] and its receptors [35], which are rapidly expanding since recently, attract considerable interest of researches. It is suggested that a complex network of TNF-like receptor–ligand pairs, which are involved in protective functions in the animal organism, has been formed during the evolution as a result of duplications and subsequent modifications of ancestor genes of a receptor and its ligand [33]. Deletion of TNF and γ-IFN genes, the well-studied cytokines, or the genes for their receptors was demonstrated to elevate considerably the sensitivity of animals to pathogenic action of the intracellular parasites, such as *Listeria*, mycobacteria, and viruses [36–39].

The interaction network of cytokines and their receptors has been so far studied only to a first approximation, and many discoveries are still awaiting researchers in this direction. Orthopoxviruses can play an important role here. The genes encoding cell-secreted (soluble) receptors for TNF, γ-IFN, α/β-IFN, IL-1β, and IL-18 were discovered in these viruses [6,8,9]. Thus, orthopoxviruses are capable of inhibiting a number of endogenous cytokines of the host organism.

The results of our experiments confirm the hypothesis [6] that the species-specific distinctions between the amino acid sequences of TNF-binding proteins of the three orthopoxvirus species differing in their host range and pathogenicity for humans are realized in differing biological properties of these proteins.

We demonstrated that in contrast to MPXV- and CPXV-CrmBs, the affinity purified recombinant VARV-CrmB formed dimers. Differences in interactions of CrmB proteins with polyclonal Ab$_{VARV-CrmB}$ were found (Fig. 2A). It is not surprising that the strongest signal was observed for binding of Ab$_{VARV-CrmB}$ to VARV-CrmB; however, the different intensities of the signals from CPXV-CrmB and MPXV-CrmB probes likely reflect a higher affinity of Ab$_{VARV-CrmB}$ for CPXV-CrmB than for MPXV-CrmB. We also found that CrmB proteins of VARV, MPXV, and CPXV inhibited the cytotoxic effects of human, mouse, and rabbit TNFs for L929 cells in different manner (Table 1). VARV-CrmB inhibited efficiently the cytotoxicity of all the tested TNFs, whereas MPXV-CrmB inhibition was extremely weak. CPXV-CrmB was weakly efficient against hTNF or rTNF but protected L929 cells against the cytotoxic effect of mTNF as efficiently as VARV-CrmB. The alignment of amino acid sequences of the studied CrmBs in the presumed regions of interaction with ligands (homologous to the 50 s and 90 s loops of cd00185 domain) displayed the variation of these regions, especially expressed in MPXV-CrmB (Fig. 1A). Presumably, this particular difference in amino acid sequences is realized in discovered *in vitro* effects of the studied CrmBs. Recently Alejo et al. [1] generated the *CrmB* gene of VARV (strain Bangladesh-1975) by a site-directed mutagenesis from *CrmB* gene encoded by camelpox virus; this recombinant CrmB was expressed in a baculovirus system. The supernatant from insect cells infected with this recombinant baculovirus was tested in a TNF cytotoxicity inhibition test. There is some discrepancy between our data (this paper and [16,18]) and the

results of Alejo et al. [1]: they showed that VARV-CrmB is more efficient against mouse TNF than against human TNF. Such discrepancy could be due to the differences in experimental conditions, different passage history of L929 cells, and/or some other effects (see Section 3.3).

Testing of CrmB proteins in the experimental model of LPS-induced endotoxic shock demonstrated that VARV-CrmB protein had a pronounced protective effect consisting in an increase in the animal survival rate (Fig. 4A). MPXV-CrmB and CPXV-CrmB failed to display such effect (Fig. 4B), although CPXV-CrmB efficiently inhibited *in vitro* the cytotoxic effect of mTNF (Table 1). Presumably, not only TNF-binding activity of CrmB, but also the chemokine-binding activity of the C-terminal SECRET domain [1] plays an important role in the development of the protection against LPS-induced endotoxic shock. VARV-CrmB differs to the greatest degree from its homologues of the other orthopoxviruses studied in the amino acid sequence of this SECRET domain (Fig. 1B).

A particular characteristic of the recombinant protein VARV-CrmB is its ability to form dimers (Fig. 2B). It is known that the dimeric forms of cellular TNF receptors and M-T2 TNF binding protein of rabbit myxoma virus (MYXV; a poxvirus of the genus *Leporipoxvirus*) bind TNF more efficiently and display a higher TNF-neutralizing activity compared with the monomeric forms [31,40,41]. VARV and MYXV are similar in a high virulence towards their hosts. It is possible that higher avidities of the dimeric (or, perhaps, oligomeric) forms result in a more efficient TNF neutralization during the primary immune response, thereby ensuring a higher virulence of the virus.

The protective role of TNF antagonists was shown in the models of experimental sepsis [42,43]. The data on TNF-neutralizing potential of VARV-CrmB protein reported here together with its newly described chemokine-binding activity [1] support the hope that this protein may serve as a basis for developing a new class of TNF antagonists for therapy of the human diseases connected with the impairments of TNF metabolism, such as septic shock, rheumatoid arthritis, allergy, and others [44–46].

Acknowledgments

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