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The neutralizing human recombinant antibodies to pathogenic Orthopoxviruses derived from a phage display immune library

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ABSTRACT

A panel of recombinant human antibodies to *orthopoxviruses* was isolated from a combinatorial phage display library of human scFv antibodies constructed from the Vh and Vl genes cloned from the peripheral blood lymphocytes of *Vaccinia virus* (VACV) immune donors. Plaque-reduction neutralization tests showed that seven selected phage-displaying scFv antibodies (pdAbs) neutralized both CPXV and VACV, and five of them neutralized *Monkeypox virus* (MPXV). Western blot analysis of VACV and CPXV proteins demonstrated that seven neutralizing antibodies recognized a 35 kDa protein. To identify this target protein, we produced a recombinant J3L protein of CPXV and showed that all the selected neutralizing antibodies recognized this protein. Neutralizing pdAb b9 was converted into fully human mAb b9 (fh b9), and scFv b9 displayed high binding affinities (K_d of 0.7 and 3.2 nM). The fh b9 reduced VACV plaque formation in a dose-dependent manner.

1. Introduction

The *Poxviridae* is a large family of complex DNA viruses that replicate in the cytoplasm of vertebrate and invertebrate cells. The genus most important for the human history, *Orthopoxvirus*, includes four viruses pathogenic for humans, namely, *Variola virus* (VARV), which causes smallpox, known as a severe generalized human disease for about 3000 years; *Monkeypox virus* (MPXV), a wildlife virus naturally occurring in West and Central Africa, which, similar to VARV, can cause a systemic disease with a generalized rash in humans; *Cowpox* and *Vaccinia* viruses (CPXV and VACV), also able to cause human infections but usually produce local skin lesions at the infection site (Moss, 2007).

Monkeypox is the most severe orthopoxvirus infection in humans after the eradication of VARV in its natural state. Until the outbreak of 2003 in the United States (Reed et al., 2004; Sejvar et al., 2004), this disease was considered endemic in certain regions of Central and West Africa. The fact that MPXV can traverse great distances renewed the interest in development of new vaccines (Earl et al., 2007; Hutson et al., 2009; Seaman et al., 2010; Von Krempelhuber et al., 2010) and therapeutics (Alkhalil et al., 2009; Baker et al., 2003; Parker et al., 2008; Yang et al., 2005) against orthopoxvirus infections. Despite that VACV is a generally safe vaccine, disseminated life-threatening infections infrequently occur, especially in immunocompromised individuals. Studies in animal models have demonstrated that antibodies play an important role in the protection against orthopoxviruses (Benhnia et al., 2008; Earl et al., 2004; Hooper et al., 2004); hence, certain vaccination complications and orthopoxvirus infections can be treated with therapeutic administration of human VACV immune globulin (VIG), produced from donor blood (Hopkins and Lane, 2004; Kempe et al., 1956; Lustig et al., 2009). Human monoclonal antibodies (mAbs) offer an obvious alternative to VIG, and some day a defined cocktail of human mAbs may replace VIG (Xiao and Isaacs, 2010).

Several orthopoxvirus proteins can elicit production of neutralizing antibodies: B5R and A33R, exposed on extracellular enveloped virus (EEV), and A27L, L1R, H3L, D8L, and A17L, exposed on intracellular mature virus (IMV) (Demkowicz et al., 1992; Pütz et al., 2006). To select human antibodies capable of neutralizing orthopoxviruses, a novel phage display scFv library was generated from variable domains of heavy (Vh) and light (Vl) chains cloned from peripheral blood lymphocytes (PBLs) of VACV immune donors (Dubrovskaia et al., 2007). This immune library was earlier panned against a recombinant VARV protein, A30L, and two human scFvs

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able to neutralize orthopoxviruses were selected (Dubrovskaia et al., 2007). In this work, the same immune library was panned against live CPXV and selected phage-displaying single-chain antibodies (pdAbs) were characterized and assayed for neutralizing activity. The most promising pdAb was converted into a fully human format.

2. Materials and methods

2.1. Cells and viruses

Vero E6 cells were grown in the Eagle's medium (DMEM, Gibco BRL) containing 5% heat inactivated fetal bovine serum (FBS). Dilution of viruses and cell infection in virus neutralization experiments were done using the DMEM with 2% FBS.

CPXV strain Grishak, VACV strain Lister, and MPXV strain Zaire 599 were obtained from the repository of the State Research Center Vector, Novosibirsk, Russia. Viruses were propagated in the chorioallantoic membrane of developing chick embryos. Virus particles were purified as earlier described (Tikunova et al., 2005). All experiments with live MPXV were conducted under BSL-4 conditions.

2.2. Virus-specific ELISA of volunteer serum

Blood samples were withdrawn from the volunteers vaccinated or boosted with the licensed VACV L-IVP vaccine (NPO Virion, Russia). The blood serum from a volunteer who had neither been vaccinated nor had had an orthopoxvirus infection was used as a negative control. The study was approved by the Ethics Committee with the State Research Center Vector (license no. IRB0001360). Microtiter plates were coated with 200 ng of VACV and control antigen, 3% bovine serum albumin (BSA, Sigma). Each serum sample was subsequently diluted in PBS/0.05% Tween 20 (initial dilution of 1:200). Each dilution (100-μl aliquot) was added to both the VACV- and control antigen-coated microtiter wells. Indirect ELISA was conducted with alkaline phosphatase-conjugated goat anti-human IgG specific antibody (Sigma) as the secondary antibody and stained with *p*-nitrophenyl phosphate. Absorbance was measured at 405 nm using a microtiter reader (Bio-Rad).

2.3. Construction of immune phage antibody library

Heparinized venous blood (100 ml) was collected from each of two vaccinated healthy volunteers and two healthy volunteers boosted with the licensed VACV L-IVP vaccine (NPO Virion, Russia). Library construction was described in detail earlier (Dubrovskaia et al., 2007). Briefly, the library was constructed with the primers (Marks et al., 1991b) complementary to the 3′- and 5′-ends of the Vh and Vl genes. Gel-purified Vh and Vl PCR products were independently connected by PCR with the DNA linker fragment encoding $S(G_4S)_2AAGSG_4S$ peptide, and the final PCR products encoding the repertoire of human scFv genes were cloned into pHEN2 plasmid (Nissim et al., 1994). The library comprised 3×10^7 independent clones, and 90% of them contained phagemids carrying correct scFv genes.

2.4. Biopanning conditions

The wells of 96-well microtiter plates were coated with 100 mkg/ml of live CPXV in PBS, pH7.4. The wells were washed twice with PBS and blocked with 5% skimmed milk in PBS at 37 °C for 1 h. An aliquot (100 μl) of the immune library containing $\sim 10^{12}$ cfu (colony-forming units) in PBS was added to immobilized antigen and incubated for 2 h at 37 °C. The unbound phages were discarded, and the wells were washed 20 times with PBS/0.05% Tween 20 followed by 20 washings with PBS. The bound phages from each well were eluted with 100 mM triethylamine and neutralized with 1 M Tris–HCl pH 7.4. The eluted phages were used to infect an exponentially growing *Escherichia coli* TG1 culture (Marks et al., 1991a). The amplified phages were subject to another round of panning. The phage particles eluted after the second round of panning were allowed to infect *E. coli* TG1 cells, and the infected cells were plated on the agar containing 100 mg/ml ampicillin and 1% glucose and incubated at 30 °C overnight. Single colonies were picked, and monoclonal pdAbs were isolated and assayed by ELISA.

2.5. Virus-specific ELISA of phage displaying antibodies (pdAbs)

For indirect ELISA, the wells of 96-well microtiter plate were coated with 100 ng of CPXV, VACV, or control antigen (3% BSA) in PBS, pH 7.4. After blocking with 3% BSA in PBS, the phage particles diluted in PBS/0.05% Tween 20 to yield $\sim 10^{10}$ cfu were added to each well. Indirect ELISA was conducted with anti-M13 polyclonal rabbit antibodies followed by alkaline phosphatase-conjugated anti-rabbit IgG (ICN, United States) and stained with *p*-nitrophenyl phosphate. Absorbance was measured at 405 nm using a microtiter reader (Bio-Rad). Anti-Thy pdAb (Griffiths et al., 1994), not binding any of the orthopoxviruses, was used as the control of nonspecific binding. Clones were selected as positive, if their OD_{405} signals were fivefold higher as compared with the control antigen.

2.6. Plaque-reduction neutralization test (PRNT)

CPXV, VACV, or MPXV suspensions at an optimal dilution were mixed with an equal volume of polyclonal or monoclonal pdAbs at selected dilutions. The mixtures were incubated at 37 °C for 1 h and inoculated onto confluent Vero E6 cell monolayers in 24-well culture plates (Nunc). After incubation at 37 °C for 1 h, the mixtures were removed, and the cells were washed with DMEM and overlaid with the DMEM containing 2% FBS and 0.24% melted agarose. Two–three days after infection, the viable cells were stained with 0.1% crystal violet with 10% formalin, and clear plaques were visualized. Anti-Thy pdAb (Griffiths et al., 1994), not binding to any of the orthopoxviruses, was used as a control of nonspecific neutralization. To assay neutralization by a complete antibody (fh b9), PRNT was performed under the same conditions. Neutralization titer was calculated according to $N = (V_0 - V_n)/V_0 \times 100\%$, where V_0 is the number of plaques in control wells and V_n, the number of plaques in tested wells.

2.7. Western blot

In order to test the selected pdAbs, CPXV, VACV, or *E. coli* proteins were fractioned by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes (Sigma). The membrane was blocked with 5% dry skim milk in PBS and incubated with 10^{10}–10^{11} pdAbs for 1 h at 37 °C. Then the membrane was incubated with secondary anti-M13 polyclonal rabbit antibodies followed by alkaline phosphatase-conjugated anti-rabbit IgG (ICN). The protein–antibody interactions were visualized by incubating the membrane in a mixture of nitro blue tetrazolium (Sigma) and 5-bromo-4-chloro-3-indolyl phosphate (Roche) for 20 min at a room temperature. To test volunteer serum samples, the membranes after blocking were incubated with the diluted serum samples for 1 h at 37 °C. After washing with PBS/0.05% Tween 20, the membranes were incubated with alkaline phosphatase-conjugated goat anti-human IgG (Sigma) and stained with a mixture of nitro blue tetrazolium (Sigma) and 5-bromo-4-chloro-3-indolyl phosphate (Roche). The blood serum from a volunteer who had never been vaccinated nor infected by orthopoxviruses was used as a negative control.