Multimeric humanized varicella-zoster virus antibody fragments to gH neutralize virus while monomeric fragments do not

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The relative roles of antibody and cell mediated immunity in the control of varicella-zoster (VZV) infection are still not clearly understood. In an early and oft cited hypothesis, Hope-Simpson (1965) speculated that periodic subclinical VZV reactivation played a pivotal role, boosting antibody titres, and thus suppressing the neuronal VZV infection. With an expanded knowledge of cell mediated immunity, especially the cytotoxic T cell response, an increased emphasis has been placed on cellular mechanisms (Abendroth & Arvin, 2000). Yet, there remain compelling reasons to suspect that antibody can alter the course of VZV infection. In particular, the large body of data from clinical trials with zoster immune globulin (ZIG) clearly established that intramuscular administration of high titre VZV antibody abrogates a nascent primary VZV infection (Zaia et al., 1983). How antibody exerts this effect is not entirely clear, but the most likely explanation relates to an expanded concept of neutralization of inoculum virus before vireaemia occurs (Grose et al., 2000).

The VZV specific antibody with the highest documented neutralization titre is directed against VZV glycoprotein gH (Grose, 1990). Of particular interest, one of these anti-gH murine monoclonal antibodies (MAb 206) (Montalvo & Grose, 1986) is capable of neutralizing VZV in vitro in the absence of human complement. Furthermore, blockage of viral egress and inhibition of cell-to-cell spread of VZV in cultured cells have also been demonstrated (Rodriguez et al., 1993). Because of the large body of knowledge about this anti-gH monoclonal antibody and its apparent biological relevance, we selected it as a candidate for humanization. Moreover, we investigated whether recombinant antibody fragments could be generated that retain these neutralizing properties.

Transfer of the 206 murine CDRs to human frameworks was achieved by oligonucleotide site-directed mutagenesis (Tempest et al., 1991; Nakamaye & Eckstein, 1986) to form the hu VH and hu VK sequences. The templates for mutagenesis were M13HuVHLYS, which contains VH framework regions from human antibody NEWM and VH CDRs from murine antibody D1.3 cloned into M13VHPCR1, and M13HuVKLYS, which contains VK framework regions from human antibody REL and VK CDRs from murine antibody D1.3 cloned into M13VKPCR1 (Riechmann et al., 1988). The oligonucleotides used were: VHCDR1, 5’ dTGTCT CACCCAGCTC ATCCA; VHCDR2, 5’ dTGTCT GGTGT; GCAGG TCAGG 3’. The VZV specific antibody with the highest documented neutralization titre is directed against VZV glycoprotein gH (Grose, 1990). Of particular interest, one of these anti-gH murine monoclonal antibodies (MAb 206) (Montalvo & Grose, 1986) is capable of neutralizing VZV in vitro in the absence of human complement. Furthermore, blockage of viral egress and inhibition of cell-to-cell spread of VZV in cultured cells have also been demonstrated (Rodriguez et al., 1993). Because of the large body of knowledge about this anti-gH monoclonal antibody and its apparent biological relevance, we selected it as a candidate for humanization. Moreover, we investigated whether recombinant antibody fragments could be generated that retain these neutralizing properties.

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regions and control elements, were used to transfect NS0 cells (European Collection of Animal Cell Cultures, Porton, UK, ECACC no. 85110505). Humanized antibody was purified by protein A chromatography.

The biological property of greatest interest is the ability of the anti-gH antibody to neutralize infectious virus in a plaque-reduction assay (Grose et al., 1979). Therefore, we sought to determine whether this property was retained in the humanized antibody. An aliquot of antibody ranging from 1-2 to 50 μg in a 1 ml volume was added to 1 ml of virus suspension and the 2 ml mixture was incubated for 1 h prior to inoculation onto a 35 mm MeWo cell monolayer. When the monolayers were examined 3 days later, no more than ten small plaques were visualized in any of the five monolayers inoculated with both virus and humanized antibody in a final concentration of at least 2.5 μg/ml. The virus control exhibited more than 100 plaques. This observation was easily reproducible and suggested that the humanization procedure had been successful because the recombinant antibody retained the same complement independent neutralization property of the murine antibody.

The laboratory strain VZV-32 (Grose & Brunell, 1978) is a low passage virus (fewer than 20 passages) isolated from a child with chickenpox. To be assured that there was no variation in susceptibility to neutralization between wild-type VZV strains, isolates from ten other children with chickenpox, collected in the USA between the years 1977–1990, were tested by plaque reduction. Among these ten wild-type isolates, there were no major differences in susceptibility to neutralization compared to strain VZV-32. In general, most VZV isolates were neutralized by 1–2–2.5 μg/ml of humanized antibody.

In order to evaluate whether the humanized MAb was superior to VZIG, we purchased two vials of VZIG (Massachusetts Public Health Biologic Laboratories, Boston, MA, USA). Each vial was selected from a different lot (MVZIG-44 and MVZIG-46). The VZV neutralization assays were repeated as previously described. The total protein content of VZIG was 165 mg; the initial plaque-reduction assays were carried out with the same concentrations of antibody protein, i.e. between 1 and 100 μg/ml. No inhibition of plaque formation was observed. Therefore, the amount of antibody was increased by directly adding VZIG at much larger volumes. Only when a final VZIG concentration of 6 mg/ml was attained was there a greater than 80% reduction in plaque formation. If a level of 2-5 μg/ml (167 nM) of humanized Ab is considered necessary for plaque inhibition, then a comparable level for VZIG is 6000 μg/ml. The biological activity of the humanized antibody is approximately 2400 times that of the standard VZIG preparation on a mg per mg basis. There is an obvious caveat to this comparison: even though human VZIG is prepared from whole IgG with a high anti-VZV titre, a major portion of the IgG will have specificities other than the VZV gH antigen.

Fig. 1. Bacterial expression vectors. All coding regions were cloned (HindIII/EcoRI) into the multiple cloning site of pUC19, downstream of the lac promoter. pPM1-His was derived from a previously described vector (McGregor et al., 1994; Molloy et al., 1995). PeBI is a leader sequence directing secretion to the E. coli periplasm (Ward et al., 1989). VH (117 amino acid residues) and VK (111 residues) are the variable heavy and light regions of the antibody, respectively. A peptide linker of 14 or 5 amino acid residues links the heavy and light chains in all vectors except p0LINK-His. Hu Ck denotes human constant light (kappa) domain (107 residues), which was used for detection in ELISA and Western blot. 6-His denotes 6 histidine residues used for purification by immobilized metal chelate affinity chromatography.

ATATA GTTTA TCGTA CTGCT ATTC TCCAA TCCACTCA 3' VHCDR3, 5' dCCTTG GCCCC AGTAG TCTAT AGGAC CACCC TCTTG CAC- AATAA 3', VKCDR1, 5' dTACCA GTGCA TATAA CTAGA GCCAG ATGTA CTGAC ACTTT TGCTG GCCCT ACGAG TGATGGT 3'; VKCDR2, 5' dCACAC CAGAT TCTAG GTTGG ATGCA AAGTAG 3'; VKCDR3, 5' dGTCC CTTGG CCGAA CGTGA ATGGA AGCTC CCTAC TGTGC TGGCA GTAGTAG 3'. Use of these oligonucleotides led to a number of additional murine residues being deliberately substituted into the human variable region frameworks. Specifically, the VHCDR1 oligonucleotide incorporated framework alterations at residues 24 (Val changed to Ala: V24A), 27 (Ser to Phe: S27F) and 28 (Thr to Asp: T28D) and the VHCDR2 oligonucleotide incorporated a framework alteration at residue 71 (Val changed to Arg: V71R).

Mammalian expression vectors pSVgpt and pSVhyg (Mulligan & Berg, 1981), containing the hu206 VH and VK genes, respectively, together with the appropriate constant
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Fig. 2. Antigen binding cell monolayer ELISA. In (a), parental hu206 MAb (▼) was compared to hu206 scAb-14 (●) binding to VZV infected cells. Binding of hu206 MAb (▼) and scAb-14 (○) to uninfected cells was also tested. In (b), binding of hu206 scAb-14 (■), scAb-5 (▲) and scAb-0 (▼) to infected cells was measured. Similarly, binding of hu206 scAb-14 (□), scAb-5 (○) and scAb-0 (▼) to uninfected cells was also tested. Each point represents a mean of two replicates, and each experiment was performed in duplicate.

To further investigate the nature of the hu206 antibody–antigen interactions and provide additional insight into the mechanism of neutralization, we cloned and expressed single-chain antibody fragments (hu206 scAb). These are similar to scFv (Bird et al., 1988), but in addition to V\textsubscript{H} and V\textsubscript{\kappa}, they also contain the whole human light chain constant region (hu C\textsubscript{\kappa}), and have a molecular mass of approximately 40 kDa (Fig. 1) (McGregor et al., 1994). A pUC19-based periplasmic expression vector, pPM1-His (Molloy et al., 1995), was used for expression in Escherichia coli XL-1 Blue (Stratagene).

Three different constructs were prepared and expressed, with linkers of length 14, 5 and 0 amino acid residues (Fig. 1). Antibody fragments were purified by immobilized metal affinity chromatography (Dooley et al., 1998). Protein concentration was determined by capture ELISA (McGregor et al., 1994) and protein samples analysed on 15% SDS-polyacrylamide gels (Sambrook et al., 1989) and size exclusion HPLC (McGregor et al., 1994) (results not shown). These antibody fragments were initially tested for antigen binding in a MeWo cell monolayer ELISA (Sugano et al., 1991). When compared to the parental MAb (Fig. 2a), the 14 residue linker hu206 scAb (scAb-14) appears to have similar functionality in binding to infected cells, while neither the MAb nor the scAb appear to bind to uninfected MeWo cells. The 5 amino acid residue linker scAb (scAb-5) appears to have reduced functionality compared to scAb-14 (Fig. 2b), while the 0 residue linker scAb (scAb-0) exhibited only a small amount of binding activity in this assay.

Despite positive results in the cell monolayer ELISA, neutralizing activity could not be detected in the standard hu206 scAb-14 at concentrations up to 2000 nM, while the parental hu206 MAB had a 50% neutralizing concentration of about 4 nM (Fig. 3). In contrast, scAb-5 and scAb-0, which were predicted to form multimeric diabodies or triabodies, had 50% neutralizing concentrations of approximately 90 and 700 nM respectively. To provide further evidence suggesting a role for valency in neutralization, hu206 scAb-14 was pre-incubated with 50 µg/ml of goat anti-hu C\textsubscript{\kappa} secondary antibody for 1 h at 4 °C, before dilution and use in a neutralization assay (Fig. 3). When pre-incubated with secondary antibody, which would be expected to induce multimerization, scAb-14 had a 50% neutralizing concentration of about 20 nM, while neither scAb-14 nor the secondary antibody alone had detectable neutralizing activity.

Papain was used to digest whole hu206 antibody (Schofield et al., 1997), and when tested for antigen binding in a cell monolayer ELISA (Fig. 4a), hu206 Fab had a very similar binding profile to that of the whole hu206 MAb, despite the monomeric nature of the Fab. However, when analysed in a neutralization test (Fig. 4b), the Fab was unable to reduce the number of plaques by 50%, even at 500 nM, while the 50%
neutralization concentration of the MAb was only 2 nM. When the hu206 Fab was pre-incubated with anti-hu Cκ secondary antibody, as for the scAb, the 50% neutralization titre was approximately 11 nM (Fig. 4b).

Humanized antibodies have several potential advantages over murine monoclonal antibodies (Winter & Harris, 1993): (i) their expected reduced immunogenicity allows repeated administration without the induction of a host anti-globulin response, (ii) the human Fc portion allows efficient activation of host effector functions and (iii) the presence of a human Fc prolongs the serum half-life of the antibody. Given the increased in vitro neutralization efficacy of the 206 antibody compared to VZIG, the availability of a humanized version may warrant the re-evaluation of the ability of antibody to modify the progression of established VZV infection in vivo.

It appears that single-chain antibody fragments expressed in E. coli can also neutralize VZV, but only when expressed as so-called diabodies or triabodies (Holliger et al., 1993; Kortt et al., 1997; Lawrence et al., 1998), or if multimerization is induced by a secondary antibody. Similar results were obtained for Fab fragments produced by papain digestion of whole hu206 MAb purified from mammalian cells. These observations raise questions about the mechanism of neutralization of the virus by antibody at this epitope. The apparent requirement for multimerization for neutralization may be explained in various ways. Firstly, it may simply be an issue of size i.e. smaller molecules are less able to block the fusogenic function of gH, due to reduced steric hindrance. The larger MAb, and antibody fragments bound by secondary antibody, may more efficiently neutralize the virus because increased steric hindrance blocks a critical step in the fusion process. Alternatively, they may cause aggregation or cross-linking of virus envelope structures. It is, however, unlikely that the neutralization effect of 206 antibody and fragments is dependent on aggregation of the virions, because, despite its name, ‘cell-free’ VZV prepared by sonic disruption of infected cells tends to be a heterogeneous mixture of virions associated with fragments of cell membrane (Cohen & Straus, 1996). The literature also suggests that virus aggregation, although possible, is not always the most likely cause of neutralization. Mason et al. (1996) described a single-chain antibody against foot-and-mouth disease virus (FMDV) that is only weakly neutralizing in vitro unless incubated with a secondary antibody. Aggregation of the FMDV did not appear to be the mechanism for neutralizing. Thullier et al. (1999) report that a recombinant Fab against dengue virus neutralizes in vitro, but to a degree substantially less than would be expected given its affinity compared to the parental antibody. Like antibody 206, this antibody also binds to an envelope protein, and the authors postulate that the reduced neutralization titre was due to a decrease in steric hindrance caused by the Fab, compared to the MAb. Similarly, Lamarre & Talbot (1995) found that while proteolytically generated Fab fragments, used at sufficiently high concentrations, were able to neutralize coronavirus infectivity both in vitro and in vivo, neutralization was significantly greater in bivalent F(ab’2) fragments, but this effect was not mediated by aggregation of virions.

The possibility that simply an increased avidity of multimeric antibody (fragments) compared to monomeric fragments is responsible for their increased neutralizing potency cannot be ruled out. Avidity refers to the apparent increase in affinity observed when multiple antigen binding sites for a given epitope are located on a single molecule. The physical basis for this effect is that when the first binding site is bound to a surface, the concentration of additional antigen binding site(s) is dramatically increased. Although any avidity effect due to multimerization forced by shortening the linker failed to manifest in the cell monolayer ELISA, it may be that the
distribution or orientation of the 206 epitope is different on cell-free VZV, such that multivalent binding would become possible.

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