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Lantto, Johan; Fletcher, Jean M; Ohlin, Mats

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A divalent antibody format is required for neutralization of human cytomegalovirus via antigenic domain 2 on glycoprotein B

Johan Lantto,[1] Jean M. Fletcher[2] and Mats Ohlin[1]

[1] Department of Immunotechnology, Lund University, PO Box 7031, S-220 07 Lund, Sweden

[2] Department of Immunology, Royal Free and University College Medical School, Royal Free Campus, London NW3 2PF, UK

Glycoprotein B (gB) of human cytomegalovirus (HCMV) is the dominating protein in the envelope of this virus and gives rise to virus-neutralizing antibodies in most infected individuals. We have previously isolated a neutralizing human antibody specific for antigenic domain 2 (AD-2) on gB, a poorly immunogenic epitope, which nevertheless is capable of eliciting potent neutralizing antibodies. In order to define parameters important for the neutralization of HCMV via gB, we have investigated the virus-neutralizing capacity and the kinetics of the interaction with AD-2 of the monomeric and dimeric forms of a single chain variable fragment (scFv) corresponding to this antibody. We demonstrate here that neutralization of HCMV via AD-2 on gB can be mediated by dimeric scFv, while monomeric fragments cannot mediate neutralization of the virus, despite a slow dissociation from the intact glycoprotein. This finding is discussed in the context of possible mechanisms for antibody-mediated virus neutralization.

Human cytomegalovirus (HCMV) is a member of the *Herpesviridae* with a very high prevalence in the population. The infection is usually asymptomatic in healthy adults, but the virus remains a major cause of morbidity in immunocompromised individuals such as AIDS patients and transplant recipients. Congenitally infected children may also suffer severe sequelae as a consequence of infection with HCMV (Ho, 1991). Thus, a functional immune system appears to be able to control the virus infection *in vivo*, but the virus has also invented numerous ways of escaping immune surveillance (Loenen *et al.*, 2001), allowing it to establish life-long latency. Both cellular and humoral responses have been reported to contribute to the limitation of HCMV infections, but their relative significance is uncertain. Several pieces of evidence indicate, however, that antibodies have an important role in protecting against HCMV infection and decreasing the severity of the disease caused by the virus. For instance, passive transfer of antibodies has been shown to improve the outcome of the disease in both transplant recipients and prematurely born infants (Snydman *et al.*, 1995; Zaia, 1993). It has also been shown that maternal seroimmunity to HCMV prior to conception provides considerable protection against harmful congenital HCMV infection in the infant (Fowler *et al.*, 1992). Many *in vitro* neutralizing monoclonal antibodies have been raised against surface components such as glycoproteins B (gB) and H of the virus (Spaete *et al.*, 1994), but there is still little known about how antibodies are able to mediate protection *in vivo*. gB is the major membrane-associated protein of HCMV and has been shown to give rise to a large fraction of all virus-neutralizing antibodies in infected individuals (Britt *et al.*, 1990). This antigen contains a set of neutralization epitopes, among which antigenic domains 1 (Utz *et al.*, 1989) and 2 (AD-2) (Meyer *et al.*, 1992) are the best characterized. AD-2 is, however, not very immunogenic and induces antibodies in only a fraction of infected individuals (Ayata *et al.*, 1994; Meyer *et al.*, 1992; Navarro *et al.*, 1997; Schoppel *et al.*, 1997). The effective neutralization mediated by AD-2-specific antibodies *in vitro* has warranted extensive studies and even clinical trials using such reagents (Azuma *et al.*, 1991), despite the fact that only very few antibodies recognizing this epitope have ever been developed (Masuho *et al.*, 1987; Ohlin *et al.*, 1993). The mechanism behind the neutralization mediated by antibodies against AD-2 is not fully understood, although they seem not to block the initial binding of the virus to the target cell (Ohizumi *et al.*, 1992). In order to shed light on the mechanism of action, we have investigated the ability of a human antibody specific for AD-2 on gB to interact with this epitope in monomeric and dimeric form.

The gene encoding the AE11 single chain variable fragment (scFv) corresponding to the human antibody ITC88 (Ohlin *et al.*, 1993), which recognizes AD-2 on HCMV gB, has been

Author for correspondence: Mats Ohlin.
Fax +46 46 2224200. e-mail mats.ohlin@immun.lth.se

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described previously (Ohlin *et al.*, 1996). This gene was recloned into the pPICZα vector (Invitrogen) for large-scale production of soluble protein carrying the FLAG epitope (Hopp *et al.*, 1988) (named AE11F) in *Pichia pastoris*. Following induction of protein expression with methanol, the scFv was purified by a combination of ultrafiltration (Filtron Ultrasette with Omega membrane, MWCO: 10 kDa; Pall Gelman Sciences), ion exchange chromatography (SP Sepharose Fast Flow; Amersham Pharmacia) and size exclusion chromatography on Superdex 75 (Amersham Pharmacia). Both the monomeric and dimeric forms of the scFv were obtained in this way. Microsequencing of the N terminus of the product confirmed that the yeast-derived leader sequence was properly removed.

The antibody neutralization assays were performed with the AD169 strain of HCMV (European Collection of Animal Cell Cultures, Salisbury, Wiltshire, UK) in 48-well plates, essentially as described by Roy & Grundy (1992). In addition to purified monomers and dimers of the AE11F scFv, dimerized monomeric scFv were also analysed. These were obtained by incubation of monomeric scFv in minimal essential medium containing 2% fetal calf serum for 30 min at 37 °C with anti-FLAG antibody (anti-FLAG M2; Sigma Chemical Company) at a molar ratio of 2:1. Serial dilutions of the various scFv preparations were incubated for 60 min at 37 °C with the concentration of virus required to produce 50–100 viral plaques per well. The various dilutions were then inoculated in triplicate for 60 min on to fibroblast monolayers. The cell monolayers were washed twice and a 2% methylcellulose overlay was added. After incubation for 12 days at 37 °C in 5% CO_2, the cells were fixed in 10% (v/v) formalin and stained with 0·03% methylene blue. The numbers of viral plaques per well were counted, and the mean number of p.f.u./ml was calculated for each dilution of a given antibody format. The plaque reduction and the effective concentration giving 50% plaque reduction (EC_{50}) were calculated using the Reed–Muench method (Thorpe *et al.*, 1987). In order to investigate the antigen-specificity of the interaction, neutralization assays were also performed in the presence of an AD-2-mimicking peptide (ANETIYNTTLKYGDV). A peptide incorporating residues known to be important for recognition by the scFv (Ohlin *et al.*, 1996) in scrambled order (YTLETYINKT) was used similarly as a negative control. The assays were performed with dimeric scFv at a concentration approximately equal to their EC_{50} value (1 μg/ml).

The reaction rate kinetics of the scFv with the peptide antigen and recombinant gB produced in eukaryotic cells (Spaete, 1991) were studied using the BIAcore technology (BIAcore AB). The antigens were bound to a CM5 sensor chip either through direct coupling using the Amine Coupling Kit (BIAcore AB) in the case of gB (approximately 15000 RU) or through binding to similarly immobilized streptavidin (approximately 800 RU) in the case of the biotinylated peptide antigen.

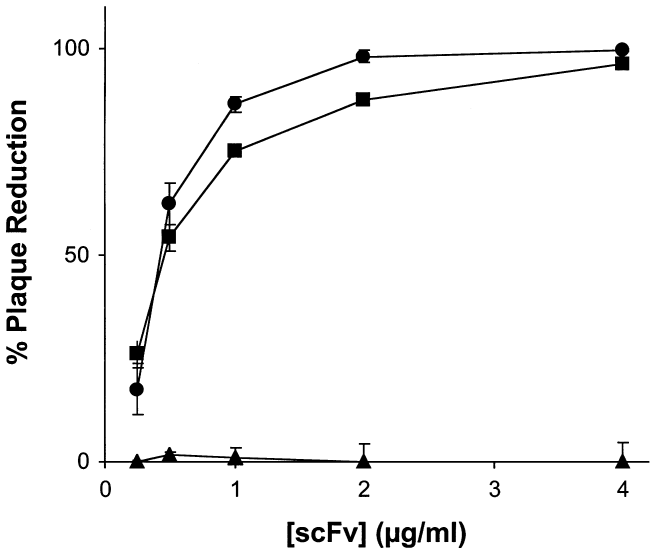

Fig. 1. Neutralization of HCMV strain AD169 infection of human embryonic lung fibroblasts in the presence of monomeric (▲) and dimeric (●) scFv, and monomeric scFv dimerized with the anti-FLAG M2 antibody (■). The plaque reduction was calculated using the Reed–Muench method (Thorpe *et al.*, 1987). Experiments for all data points were performed in triplicate. The error bars indicate SEM.

Immunofluorescence studies showed that both monomers and dimers of the AE11F scFv bound to virus-infected cells and displayed similar titration endpoints (data not shown). When assessing the neutralizing capacity of the various preparations, it became apparent that the dimeric but not the monomeric scFv format could mediate virus neutralization *in vitro* as assessed by their effect on the ability of the virus to infect human embryonic lung fibroblasts (Fig. 1). The EC_{50} value of the dimeric scFv was calculated at 0·6 μg/ml, while virus neutralization could not be detected with the monomeric form, even at a concentration of 10 μg/ml (data not shown). The neutralizing activity of the monomeric protein could, however, be restored on addition of a cross-linking antibody, which recognizes the C-terminal FLAG epitope on each scFv (Fig. 1). The EC_{50} value of this format was found to be 0·5 μg/ml, i.e. more or less the same as for the dimeric scFv. We thus concluded that neutralization of HCMV with AD-2-specific antibodies or fragments thereof requires a bulkier, divalent construct to be effective. The antigen specificity of the interaction was demonstrated by the fact that the scFv-mediated neutralization could be inhibited by the addition of a soluble 15-mer peptide recognized by the scFv (Fig. 2).

The fact that only dimeric constructs were able to mediate virus neutralization may have several explanations. It is not likely that the association rate constant, which has previously been demonstrated to be important for neutralization of some viruses (Roost *et al.*, 1995), is the critical factor determining virus neutralization in this study, since association rates are not greatly affected by the valency of the reagents. In fact, a

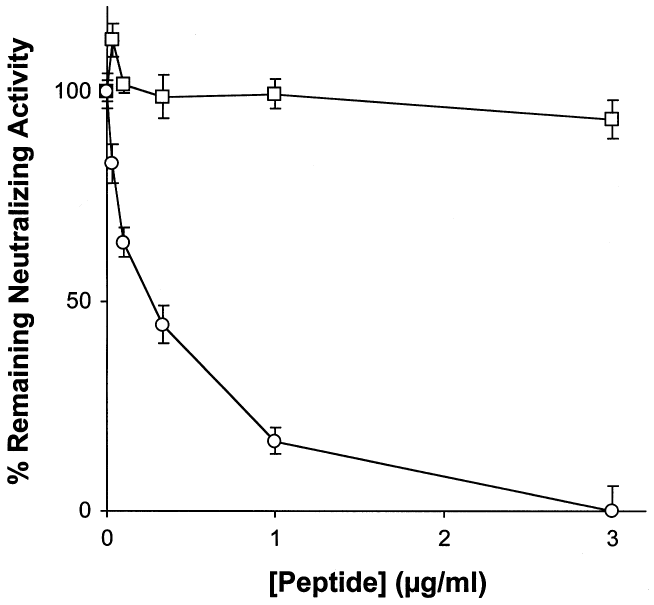

Fig. 2. Inhibition of the neutralizing activity of dimeric scFv by the addition of a soluble AD-2-mimicking peptide (○), or a negative control peptide (□). Experiments for all data points were performed in triplicate. The error bars indicate SEM.

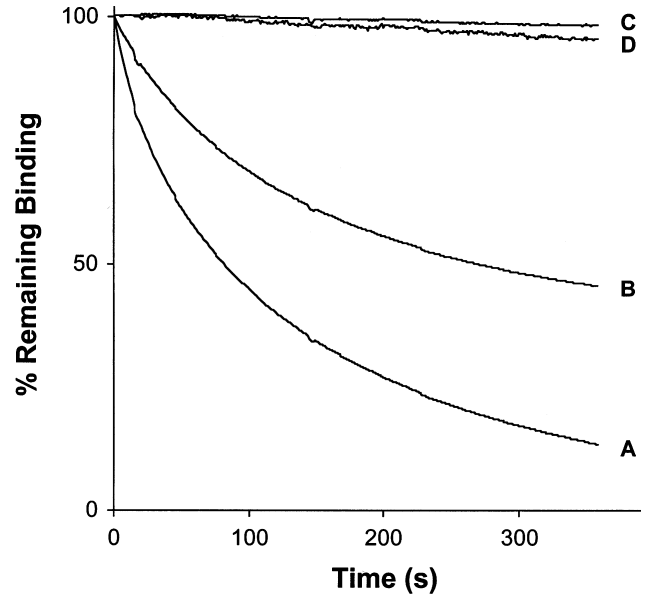

Fig. 3. Dissociation of monomeric and dimeric AE11F scFv from a 15-mer peptide antigen mimicking AD-2 (A and B, respectively) and from recombinant gB (C and D, respectively) immobilized on to a BIAcore CM5 sensor chip. Both scFv formats were analysed at a concentration of 34 μg/ml and the obtained curves were normalized along the *y*-axis to facilitate comparison.

smaller protein molecule, in this case the monomeric scFv, can be expected to find its target more rapidly as a consequence of its higher diffusion rate. Rather, it is suggested that the neutralizing activity depends either on: (i) the ability of dimeric scFv to remain for an extended period of time on the surface of the virus particle; (ii) the larger size of the dimeric format; or (iii) the capability of dimeric scFv to cross-link epitopes. In light of the fact that AD-2-specific antibodies seem to neutralize the virus without preventing binding of the virus to the cell surface (Ohizumi *et al*., 1992), the time of residence of the scFv on the virus particle is a particularly important parameter to review. The half-life of the scFv on the short (15-mer), synthetic AD-2-mimicking peptide was relatively short (Fig. 3), in the order of minutes ($k_d = 6 \times 10^{-3}$ s^{-1}), suggesting that dissociation of monomeric scFv from the antigen may prevent their effectiveness in virus neutralization. However, as the peptide only partly mimics the natural antigen, we also investigated the half-life of the scFv on recombinant gB to understand better this factor. As shown in Fig. 3, it was evident that the time of residency of the monomeric scFv on the intact, extracellular part of gB was much longer, in the order of hours ($k_d = 1 \times 10^{-4}$ s^{-1}), than on the peptide antigen. This strongly suggests that monomeric scFv will remain bound to virus particles for extended periods of time. As the adsorption and infection phase was allowed to proceed for only 1 hour, it also indicates that dissociation from the epitope is not the most likely explanation for the inability of monomeric scFv to mediate virus neutralization. The fact that virus may bind to target cells even in the presence of AD-2-specific antibodies (Ohizumi *et al*., 1992) suggests that bound virus may remain on the cells for a long enough time to allow sufficient amounts of the monomeric scFv to dissociate, allowing infection to proceed. However, we consider this scenario an unlikely one, as the difference in half-life on gB between monomeric and dimeric scFv as determined by BIAcore measurements was rather small (Fig. 3). Instead, other factors may be at play here. It has recently been strongly argued that antibodies mediate virus neutralization mainly through steric hindrance and that the level of occupancy of epitopes on the virus is the critical factor for neutralization (Burton *et al*., 2000, 2001). In this context it may be suggested that the monomeric scFv is too small to exert a blocking effect, while the dimeric form may reach above a critical size limit at which the blocking of infection may occur. Similarly, the binding of the anti-FLAG mouse IgG to the scFv may provide the volume and size that prevents infection from occurring. However, this explanation can at present not be distinguished from the possibility that cross-linking of gB mediates a conformational change in the protein, making it unable to participate in the infection process. Further studies of these mechanisms will be required to resolve this issue fully, and given that gB is known to be a homodimer in its mature, membrane-bound form (Britt & Vugler, 1992), the latter possibility should perhaps be rewarded particular attention.

Irrespective of the precise molecular mechanism, our results show that low molecular mass, monomeric scFv against AD-2 on gB are unable to neutralize the virus. This agrees with

recent findings of antibodies against another herpesvirus, varicella-zoster virus, where scFv or Fab fragments against glycoprotein H were unable to mediate a virus-neutralizing activity *in vitro* (Drew *et al*., 2001). Multimerized scFv are, as in the case of dimerized anti-AD-2 scFv, able to neutralize virus infection. This is in contrast to murine vesicular stomatitis virus (VSV), which can be neutralized with monomeric scFv (Kalinke *et al*., 1996). It is possible that the difference in architecture of the two types of viruses – almost crystalline VSV particles versus membranous herpes virus particles – in some way accounts for the differences in neutralization effectiveness of low molecular mass antibody fragments. Naturally, the role of the target antigen in the infection process may also be crucial in determining the ability of monovalent antibody fragments to mediate virus neutralization, and it is a factor that warrants further investigation in additional virus systems.

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