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A recombinant Fab neutralizes dengue virus in vitro

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Abstract

A recombinant Fab that recognizes a neutralizing epitope located in the (296–400) region of protein E of dengue virus was obtained from cloned hybridoma cells secreting the mouse monoclonal antibody (mAb) 4E11. The Fd and light chain antibody genes were amplified by polymerase chain reaction, cloned into the phagemid vector pMad, expressed in bacteria to produce Fab fragments and sequenced. The mAb 4E11, in particular its light chain complementary-determining regions, shared homologies with two other anti-viral mAbs. The affinity of the parental mAb and the cloned Fab to the MalE-E(296–400) fusion protein were shown to be of the same magnitude, i.e. nanomolar. Fab 4E11 neutralization capacity was found between 8 and 4-times or less lower than that of mAb 4E11, depending on serotypes, thus the Fab could have a smaller antiviral activity than the mAb in vitro. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

Dengue virus is a member of the *Flaviviridae* family. This virus causes a disease prevalent between 30th north and 20th south parallels and its incidence has been estimated to 60 million per year, causing 30 000 deaths (Tolou et al., 1997), with appearance of hyper endemic transmission in the Americas (Gubler, 1998). It is transmitted among humans by mosquitoes, mainly *Aedes aegypti*.

A wide spectrum of symptoms can be encountered, ranging from a benign fever with occasional hemorrhages to a potentially fatal syndrome. Without medical intervention, the case fatality rate of the hemorrhagic and shock syndrome can reach 40–50% (Igarashi, 1997). The mechanisms of this syndrome are still under discussion: (i) specific strains could be involved (Murgue et al.,

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1997); and (ii) preexisting subneutralizing antibodies could enhance the antibody-mediated uptake of the virus by monocytes or macrophages which are designated as host cells of the virus (antibody dependent enhancement: ADE) (Hawkes and Lafferty, 1967; see review in Porterfield, 1986). The requirement for the Fc region of IgG in ADE has been demonstrated by Halstead and O'Rourke (1977) who compared whole human serum, that had enhancing activity and Fab'_2 of the same origin that had not. The requirement for fonctional Fcγ receptors in ADE has also been shown (Littaua et al., 1996). These receptors, present at the surface of macrophages, play a major role in the phagocytosis of antibody-coated particles (Paul, 1993) and are dependent on the Fc binding of IgG.

No specific prophylactic or therapeutic agent is currently available against dengue disease. Intravenous infusion to correct hypovolemia and eventually blood transfusion, can reduce the mortality rate. Monoclonal antibodies passively transferred to mice challenged with a lethal intracerebral dose of dengue 2 virus have been shown to be protective (Kaufman et al., 1987). In the case of another virus (vesicular stomatitis virus) protection against lethal infection has been shown to be mediated by neutralizing antibodies (Zinkernagel, 1997). Immunotherapy of dengue, as prophylaxis or treatment, could thus be of interest. IgG fragments lacking Fc (scFv or Fab) should be used so that they do not participate in any enhancing effects (ADE). For this work, Fab were prefered to scFv because they have a better stability, resulting in a longer half-life. A Fab has recently been shown to have a 55-fold greater half-life than its corresponding scFv (Lamarre et al., 1997).

Recombinant IgG fragments have already been obtained against influenza virus (Caton and Koprowski, 1990; Stark and Caton, 1991), HIV virus (Burton et al., 1991), rabies virus (Cheung et al., 1992; Muller et al., 1997), respiratory syncitial virus (Tempest et al., 1991; Barbas et al., 1992), hepatitis B virus (Zebedee et al., 1992), hantaan virus (Liang et al., 1996), coronavirus (Lamarre and Talbot, 1995) and against such flaviviruses as looping ill and tick-borne encephalitis (Jiang et al., 1993) but not against dengue virus.

In this study, we have cloned in a phagemidic vector the Fab region of the murine monoclonal antibody (mAb) 4E11, raised against dengue virus. Immunochemical and serological properties of the mAb and the Fab were compared.

2. Materials and methods

2.1. Preparation and characterization of the hybridoma

Hybridoma cells producing a murine monoclonal antibody 4E11, recognizing dengue virus, serotype 1 envelope protein, were prepared according to protocols published previously (Morens et al., 1987). These cells were a generous gift from Dr D.M. Morens. The mAb is of the IgG2a, k, isotype and is protective in vivo against dengue virus, serotype 1: 0.4 mg protected all 3 weeks-old BALB/c mice challenged with 100 DL_{50} (data not shown).

2.2. Production of phage-displayed Fab fragments

Total RNAs were isolated from 5×10^6 cells of the mouse 4E11 hybridoma (RNAxel®, Eurobio, Les Ulis, France). cDNA was synthetized using random hexanucleotides (Lafaye et al., 1995) and MLV enzyme (Eurobio), then PCR-amplified (Saiki et al., 1985). The PCR primers harboured a restriction site that enabled the double-stranded PCR products to be cloned into the vector. The 5′ primers were designed to hybridize to partially conserved sequences coding for the N-terminus of variable regions. Four 5′ primers for light chain and eight 5′ primers for Fd had to be tested, depending on the family to which belonged the template. The 3′ primers hybridize in the region corresponding to the light chain C-terminus and to the hinge region of Fd (Kang et al., 1991). PCR conditions were described elsewhere (Lafaye et al., 1995). After digestion by *Sac*I and *Xba*I (Gibco, Gaithersburg, MD), the light chain gene was cloned into the phagemidic expression vector pMad (kindly given to us by

G. Orfanoudakis, E.S.B.S., Strasbourg). After digestion with *Xho*I and *Spe*I (Boehringer, Mannheim, Germany), the Fd gene was cloned in frame with the gene for minor coat protein III (PIII) of filamentous phages, in the vector having already received the light chain gene (for a schematic drawing of the construct, see Barbas et al., 1991).

2.3. E. coli expression and purification of phages-Fab

The phagemid was then introduced into competent *E. coli* cells by $CaCl_2$ transformation (Sambrook et al., 1989) at an efficiency rate of 2×10^5 transformants per mg phagemid. The phage-Fab was obtained as described elsewhere (Lafaye et al., 1995), then it was titrated (Parmley and Smith, 1988) and analysed by ELISA, as described below.

2.4. Production of Fab fragments

The selected phagemid was digested with *Nhe*I and *Spe*I (Boehringer) to remove the gene coding for PIII, then ligated and transformed (Barbas et al., 1991). This clone was cultivated in 2YT/ampicillin at 37°C until a OD_{600} of 1.8 was reached. IPTG was added at a concentration of 1 mM and the culture was grown overnight at 30°C, with stirring. Cells were pelleted by centrifugation and soluble Fab were recovered from the periplasm by osmotic shock. One ml of buffer (8.6 g saccharose, 100 μl EDTA 0.5M pH 8.0, 10 ml Tris 1 M pH 8.0, milli-Q purified water for a total volume of 50 ml) was used for the extraction of Fab expressed in 70 ml culture. The periplasmic fraction was cleared by centrifugation ($10\,000 \times g$, 4°C, 20 min) and Fab were purified with an affinity column prepared by covalently coupling anti-mouse Fab antibodies (Sigma, St. Louis, MO) to protein G-sepharose 4B (Sigma) with 20 mM dimethylpimelimidate (Sigma) (Harlow and Lane, 1988). The Fab were eluted with 100 mM glycine pH 2.5, neutralized by adding 3M Tris–HCl pH 9.0, dialysed agaist PBS and filtered (Millex-GP 0.22 μm, Millipore, Bedford, MA).

2.5. ELISA

A protein E fragment of dengue virus, strain FGA/89 (Després et al., 1993), located between residues 296 and 400 (E(296–400)), was cloned as a MalE fusion protein in p-MALc plasmid (Biolabs, Beverly, MA) following the procedure described previously (Megret et al., 1992). The protein was produced in *E. coli* and purified on a maltodextrin column according to the manufacturer procedures (Biolabs). This protein at a concentration of 1 μg ml^{-1} in PBS was coated for 2 h at 37°C on microtiter plates (Nunc, Roskilde, Denmark). Plates were washed six times in PBS/0.5% gelatin/0.1% Tween (PGT), then non specific binding was blocked for 1 h at 37°C with PGT. Two-fold dilutions of 100 μl of phage-Fab, starting with 10^{12} Transforming Units per ml were distributed in the wells. The negative control was a non recombinant phage. After 2 h at 37°C, plates were washed and each well was incubated with 100 μl of an anti-phage M13 horse radish peroxydase (HRP) conjugate (Pharmacia, Uppsala, Sweden) and revealed with orthophenylenediamine dihydrochloride (Dako, Glostrup, Denmark). The 490 nm absorbance was measured on an ELISA reader (Dynatech, Guernsey). Concentration of soluble Fab in periplasmic extract were ELISA assayed using a rat anti-mouse chain (Immunotech, Marseille, France) that was coated and a goat anti-mouse Fab HRP conjugate (Sigma) for revelation. The result was established by comparison with the purified recombinant Fab.

2.6. Affinity measurements

Affinity was measured in solution (Friguet et al., 1985). Samples of Fab 4E11 (as periplasmic extract diluted 1/640 in PGT) or mAb 4E11 (5 ng ml^{-1}) were incubated overnight with increasing amounts of MalE-E(296–400), at concentrations ranging from 10^{-11} to 10^{-7} M. The remaining free antigen-binding sites were then quantified by ELISA, using an anti-mouse β-galactosidase conjugate (J. Gregoire, Pasteur Institute, Paris) and 4-methylumbelliferyl β-D galactoside (Sigma). Fluorescence was read (Fluoroskan,

Labsystem, Finland) at 460 nm, after excitation at 355 nm.

2.7. Sequencing, analysis and modeling of the Fab-DNA fragment

Automatized sequencing was performed by Genome Express S.A. (Grenoble, France), using P13 (5′ GCC GCT GGA TTG TTA TTA CTC 3′) and P21 (5′ CAC CCT CAG AGC CAC CAC CCT 3′) for Fd sequencing and KEF (5′ GAA TTC TAA ACT AGC TAG TCG 3′) and Universal Primer (5′ TGA CCG GCA GCA AAA TG 3′) for light chain sequencing.

The DNA sequences in the Genbank/EMBL nucleotide sequence data base were compared using Genetics Computer Group software (University of Wisconsin, WI). The DNA sequences of the Fab heavy and light chains are accessible in Genbank under the following numbers, respectively, AJ131288 and AJ131289.

2.8. Plaque-reduction neutralization tests

Neutralization tests were performed on Vero cells, in 24-wells culture plates with prototype dengue viruses of the four serotypes. Dengue virus strains were the following: Hawaii 1944 for serotype 1, New Guinea C 1944 for serotype 2, H 87 for serotype 3, H 241 for serotype 4. Periplasmic extract containing Fab 4E11, periplasmic extract of non-transformed TG1, purified Fab 4E11 (all stored at −20°C) and parental mAb (stored at high concentration at 4°C) were incubated in two-fold serial dilutions with 100 Focus Forming Units (FFU) of virus overnight at 4°C (Després et al., 1993). The mixture was then incubated, in duplicate, on Vero cells for 2 h at 37°C with Iscov-carboxycellulose 1.6% and then re-incubated at 37°C in a 5% CO_2 incubator during 5 days for serotypes 2 and 4 and 6 days for serotypes 1 and 3. After fixation, plaques were revealed with anti-dengue virus specific hyperimmunized mouse ascitic fluids, then with an anti-mouse HRP conjugate. The neutralization capacity was estimated as the mAb or Fab concentration causing 50% reduction of FFU.

3. Results and discussion

In this work, starting with the murine hybridoma 4E11, we have prepared a recombinant Fab and compared its properties with those of the parental mAb.

3.1. Isolation of Fab cDNA

mRNA was extracted from a culture of hybridoma cells 4E11 and utilized as a template for RT-PCR to obtain cDNA. The 3′ primer CkDNA and the 5′ primer LC7 allowed the amplification of a DNA fragment of a length of 650 bp, which is the size for a DNA fragment coding for a light chain. The 3′ primer IgG2a and the 5′ primer VHIIC allowed the amplification of DNA coding for Fd. The DNA fragment coding for the light chain was inserted first in the pMad phagemidic vector and then the fragment coding for the heavy chain.

One clone constructed with the light chain and Fd genes from 4E11 was ELISA tested for binding to MalE-E(296–400) fusion protein and compared to a phage having incorporated no heterologous gene. The signal obtained with the phage-Fab was constantly above the negative phage and linearly dependent on the phage-Fab concentration (data not shown). For soluble Fab production, the gene coding for minor coat protein III was removed by *Nhe*I and *Spe*I digestion.

3.2. DNA sequence analysis

When compared to the data base, Fd belonged to the VH14 family consisting of four members: X62705 (Moncharmont et al., 1982) with which most identity (90%) was shared, X07144 (Rocca-Serra et al., 1983), M13068 (Roth et al., 1985) and Z22138 (Tillman et al., 1992). These antibodies were directed against an oestrogen receptor (X62705), against an hapten (GAT) (X07144 and M13068) and against DNA (Z22138). The D segment of Fd corresponded to the DQ52 genetic element and the junction segment corresponded to the JH3 genetic element.

The sequence of the light chain of 4E11 belonged to the VK 21 group III subgroup. When compared with sequences of other light chain coding genes, two of the three genes presenting most homologies were from anti-viral IgG. The non anti-viral IgG was directed against CD 18 (95.2% identity). The anti-viral IgG were directed against influenza virus (X59209, Stark and Caton, 1991) (96% identity) and Hantaan virus (L46814, Liang et al., 1996) (95.2% identity); the identity between CDRs being 77 and 95%, respectively. Because the third light chain CDR (L3) plays a significant role in antigen recognition, the conservation of six of its nine residues is noteworthy. Identities between the Fd of these IgG were less remarquable, though 40% of the CDR 2 (H2) was identical.

The fine mapping of 4E11 epitope could provide clues to test the hypothesis that the use of variants of this light chain among three antiviral antibodies could eventually signal the presence of comparable epitopes at the surface of the three different viruses.

3.3. Immunochemical and serological properties

3.3.1. Affinity measurements

The affinity of the mAb and the Fab for MalE-E(296–400) binding were, respectively, measured as 3.77×10^{-9} and 1.7×10^{-9} M and can be considered as equivalent due to experimental variations and precision of the method.

3.3.2. Quantification and purification of the soluble Fab

The Fab was harvested in periplasmic extract, purified and electrophoresed on a 10% SDS-PAGE gel. Two narrowly separated bands of approximately 25 kD were shown, corresponding to light chain and Fd present in similar quantities (Fig. 1). No contaminant was present and the concentration was measured as 9 μg ml^{-1} by Bradford's method.

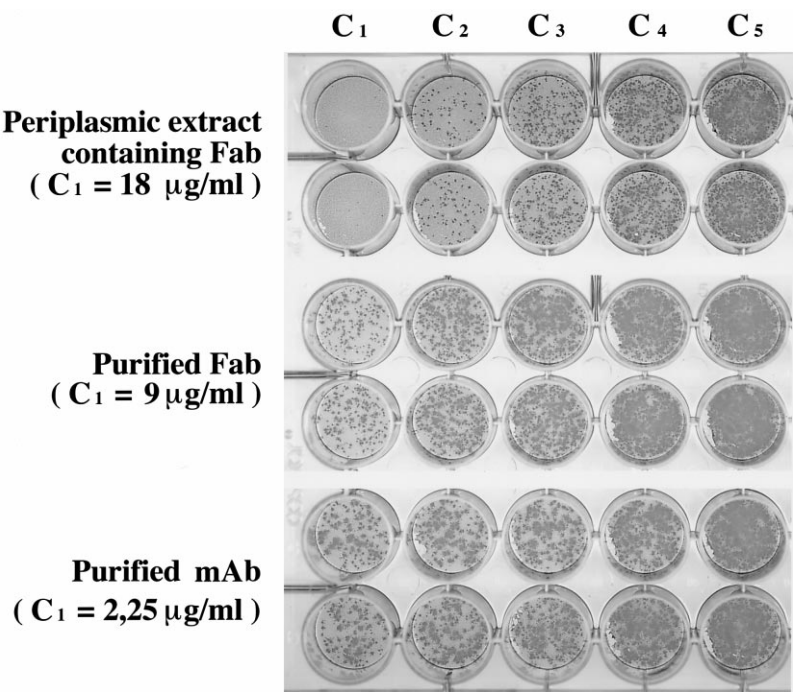

Fig. 1. Coomassie stained 10% SDS PAGE gel of Fab 4E11. The two narrowly separated bands of an approximate apparent molecular weight of 25 kD correspond to the light chain and Fd.

Table 1
Plaque reduction neutralization test of 4E11 against the four serotypes of dengue virus[a]

Dengue virus	mAb 4E11 (μg ml^{-1})	Fab 4E11 (purified form) (μg ml^{-1})	Fab 4E11 (in periplasmic extract) (μg ml^{-1})
Serotype 1	0.28	2.25	2.25
Serotype 2	2.25	ND	18
Serotype 3	2.25	9	9
Serotype 4	>2.25	ND	18

[a] Titers are shown as the concentration of mAb or Fab giving a 50% reduction in the number of FFU. Such a titer could not be determined (ND) utilizing purified Fab 4E11 against serotypes 2 and 4, because the concentration of this preparation (9 μg ml^{-1}) was too low.

3.3.3. PRNT

PRNT (50% inhibition) were performed for the four serotypes during the same experiment in order to allow comparisons to be made between Fab (purified Fab and Fab in periplasmic extract) and mAb and between serotypes. This experiment was preceded by preliminary experiments, whose results are in accordance with those given in Table 1 (data not shown). mAb concentration which neutralized serotype 1 (0.28 μg ml^{-1}) (Fig. 2) was lower than that neutralizing serotypes 2 and 3 (2.25 μg ml^{-1}) and serotype 4 (> 2.25 μg ml^{-1}) (Table 1). Periplasmic extract without Fab did not show any neutralizing properties. Fab neutralization capacity could be estimated as 8 times (serotypes 1 and 2) to four times (serotype 3) and four times or less (serotype 4) lower than that of mAb 4E11.

Despite experimental variations observed between the tested serotypes, some reduction of Fab neutralizing capacity when compared with the parental mAb was observed. It has already been reported that neutralization by a Fab is usually reduced by at least one order of magnitude compared with its parental IgG (Schofield et al., 1997), but the observed reduction was not in agreement with the conserved affinity. Technical reasons for this appearent loss of activity, such as alterations during freeze-thaw, or defects in the association of the light chain and Fd could not be ruled out despite the taken precautions. These reasons would not have hampered the affinity measurements, which were not dependent on the quantity of active Fab present in the tested solution, but only on the linearity of the signal with respect to the dilution. Reasons based on the mechanism of neutralization could also be proposed. Because Fab 4E11 bound to the envelope protein of the virus, it should block the attachment of the virus to the host cell, by binding close to the attachment site (see review in Dimmock, 1993). This blocking could be less complete by the Fab than by the Mab, due to reduction in Fab steric hindrance. Because no effector for Fc is present in the in vitro neutralization essay, the fact that the lack of Fc seems to cause a reduction of neutralization properties can be mostly explained by this reduction of Fab steric hindrance.

Our in vitro neutralization experiments by recombinant Fab 4E11 proved that Fc and diva-

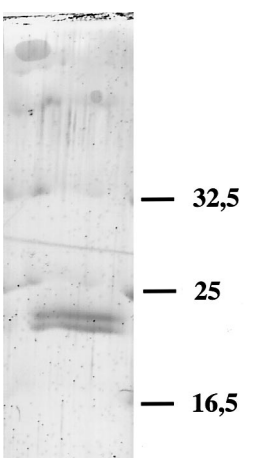

Fig. 2. PRNT of dengue 1 virus by periplasmic extract containing Fab 4E11, purified Fab 4E11 and purified mAb 4E11 diluted 2-fold. Initial concentrations (C1) are indicated. Tests were realized in duplicate.

lence of the parental mAb were not necessary to the neutralization process. Such results have already been established by Fab obtained by papain digestion, but could be subject to caution due to incomplete digestion (Dimmock, 1993). These results cannot be generalised to all monoclonal antibodies (Ubol et al., 1995).

In a medical perspective, more work could be done to improve the production of Fab 4E11 and to test it in vivo. The humanization of Fab 4E11, or the search for an antibody of similar properties but of human origin, that would be better tolerated in medical use, can also be pursued. Recent availability for medical practice of a recombinant mouse-human chimera Fab (Abciximab® used as an anti-aggregant) gives ground for hope that long-pursued antiviral seroprophylaxis or serotherapy may some day become reality.

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