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# Antigenicity and immunogenicity of the HIV-1 gp41 epitope ELDKWA inserted into permissive sites of the MalE protein

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**Abstract**

The highly conserved amino acid sequence ELDKWA of HIV-1 gp41 has been inserted into *Escherichia coli* MalE protein which had been shown to be an adequate carrier to present foreign epitopes to the immune system. We first investigated whether eight different permissive sites of MalE are able to tolerate an insertion of 7–50 residues encoding this epitope. Secondly, antigenicity of the epitope inserted in MalE protein was estimated from monoclonal antibody 2F5 binding analysis using the BIAcore® technology and its immunogenicity in mice was measured as the ability of hybrid proteins to elicit antibodies against a synthetic peptide containing this epitope. This study revealed a good correlation between the antigenicity of the inserted epitope and its immunogenicity. Increasing the length of the inserted epitope, as well as inserting multicopies of this epitope increased both its antigenicity and immunogenicity. However, none of the MalE hybrid proteins tested induced anti-HIV-1 neutralizing antibodies. This study strongly suggests that the capacity of the 2F5 epitope to induce neutralizing antibodies depends on the molecular context in which it is presented. © 2000 Elsevier Science Ltd. All rights reserved.

*Keywords:* HIV-1; gp41 epitope; MalE protein

## 1. Introduction

The determinant recognized by the anti-gp41 human monoclonal antibody 2F5, isolated from an HIV seropositive subject, has been mapped and corresponds to aminoacids 662–667 (ELDKWA) located near the C terminus of the gp41 ectodomain [1]. The amino acid sequence of this epitope was found to be conserved in 72% of otherwise highly variable HIV-1 isolates [1]. This antibody 2F5 is the only anti-gp41 monoclonal antibody described that exhibits potent neutralizing activity [2,3]. This antibody neutralizes genetically divergent HIV-1 isolates, including the laboratory strains and several clinical isolates [2–5]. No other monoclonal antibodies with similar properties have been described so far and antibodies to the 2F5 epitope are poorly represented in the human sera. This suggests that this epitope is either not well-exposed or only weakly immunogenic [6]. Passive administration of 2F5 to chimpanzees before HIV-1 challenge delayed the appearance of viremia and reduced virus load [7,8]. This neutralizing monoclonal antibody blocks HIV-1 infection of dendritic cells and the cell-to-cell transmission of virus from infected dendritic cells to T cells [9]. Altogether, these data indicate that the epitope recognized by the 2F5 antibody represents an important target for developing immunogens able to induce anti-HIV neutralizing antibodies.

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In the present study, the determinant recognized by the anti-gp41 human monoclonal antibody 2F5, ELDKWAS, has been inserted into the MalE protein which has been previously demonstrated to be an adequate carrier for foreign B-cell epitope presentation to the immune system [10–15]. This *E. coli* periplasmic protein is an attractive carrier protein for several reasons: it can be expressed at high levels, may be released from the periplasm by osmotic shock and purified by a simple and mild chromatography [16,17]. Permissive sites have been identified into the MalE protein which accept the insertion of heterologous peptides without major deleterious consequences for the activities, structure and cellular location of the hybrid proteins [16,18,19]. Moreover, following immunization with chimeric MalE proteins, high antibody titers have been obtained against the inserted foreign epitopes [11–15].

In the present study, we investigated whether eight MalE insertion sites were able to tolerate insertions of 7–50 residues-long polypeptides encoding the continuous gp41 epitope recognized by the 2F5 monoclonal antibody. Secondly, we examined the antigenic and immunogenic properties of this epitope inserted in the different molecular environments provided by the various MalE hybrid proteins. Antigenicity was tested by analyzing kinetic parameters of interaction between monoclonal antibody 2F5 and recombinant proteins using the BIAcore® technology. Immunogenicity was tested by measuring antibody titers in mice injected with the different purified MalE hybrid proteins. Our results suggest that the length of the inserted sequence containing the gp41 epitope and its flanking regions are important parameters to ensure an adequate conformation of the inserted epitope which could mimic its native structure.

## 2. Materials and methods

### *2.1. Bacterial strains, plasmids, culture conditions and standard procedures*

*E. coli* pM9F' [12] which carries the Δ*malE444* nonpolar deletion in the chromosomal *malE* gene was used as host in transformation experiments. *E. coli* strains were grown in L-agar plates (containing 1.5% agar) at 37°C. Antibiotics for the selection of transformants were ampicillin (100 μg/ml) and carbenicillin (100 μg/ml).

Plasmid pNTE in which the *malE* gene is under the control of the *ptac* promoter was previously described [14] whereas plasmid pMAL-c was obtained from New England Biolabs (Beverly, MA, USA). All the plasmids were sequenced in a region covering the insert to check the fidelity of the constructs. Sequencing of double-stranded DNA was performed by dideoxynucleotide chain termination with the Sequenase kit (U.S. Biochemical, Cleveland, OH).

### *2.2. Epitope insertion*

Epitope insertion into eight different sites of MalE protein has been previously described [12,14]. These sites correspond to substitutions of short DNA sequences within *malE* gene by an oligomer carrying a *Bam*HI site. Four oligonucleotides encoding the monomeric and dimeric peptides ((ELDKWAS), (LLELDKWAS), (ELDKWAS)2, (LLELDKWAS)2) were synthesized by Genset SA (Paris, France). The codons chosen were the most frequently used in bacteria. These oligonucleotides were inserted into the *Bam*HI sites of the *malE* mutants. A *Sac*I restriction enzyme site was introduced into the coding strands for an easy detection of recombinants. After insertion, only one *Bam*HI site is restored at the beginning of the insert so that further insertions of the same gp41 oligonucleotides were possible and led to the construction of recombinant proteins carrying up to four monomers of the epitope.

The monomeric peptides were named QC and QL, the dimeric peptides QC2 and QL2 respectively, the multimeric peptides QL3 and QL4 and the names of the corresponding hybrid proteins are given in Table 1.

All the constructs were initially made in pNTE vector. For protein purification convenience, most of the recombinant MalE carrying HIV epitopes were transferred onto a modified version of pMAL-c in which MalE with the wild type C-terminal sequence was restored and is expressed under the control of *ptac* promoter. These constructs led to a cytoplasmic production of the recombinant proteins whereas pNTE ensures secretion of the same proteins into the periplasm of bacteria.

### *2.3. Expression and purification of hybrid proteins*

*E. coli* strains containing the hybrid plasmids were grown in Luria Bertani Medium. Cells were harvested at $O.D._{600} = 2$. MalE hybrids proteins were released from the periplasm by osmotic shock. Cytoplasmic proteins were prepared by lysis of bacteria using a French press. Inclusion bodies were pelleted by centrifugation (30 min at 15 000 rpm) and denatured by 8 M urea. After centrifugation, supernatants were diluted with 20 mM Tris pH 7.5, NaC1 150 mM to allow renaturation. Recombinant MalE proteins were purified from extracts on a cross-linked amylose column as described [20].

### 2.4. Western blot

Purified heat denatured recombinant proteins were electrophoresed on 12% polyacrylamide gels and either Coomassie stained or transferred onto nitrocellulose and revealed by Western-blot using polyclonal anti-MalE and monoclonal anti-gp41 antibodies (2F5, kindly given by Dr Katinger) as described [14].

### 2.5. Binding analysis of monoclonal antibody 2F5 to the different MalE-hybrid proteins

Mouse monoclonal antibodies against human IgG1 (Zymed, San Francisco, USA) were covalently immobilized on the carboxymethylated dextran surface of a CM5 sensorchip using a BIAcore®2000 system (BIAcore® AB, Uppsala, Sweden). Monoclonal antibody 2F5 was coupled by injecting 90 μl of a 50 μg/ml solution at a flow rate of 10 μl/min. The resulting derivatized surface, CM5-anti IgG1-2F5, was equilibrated with buffer (HEPES 10 mM, pH 7.4; NaCl 150 mM; EDTA 3.4 mM; BIAcore® surfactant P20 0.05%) which was used for all subsequent dilutions. Binding analysis of the different MalE-hybrid proteins was tested by injecting 90 μl of a 50 μg/ml solution at a flow rate of 30 μl/min. Kinetic constants, $K_a$ (association rate constant) and $K_d$ (dissociation rate constant), for the interaction of MalE-hybrid proteins with immobilized 2F5 were calculated using Bia Evaluation 3.0 software.

### 2.6. Mice

BALB/c, C3H/He, C57BL/6 and DBA/1 female mice, 6–8 weeks old, were used in this study. BALB/c, C3H/He and C57BL/6 were obtained from Iffa Credo (L'Arbresle, France). DBA/1 mice were from the animal colony of Pasteur Institute (Paris, France).

### 2.7. Immunization of mice and ELISA assay

Mice (five per group) were injected intraperitoneally (i.p.) at days 0, 21 and 42 with 10 μg of wild type MalE or MalE hybrid proteins mixed with 1 mg alum. Mice were bled on days 20, 31, 52 and mouse sera were tested for the presence of antibodies against the MalE protein and the L-11-K peptide (LLELDKWASL) synthesized by Neosystem (Strasbourg, France), by ELISA as previously described [15]. The negative control consisted of sera from mice immunized with an irrelevant non-cross-reactive antigen. The titers were calculated to be the $\log_{10}$ highest dilution which gave twice the absorbency of pooled control sera diluted 1/100. Titers were given as the arithmetic mean ± S.E.

Table 1
Hybrid proteins, vectors and plasmids used in this study

| Hybrid protein | Plasmid | Vector | Sequence of inserted peptide |
|---|---|---|---|
| $MalE_{133}$-QC | $pNTE_{133}$-QC | pNTE | DP ELDKWAS DP |
| $MalE_{303}$-QC | $pNTE_{303}$-QC | pNTE | PDP ELDKWAS DP |
| $MalE_{133}$-QL | $pNTE_{133}$-QL | pNTE | DP LLELDKWASL DP |
| $MalE_{303}$-QL | $pNTE_{303}$-QL | pNTE | PDP LLELDKWASL DP |
| $MalE_{133}$-QC2 | $pNTE_{133}$-QC2 | pNTE | DP ELDKWASELDKWAS DP |
| $MalE_{303}$-QC2 | $pNTE_{303}$-QC2 | pNTE | PDP ELDKWASELDKWAS DP |
| $MalE_{133}$-QL2 | $pNTE_{133}$-QL2 | pNTE | DP LLELDKWASLLLELDKWASL DP |
| $MalE_{303}$-QL2 | $pNTE_{303}$-QL2 | pNTE | PDP LLELDKWASLLLELDKWASL DP |
| $MalE_{133}$-$QC2_{DP}$ | $pNTE_{133}$-$QC2_{DP}$ | pNTE | DP ELDKWAS DP ELDKWAS DP |
| $MalE_{303}$-$QC2_{DP}$ | $pNTE_{303}$-$QC2_{DP}$ | pNTE | PDP ELDKWAS DP ELDKWAS DP |
| $MalE_{133}$-$QL2_{DP}$ | pmalc133-$QL2_{DP}$ | pmalc | DP LLELDKWASL DP LLELDKWASL DP |
| $MalE_{161}$-$QL2_{DP}$ | pmalc161-$QL2_{DP}$ | pmalc | RI LLELDKWASL DP LLELDKWASL QIR |
| $MalE_{206}$-$QL2_{DP}$ | pmalc206-$QL2_{DP}$ | pmalc | RI LLELDKWASL DP LLELDKWASL QIR |
| $MalE_{211}$-$QL2_{DP}$ | pmalc211-$QL2_{DP}$ | pmalc | GS LLELDKWASL DP LLELDKWASL GSE |
| $MalE_{285}$-$QL2_{DP}$ | pmalc285-$QL2_{DP}$ | pmalc | RI LLELDKWASL DP LLELDKWASL QIR |
| $MalE_{296}$-$QL2_{DP}$ | pmalc296-$QL2_{DP}$ | pmalc | TDP LLELDKWASL DP LLELDKWASL DP |
| $MalE_{303}$-$QL2_{DP}$ | pmalc303-$QL2_{DP}$ | pmalc | PDP LLELDKWASL DP LLELDKWASL DP |
| $MalE_{339}$-$QL2_{DP}$ | pmalc339-$QL2_{DP}$ | pmalc | SDP LLELDKWASL DP LLELDKWASL DP |
| $MalE_{133}$-$QL3_{DP}$ | pmalc133-$QL3_{DP}$ | pmalc | DP LLELDKWASL DP LLELDKWASL DP LLELDKWASL DP |
| $MalE_{303}$-$QL3_{DP}$ | pmalc303-$QL3_{DP}$ | pmalc | PDP LLELDKWASL DP LLELDKWASL DP LLELDKWASL DP |
| $MalE_{161}$-$QL4_{DP}$ | pmalc161-$QL4_{DP}$ | Pmalc | RI LLELDKWASL DP LLELDKWASL QI LLELDKWASL DP LLELDKWASL QIR |
| $MalE_{206}$-$QL4_{DP}$ | pmalc206-$QL4_{DP}$ | pmalc | RI LLELDKWASL DP LLELDKWASL QI LLELDKWASL DP LLELDKWASL QIR |
| $MalE_{211}$-$QL4_{DP}$ | pmalc211-$QL4_{DP}$ | pmalc | GS LLELDKWASL DP LLELDKWASL GS LLELDKWASL DP LLELDKWASL GSE |
| $MalE_{285}$-$QL4_{DP}$ | pmalc285-$QL4_{DP}$ | pmalc | RI LLELDKWASL DP LLELDKWASL QI LLELDKWASL DP LLELDKWASL QIR |
| $MalE_{296}$-$QL4_{DP}$ | pmalc296-$QL4_{DP}$ | pmalc | TDP LLELDKWASL DP LLELDKWASL DP LLELDKWASL DP LLELDKWASL DP |
| $MalE_{303}$-$QL4_{DP}$ | pmalc303-$QL4_{DP}$ | pmalc | PDP LLELDKWASL DP LLELDKWASL DP LLELDKWASL DP LLELDKWASL DP |
| $MalE_{339}$-$QL4_{DP}$ | pmalc339-$QL4_{DP}$ | pmalc | SDP LLELDKWASL DP LLELDKWASL DP LLELDKWASL DP LLELDKWASL DP |

### 2.8. Virus neutralization

All the assays were performed with the indicator cell line MT4 and, as virus inoculum, frozen stocks of HIV-1 MN and RF. For the primary isolate of HIV-1, Bx08, phytohaemagglutinin (PHA)-stimulated PBMC from healthy HIV-seronegative blood donors were used. Virus were previously titrated within the assay format to determine the amount that yields submaximal p24 antigen production. Titer of viral solution was calculated by the Karber method as TCID50 (50% tissue culture infectious dose). In neutralization assays, antisera were diluted in culture medium and distributed into 96-well microtiter plates with conical bottom wells shape (Nunc, Roskilde, Denmark); 50 μl of three virus dilutions (0.1–100 TCDI50) were added to 50 μl of diluted antisera, and the virus-antibody mixture was preincubated for 1 h at 37°C. For infection, MT4 or PHA-stimulated PBMC ($8 \times 10^5$ cells/100 μl) were added to each well and incubated for 1 h at 37°C. After three washes, pellets were resuspended in complete medium, transferred to 96-well microtiter plates with flat well bottom shape and cultivated at 37°C. Presence of syncytia was recorded after 5 days as an indication of HIV-1 infection. Culture supernatants were analyzed for p24 production at days 6 and 10. Detection of p24 was determined using a double-antibody sandwich ELISA (Coulter HIV-1 p24 antigen assay, Coulter Corporation, Miami, USA). Neutralization was determined by the percentage of reduction in virus-induced p24 production in the presence of two-fold dilutions of serum.

## 3. Results

### 3.1. Production and purification of MalE hybrid proteins

To examine the antigenicity and immunogenicity of the gp41 epitope (ELDKWAS sequence), we constructed hybrid proteins where polypeptides of varying lengths encompassing this epitope were genetically inserted into 8 different sites of the *E. coli* periplasmic MalE protein. Since our preliminary results indicated that increasing the length of the ELDKWAS peptide could potentiate its immunogenicity, we compared the immunogenicity of a short (ELDKWAS) and a long (LLELDKWASL) sequence containing the minimal gp41 epitope sequence. These sequences were inserted either in a monomeric or a multimeric form. The different inserted peptides and the hybrid proteins constructed in this study are detailed in Table 1. Depending on the reading frame of the inserted *Bam*HI linker, the peptides inserted in MalE are surrounded by sequences DP … DP, or RI … QIR, or GS …GSE (Table 1). The peptides were inserted into the potentially permissive *Bam*HI sites genetically introduced after residues 133, 161, 206, 211, 285, 296, 303 and 339 of MalE protein [14]. Insertion of these foreign sequences into the different sites of MalE used in this study did not modify the properties of the carrier protein: all hybrid proteins allowed maltose transport in *E. coli* (data not shown) indicating that they were still active, correctly folded and exported to the periplasm. However, some of these proteins were poorly expressed in the periplasm when pNTE vectors were used (data not shown). In contrast, cytoplasmic MalE hybrid proteins, produced by the pMAL-c vector, were abundantly expressed and were synthesized as inclusion bodies. Correctly folded proteins could be recovered from these inclusion bodies (see Section 2), as judged from the efficient binding to cross-linked amylose columns which allowed their purification. The only exceptions were MalE339 and its derivatives, which does not bind amylose but could be purified by a Q-Fast-flow chromatography as previously described [14]. Since elution of the proteins from the amylose column was performed under non-denaturing condition, the hybrid proteins used in this study kept their native conformation. Coomassie-stained-SDS-polyacrylamide gel of the purified MalE hybrids shows that the preparations were homogeneous (Fig. 1a and b).

### 3.2. Antigenicity of MalE hybrid proteins

Western blot were realized to analyze the recognition of the hybrid proteins by polyclonal anti-MalE antibodies. All the hybrid proteins were recognized by anti-MalE antibodies, showing that insertion of foreign epitope did not modify the overall antigenicity of the carrier protein (Fig. 1a and b).

To verify if the inserted epitope was recognized by the anti-gp41 mAb 2F5, hybrid proteins were tested for reactivity with human mAb 2F5 by immunoblotting (Fig. 1a and b). In contrast to recombinant MalE constructs that contain the monomeric or polymeric forms of the gp41 epitope and which were reactive with the human mAb 2F5, no binding to mAb 2F5 was observed with the wild type (wt) MalE protein. Furthermore, the MalE proteins containing the longer forms of the gp41 epitope (QL: LLELDKWASL) were better recognized than the smaller forms (QC:ELDKWAS) (Fig. 1a). The strongest binding of mAb 2F5 was observed with MalE133QL2DP, MalE211QL2DP, MalE211QL4DP, MalE296QL2DP and MalE296QL4-DP hybrid proteins (Fig. 1b). MalE133-QL3DP and MalE303-QL3DP were also recognized by anti-MalE antibodies and the anti-gp41 mAb 2F5 (data not shown). These results indicate that all the hybrids containing the epitope ELDKWAS are recognized by the human mAb 2F5, but with different efficiencies.

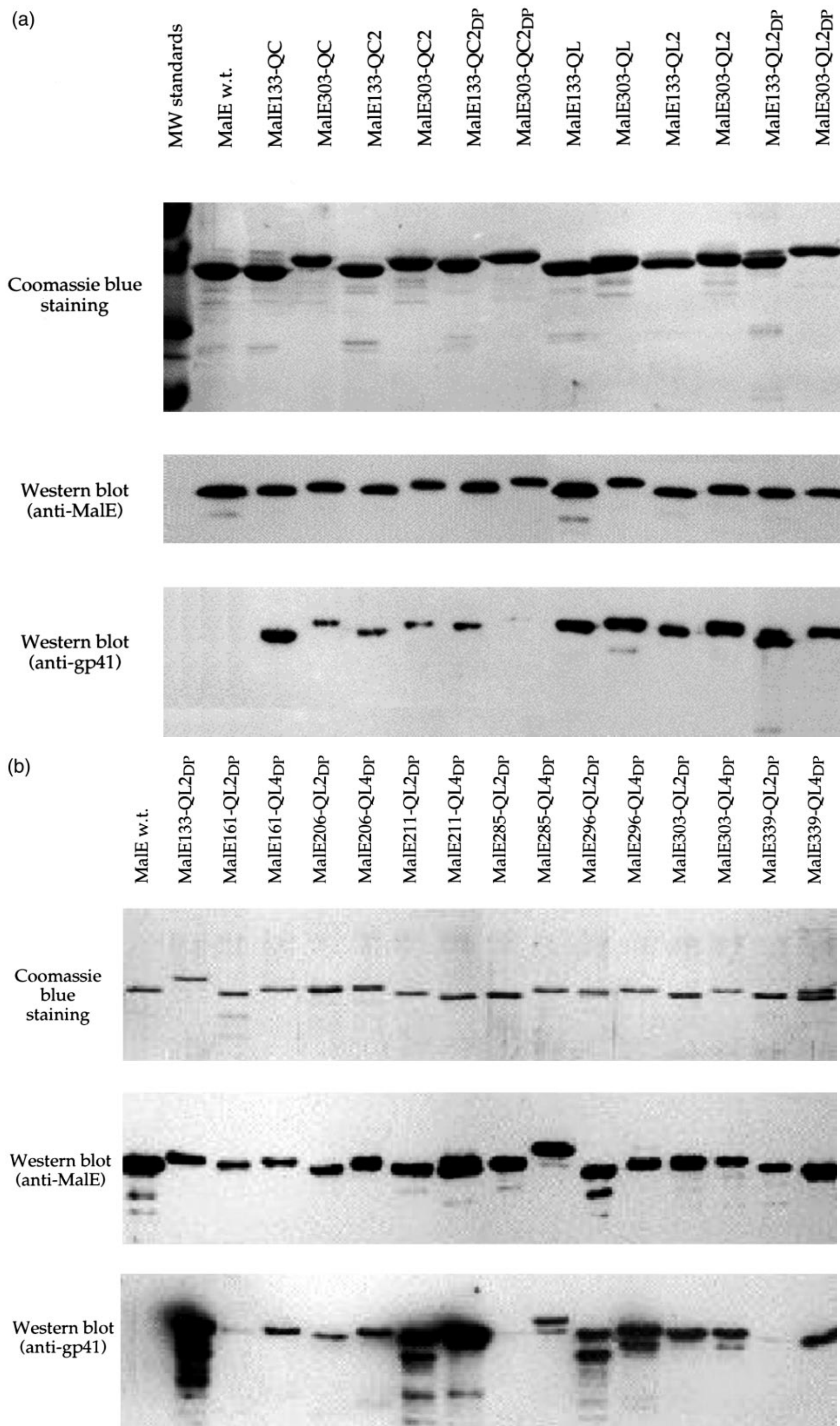


Fig. 1. Purified hybrid proteins (2 μg of each protein) were loaded on SDS-polyacrylamide gel (12%) subsequently coomassie-stained (top) or analyzed by Western blot with anti-MalE antibody (middle) and with anti-gp41 antibody (bottom). 1a: MalE (wild type) and MalE hybrid proteins with monomers or dimers of the gp41 epitope. 1b: MalE (wild type) and MalE hybrid proteins with dimers and tetramers of the gp41 epitope.

The difference in reactivity of the monoclonal antibody 2F5 towards the different MalE hybrid proteins were analyzes more precisely with the BIAcore® technology.

### 3.3. Monoclonal antibody 2F5 binding to the different MalE-hybrid proteins

The BIAcore® technique allowed us to quantify the interaction between the monoclonal antibody 2F5 and the different MalE-hybrid proteins under fixed conditions, by measuring and comparing kinetic constants: $K_a$ (association rate constant) and $K_d$ (dissociation rate constant).

As shown in Fig. 2, 206QL2DP, 211QL2DP, 296QL2DP, 303QL3DP, 206QL4DP, 285QL4DP, 296QL4DP and 303QL4DP MalE hybrid proteins bound very quickly to 2F5 and dissociated very slowly, the fastest binding being obtained with 211QL2DP, 303QL3DP and 296QL4DP. MalE hybrid proteins 133QL2DP, 303QL2DP, 133QL3DP and 161QL4DP bound less quickly than the previous proteins to 2F5 and dissociated less slowly. MalE hybrid proteins 285QL2DP, 339QL2DP and 211QL4DP bound even more slowly to 2F5 and dissociated very rapidly. Taken together, our results show that insertion in sites 133, 296 and 303 corresponded to a rapid binding and a slow dissociation. In contrast, insertion 339 corresponded to a rapid dissociation.

The MalE hybrid protein 211QL2DP bound very quickly to 2F5 and dissociated very slowly whereas the MalE hybrid protein 211QL4DP bound slowly to 2F5 and dissociated very rapidly. Thus, for position 211, increasing the length of the inserted epitope led to a decrease in the recognition by the monoclonal antibody 2F5. In contrast, for the other positions, 161, 285, 296, 303 and 339, increasing of the length of inserted epitope led to an increase in the recognition by the monoclonal antibody 2F5.

### 3.4. Immunogenicity of MalE hybrid proteins in mice

We then analyzed the antibody responses of BALB/c mice immunized with these different MalE hybrid proteins. We analyzed anti-MalE and anti-L-11-K antibody responses obtained at days 20, 31 and 52 after immunization. The antibody response against the inserted gp41 epitope was monitored by ELISA, using the peptide L-11-K (LLELDKWASLK), which binds optimally plastic microplates. We compared the primary, secondary and tertiary anti-L-11-K antibody responses obtained in BALB/c immunized with 10 μg of MalE hybrid proteins containing peptides QC, QC2, QC2DP, QL, QL2, QL2DP, QL3DP and QL4DP inserted in eight different positions in MalE. The tertiary anti-L-11-K antibody response obtained at day 52, corresponding to the maximal antibody responses, is shown in Fig. 3.

MalE133-QC and MalE303-QC proteins failed to induce any anti-L-11-K response in BALB/c. In contrast, a significant anti-L-11-K antibody response was

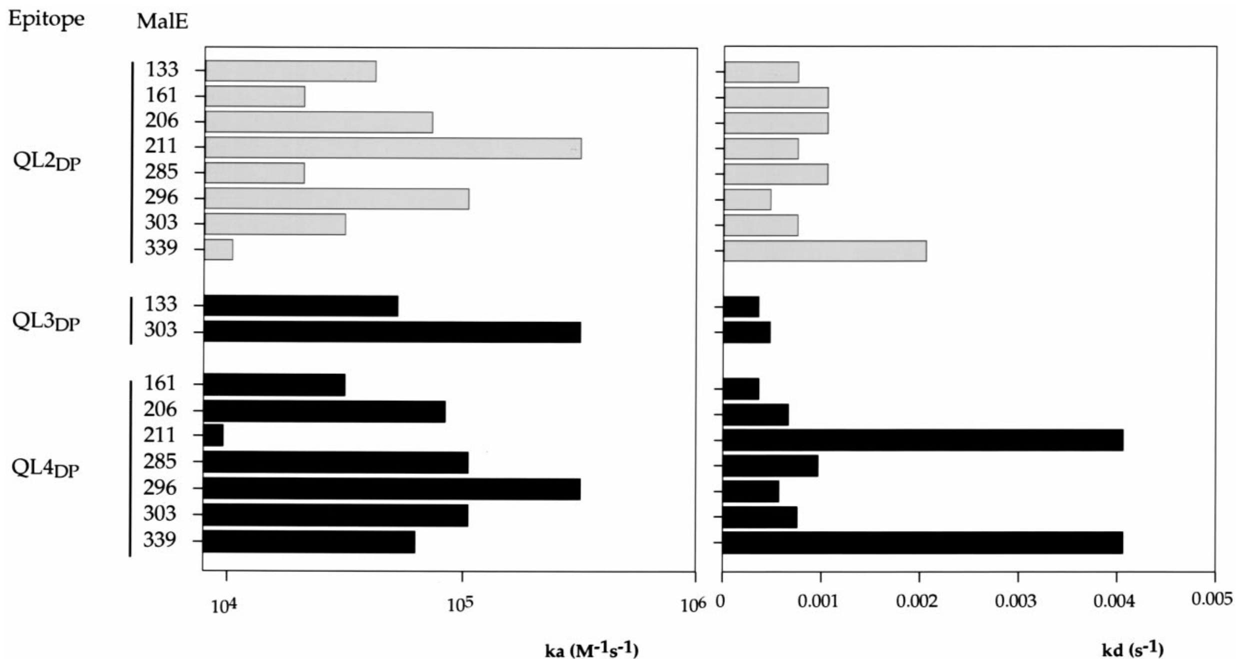


Fig. 2. BIAcore® analysis of binding between the human anti-gp41 monoclonal antibody 2F5 and the different MalE-hybrid proteins. Mouse monoclonal antibodies specific for human IgG1 were covalently immobilized on the carboxymethylated dextran surface of a CM5 sensorchip using a BIAcore® 2000 system. Then, the monoclonal antibody 2F5 was added and the resulting derivatized surface, CM5-anti IgG1-2F5, was equilibrated. The binding of the different MalE-hybrid proteins was then analyzed and the kinetic constants obtained in fixed conditions, and $K_a$ (association rate constant, left pannel) and $K_d$ (dissociation rate constant, right pannel) were calculated.

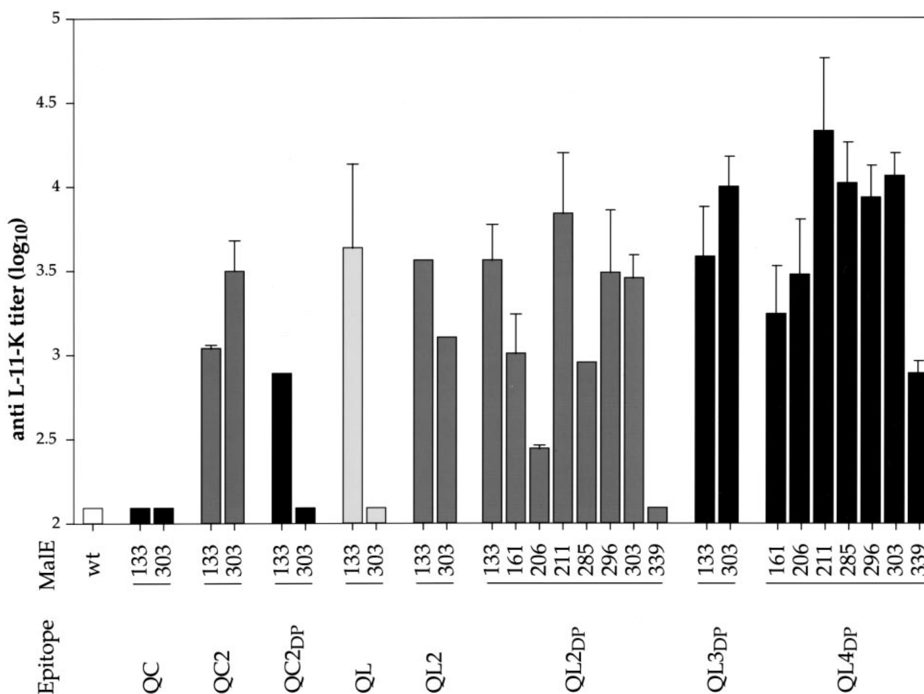


Fig. 3. Antibody responses obtained in BALB/c mice immunized with the different MalE-hybrid proteins. BALB/c (five mice per group) were injected i.p. on days 0, 21 and 42 with 10 μg of MalE wild type or MalE hybrid proteins mixed with 1 mg of alum. The antibody response against the inserted gp41 epitope was monitored by ELISA, using the peptide L-11-K (LLELDKWASLK). Results were expressed as the mean ± S.E. of $\log_{10}$ titers of the tertiary response at day 52 of individual sera. The bars represent the mean of 2–5 independent experiments (five mice per experiment).

obtained after immunization by the proteins expressing dimers of the short version of the epitope (QC2, QC2DP), except for QC2DP in position 303. The proteins expressing the longest version (QL) as a monomer or as a polymer (QL, QL2, QL2DP, QL3DP, QL4DP) induced in most cases higher anti-L-11-K antibody responses than the proteins expressing the short one (QC). The anti-L-11-K antibody responses obtained in BALB/c thus increased with the length and with the number of copies of the inserted epitope. The insertion site of gp41 epitope also plays an important role on the level of anti-L-11-K antibody responses. Except for proteins expressing the short monomer QC, a very good anti-L-11-K antibody response was obtained for positions 133, 161, 211, 285, 296 and 303 (titers in $\log_{10}$ included between 3 and 4). When the gp41 epitope was inserted at position 339, the anti L-11-K antibody responses were weaker.

The antibody response against the MalE proteins was also monitored to investigate the possibility that the lack of antibody response to the gp41 epitope was due to a lack of immunogenicity of the MalE carrier protein. BALB/c developed high anti-MalE antibody titers after three injections of the different MalE hybrid proteins ($\log_{10}$ titers equal 6–7). Moreover, comparable primary, secondary and tertiary anti-MalE responses were observed in mice immunized with the different MalE hybrid proteins (data not shown). These results, therefore, excluded that the lack of response to the inserted gp41 epitope was due to unresponsiveness to MalE and demonstrated that the immunogenicity of the carrier protein, MalE, was not modified by the insertion of the foreign epitope.

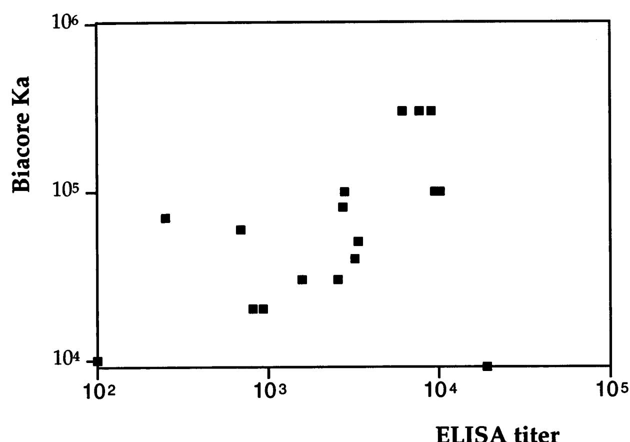


Fig. 4. Correlation between antigenicity of MalE-hybrid proteins, measured by the binding (association constant ka) between the human anti-gp41 monoclonal antibody 2F5 and the different MalE-hybrid proteins, and immunogenicity of MalE-hybrid proteins, expressed by the antibody response (anti-peptide ELISA titer) obtained in BALB/c immunized with the different MalE-hybrid proteins. This correlation was significant ($*P < 0.05$; Spearman $r = 0.5345$ and $P = 0.0223$).

As shown in Fig. 4, a positive correlation was observed between immunogenicity and antigenicity of MalE hybrid proteins according to BIAcore®. This correlation was significant (*$P < 0.05$; Spearman $r = 0.5345$ and $P = 0.0223$).

In an earlier study, we have shown that mouse capacity to elicit antibody responses against a well-defined B cell determinant is the function of the genetic background of the strain of mice and more precisely of the IgH haplotype [15]. We therefore compared antibody responses of four different mouse strains immunized with the different MalE hybrid proteins carrying the gp41 epitope. MalE133-QC protein failed to induce any anti-L-11-K response in BALB/c, DBA/1, C3H and C57BL/6 mice (data not shown). No antibody response was observed in C3H and C57BL/6 mice after immunization with MalE hybrid proteins expressing the QC peptide under dimeric form (QC2, QC2DP) whereas BALB/c and DBA/1 mice developed a weak response. The proteins expressing the QL peptide under monomeric or dimeric form induced higher anti-L-11-K antibody response in BALB/c mice than in DBA/1, C3H and C57BL/6 mice (data not shown).

### 3.5. Lack of neutralizing antibodies in mice immunized with MalE hybrid proteins

Sera from BALB/c and DBA/1 mouse immunized with MalE133-QL2DP and from BALB/C mouse immunized with MOLE Z11-QLZDP, MOLE 211-QL4DP and MalE285-QL4DP were assayed for neutralizing activity. The monoclonal antibody 2F5 was used as control. The HIV-1 clade B primary isolate Bx08 and the HIV-1 laboratory strains MN and RF, in which the amino acid sequence ELDKWA of the HIV-1 gp41 was strictly identical, were chosen for neutralization assays [1,5]. Sequence analysis of a variety of primary isolates have suggested that the major determinant of mAb 2F5 binding corresponds to the LDKW sequence [21].

No neutralization of HIV-1 MN, RF or Bx08 was observed with sera from BALB/c and DBA/1 mice immunized with MalE hybrid proteins under conditions where 2F5 at 2 μg/ml inhibited totally p24 production by cells infected by HIV-1 (data not shown).

## 4. Discussion

To examine the antigenicity and immunogenicity of the gp41 epitope inserted into the MalE protein, we constructed MalE hybrid proteins carrying the ELDKWAS sequence of the C-terminal region of HIV-1 gp41 envelope glycoprotein by genetically inserting sequences of varying lengths at eight different sites of this carrier protein.

This study demonstrated that it is possible to produce and purify MalE hybrid proteins containing ELDKWAS or LLELDKWASL sequences being expressed either in a monomeric or a multimeric form (7–50 amino acids). Insertion of these foreign peptides into different sites of MalE did not alter the biological properties of this protein. Seven of the mutant proteins, (133, 161, 206, 211, 285, 296 and 303) MalE hybrid proteins had retained affinity for amylose and confirmed the permissive nature of these sites [12,14,16,18]. One exception, MalE339, had lost the affinity for amylose and did not correspond to a permissive site.

When analyzed by Western blotting, differences in the recognition of the chimeric protein by the gp41 epitope specific monoclonal antibody were apparent. The highest binding of mAb 2F5 was observed with proteins containing peptides QL2DP and QL4DP inserted into positions 133, 211 and 296; proteins containing peptide QL2DP at positions 161, 285 and 339 reacted weakly whereas the others proteins presented intermediate reactivities. This indication of possible differences in the antigenic properties of hybrid proteins led us to quantify their interaction with the monoclonal antibody 2F5 using the BIAcore® technology.

All MalE hybrid proteins bound 2F5. Three hybrid proteins (211QL2DP, 303QL3DP and 296QL4DP) bound very quickly to 2F5 with very slow dissociation. For the other hybrid proteins, different situations were observed, namely rapid binding and slow dissociation or slow binding and rapid dissociation. These results indicated that the gp41 epitope, inserted into most MalE sites, was able to efficiently adopt the conformation which is recognized by the monoclonal antibody, the first step towards the formation of a stable complex. Since the association constant depends in particular on the accessibility of the peptide to the antibody and on the constraints exerted on its conformation, the differences we observed suggest that the inserted peptides were submitted to different constraints in different configurations. These constraints could result in different 3-D conformation and/or partial burying of the epitope.

One important conclusion of this study is that a significant correlation was observed between immunogenicity in BALB/c of MalE hybrid proteins and antigenicity according to association to 2F5 in BIAcore®, the two analyzed variables tending to increase or decrease together. For positions 133, 285, 296 and 303, an increase in the length of insert is followed by an increase in recognition by the monoclonal antibody 2F5 and also by an increase in immunogenicity. The multimerization of the epitope could thus change the conformation of the epitope and perhaps help to mimic the native one.

This study, therefore, shows that the immunogenicity of the gp41 epitope probably depends of its conforma-

tion. Martineau et al. [12] had earlier shown that the antigenic properties of different B epitopes inserted into position 133 and 303 of MalE were different at these two sites. In contrast, the inserted peptides elicited strong and comparable antibody responses in mice against the corresponding peptides. Extending this analysis by inserting the poliovirus C3 B-cell epitope into other positions in MalE showed that antigenicity varies with insertion sites [14]. NMR studies of the different MalE hybrid proteins with poliovirus C3 epitope inserted into different permissive sites showed that the inserted residues adopt a loop conformation. In the case of the hybrid protein MalE339C3, which does not present any detectable affinity for the monoclonal antibody [14], the C3 insert is included in the flexible C-terminal part of the molecule and is totally unrestrained and free to adopt a range of conformations similar to those of the free C3 peptide. This excessive mobility was interpreted as the reason for the lack of antigenicity of the MalE339C3 hybrid [22]. Similarly, this mobility in the C-terminus of the molecule could explain the very poor immunogenicity found when gp41 epitope was inserted into position 339.

The induction of an immune response against a given antigen is controlled both by the intrinsic characteristics of the antigen and by the genetic background of the host. We previously analyzed the capacity of various strains of mice to develop antibody responses against the poliovirus C3 epitope using either genetic or chemical coupling of a peptide containing this epitope to three different carrier molecules, and demonstrated that some strains of mice are unable to produce an antibody response against this epitope [15]. In the present study, we thus compared the antibody responses obtained in different mouse strains immunized with MalE-133 and MalE-303 hybrid proteins expressing the gp41 epitope. BALB/c and DBA/1 mice developed a significant antibody response. MalE-133 and MalE-303 hybrid proteins failed to induce any significant antibody response in C57BL/6 mice. Thus, like for the poliovirus C3 epitope, certains strains of mice are unable to produce any antibody response against the gp41 epitope.

A previous study showed that a B-cell epitope from poliovirus, inserted in different permissive sites of the MalE protein, led to the induction of antibodies directed against the native virus and which neutralized it in vitro [10]. However, in the present study, we failed to detect anti-HIV-1 neutralizing antibodies following immunization with MalE carrying the gp41 epitope. Since we did not test if the induced antibodies bind to the native gp41, we cannot determine if the lack of neutralizing activity is due to a lack of gp41 recognition or whether the sera bind to gp41 but do not neutralize the virus. However, several studies have previously demonstrated that mAb 2F5 binds to native gp41 [1,2] and the present study also clearly established that 2F5 binds to the different MalE hybrid proteins.

It should be noticed that the antibody 2F5 has a remarkably long CDR3 loop and this unusual heavy chain CDR3 may probably account for 2F5 rare occurrence in patient sera [23]. No Ab with this CDR3 length have been found so far in mice, therefore, opening the possibility that immunogens failing to induced neutralization in mice may do so in humans.

It was previously shown that immunization in mice with the gp41 sequence ELDKWAS inserted as a monomer into the antigenic site B of the hemagglutinin of WSN virus induces cross-neutralizing activity against divergent HIV-1 isolates [3]. In contrast, the ELDKWAS gp41 epitope presented on recombinant surface antigen of hepatitis B virus failed to induce antibodies able to neutralize HIV-1 in vitro [24].

The fact that the gp41 epitope failed to induce neutralizing antibodies when inserted into permissive sites of the MalE protein or after fusion to the hepatitis B virus surface, antigen, while it did when presented on recombinant influenza virus haemagglutinin, indicates that its capacity to stimulate the induction of neutralizing antibodies depends on the molecular context in which it is presented. Therefore, stabilization of this epitope in a favorable secondary and tertiary structure may be crucial for its capacity to induce neutralizing antibodies and for the full exploitation of its potential as a vaccine component.

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