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Immunization with Peptide-Functionalized Carbon Nanotubes Enhances Virus-Specific Neutralizing Antibody Responses

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Summary

Functionalized carbon nanotubes (CNTs) hold a lot of promise for application in medicinal chemistry. Based on a method for preparation of water-soluble CNTs, we covalently linked a neutralizing B cell epitope from the foot-and-mouth disease virus (FMDV) to mono- and bis-derivatized CNTs. Immunological characterization of these conjugates revealed that the epitope was appropriately presented after conjugation to CNTs for recognition by antibodies as measured by BIAcore technology. Moreover, peptide-carbon nanotubes elicited strong anti-peptide antibody responses in mice with no detectable cross-reactivity to the carbon nanotubes. However, only the mono-derivitized CNT conjugate induced high levels of virus-neutralizing antibodies. These findings highlight for the first time the potential of CNTs to present biologically important epitopes in an appropriate conformation both in vitro and in vivo and open up the possibility for their use in vaccine delivery.

Introduction

Carbon nanotubes have recently attracted considerable attention because of their structural properties (e.g., high stability, strength, and stiffness, combined with low density and elastic deformability) and potential biomedical and biotechnological applications [1]. Since their discovery [2], there have been several advances in preparation techniques, which can provide quite pure single- and multiwalled carbon nanotubes (SWNTs and MWNTs) in gram quantities. Moreover, the development of oxidative and synthetic protocols has opened up the possibility of generating or attaching functional groups to the end groups and/or to the side walls of the tubes to obtain highly soluble carbon nanotubes in organic solvents and aqueous solutions [3–8]. In particular, 1,3-dipolar cycloaddition reaction of azomethine ylides has emerged as a powerful methodology for the generation of amino-functionalized carbon nanotubes with a remarkable solubility, particularly in water [8]. The organic modification of carbon nanotubes provides multiple sites for the attachment, through stable covalent bonds, of drugs, amino acids, sugars, DNA, oligonucleotides, peptide nucleic acids (PNAs), peptides, proteins, or enzymes [8–17]. Such nanovehicles could have potential applications as delivery systems and as biosensors [17–21].

In the field of vaccination there is considerable interest in developing new and effective delivery approaches to administer protective antigens. Carbon nanotubes could serve as an excellent vehicle to administer vaccines. Their use for vaccination may allow effective utilization of antigens that have previously not been able to induce adequate or appropriate responses, as well as providing significant means of enhancing and modulating immune responses. In vaccine delivery, one important parameter to consider is the retention of antigen conformation, which is required for the induction of antibody responses with the right specificity. In a recent study, we demonstrated the development of a versatile method for the functionalization of carbon nanotubes with a peptide representing a protective B cell epitope from the VP1 coat protein of FMDV [15]. Based on this finding, in this study we sought to evaluate the capacity of mono- and bis-derivatized CNTs to present this B cell epitope in vivo and examined the possibility of enhancing its neutralizing potential.

Results and Discussion

Design and Characterization of Peptide-Carbon Nanotube Conjugates

Recently the synthesis of a water-soluble carbon nanotube functionalized with a pyrrolidine ring through the 1,3-dipolar cycloaddition of azomethine ylides was described [8]. The amino-derivatized carbon nanotube **1** (Figure 1) presenting 0.50 mmol of active group per gram of material (loading), as established by the quantitative Kaiser test [22], was subsequently used to covalently link a series of peptides by different strategies [15]. In particular, a peptide corresponding to the sequence 141–159 derived from VP1 protein of FMDV was coupled through a stable bond to functionalized SWNTs, employing a selective chemical ligation to obtain peptide-carbon nanotube **2** (mono-conjugate **2**) (Figure 1). Then we explored the possibility of increasing the amount of peptide around the tube surface by conceiving and preparing the conjugated system **3** (Figure 1). This consists of a single-walled carbon nanotube bearing a double functionalization with the FMDV peptide (bis-conjugate **3**).

To prepare the bis-FMDV peptide-carbon nanotube

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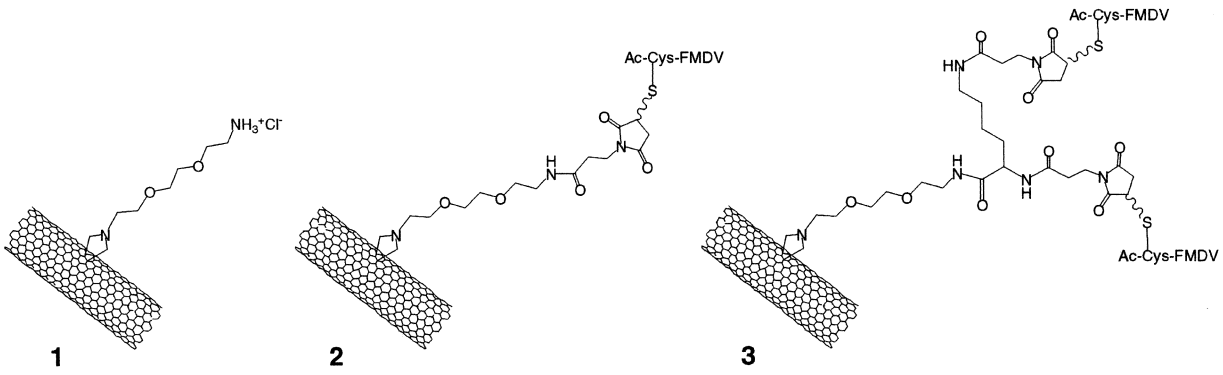

Figure 1. Molecular Structures of the CNT Derivative and Mono- and Bis-Conjugates

3, the free amino groups of SWNT **1** were first derivatized using an excess of Boc-Lys(Boc)-OH activated with DIC (diisopropylcarbodiimide) and HOBt (1-hydroxybenzotriazole) in DMF, followed by the cleavage of Boc (*tert*-butyloxycarbonyl) with TFA (trifluoroacetic acid). The quantitative Kaiser test [22] confirmed that the amount of amines increased from 0.50 to 0.92 mmol per gram of carbon nanotube. This derivative displayed a remarkable solubility in water (~150 mg/ml). The free amino functions were subsequently neutralized with DIEA (diisopropylethylamine) and coupled with *N*-succinimidyl 3-maleimidopropionate in DMF [23]. After stirring overnight, the excess reagent was removed by addition of amino-PEGA resin [24] as a scavenger to block the remaining carboxylic active ester. A negative Kaiser test confirmed the complete acylation of the amino groups [25]. The N-terminal acetylated FMDV 141-159 peptide, bearing an additional cysteine at position 1 for the chemo-selective ligation, was dissolved in water, and linked to the two maleimido moieties of the wires in 2.6-fold excess to obtain peptide-carbon nanotube **3** after stirring for 9 hr. Unreacted peptide was removed again using a scavenger resin. For this purpose we recycled the previous PEGA resin derivatized with the maleimido function. The formation of the peptide covalent bond to the SWNTs was monitored by reverse-phase HPLC as previously described [15]. The mono-conjugate **2** and the bis-conjugate **3**, to a lower extent, were highly soluble in water, reaching a concentration of 18.0 and 12.5 mg/ml, respectively. To prove the formation of the peptide-SWNT covalent bond, CNT **1** and its derivative with the deprotected lysine were added to a solution of FMDV peptide in water. The mixture was analyzed by HPLC before and after the addition of CNTs without observing any decrease of peptide concentration due to the spontaneous adsorption of peptide at the surface of the wires by noncovalent interactions. Recent studies have shown that designed amphiphilic helical peptides or hydrophobic peptides derived from phage displayed peptide libraries may posses a selective affinity for pristine CNTs [26, 27].

Bis-conjugate **3** was examined by TEM (transmission electron microscopy). Figure 2 shows the bundles of different diameters obtained after deposition of the sample dispersion onto a TEM grid and evaporation of the solvent.

To complement the TEM structural characterization, a series of bidimensional NMR experiments were acquired in a solution of 9:1 water/*t*-BuOH-d_9. TOCSY and ROESY spectra allowed the full assignment of the 40 amino acids that constitute the peptide moiety of bis-conjugate **3** (Figure 3). Despite the number of residues, the attribution was facilitated by the presence of a copy of the same peptide sequence at the external surface of the carbon nanotube. The chemical shift dispersion, and the intensity and position of NOEs were identical to those of the mono-conjugate **2** that we recently characterized [15]. It is evident that the peptide was linked to the CNTs as a single chain (mono-conjugate **2**) or as a double chain (bis-conjugate **3**) displaying the same secondary structure. Finally, bis-conjugate **3** was characterized by amino acid analysis that confirmed the presence of lysine, necessary to obtain the peptide bis-adduct, in a correct ratio to the amino acid composition of FMDV peptide.

Recognition of FMDV 141-159 Peptide Linked to Carbon Nanotubes by a Monoclonal Antibody

SPR (surface plasmon resonance) measurements allow the study of antigen-antibody interactions in real time [28]. To avoid artifacts due to immobilization of one of

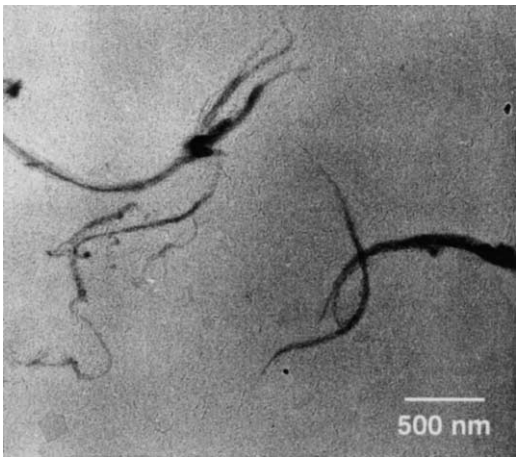

Figure 2. TEM Image of Peptide-Carbon Nanotubes **3**
The diameter of the bundles is comprised between 15 and 60 nm.

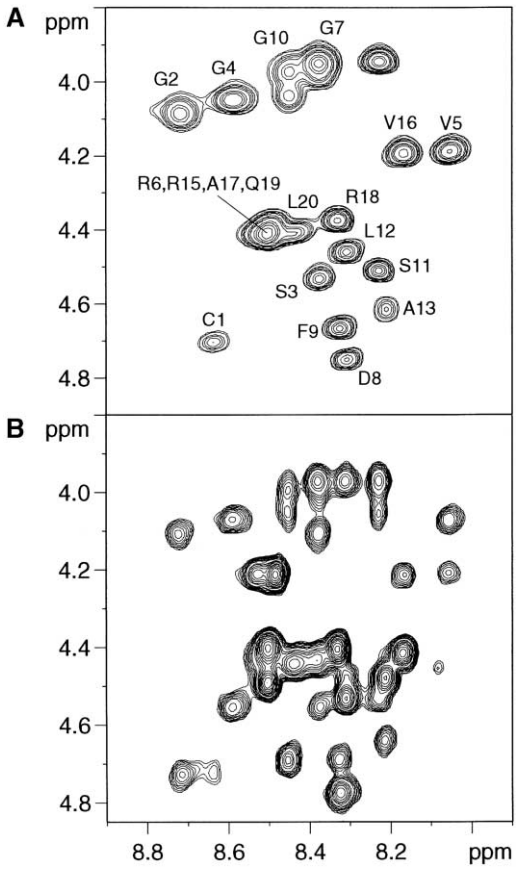

Figure 3. 2D-NMR Spectra of Peptide-Carbon Nanotube **3**

Partial (A) TOCSY and (B) ROESY ^{1}H NMR spectra of peptide-carbon nanotube **3** in H_2O/*t*-BuOH-d_9 9:1 solution. Peptide residues are numbered from Cys^1 to Leu^{20}.

the partners [29], we chose to present the anti-FMDV 141-159 peptide mAb to a covalently linked anti-mouse Fcγ antibody and to measure the increase in mass due to the interaction of peptide or carbon nanotube-linked peptide present in the fluid phase. Corrected sensorgrams are shown in Figure 4. Carbon nanotubes without any bound peptide did not react with the mAb, whereas free peptide, mono-conjugate **2**, and bis-conjugate **3** interacted with increasing mass. A qualitative comparative analysis of the three sensorgrams showed no differences in the association rate constants and only a small decrease of the dissociation rate constant for the bis-conjugate **3**, which might be ascribed to the avidity of the bivalent reagent. Nanotube-linked peptide thus cover the same conformational space as the free peptide.

Induction of Anti-Peptide and FMDV-Neutralizing Antibody Responses Using the Peptide-Carbon Nanotube Conjugates

Immune responses to FMDV 141-159 peptide are major histocompatibility complex restricted, and when the peptide is injected alone with an adjuvant in BALB/c mice it is not immunogenic [30]. Its capacity to elicit antibodies improves when the peptide is coupled to a

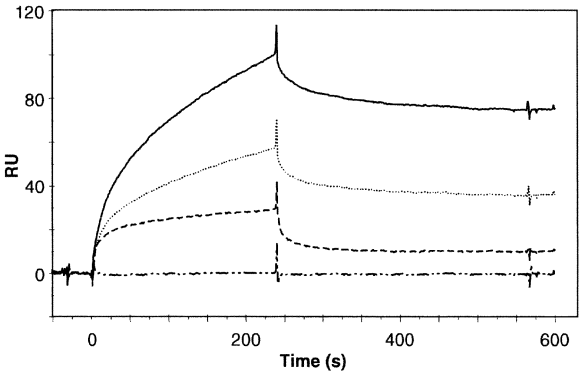

Figure 4. Sensorgrams Obtained by Allowing the Analytes to React with an Anti-Peptide mAb

The association phase required 4 min, the dissociation phase 5 min. Response with the bis-conjugate **3** (—) (5 μM); response with the mono-conjugate **2** (·····) (5 μM); response with free FMDV peptide (-----) (5 μM); response with acetylated CNT **1** (- ·· -) (5 μM). RU value corresponds to the resonance unit (1000 RU = 1 ng/mm^2 of analyte).

carrier protein or to a T helper epitope(s) [31]. However, in the latter case, and particularly in BALB/c mice, it was observed that not all anti-FMDV 141-159 peptide antibodies are capable of neutralizing virus infectivity [31]. Since the peptide was coupled to the CNTs to circumvent the problem of its poor immunogenicity, we tested an immunization protocol that has previously been shown to overcome the requirement of coupling nonimmunogenic peptides to carrier proteins or to T helper epitopes [32]. Indeed, coinjection of OVA (ovalbumin) with the FMDV 141-159 peptide renders it immunogenic. Anti-peptide antibody responses were measured by ELISA using the BSA-conjugated FMDV 141-159 peptide as a solid-phase antigen (Figure 5). In preliminary experi-

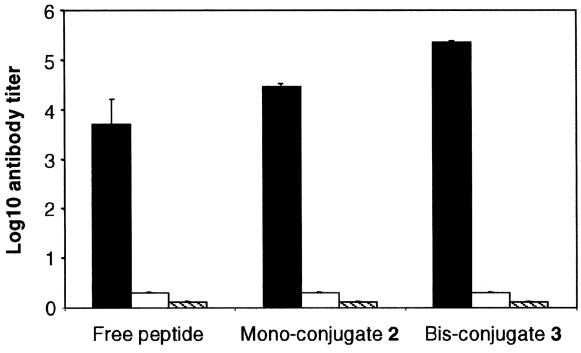

Figure 5. Anti-Peptide Antibody Responses Following Immunization with Peptides and Peptide-Carbon Nanotubes

Groups of BALB/c mice were coimmunized intraperitoneally with OVA and free FMDV 141-159 peptide, mono-conjugate **2**, or bis-conjugate **3** in Freund's adjuvant emulsion. Serum samples collected 2 weeks after the booster immunization (on day 14 post priming) were screened by ELISA for the presence of antibodies using FMDV 141-159 peptide conjugated to BSA (solid bars), control peptide conjugated to BSA (open bars), or CNT **1** functionalized with maleimido group without peptide (hashed bars) as solid-phase antigens. Data represent mean of log10 antibody titers from five mice per group ± SD.

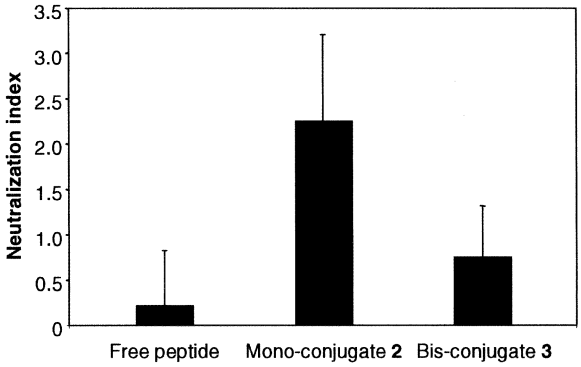

Figure 6. Neutralization Indices of Serum Samples of Immunized Mice

Serum samples were collected 2 weeks after the boost of mice coimmunized intraperitoneally with OVA and free FMDV 141-159 peptide, mono-conjugate **2**, or bis-conjugate **3** in Freund's emulsion. Data represent mean of antibody titers from five mice per group ± SD.

ments, we have established that the use of conjugated peptide as a solid-phase antigen increases the sensitivity of the ELISA as compared to the nonconjugated peptide (data not shown). The anti-FMDV 141-159 antibody response was further enhanced but not significantly ($p = 0.098$, according to Student's t test, a value smaller than 0.05 was considered significant) when OVA was coinjected with the mono-conjugate **2** (Figure 5). However, when the bis-conjugate **3** was used as an immunogen, the anti-peptide antibody titers were significantly elevated ($p = 0.001$) (Figure 5). The observed antibody responses were peptide specific and were not directed to the functional group linking the FMDV 141-159 peptide to the carbon nanotubes. This was confirmed by the lack of reactivity of serum antibodies with a control peptide conjugated to BSA (Figure 5).

The demonstration that the carbon nanotube peptide conjugates elicit antibodies of the IgG isotype is compatible with the notion that these constructs together with OVA are sampled by antigen-presenting cells. After their processing, the bystander help provided by the OVA-specific T helper cells assists the B cells to produce antibodies that recognize the FMDV 141-159 peptide. Furthermore, we did not detect any anti-carbon nanotube antibodies (Figure 5). In a recent publication, Erlanger et al. have demonstrated that anti-fullerene antibodies [33] can recognize carbon nanotubes [34]. However, the fullerene was immunogenic only after being coupled to a carrier protein [33]. In our immunization protocol, the carbon nanotube peptide-conjugates were coinjected with the carrier protein. Therefore, it appears that the bystander help provided by the carrier (OVA) is directed to the covalently attached peptide and not to the carbon nanotubes. This suggests that carbon nanotubes do not possess intrinsic immunogenicity.

In subsequent experiments, we tested the capacity of anti-peptide antibodies to neutralize FMDV. As shown in Figure 6, the mono-conjugate **2** elicited virus-neutralizing antibody responses in all mice, which were significantly higher than those induced by the bis-conjugate **3** ($p = 0.009$), and control free peptide ($p = 0.001$). Coimmunization with the control free peptide elicited detectable neutralizing antibodies only in one mouse. Thus, the increase of the number of epitopes at the surface of CNTs, although it enhances the immunogenicity of the attached peptide as previously shown [35], does not improve the neutralizing titers. On the contrary, it had a detrimental effect, suggesting that the two copies of the FMDV peptide displayed by the bis-conjugate **3** might interact in vivo and adopt a conformation different from that displayed by the native epitope on the virus. Therefore, the antibody responses do not have the correct specificity. These findings are in a good agreement with observations highlighting the limitation of multiple presentation of epitopes to elicit antibodies cross-reactive with the native protein [36].

The preferential induction of peptide-specific antibodies with enhanced virus neutralizing capacity using the mono-conjugate **2** as immunogen, combined with the observed nonresponsiveness to the attached carbon nanotube could be advantageous in the context of vaccine delivery for two main reasons: (1) it demonstrates the successful presentation of the attached peptide in vivo, and (2) it highlights the potential of carbon nanotubes as an antigen delivery system since no anti-carbon nanotube antibodies were elicited that could influence immune responses to the attached peptide (epitopic suppression) [37]. Although the biological relevance of these antibodies has not been tested in vivo, a recent study has demonstrated that there was a good correlation between serum anti-FMDV neutralizing antibodies and protection [38].

Significance

The study of functionalized carbon nanotubes has been intensified in recent years due to the realization that they hold a lot of promise for potential biomedical and biotechnological applications. Organic modification of carbon nanotubes generates multiple sites for the attachment of bioactive molecules, and the nanotube scaffold could be used as a novel delivery system or as a biosensor. We have recently devised a new method to prepare a series of peptide-carbon nanotube conjugates based on covalently linking a neutralizing and protective B cell epitope from the FMDV VP1 coat protein to a maleimido-derivatized water-soluble carbon nanotube using the selective chemical ligation. To increase peptide concentration around the tube, we also designed a bis-maleimido derivative for peptide conjugation. The nanotube displayed B cell epitope retained its conformational characteristics since it was recognized by specific antibodies using BIAcore technology. More importantly, both the mono- or bis-maleimido peptide-derivatized carbon nanotubes elicited strong anti-peptide antibodies, but only the mono-peptide CNT enhanced significantly the virus neutralizing antibody titers. Carbon nanotubes did not have any detectable immunogenicity. These findings pave the way for the potential application of carbon nanotubes as a delivery system of candidate vaccine antigens.

Experimental Procedures

SWNTs were purchased from Carbon Nanotechnologies (Houston, TX). CNT derivative **1** and mono-conjugate **2** were prepared ac-

cording to previously reported procedure [8, 15]. NMR spectra were recorded on a Bruker ARX 500 MHz spectrometer. The sample was dissolved in H_2O/*t*-BuOH-d_9 9:1. The spectra were acquired at a temperature of 300 K and referenced to the peak of the solvent. The identification of amino acid spin systems and sequential assignment were made using a combination of TOCSY [39] and ROESY [40] experiments. WATERGATE pulse sequence was applied for the suppression of water signal [41]. TEM images were recorded on a Hitachi 600 HS instrument at 110kV. Bis-conjugate **3** was suspended in diethyl ether, deposited on the grid (Formvar/carbon support film on copper 400 mesh-grid), and analyzed after evaporation of the solvent. Amino acid analysis was performed on an Applied Biosystem model 130A separation system coupled to an Applied Biosystem model 420A derivatizer.

Preparation of Bis-Conjugate 3

Carbon nanotube **1** (5.0 mg, 2.5 μmol) was dissolved in 1 ml of dichloromethane and neutralized with DIEA (4 μl, 22.5 μmol). A solution of Boc-Lys(Boc)-OH (2.6 mg, 7.5 μmol) in 1 ml of DMF was activated with DIC (1.2 μl, 7.5 μmol) and HOBt (1.2 mg, 7.8 μmol) for 10 min and subsequently added to carbon nanotube **1** solution. The mixture was stirred for 3 hr. The solvent was evaporated and the product precipitated several times from methanol/diethyl ether and dried under vacuum. The disappearance of the excess of Boc-Lys(Boc)-OH was followed by thin-layer chromatography (dichloromethane/methanol 8:2). The Boc-protecting group was removed from the functionalized CNT by treatment with 2 ml of trifluoroacetic acid (TFA) overnight. The product was recovered as a brown solid after several precipitations in cold diethyl ether. The deprotected carbon nanotube was dissolved in 1 ml of DMF and neutralized with DIEA (30.0 μl, 169.5 μmol). *N*-succinimidyl 3-maleimidopropionate (13.0 mg, 48.8 μmol) dissolved in 1 ml of DMF was added and the reaction mixture stirred at room temperature overnight. The excess of maleimido derivative was removed overnight by adding 70 mg of PEGA-NH_2 resin (Novabiochem, Läufelfingen, Switzerland), which was removed by filtration, and the solvent was evaporated. The product was dissolved in methanol and precipitated several times with cold diethyl ether. To a solution of the carbon nanotubes functionalized with the maleimido group (4.0 mg, 2.0 μmol) in 2 ml of water, Ac-Cys-^{141}GSGVRGDFGSLAPRVARQL^{159} (Ac-Cys-FMDV peptide) (11.0 mg, 5.2 μmol), prepared by standard Fmoc/*t*Bu (Fmoc, fluorenylmethyloxycarbonyl; *t*Bu, *tert*-butyl) solid phase chemistry on an automatic peptide synthesizer (15), was added. The reaction was stirred for 9 hr at room temperature and 70 mg of PEGA-NH_2 resin previously derivatized with *N*-succinimidyl 3-maleimidopropionate were added to remove the excess of peptide after 5 hr. The resin was removed by filtration and the solvent was lyophilized to provide carbon nanotube (bis-conjugate) **3**. The removal of the free FMDV peptide after the addition of the scavenger resin was monitored by RP-HPLC on a Macherey-Nagel C_{18} column using a linear gradient of A (0.1% TFA in water) and B (0.08% TFA in acetonitrile), 5%–65% B in 20 min at 1.2 ml/min flow rate.

Animal Immunization

Groups of five female BALB/c mice (6–8 weeks old) were coimmunized intraperitoneally (i.p.) with 100 μg of FMDV 141-159 peptide either free (N-terminal acetylated) or attached to carbon nanotubes (mono-conjugate **2** and bis-conjugate **3**) together with 100 μg of ovalbumin (Sigma, St. Louis, MO) in a 1:1 emulsion in complete Freund's adjuvant (Sigma). A booster injection was given i.p. in incomplete Freund's adjuvant (Sigma) 3 weeks later. Mice were bled at various time intervals after the boost and collected serum samples were stored at −20°C until use.

ELISA for Detection of Antibodies

The presence of anti-peptide antibodies in serum samples was determined by an enzyme-linked immunosorbent assay (ELISA). Briefly, 96-well microtiter plates (Falcon) were coated overnight with 2.5 μg/ml of bovine serum albumin (BSA)-conjugated FMDV 141-159 or control peptide (peptides were conjugated using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide [MBS] as a linker according to a protocol provided by Pierce, Rockford, IL), or with carbon nanotube **1** in 0.05 M carbonate/bicarbonate buffer, pH 9.6 at 37°C. The plates were blocked with 1% (w/v) BSA in phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS-T) at 37°C for 2 hr. Following washing with PBS-T, serial 2-fold dilutions of serum in PBS-T containing 0.25% BSA were made across the plate (final volume 50 μl) and plates were incubated at 37°C for 1 hr. At the end of the incubation period, plates were washed with PBS-T and incubated with 50 μl/well of horseradish peroxidase-conjugated goat anti-mouse IgG Fc specific (Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 hr at 37°C. Unbound conjugate was removed by thorough washing with PBS-T, and the enzymatic activity was determined by adding 150 μl/well substrate solution (10% v/v citric phosphate buffer at pH 5 + 0.04% H_2O_2 + 90% v/v of a solution containing 72 ml dimethylsulfoxide + 18 ml glycerol + 300 mg 3,3′,5,5′-tetramethyl benzidine) for 15 min at 37°C. The reaction was stopped with 1 N HCl, and absorbance was measured at 450 nm. Data are expressed as antibody titers corresponding to the reciprocal dilution giving an OD value equal to 0.2.

BIAcore Measurements

The immunological reactivity of the FMDV peptide coupled to carbon nanotubes, with the specific monoclonal antibody (mAb) 21 × 27 (generated after injecting mice with conjugated FMDV 147-156 peptide [our unpublished data]), was assessed using SPR technology on a BIAcore3000 instrument. Rabbit anti-mouse Fcγ IgG (BIAcore) was immobilized on a CM5 carboxylated, dextran-coated chip by the standard amino-coupling procedure recommended by BIAcore. Supernatants of hybridoma cultures secreting the 21 × 27 anti-FMDV peptide mAb and a control mAb of the same isotype (IgG2a) were allowed to react for 5 min at a flow rate of 5 μl/min to prepare the experimental channel and the control channel on the chip, respectively. The reaction step was followed by the injection of the analytes (solvent, acetylated carbon nanotube **1**, mono-conjugate **2**, bis-conjugate **3** and FMDV peptide in HBS [150 mM NaCl, 10 mM HEPES, pH 7.4, NP20 at 0.005% v/v]) at a flow rate of 30 μl/min for 4 min followed by a dissociation phase of 5 min. The anti-mouse Fcγ surface was regenerated by a 10 mM HCl solution passing for 30 s over the two channels. The results expressed as resonance units (RU) were corrected by subtracting from the experimental sensorgram that obtained with the control antibody (to take into account nonspecific interactions) and with the solvent to take into account the differential dissociation rate of the two mAbs from the anti-mouse Fcγ IgG.

In Vitro FMDV Neutralization

Serum samples were assayed by determining the neutralization index. Equal volumes of 10-fold dilutions of virus and 1/50 serum were incubated at 37°C for 20 min before placing the mixtures onto monolayers of BHK 21 cells in 96-well plates. After incubation in the presence of 5% CO_2 for 3 days, cells were stained with crystal violet-formaldehyde. The difference between the titer of the virus alone and the virus serum mixtures was taken as the neutralization index of 1/100 dilution of serum (a neutralization index of 2.0 logs means that 1/100 dilution of the antiserum reduces the titers of the homologous virus by 2 $\log_{10}$ units).

Acknowledgments

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