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Use of a Gel-forming Dipeptide Derivative as a Carrier for Antigen Presentation

ROLANDS VEGNERS[1], IRINA SHESTAKOVA[1], IVARS KALVINSH[1], ROBERT M. EZZELL[2] and PAUL A. JANMEY[3]

[1]Latvian Institute of Organic Synthesis, Riga, Latvia
Departments of [2]Surgery, Massachusetts General Hospital and
[3]Medicine, Brigham and Women's Hospital, and Program in Biological and Biomedical Sciences, Harvard Medical School, Boston, USA

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Abstract: A dipeptide of the formula Fmoc-Leu-Asp and some other related dipeptides were synthesized in solution by standard methods. When such peptides are dissolved in water at concentrations below 1% at 100 °C and cooled below 60 °C they form turbid solutions and eventually viscoelastic gels at lower temperatures. Such gels are thermoreversible and can also be disrupted by mechanical agitation. At a concentration of 2 mg/ml the peptide Fmoc-Leu-Asp forms an aqueous gel at 60 °C with a shear modulus of 80 Pa measured at a frequency of 1 rad/s. Peptide solutions undergo an abrupt increase in light scattering between 1 and 1.5 mg/ml at both 23 and 60 °C. By analogy with previous observations of other systems, this increase appears to be due to the formation of filamentous micelles and the aggregation of filaments into a three-dimensional network. When low molecular weight adamantanamine derivatives, which are inherently non-antigenic antiviral drugs, were incorporated into the Fmoc-Leu-Asp gel and injected into rabbits, high titre specific antibodies were efficiently produced without the need for additional adjuvant. Both the physical properties of the gel and its effect on the antigenicity of low molecular weight compounds suggest a number of practical applications.

Keywords: Gel; viscoelasticity; antigen; dipeptide; drug delivery

Abbreviations

Ada2Me, 3,5-dimethyl-1-adamantanamine hydrochloride; AdaMeC, 5-methyl-1-adamantanamine 3-carboxylic acid hydrochloride; AdaMeC-BSA, conjugate of bovine serum albumin and AdaMeC prepared by the carbodiimide method; AdaMeC-Bal-BSA, conjugate of bovine serum albumin and AdaMeC-(beta-alanine) with a free amino group; Bal, beta-alanine; BSA, bovine serum albumin; P buffer, 0.01 M phosphate buffer, pH 7.4; PS buffer, 0.01 M phosphate-buffered saline (150mM NaCl), pH 7.4; SRBC, sheep red blood cells.

Address for correspondence: Dr Paul A. Janmey, Division of Experimental Medicine, Brigham and Women's Hospital, LMRC 301, 221 Longwood Ave., Boston, MA 02115, USA. Tel: (617) 278-0382, Fax: (617) 734-2248, e-mail: janmey@calvin.bwh.harvard.edu.

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INTRODUCTION

Aqueous gels formed by biocompatible materials have many applications in medicine and industry as thickening agents or in drug delivery, but easily synthesized molecules rarely form gels in water except at high concentrations or very high molecular weights. Previous reports of peptides that form gels in organic or mixed solvents [1–4], suggest that they do so by forming micelles that attain lengths great enough to form systems of interacting filaments similar to entangled polymer solutions. The chemical basis for the self-assembly and gelling properties of

peptide derivatives can be understood by analogy with the assembly into fibers and other structures of bilayer-forming lipid derivatives reviewed in [5, 6]. If similar materials could be formed by peptides in aqueous solvents, they would have many potential applications in clinical or pharmaceutical settings. A biological advantage of peptides is that their toxicity is often very low and they can be degraded by the normal cellular metabolic pathways. One example of such a use is the report of a lipid/peptide/drug conjugate that may have utility for drug delivery *in vivo* [7]. The chemistry of peptides is easily modified by standard methods, and peptides of known structure and function can be linked to the ends of the peptides forming the filamentous network of the gel. By this means multivalent particles could conveniently be formed.

Here we show that the N-terminal Fmoc-protected anionic dipeptide Leu-Asp forms gels in aqueous solution at peptide concentrations as low as 2 mg/ml. The concentration at which Fmoc-Leu-Asp forms a gel is in the range of the most efficient gelation agents known [8, 9], and this implies that highly elongated structures must form. Thin rod-like filaments with lengths of 10 μm have been visualized by incorporation of trace amounts of rhodamine-Leu-Asp into the Fmoc-Leu-Asp gel. The potential utility of this material is demonstrated by its ability to incorporate antiviral adamantanamine derivatives and thereby produce specific antibodies against these drugs, which otherwise remain non-antigenic unless covalently coupled to protein carriers and injected with adjuvant by conventional methods.

MATERIALS AND METHODS

Reagents

1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride and glutaric dialdehyde, 50% solution in water, were purchased from Aldrich Chemical Company. Bovine serum albumin, complete Freund's adjuvant, Coomassie brilliant blue and thimerosal were obtained from Sigma Chemical Company. 1-Adamantanamine derivatives were a gift from M. Gold, Merz & Co., GmbH, Frankfurt/Main, Germany.

Peptide synthesis

The partially protected dipeptide Fmoc-Leu-Asp and its analogues (Table I) were synthesized in 60–80% yield by reaction of the corresponding Fmoc-amino acid-*O*-succinimidyl esters with sodium salts of amino acids or their derivatives. The reaction was carried out in aqueous DMF, and the peptides were purified by HPLC in an acetonitrile/water gradient.

Gel Formation and Incorporation of Adamantanamine Derivatives

Aqueous solutions of Fmoc-Leu-Asp and other peptides were formed by suspending desiccated peptide in boiling solutions of 10 mM phosphate pH 7.4 (P buffer) followed by vigorous stirring or shaking. Upon cooling to below 60 °C gels form in some cases and turbid solutions in others, as discussed in the text. Adamantanamine derivatives and rhodamine-Leu-Asp were incorporated into Fmoc-Leu-Asp aggregates by adding these to the cooling solutions before gelation at concentrations denoted in the text.

Physical Methods

Light scattering from 1 ml solutions in 1 cm diameter cylindrical cells was measured with a Brookhaven Instruments BI30ATN apparatus using a 633 nm 10 mW laser. Scattering intensity is reported in arbitrary units (total counts per 60 s). Viscoelasticity was measured by oscillatory deformation with a Rheometrics RFSII instrument by standard methods [10]. Values of maximal strain amplitude and frequency are given in the figure legends. Confocal

Table I. Fmoc-Dipeptides Tested for Aqueous Gel Formation

Sequence	Melting point, (°C)	Optical rotation, $c = 1$; ethanol	Gel formed (minimum conc., %)
Fmoc-Leu-Asp	158–160	− 8.4	Yes (0.5)
Fmoc-Ala-Asp	135–137	− 5.2	Yes (6.7)
Fmoc-Ile-Asp	162–168	− 11.6	Yes (0.4)
Fmoc-Leu-Ala	168–170	− 26.5	No
Fmoc-Leu-Bal	150–152	− 25.3	No
Fmoc-Leu-Glu	100–102	− 16.9	No
Fmoc-Leu-Lys(Cbz)	98–100	− 12.3	No

microscopy was performed on a 10 μl sample of Fmoc-Leu-Asp gel containing 2% by weight rhodamine-Leu-Asp with a BioRad MRC 600 confocal imaging system attached to a Zeiss inverted microscope with a 100 × objective.

Derivitization of Antigens

AdaMeC-BSA. BSA was conjugated with AdaMeC (through both of its functional groups) by the carbodiimide method [11]. BSA (0.1 g) and AdaMeC hydrochloride (0.1 g) were dissolved in 4.5 ml of water, and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (0.16 g; 0.83 mM) was added. The reaction mixture was brought to pH 8.5 with 1 M sodium carbonate solution, stirred for 60 min at 20 °C, and neutralized with dilute hydrochloric acid. Some insoluble material was removed by centrifugation, the solution was dialysed against phosphate buffer and concentrated using Amicon filters. The BSA conjugate was quantified colorimetrically with Coomassie Brilliant Blue [12]. Yield of the protein in concentrate was 12 mg. The concentrate was diluted with P buffer to a protein concentration of 1 mg/ml, treated with thimerosal and stored at 4 °C.

-AdaMeC__-BSA. The BSA conjugate with AdaMeC through its amino group was prepared using glutaric dialdehyde in a one step procedure [13].

AdaMeC-Bal-BSA. BSA was conjugated with AdaMeC either directly to its carboxyl group or through a beta-alanine spacer by the *N*-hydroxysuccinimide ester method [14]. In the latter case a derivatized hapten was synthesized in several steps by standard solution methods: AdaMeC→Boc-AdaMeC→Boc-AdaMeC-hydroxysuccinimide ester→Boc-AdaMeC-Bal sodium salt→Boc-AdaMeC-Bal→Boc-AdaMeC-Bal-hydroxysuccinimide ester.

A solution of Boc-AdaMeC-Bal-hydroxysuccinimide ester (0.14 g; 0.29 mM) in 2 ml dimethylformamide was added to a solution of BSA (0.1 g) in 3 ml water. The reaction mixture was brought to pH 8.5 with 1 M sodium carbonate, stirred for 120 min at 20 °C, neutralized with dilute hydrochloric acid, and dried *in vacuo*. The residue was treated with trifluoroacetic acid for 30 min at 20 °C to remove the *t*-butoxycarbonyl group. The solution was then evaporated again and neutralized with 1 M sodium carbonate. After neutralization the solution was ligated to BSA by the same method using glutaric dialdehyde [13]. Yield of the protein in concentrated solution was 22 mg.

Antibody Production. Female random-bred rabbits were immunized three times, at weekly intervals, and bled on the 24th day. A full dose was used for the first two immunizations, and a half of the full dose for the third immunization. Full doses of the BSA conjugates were 100 μg total protein. Before injections, AdaMeC-BSA and AdaMeC-Bal-BSA solutions were diluted with equal volumes of complete Freund's adjuvant, but AdaMeC + dipeptide gel was diluted with an equal volume of 0.01 M phosphate buffer; pH 7.4. The volume of immunogen solutions injected each time was 1 ml.

Determination of Specific Antibodies. Immune sera were analysed by two immunoprecipitation assays [15] (the double diffusion in agarose gel and the reverse radial immunodiffusion) and by the passive haemagglutination test [16]. In the first two assays, the solid phase was 1% agarose containing 0.8% sodium chloride. The sample volumes were 25 μl. The probes were incubated for 72 h at 37 °C. In double diffusion in agarose gel, the antigens were placed in central wells and the antisera in surrounding wells in concentrations between 20 and 1000 μg/ml which also included the equivalent balanced concentration. In the reverse radial immunodiffusion assay, Ada2Me or AdaMeC was mixed with agarose in concentrations between 20 and 200 μg/ml, and the antisera were placed in wells in various concentrations. In control experiments, sera of non-immunized animals were used. In the passive haemagglutination reaction, 0.5% suspensions of sheep red blood cells were treated with seven preparations: two BSA conjugates [AdaMeC-BSA, AdaMeC-Bal-BSA], BSA alone, Ada2Me + dipeptide gel, the dipeptide gel alone, and Ada2Me or AdaMeC alone. The protein concentration in the BSA preparations was 1 mg/ml. In the case of the dipeptide gel preparations, 0.5 ml of the gel was mixed with 4.5 ml of a suspension of SRBC. Ada2Me was applied in a concentration of 200 μg/ml. The mixtures were incubated for 1 h at 20 °C and the unadsorbed antigens were removed by centrifugation. SRBC were then washed carefully with PS buffer. Samples of 50 μl were placed in the wells of the trays. Antisera and purified antibodies were diluted with 0.01 M phosphate-buffered saline, pH 7.4 starting from 1:100 with a step of 1/2. Untreated SRBC, sera and antibodies from non-immunized animals were used in control.

RESULTS

Optical and Viscoelastic Measurements

At concentrations between 1 and 1.5 mg/ml solutions of Fmoc-Leu-Asp undergo a large increase in light scattering at both 20 and 60 °C, as shown in Figure 1A. When a solution of 2 mg/ml dipeptide is cooled from 100 to 60 °C, viscoelastic parameters characteristic of a gel are obtained by low strain oscillatory measurements. The storage shear modulus, G', a measure of the elastic strength of the material, reaches a stable level of 80 Pa within several minutes (Figure 1B). This value is comparable to that of the strongest biopolymer gels, such as fibrin [8] or F-actin [9]. In contrast, gelatin solutions remain sols at this concentration, and the lowest concentration of other synthetic polymer gels with comparable shear moduli are at least an order of magnitude higher. G' depends weakly on frequency from 0.1 to 100 rad/s, and is much larger than the loss modulus G'' (Figure 1C). It is also insensitive to temperature, suggesting that the structures formed in the dipeptide gel are thermally stable over a physiologically relevant range. However, as Figure 1D shows, the shear moduli depend very strongly on the magnitude of shear deformation. The gels are strain-weakening at the smallest measurable strains (0.3%), and although G' remains $> G''$, both moduli fall by a factor of 100 when the samples are strained to 10%. In this sense, Fmoc-Leu-Asp gels differ from fibrin [17] or F-actin gels [9], as the latter both show strain hardening at 10% followed by rupture and weakening at larger strains.

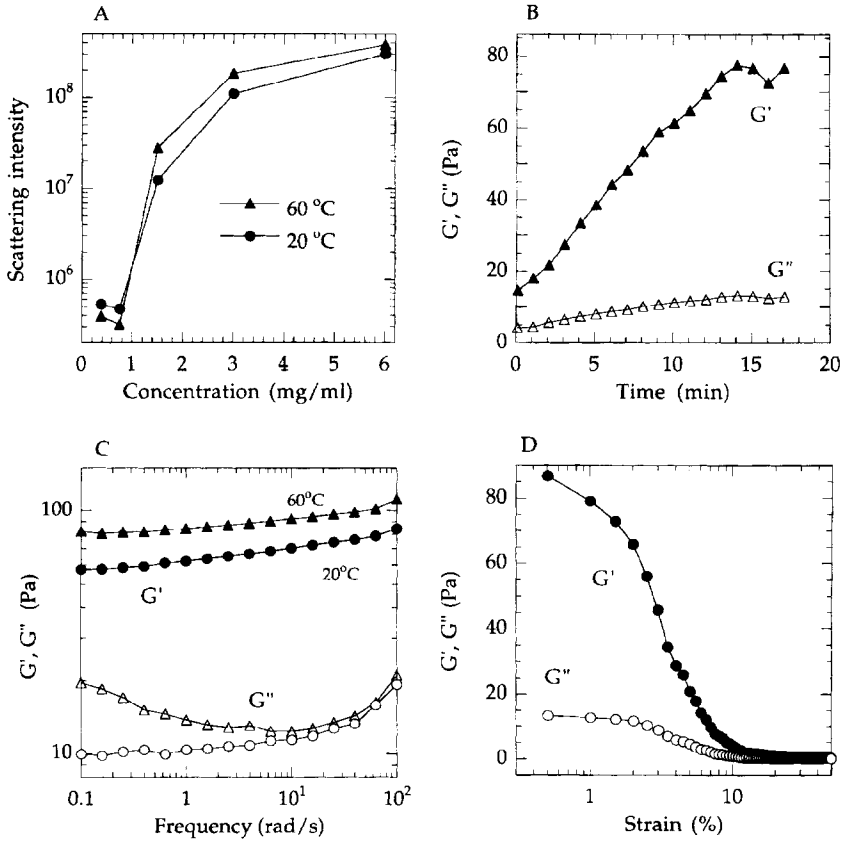

Figure 1. (A) Light scattering intensity of various concentrations of Fmoc-Leu-Asp in 10 mM Tris pH 7.0, measured at 20 °C (circles) and 60 °C (triangles). (B) The storage (closed symbols) and loss shear moduli (open symbols) of 2 mg/ml Fmoc-Leu-Asp in 10 mM Tris pH 7.0 at 60 °C. The maximal shear strain was 1% for oscillatory deformation at a frequency of 1 rad/s. (C) G' (closed symbols) and G'' (open symbols) were measured at 1% maximal strain over a range of frequencies at 60 °C (triangles) and 20 °C (circles). Other experimental conditions are as described for (B). (D) G' and G'' measured at various maximal shear strains at a frequency of 1 rad/s. Other experimental conditions and symbols are as described for (C).

Chemical Requirements for Gel Formation

The structural specificity of the gel-forming peptide is summarized in Table I. Gels are formed only by dipeptides with a C-terminal Asp residue. Either Leu or Ile can be the N-terminal residue for aqueous gels. One per cent Fmoc-Leu-Asp also forms a gel in ethyl ether and in 80% glycerol/20% water, although with a ten times lower shear modulus (data not shown). Fmoc-Ala-Asp has a higher critical gelation concentration, presumably because of its better solubility in water. Surprisingly, Fmoc-Leu-Glu does not form a gel at the concentrations tested, probably because of the greater flexibility of the Glu sidechain. Replacement of the Fmoc group with Boc- or Cbz-groups eliminates gelation activity (not included in Table I).

Confocal Microscopy of Peptide Filaments

Figure 2 shows that filaments longer than 10 μm can be observed by confocal fluorescence microscopy when the Fmoc-Leu-Asp gel is formed in the presence of a 2% weight ratio of rhodamine-Leu-Asp. Since rhodamine-B bears a crude structural similarity to Fmoc, this fluorescent derivative is able to partition into the Fmoc-Leu-Asp structures without perturbation. Control measurements confirm that rhodamine-Leu-Asp does not form elongated structures itself and has no effect on the elastic modulus of the Fmoc-Leu-Asp gel (data not shown). A plausible model for packing of the dipeptide into the filaments is a rod-like micelle with the hydrophobic Fmoc-Leu in the centre and the doubly negatively charged C-terminal Asp on the exterior. Other molecular arrangements such as tubular bilayer vesicles may also account for the structures observed [18]. Similar rod-like micelles have recently been observed in solutions of some larger peptide derivatives, but only in organic solvents [2, 3].

A minimal requirement for gel formation is that the solute molecules be large enough to interact with each other sterically or electrostatically. The steric requirement can be estimated by the relation defining semi-dilute solutions in which extensive steric overlap occurs [19]:

$$c^*(\text{in mg/ml}) \gg (M_r/Na)L^3$$

where (M_r/Na) is the weight of the polymeric filament and L is its length. To estimate the mass of a filament we assume the rod is composed of peptide at a mass density $\rho = 1$ g/ml. Since the true diameter of the filaments cannot be resolved by the fluorescence microscope, we assume a diameter of 10 nm, by analogy with micellar rods formed by amphiphiles of similar molecule weight [5]. For filaments of $L = 10$ μm, therefore, the mass is $\pi r^2 L\rho = 10^{-15}$ g or equivalently a molecular weight of 6×10^8. For a system of rods 10 μm long, $c^* = (10^{-15}\text{ g})/(10^{-3}\text{ cm})^3 = 0.001$ mg/ml. Therefore a concentration of 2 mg/ml is well above the critical overlap concentration for such solutions even accounting for underestimates of the average filament length and the filament diameter. This model of steric overlap

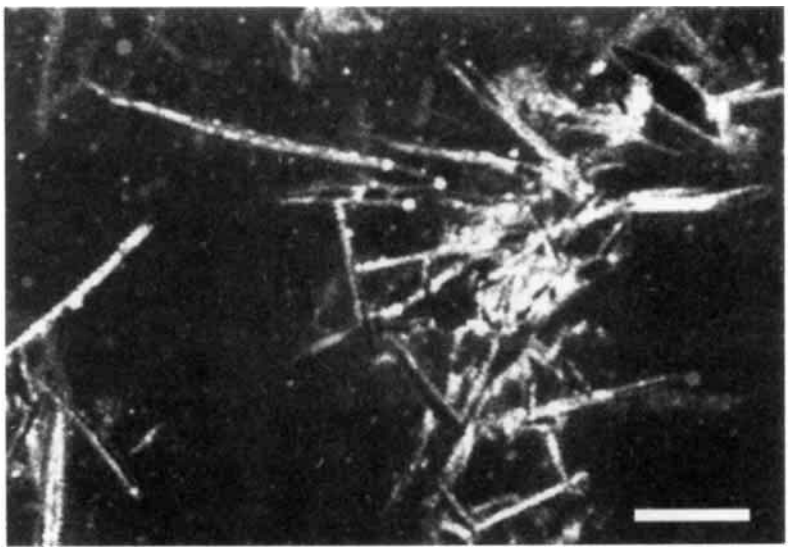

Figure 2. Confocal scanning micrograph of 4 mg/ml Fmoc-Leu-Asp in 10 mM Tris pH 7.0 containing also 0.02 weight fraction rhodamine-Leu-Asp. The scale bar denotes 5 μm.

provides the basis for viscoelasticity of other filamentous systems such as F-actin [10]. In addition to steric effects, the molecular structure of the peptide comprising the filaments and the large negative charge suggest that hydrogen bonding, electrostatics and van der Waals interactions may also be significant.

Incorporation of Drugs into Peptide Aggregates and Enhancement of Antigenicity

The low molecular weight drugs 3,5-dimethyl-1-adamantanamine hydrochloride (Ada2Me) and 5-methyl-1-adamantanamine 3-carboxylic acid (AdaMeC) can be incorporated into the Fmoc-Leu-Asp

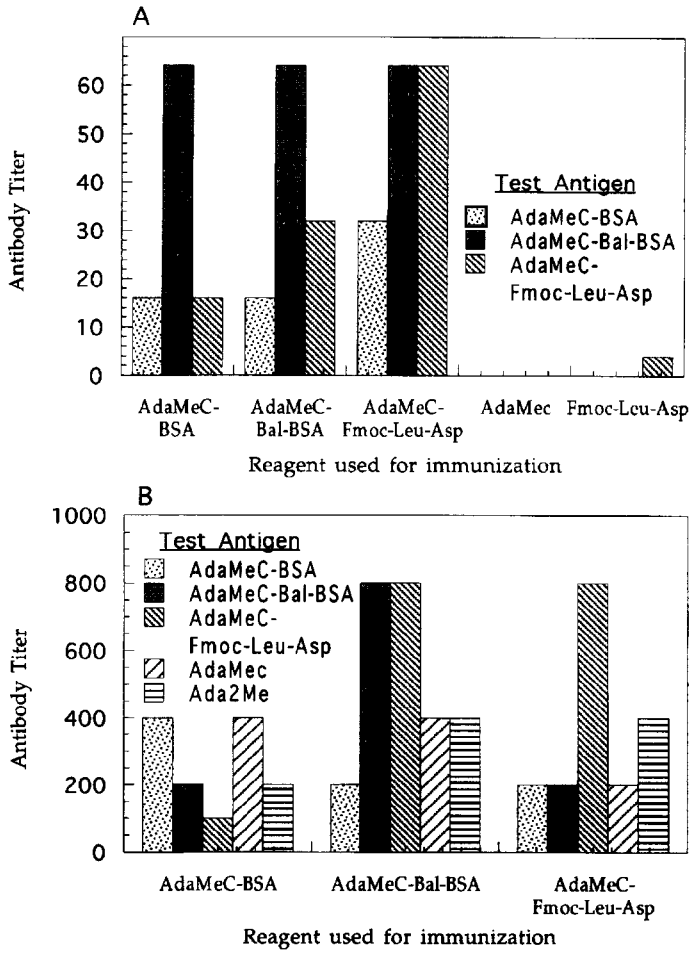

Figure 3. Use of Fmoc-Leu-Asp to develop antibodies against 5-methyl-1-adamantanamine 3-carboxylic acid hydrochloride (AdaMeC). (A) Double diffusion in agarose was used to determine the titre of antibodies recognizing AdaMeC-containing conjugates in sera of rabbits immunized with AdaMeC-coupled with or without a beta-alanine (Bal) spacer to BSA in complete Freunds adjuvant or with AdaMeC mixed with Fmoc-Leu-Asp without adjuvant. The antisera were tested against each of the three immunogens and compared with sera of rabbits injected with either AdaMeC or Fmoc-Leu-Asp alone. (B) Haemagglutination was used to analyse antisera of rabbits immunized with each of the three AdaMeC conjugates after challenge with the three AdaMeC conjugates, AdaMeC alone or Ada2Me.

gels at concentrations of 1 and 33 Mm, respectively. At higher concentrations, (>5 mM Ada2Me or >33 mM AdaMeC) these agents inhibit gelation. When the Fmoc-Leu-Asp gel containing AdaMeC in phosphate-buffered saline was injected into rabbits, without adjuvant, antibodies were raised against this drug to produce antisera with titres as high or higher than those of animals immunized with AdaMeC-BSA conjugates in equal volumes of complete Freund's adjuvant (Figure 3). Three methods of antibody determination, double diffusion in agarose gel, reverse radial diffusion (data not shown) and passive haemagglutination gave comparable results. Injected alone, neither Ada2Me (5 mM) nor AdaMeC (33 mM) produced specific antibodies. Fmoc-Leu-Asp produced antibodies with titres no greater than 1:4.

DISCUSSION

Several peptide derivatives form long micelles and gels in certain organic solvents [1–3]. The dipeptide derivative Fmoc-Leu-Asp is unique in that it is smaller and forms gels in water by assembling into long, amphipathic polyanionic filaments. Such structures have a variety of potential practical applications. The physical properties of the gel, notably its high elastic modulus and strain-weakening, lend themselves to preparation of cremes and lotions. The amphipathic nature of the micelles also facilitates the loading of small hydrophobic or amphiphilic molecules into the micelle which may be exploited for purposes of drug delivery and targeting.

Fmoc-derivitized amino acids, in particular Fmoc-Leu [20] and anionic polyelectrolytes [21] have been reported to influence the immune system. Boc-Leu-Asp dipeptides also may inhibit platelet aggregation [22]. These features, together with the ability of compatible low molecular weight compounds such as rhodamine-Leu-Asp or Ada2Me to incorporate into the Fmoc-Leu-Asp gel, suggest applications of the peptide for antigen presentation. This possibility is confirmed by the facile use of the Fmoc-Leu-Asp gel to prepare specific antibodies against adamantanamine derivatives which are otherwise very poor antigens. In comparison with the covalent conjugation of these compounds to macromolecular carriers such as bovine serum albumin, the benefits of this entrapment approach are the simplicity of the preparation of antigenic aggregates, ease of standardization and the lack of need to modify the hapten chemically or to use an additional adjuvant.

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