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Induction of Biologically Active Antibodies by a Polyvalent Synthetic Vaccine Constructed without Carrier

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Four synthetic peptides that copy fragments of two bacterial antigens (*Streptococcus pyogenes* M protein and diphtheria toxin), one viral antigen (hepatitis B surface antigen), and one parasitic antigen (circumsporozoite protein of *Plasmodium knowlesi*) were covalently bound within the same construct. This totally synthetic polyvalent administered to mice with Freund complete adjuvant or in saline with murabutide (an adjuvant-active muramyl peptide) elicited high levels of antibodies which, in certain cases, were shown to be biologically active. The results indicated that these antibodies recognized specifically the four peptides. None of the epitopes were immunodominant. It was also demonstrated that the association of several peptides enhanced their respective immunogenicities as compared with those of their homopolymers. Finally, this study shows that a totally synthetic vaccine administered in saline with a synthetic adjuvant can be immunogenic in the absence of a protein carrier.

We have demonstrated previously that peptides of different specificities conjugated to tetanus toxoid produced non-cross-reactive antibodies of each specificity when administered with Freund complete adjuvant (FCA) (8). Moreover, these antibodies were shown to be biologically active. More recently, similar results were obtained with type 24 streptococcal M protein as a carrier. The polyvalent conjugate was administered in saline with a synthetic adjuvant called murabutide (*N*-acetylmuramyl-L-alanyl-D-glutamine-α-*n*-butyl-ester) (3).

In the present report, we show that biologically active antibodies can be obtained in the absence of any protein or polypeptide carrier by immunizing mice with a heteropolymer containing only synthetic peptides of different specificities. In these studies, two bacterial peptides, a parasitic peptide, and a viral peptide were copolymerized. These were the following (Fig. 1): (i) a streptococcal peptide hereafter referred to as S-34 which consists of a repetitive sequence along the structure of the M protein of *Streptococcus pyogenes* type 24 (5), (ii) a diphtheric peptide termed D(A-A-186-201) which represents amino acids 186 to 201 of the diphtheria toxin elongated at the N-terminal end by two alanyl residues (2), (iii) a malarial peptide called PK 26 which represents the tandem repeat of a repetitive sequence of the *Plasmodium knowlesi* circumsporozoite coat protein to which one tyrosine and one cysteine have been added to the N and C termini, respectively (12), and (iv) a hepatitis B virus peptide designated as H(99-121) which represents a 23-amino-acid epitope of the surface antigen of the hepatitis B virus (4). The three former peptides have been shown to be capable of inducing biologically active antibodies (1, 6, 9, 12, 14). Mice were immunized with the copolymer of these four peptides and also with homopolymers of PK 26 or S-34 peptides. The polyvalent vaccine and homopolymer controls were administered to mice either in FCA or in saline in the absence or presence of murabutide.

MATERIALS AND METHODS

Synthetic peptides. The primary structures of the four peptides are shown in Fig. 1. S-34 (a streptococcal peptide of 34 amino acids) represents the first 34 amino acids of a peptide which has also been described as S-CB7 (synthetic cyanogen bromide fragment number 7). This peptide represents a repetitive structure of the M protein of *S. pyogenes* type 24 and has been shown to elicit protective antibodies (6). Because its terminal methionine has been shown to be unnecessary for raising protective antibodies (16), S-34 has been synthesized according to previously reported methods (13). D(A-A-186-201) is the 16-amino-acid segment of the N-terminal loop of the diphtheria toxin. It has been elongated at the N-terminal end by two alanyl residues which play the role of spacers. This peptide has been demonstrated to contain at least one protective epitope (1) and has also been referred to as SODP (synthetic octodecapeptide) (2). The peptide was synthesized by J. Diaz (Clin-Midy, Montpellier, France). H(99-121) is composed of amino acids 99 to 121 of the hepatitis B surface antigen. It has been synthesized as previously described (4) and has been shown to induce antibodies recognizing the natural antigen (4). PK 26 is the *P. knowlesi* tandem repeat of the dodecapeptide circumsporozoite coat protein and contains a tyrosine residue at its N terminus and a cysteine residue at its C terminus. This peptide has also been shown to induce biologically active antibodies (10, 14). The chemical synthesis and characterization of the tandem repeat have been previously reported (18). All four peptides were amidated at the C-terminal end.

Adjuvants. FCA and Freund incomplete adjuvant were purchased from Difco Laboratories, Detroit, Mich. Murabutide was synthesized as described previously (17) by P. Lefrancier et al. (Institut Choay, Paris, France).

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STREPTOCOCCUS PYOGENES PEPTIDE S-34

N-F-S-T-A-D-S-A-K-I-K-T-L-E-A-E-K-A-A-L-A-A-R-K-A-D-L-E-K-A-L-E-G-A

DIPHTHERIA TOXIN PEPTIDE D(A-A-186-201)

A-A-C-A-G-N-R-V-R-R-S-V-G-S-S-L-K-C

HEPATITIS B VIRUS PEPTIDE H(99-121)

D-Y-Q-G-M-L-P-V-C-P-L-I-P-G-S-S-T-T-S-T-G-P-C

PLASMODIUM KNOWLESI SPOROZOITE PEPTIDE PK 26

Y (Q-A-Q-G-D-G-A-N-A-G-Q-P)$_2$C

FIG. 1. Primary structure of the four peptides S-34, D(A-A-186-201), H(99-121), and PK 26.

Construction of the polyvalent vaccine. Peptides were copolymerized with glutaraldehyde as follows: 12.6 mg of S-34 (corresponding to 20 NH_2 μeq), 10.1 mg of D(A-A-186-201) (corresponding to 10 NH_2 μeq), 12.1 mg of H(99-121) (corresponding to 5 NH_2 μeq), and 14.2 mg of PK 26 (corresponding to 5 NH_2 μeq) were mixed in 9.8 ml of 0.1 M sodium bicarbonate for 1 h. Glutaraldehyde (25% in water; grade I; Sigma Chemical Co., St. Louis, Mo.) was then added to a final concentration of 2.63 mM with continuous stirring at room temperature. After 2 weeks of incubation, the resulting mixture was dialyzed exhaustively (molecular weight cutoff of 12,000) against phosphate-buffered saline (PBS). Under these conditions, 90% of the total amount of peptides were recovered, as shown by the method of Folin. To prepare poly-S-34 and poly-PK 26, S-34 and PK 26 were autopolymerized with glutaraldehyde as described previously (9, 15). S-34–tetanus toxoid conjugate and PK 26–tetanus toxoid conjugate were prepared with glutaraldehyde as described previously (2, 15) by using tetanus toxoid (5,000 limit flocculation by ml; Pasteur Production).

HPLC. High-performance liquid chromatography (HPLC) was performed by using a system from LKB Instruments, Inc., Rockville, Md. Eluates were monitored by UV A_{220} or A_{230} with a variable wavelength detector (LKB). Analytical reverse-phase HPLC was performed with an Ultropac column (LKB) Lichrosorb RP18 (10 μM). The elution gradient was 3 to 45% aqueous acetonitrile containing 0.025 M sodium phosphate (pH 3.2) for 25 min at a flow rate of 1 ml/min.

High-performance size exclusion chromatography was performed on a TSK G 3000 SW column (7.5 [inner diameter] by 600 mm; LKB) by isocratic elution with 0.01 M sodium phosphate (pH 7.0) containing 0.2 M sodium chloride. Eluates were monitored by UV A_{206} for the poly-S-34 and A_{220} for the poly-PK 26 and polyvalent vaccine. Molecular weight markers (Pharmacia) were used to standardize the column. The eluant of the polyvalent vaccine was collected and divided into five fractions before their antigenic compositions were studied.

Immunization. Female Swiss mice (7 to 8 weeks old; Iffa Credo, St. Germain-sur-l'Arbresle, France) were immunized subcutaneously with 100 μg of the polyvalent vaccine in PBS (Bio-Merieux, Lyon, France) with or without adjuvants, according to protocols described in Results. Sera were collected and pooled by groups during the experiment, with the exception of day 78, when sera were collected individually.

Studies of antigenicity and antibody titration. The antigenicity of the polyvalent vaccine and the measurement of antibody levels were performed by enzyme-linked immunosorbent assay (ELISA). Antigens were used at 2 μg per well for peptides and 0.5 μg per well for M24 protein, prepared by E. H. Beachey (Veterans Administration, Memphis, Tenn.). The antigen-coated plates (Nunc, Roskilde, Denmark) were washed and incubated for 1 h with serial dilutions of sera. The wells were then washed and treated for 1 h with a goat anti-mouse immunoglobulin G peroxidase conjugate (Sigma). After the wells were washed extensively, the substrate was added (*o*-phenylenediamine [50 mg/100 ml; Sigma], 0.05 M citrate-phosphate buffer [pH 5.2], 20 μl of H_2O_2 [35%]). The enzyme reaction was stopped after 10 min by the addition of 12.5% (vol/vol) sulfuric acid. Absorbances were read at 492 nm on a Titerteck Multiskan ELISA reader (Flow Laboratories, Inc., McLean, Va.). The negative control was a pool of 50 normal mouse sera diluted 100-fold. Titers were expressed as the maximal dilution giving an absorbance threefold higher than that of the negative control (background had an optical density of 0.025). The inhibition studies were performed with the monomeric peptide (S-34) as the antigen and immune sera diluted (with 1% bovine serum albumin) at the ratio of 1:15,000. The inhibiting antigens, monomeric S-34, homopolymer S-34, polyvalent vaccine, and diphtheric peptide, were each incubated with the sera for 20 h at 4°C and then tested with indirect ELISA.

To study the antigenic composition of the polyvalent vaccine samples, five fractions collected by high-performance size exclusion chromatography were distributed into microtiter plates in duplicate, and monovalent antipeptide sera were used as reagents according to methods previously described (8). The sera were prepared as reported for anti-S-34 (16), anti-D(A-A-186-201) (2), anti-H(99-121) (4), and anti-PK 26 (9).

Bacterial challenge. Mice were injected intraperitoneally with approximately 3.6×10^5 *S. pyogenes* type 24 (6) suspended in PBS. Bacterial viability and numbers of organisms were determined by plating on tryptose agar and counting the number of CFU. The number of mice that survived was recorded during a period of 10 days.

CSP assay. The circumsporozoite precipitation (CSP) assay was performed according to the general method of Vanderberg et al. (20) by using live sporozoites and undiluted sera.

RESULTS

Analysis of the polyvalent vaccine by HPLC and by specific antipeptide antisera. The analysis of the polyvalent synthetic vaccine by reverse-phase HPLC revealed one broad peak (integration surface, 92%) and a minor peak (8%). The four free peptides and their corresponding homopolymers were used as controls and were analyzed by using the same column. The comparison of the different elution profiles obtained showed that no overlapping could occur between the time of elution of the polyvalent vaccine and those of the controls since the time of elution of the former was separated by more than 1.5 min from any of the other times of elution.

The polyvalent vaccine was also analyzed by high-performance size exclusion chromatography and its diagram of elution was compared (Fig. 2) with the diagrams of the homopolymers of PK 26 (poly-PK 26) and of S-34 (poly-S-34). It was observed that the polyvalent vaccine was eluted as a single peak corresponding to an average molecular weight of 24,000. The times of elution of poly-S-34 and

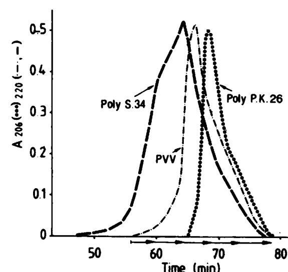

FIG. 2. Elution pattern of polyvalent vaccine (PVV), streptococcal homopolymer (poly-S-34), and malarial homopolymer (poly-PK 26) by high-performance size exclusion chromatography on a TSK G 3000 SW column equilibrated in 0.01 M sodium phosphate (pH 7.0) containing 0.2 M NaCl. Each vaccine was tested separately. Sample volume was 20 μl containing 75 μg of each compound. Eluates were monitored for UV A_{206} for the poly-S-34 and for UV A_{220} for the PVV and poly-PK 26. The flow rate was 0.3 ml/min at room temperature. Polyvalent vaccine was collected and divided into five fractions (indicated by arrows).

poly-PK 26 were shorter and longer, respectively. The peak of the polyvalent vaccine was collected in five different fractions and studied for the antigenic composition by ELISA with antisera that were monovalent for each peptide (8). All five fractions were shown to contain the four antigens in approximately the same proportions.

Immunogenicity of the polyvalent synthetic vaccine and production of anti-peptide antibodies. Three groups of eight outbred mice were immunized as follows: (i) controls received 100 μg of the conjugate in PBS on days 1, 30, and 70; (ii) a group received 100 μg of the conjugate in FCA on day 1, in Freund incomplete adjuvant on day 30, and in PBS on day 70; and (iii) a group received 100 μg of the conjugate in PBS with 100 μg of murabutide on day 1 and the same amount of conjugate in saline on days 30 and 70. Sera were collected on days 28, 44, and 78. In the controls which were immunized with no adjuvants, no antibodies could be detected even after three injections (Table 1). However, FCA-treated mice produced antibodies against the four peptides after the primary injection, and higher titers were observed on days 44 and 78. In the murabutide-treated group, high levels of antibodies against the four peptides were also produced, but only after the third injection. Similar results were observed in three identical experiments. Individual titers measured on day 78 showed that all the FCA- and murabutide-treated mice responded to the four peptides (Table 2). These results demonstrate the following. (i) Although the titers were higher in the FCA-treated group, several mice treated with murabutide also had very high antibody levels. (ii) All the mice in the FCA group showed high titers of the same order of magnitude against all four specificities except for mice 2 and 6, which did not respond well to D(A-A-186-201). (iii) It can also be noted that mice in the murabutide-treated group responded well to the four specificities, although the responses were more disperse and selective. For example, mice 2 and 3 responded preferentially to the diphtheric peptide, and mouse 8 responded preferentially to the streptococcal peptide. (iv) As indicated in the following experiments, immunization with the polyvalent vaccine produced biologically active antibodies.

TABLE 1. Antibody response of mice immunized with the polyvalent synthetic vaccine

Group[a]	Day	Titer of antibody against[b]:			
		S-34	D(A-A-186-201)	H(99-121)	PK 26
Controls	28	<100	<100	<100	<100
	44	<100	<100	<100	<100
	78	<100	<100	<100	<100
FCA	28	5,850	350	400	1,900
	44	100,000	25,000	75,000	70,000
	78	150,000	60,000	96,000	176,000
Murabutide	28	<100	160	<100	<100
	44	250	500	150	150
	78	117,000	41,000	13,000	31,500

[a] Eight Swiss mice received 100 μg of polyvalent vaccine on days 1, 30, and 70. The vaccine was administered alone, with FCA, or with murabutide under the conditions described in Results.
[b] Antibodies were measured by ELISA.

TABLE 2. Individual responses of mice to the polyvalent vaccine measured on day 78

Group	Mouse no.	Titer of antibody against:			
		S-34[a]	D(A-A-186-201)	H(99-121)	PK26
FCA	1	60,000	93,000	68,000	220,000
	2	80,000	7,500	100,000	250,000
	3	98,000	94,000	40,000	54,000
	4	140,000	52,000	110,000	290,000
	5	144,000	120,000	30,000	160,000
	6	305,000	15,000	140,000	300,000
	7	345,000	52,000	85,000	48,000
	8	390,000	50,000	200,000	350,000
Murabutide	1	1,900	20,000	1,000	7,500
	2	2,000	43,000	4,700	4,000
	3	2,800	38,000	3,600	8,000
	4	8,000	7,500	1,750	3,100
	5	10,700	18,000	1,200	9,000
	6	14,000	130,000	15,000	110,000
	7	100,000	105,000	80,000	106,000
	8	400,000	6,200	1,700	5,000

[a] Sera have been ranked according to their level of anti-S-34 antibodies.

Production of biologically active antibodies. (i) Antibody response against *P. knowlesi* sporozoites. In the following experiment, sera were pooled by groups for the controls and for the adjuvant-treated mice that had the highest titers. All three samples were tested in the CSP assay with at least 25 sporozoites for each test. This assay is considered to give a good estimate of the efficacy of antibody production after vaccination with sporozoites (11). The results given in Table 3 show that the control sera were negative, whereas high titers of functional antibodies were found in both the FCA- and murabutide-treated groups.

(ii) Antibody response against homologous *S. pyogenes*. In

TABLE 3. CSP activity of sera obtained after immunization with the polyvalent vaccine

Group	Anti-PK 26 titer	CSP	
		No. of CSP/ no. of sporozoites	Score[a]
Controls	<100	0/25	−
FCA	275,000	16/25	++/++++
Murabutide	110,000	11/25	++

[a] ++, A granular precipitate on the surface of the sporozoites; ++++, a long threadlike filament at the end of the sporozoite.

TABLE 4. Protection against *S. pyogenes* of mice treated by polyvalent synthetic vaccine

Mouse no.	Anti-M24 titer[a] for:		
	Controls	FCA group	Murabutide group
1	<100[b]	5,400[b]	2,500[b]
2	<100[b]	11,000	4,800
3	150[b]	25,000[b]	2,200[b]
4	<100[b]	27,000	3,000
5		48,000[b]	8,000
6		61,000	6,000
7		63,000	12,000
8		92,000	20,000

[a] Individual anti-S-34 antibody titers for the mice from the FCA and murabutide groups are given in Table 2. All eight nonimmunized controls had no anti-M24 titer and died by day 3; all four immunized controls died by day 3; three of eight mice from the FCA group died on days 3, 6, and 7; two of eight mice from the murabutide group died on days 3 and 5.

[b] Animal did not survive.

the following experiment, four groups of mice were challenged with a lethal amount of *S. pyogenes* type 24. These groups are as follows: (i) nonimmunized mice were used as controls, (ii) four mice which were immunized without adjuvant were also used as controls, (iii) eight FCA-treated immunized mice, and (iv) eight murabutide-treated immunized mice. After the levels of antibodies against M protein were measured, all the immunized animals were challenged 80 days after the first administration of the vaccine. All the control mice which had no detectable anti-M24 protein antibodies died within 3 days (Table 4). In contrast, most of the adjuvant-treated mice had anti-M24 protein titers and a degree of protection. Antibody titers of the murabutide-treated survivors were often lower than those of the FCA-treated mice which did not survive.

Comparison of the immunogenicity of S-34 and PK 26 when administered within the polyvalent vaccine, homopolymers, or hapten-carrier conjugate. Five groups of eight mice received antigens emulsified in FCA as follows: (i) 100 μg of the polyvalent conjugate (containing less than 50 μg of S-34 or PK 26), (ii) 50 μg of poly-S-34, (iii) 50 μg of poly-PK 26, (iv) 100 μg of S-34–tetanus toxoid conjugate, and (v) 100 μg of PK 26–tetanus toxoid conjugate. After 1 month, the animals received a booster injection of the same amount of immunogen in Freund incomplete adjuvant. The highest antibody titers were observed with peptide-tetanus toxoid conjugates, but the polyvalent conjugate also induced good primary and secondary responses (Fig. 3). In contrast to the copolymer, the immunogenicity of the poly-S-34 and poly-PK 26 was greatly reduced or even nondetectable after autopolymerization. Competitive-binding inhibition ELISA (Table 5) showed that S-34 remained antigenic after autopolymerization or copolymerization. The polyvalent vaccine is a less potent inhibitor than is monomeric or polymeric peptide S-34 because the amount of S-34 present in the polyvalent vaccine represents only a small part of the conjugate.

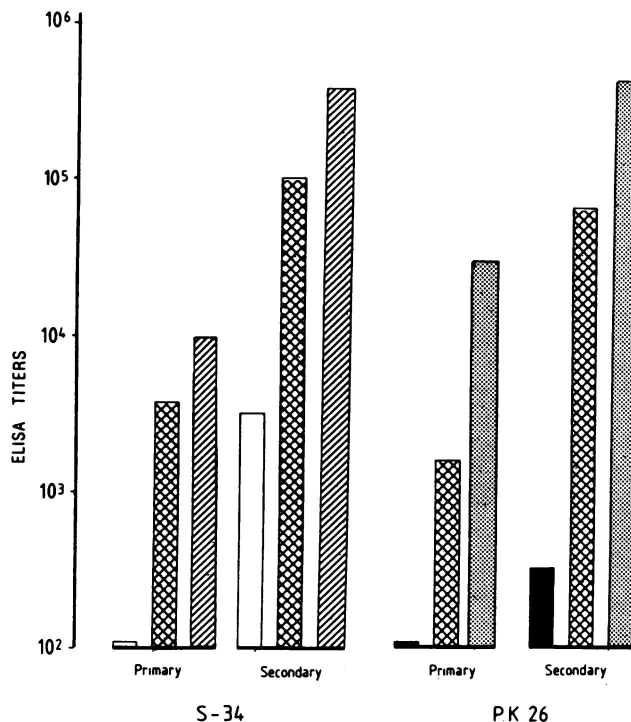

FIG. 3. Eight mice per group were injected subcutaneously with the following (which were emulsified in FCA): 100 μg of polyvalent conjugate (▩), 50 μg of poly-S-34 (□), 100 μg of S-34–tetanus toxoid conjugate (▨), 50 μg of poly-PK 26 (■), and 100 μg of PK 26–tetanus toxoid conjugate (▤). A second injection was given on day 30. Sera were collected on days 28 and 44. Antibodies were measured by ELISA titration. The results shown here give the antibody titers found in pooled sera from each group. These data correspond to two separate experiments.

DISCUSSION

The immunogenicity of four peptides covalently linked together in the absence of a carrier was studied. Analysis by HPLC and by specific antipeptide antisera showed that this conjugate did not contain detectable amounts of free peptide or homopolymers and that the same ratio of all four specificities was present in the different fractions of its elution peak. This low-molecular-weight conjugate (approximately 24,000) was found to be immunogenic if administered with FCA or murabutide in saline and induced high antibody response to the four peptides. Our results also suggest that no immunodominant epitope can be evidenced in this structure because individual animals gave very different patterns of responses and because some animals responded equally well to the four specificities. Such a polyvalent construct may be useful for immunogenic studies in appropriate inbred strains. In separate experiments anti-H(99-121) antibodies were shown to recognize natural hepatitis B surface antigens (4), and anti-D(A-A-186-201) was shown to recognize the native protein and to neutralize its toxicity (2). Furthermore, protection experiments demonstrated that the antibodies

TABLE 5. Antigenicity of free peptide S-34, homopolymer, and polyvalent vaccine by the competitive-binding inhibition ELISA test

Inhibitor	Antigenicity (OD) in serum at[a]:			
	100 μg/ml	20 μg/ml	4 μg/ml	0.8 μg/ml
S-34	0.020 (100)	0.138 (87)	0.678 (36)	0.901 (15)
Poly-S-34	0.020 (100)	0.116 (89)	0.572 (43)	0.869 (18)
Polyvalent vaccine	0.096 (91)	0.561 (47)	0.880 (17)	1.060 (0)
D(A-A-186-201)	1.015 (4)	1.066 (0)	1.052 (0)	1.064 (0)

[a] Sera were obtained during secondary response from mice immunized with S-34–tetanus toxoid conjugate with 100 μg of murabutide. Data are the means of duplicate assays from two different experiments (standard deviation, 5%). Percent inhibition is indicated in parentheses. OD, Optical density.

induced against PK 26 and S-34 could bind to the natural structure and were also biologically active. Nevertheless, results reported here show that the immunogenicity for a given peptide is increased when it is associated with peptides of different specificities as compared with its homopolymer. A possible explanation for this result may be that a given epitope can adopt a higher number of configurations in a heteropolymer than in a homopolymer. However, antigenic studies showed that either poly-S-34 or polyvalent vaccine inhibits antigen-antibody binding as effectively as the free S-34 peptide does. This suggests that the homopolymer and copolymer keep the same epitopes as the monomeric peptide does. Because there was no strict correlation between the biological activity of antibody and titers as measured by ELISA, the latter method can be misleading with respect to the efficacy of a vaccine. An alternate explanation for the decreased immunogenicity observed between the two preparations could be due to a monotonous antigenic structure.

Homopolymers of small peptides may lack sufficient information to trigger all the immunocompetent cells necessary to induce a good antibody response. On the other hand, the association within the same structure of different epitopes, some of them being B epitopes and some T epitopes, could be more effective for each entity present in the conjugate (7). Separate experiments suggest that the S-34 peptide or H(99-121) peptide could play the role of a T epitope under certain conditions of conjugation (unpublished data). Whatever the explanation, results reported here show that biologically active antibodies can be raised after immunization by a totally synthetic conjugate containing only four peptides linked together and administered in saline with an acceptable adjuvant. It must be mentioned that the adjuvant activity of murabutide has also been evaluated in clinical trials (19). Such a synthetic vaccine could be very useful for future application because it can overcome many problems related to the use of natural protein carriers.

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