

Effect of Carrier Selection on Immunogenicity of Protein Conjugate Vaccines against *Plasmodium falciparum* Circumsporozoites

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Conjugate vaccines against the sporozoite stage of *Plasmodium falciparum* were synthesized by covalently coupling the recombinant protein R32 {with the one-letter amino acid code of MDP-[(NANP)₁₅NVDP]₂LR} to tetanus toxoid, cholera toxin, cholera toxin, cholera toxin, and *Pseudomonas aeruginosa* toxin A. Conjugates were produced by using adipic acid dihydrazide as a spacer molecule and carbodiimide as a coupling agent. The molar ratio of R32 to carrier protein ranged from 2.5:1 to 8.4:1. These conjugates were found to be stable, nontoxic, and nonpyrogenic. When adsorbed onto Al(OH)₃, all conjugates were capable of inducing anti-R32 antibody. Conjugates made with either cholera toxin or *Pseudomonas aeruginosa* toxin A were significantly more immunogenic than those constructed with tetanus toxoid or cholera toxin. However, the magnitude of the immune response to the R32 moiety was not governed by the antibody response to the carrier protein.

Malaria is a leading cause of morbidity and mortality in tropical and subtropical areas, affecting an estimated 300 million individuals annually. Attempts to control malaria have centered around eradicating the mosquito vector and, to a lesser extent, drug chemoprophylaxis. Vector resistance to chemical insecticides, as well as emerging resistance to currently available antimalarial agents, has led to a resurgence of the disease in the past decade. In addition, the toxicity of certain antimalarial drugs limits the length of time for which they can be taken prophylactically. Therefore, attempts to control malaria have now focused upon immunization as an alternative to the measures described above.

A major surface protein of *Plasmodium falciparum* sporozoite, termed the circumsporozoite protein (CSP), has been cloned and sequenced (7, 8). The central domain of CSP is composed of 37 tetrapeptides, NANP (corresponding to Asn-Ala-Asn-Pro), interspersed with 4 tetrapeptides with the sequence NVDP (corresponding to Asn-Val-Asp-Pro). Antibody directed against the conserved immunodominant repeat region (NANP) of the protein is protective (17, 22, 25).

Attempts have recently been made to elicit an immune response in humans to the NANP repeat by using defined vaccines. In one report, the CSP gene was fused to the first 32 amino acids of the gene coding for tetracycline resistance and the fusion protein (R32tet32) synthesized in *Escherichia coli* (24). Partial protection against a sporozoite challenge was obtained only after repeated injection with 800 µg of purified antigen (1, 23). Independently, (NANP)₃ has been covalently coupled to tetanus toxoid (TT) to form a conjugate vaccine (10). Two doses of vaccine administered to volunteers evoked a moderate but variable immune response, with few subjects attaining antibody levels protective against a sporozoite challenge (10). These studies demonstrated the protective effect of high levels of anti-NANP antibody in humans.

The purpose of this study was to construct vaccines by using an NANP-containing antigen, R32, coupled to various proteins suitable for human use, to determine the effect of

carrier selection on conjugate immunogenicity. While conjugates could be readily synthesized with TT, cholera toxin (CT), cholera toxin, (CG), and *Pseudomonas aeruginosa* exotoxin A (TA) with similar characteristics, the immune response to the R32 moiety was significantly higher when CT or TA was used.

MATERIALS AND METHODS

TT. TT (lot ppT-1, Swiss Serum & Vaccine Institute, Berne, Switzerland) for human use was further purified by ultrafiltration, ion-exchange chromatography on DEAE-cellulose (Pharmacia, Uppsala, Sweden), and gel filtration over Ultrogel ACA34 (LKB, Bromma, Sweden). This preparation had 500 LF/mg of total protein, where LF is the limit of flocculation.

CT. CT was purified by the methods described by Finkelshteyn and LoSpalluto (9). This preparation contained >90% intact CT.

CG. CG was prepared by the methods of Holmgren et al. (12). Briefly, CT in Tris-EDTA (0.05 M Tris, 0.001 M EDTA, 0.003 M NaN₃, 0.2 M NaCl [pH 7.5]) was run over an ascending Sepharose G-75 column equilibrated with 5% HCOOH and eluted with 5% HCOOH at room temperature. The fractions were monitored at 280 nm, and the CG-containing peak was pooled, concentrated over a YM-10 filter (Amicon Corp., Danvers, Mass.), dialyzed against Tris-EDTA, and filtered through a 0.45-µm-pore-size sterile filter (Millipore S.A., Molsheim, France). The solution was then stored at 4°C. Purity of this material was greater than 95%, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

TA. TA was purified from the supernatant of fermentor-grown cultures of *Pseudomonas aeruginosa* PA103 as described by Cryz et al. (4). The purity of the final preparations was between 92 and 98% as determined by high-pressure liquid chromatography with a Du Pont GF-250 column.

Sporozoite antigen. R32 is a purified recombinant protein which contains only the first two amino acids of the tetracycline resistance gene of R32tet32 (7, 11). The one-letter code for the peptide sequence is MDP-[(NANP)₁₅-NVDP]₂LR (M, methionine; L, leucine; R, arginine).

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Carrier-ADH coupling. Adipic acid dihydrazide (ADH; Fluka AG, Buchs, Switzerland) was introduced onto TT, CT, CG, and TA in phosphate-buffered saline (PBS) by using carbodiimide. Briefly, the carrier protein was added to twice its weight of ADH and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDEC; Sigma Chemical Co., St. Louis, Mo.). The solution was mixed by continuous stirring, and the pH was adjusted to 4.85 with 0.3 N HCl. The pH was kept at 4.85 for 2 h. EDEC at the above concentration was again added, and the pH was maintained at 4.85 for an additional 2 h. After incubation, the solution was sterilized by filtration through a 0.45- μ m-pore-size Millipore filter. The solution was then extensively dialyzed against sterile PBS (pH 7.4) and stored at 4°C until required.

Coupling of carrier protein-ADH to R32. Coupling of carrier protein-ADH to R32 was performed as described by Sadoff et al. (manuscript in preparation). TT(ADH), CG(ADH), CT(ADH), or TA(ADH) in PBS (pH 7.4) was added to a 10-fold (wt/wt) excess of EDEC. The pH of the solution was adjusted to 4.85. R32 in PBS, equal to twice the weight of the carrier, was slowly added to the reaction mixture, and the pH of the solution was maintained at 4.85 for 3 h at 22°C with continuous mixing. In another experiment, a sixfold (wt/wt) excess of R32 was added to the TT(ADH) to determine whether a higher ratio of the R32 to carrier could be achieved. The solution was filter sterilized and concentrated by evaporation under reduced pressure. The concentrated solution was then run over a sterile Sephadex G-75 column (Pharmacia) equilibrated with PBS. The conjugates were recovered in the void volume, filtered through a sterile 0.22- μ m-pore-size Millipore filter, and stored at 4°C.

SDS-PAGE. SDS-PAGE analysis of the conjugates was performed by the method of Laemmli (14), with a 6.5 to 12% gradient gel. The gels were stained with Coomassie blue.

Analysis of amino acid composition of the conjugates. Amino acid analysis was performed after acid hydrolysis of samples with a Beckman 6300 amino acid analyzer (Beckman Instruments, Inc., Fullerton, Calif.). The peptide-to-carrier protein ratio of the conjugate vaccines was calculated on the basis of the difference between the number of amino acids of a given type in the carrier protein, the peptide, and the conjugate. The molecular masses of the carrier proteins used for the calculation were as follows: TT, 150 kilodaltons (kDa); TA, 66 kDa; CT, 84 kDa; and CG, 57.5 kDa; R32 has a molecular weight of 13.1 kDa.

Molecular mass determination of the conjugates. Molecular masses of the conjugates were determined by gel filtration by using a Sephadex G-200 column previously calibrated with molecular mass standards.

Biological activities of conjugates. The conjugates were tested for pyrogenicity, sterility, and general safety by the method described in the Code of Federal Regulations 610.11 and 610.12 (Food and Drug Administration, U.S. Department of Health and Human Services, 1 April 1987 edition). In addition, the R32CT(ADH) was tested for activity in a Y-1 adrenal cell assay system after 1, 2, 3, and 4 weeks of incubation at 37°C (18). The R32TA(ADH) was analyzed for the presence of ADP-ribosyl transferase activity after 1, 2, 3, and 4 weeks of incubation at 37°C as described by Cryz et al. (5).

Immunogenicity of peptide-carrier conjugate. New Zealand rabbits were immunized intramuscularly with 100 μ g of Al(OH)₃-adsorbed conjugate in 0.5 ml of PBS at days 0 and 14. Serum samples were obtained on days 0, 14, and 28. In some experiments, a further immunization with R32 coupled to a homologous or heterologous carrier protein was given

on day 42. Sera were kept at -20°C. In additional experiments, rabbits were simultaneously immunized with either R32TT(ADH) and R32CG(ADH) conjugates or R32CT(ADH) and R32TA(ADH) conjugates.

Anti-NANP antibody ELISA. The enzyme-linked immunosorbent assay (ELISA) used for the detection of antibodies to the *P. falciparum* sporozoite antigen was performed as follows. Titertek immunoassay plates (Flow Laboratories, Lelystad, The Netherlands) were coated with 100 μ l of R32 (10 μ g/ml in PBS [pH 7.4]) per well. The plate was incubated at 37°C for 3 h, after which time it was stored at 4°C. Two hundred microliters of blocking solution (0.5% boiled casein [BC] in PBS [pH 7.4]) was added to each well, and the plate was incubated at 22°C for 1 h. Wells were then washed with PBS-0.05% Tween 20 (PBS-Tween). A standard anti-NANP rabbit serum, together with test serum, was serially diluted in BC-0.05% Tween 20 (BC-Tween), and 100 μ l of each dilution was added to duplicate wells. The plates were incubated at 22°C for 3 h, after which time they were washed three times with PBS-Tween. One hundred microliters of a horseradish peroxidase-immunoconjugated goat anti-rabbit immunoglobulin G (IgG) (heavy- and light-chain specific; Nordic Immunology, Tilburg, The Netherlands), diluted 1:2,500 in BC-Tween, was added to each well, and the plates were incubated for 1 h at 22°C. The plates were washed three times with PBS-Tween, and 100 μ l of substrate solution [10 mg of 2,2'-azino-di(3-ethyl-benzylthiozoline)sulfonic acid 6 (ABTS; Boehringer GmbH, Mannheim, Federal Republic of Germany) in 50 ml of NaHPO₄ buffer (pH 4.0)-125 μ l of 10% H₂O₂] was dispensed into each well. Color was allowed to develop for 30 min at 22°C, at which time the optical density at 405 nm was determined by using a Titertek Multiskan (Flow Laboratories, Hamden, Conn.). Antibody titer is defined as the reciprocal of the highest dilution of sera giving an optical density at 405 nm of 0.400, which is at least twice the standard deviation of the negative control wells which contained all reagents except sera.

Immunofluorescent antibody (IFA) assay. Antibody against native CSP on *P. falciparum* sporozoites was tested by indirect IFA assay as described by Young et al. (24). Briefly, 2,000 to 5,000 sporozoites in 10 μ l of 0.5% bovine serum albumin were spread onto slides, air dried at room temperature, and stored at -80°C. The IFA assays were initiated by spreading 20 μ l of rabbit serum, serially diluted in blocking solution (1.0% bovine serum albumin, 0.5% casein, 0.005% thimerosal, 0.0005% phenol red in PBS), onto the dried sporozoites. The slides were incubated at 22°C for 20 min in a moist chamber and washed with PBS. Twenty microliters of a 1:40 dilution of fluorescein isothiocyanate-conjugated goat antiserum to rabbit antibody was added to each spot, and the slides were incubated for 20 min and washed in PBS. The slides were mounted in glycerol and examined under UV light at \times 500 magnification. IFA titers were assigned the reciprocal of the highest dilution of serum which gave a strong positive reaction (24).

Anti-carrier antibody ELISA. Antibody titers to the different carrier proteins were determined by ELISA. Briefly, 200 μ l of CT (20 μ g/ml in PBS [pH 7.2]), CG (20 μ g/ml in PBS [pH 7.2]), TT (20 μ g/ml in PBS [pH 7.2]), or TA (20 μ g/ml in Na₂CO₃ buffer [pH 9.6]) was added to each well of a microtiter plate (Dynatech, Buchs, Switzerland) and incubated at 37°C for 3 h. The plates were washed three times with PBS-Tween. Serial twofold dilutions (150 μ l) of rabbit sera in PBS-Tween were added to duplicate wells, and the plates were incubated at 22°C for 3 h. The wells were washed three times with PBS-Tween, and 150 μ l of a 1:2,500 dilution

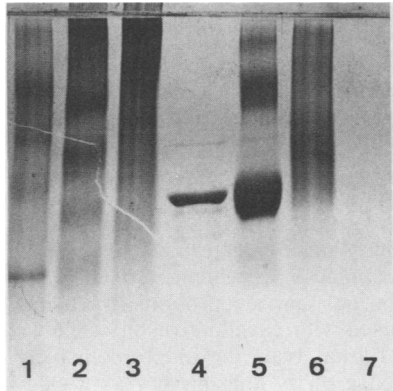


FIG. 1. SDS-PAGE profile of R32-carrier protein conjugates. Lane 1, TT; lane 2, TT(ADH); lane 3, R32TT(ADH); lane 4, TA; lane 5, TA(ADH); lane 6, R32TA(ADH); lane 7, R32.



FIG. 2. SDS-PAGE profile of R32-carrier protein conjugates. Lane 1, CT; lane 2, CT(ADH); lane 3, R32CT(ADH); lane 4, CG; lane 5, CG(ADH); lane 6, R32CG(ADH).

of goat anti-rabbit IgG peroxidase conjugate in PBS-Tween was added. The plates were incubated for 1.5 h at 22°C. Color was allowed to develop as described above. Anti-carrier antibody titer is defined as the reciprocal of the highest dilution giving an optical density at 405 nm of 0.500.

RESULTS

R32 consisting of NANP repeats could readily be coupled to TT, CT, CG, and TA with ADH as a spacer molecule and carbodiimide as a linker. When the initial coupling reaction contained a 2:1 R32/carrier ratio, the molar ratios of R32 to carrier protein were 2.6:1 for CG, 6.1:1 for CT, 6.6:1 for TA, and 8.4:1 for TT. An increase in the ratio of the R32 to carrier protein was achieved (10.8:1) when a 6:1 ratio (wt/wt) of R32 to TT(ADH) was used during the initial coupling process. The molecular masses of R32CG(ADH), R32TA(ADH), R32CT(ADH), and R32TT(ADH) conjugates were 89, 119, 178, and 251 kDa, respectively. All four conjugates were nonpyrogenic when 5 µg/kg of rabbit body weight was administered intravenously. The conjugates were also non-toxic for mice and guinea pigs upon intraperitoneal injection of 100 µg of antigen. CT and CG conjugates were biologically inactive in a Y-1 cell assay system when tested at a dose of 10 µg/ml. In comparison, native CT was active at 33 pg/ml. R32CT(ADH) was stable to toxic reversion when stored at 37°C for ≥28 days as determined in the Y-1 cell assay. The ADP-ribosyltransferase activity of TA was destroyed upon coupling of ADH to R32. Incubation of the

R32TA(ADH) conjugate at 37°C for up to 28 days did not result in the appearance of ADP-ribosyltransferase activity (<0.1% of native TA).

Carrier protein, carrier protein-ADH, and their corresponding R32 conjugates were analyzed by SDS-PAGE (Fig. 1 and 2). The coupling of ADH to carrier proteins resulted in a variable degree of cross-linking as evidenced by the formation of several higher-molecular-mass species. All R32 conjugates were visualized as a broad diffuse band with a molecular mass greater than that seen for the carrier alone or for the carrier-ADH complex. This would indicate that a substantial degree of irregular cross-linking occurs during conjugate formation.

Preliminary experiments showed that maximal anti-R32 antibody levels were achieved with 30- to 100-µg immunizing doses (data not shown). Therefore, all experiments described below were performed with 100-µg doses for monovalent immunization and 50-µg doses when two conjugates were simultaneously administered.

The ability of the various vaccines to elicit either an anti-R32 or an anti-carrier protein antibody response is shown in Table 1. All conjugates were capable of stimulating an anti-R32 IgG antibody response. However, the magnitude of the response was dependent upon the carrier protein used. Therefore, conjugates produced with TT or CG were significantly less immunogenic than those prepared with TA

TABLE 1. IgG immune response after vaccination with R32 conjugates

Immunogen ^a	IgG ELISA titer (geometric mean with range)					
	R32 on day:			Carrier protein on day:		
	0	14	28	0	14	28
R32TT(ADH)	<80	724 (456-1,148)	2,442 (1,640-3,112)	<10	21 (<10-130)	103 (90-120)
R32CG(ADH)	<80	1,898 (552-2,288)	1,652 (728-2,916)	<20	42 (<20-106)	2,974 (2,340-4,794)
R32TA(ADH)	<80	1,920 (1,644-2,520)	7,642 (3,900-15,220)	<20	132 (66-240)	749 (500-1,400)
R32CT(ADH)	<80	2,033 (432-6,544)	10,985 (3,900-29,496)	<10	20 (<10-100)	1,227 (524-7,120)
R32TT(ADH) + R32CG(ADH) ^b	<80	3,388 (2,024-7,640)	3,678 (1,344-8,180)	<10	21 (<10-150)	18 (10-100)
R32TA(ADH) + R32CT(ADH) ^b	<80	1,745 (1,320-2,388)	11,558 (8,360-15,920)	52 (48-60)	86 (68-128)	655 (560-740)
				20	18 (<20-62)	869 (616-1,232)

^a Rabbits were immunized on days 0 and 14 with 100 µg of vaccine.

^b Rabbits were immunized simultaneously on days 0 and 14 with 50 µg of each vaccine.

TABLE 2. Antibody response against *P. falciparum* CSP

Immunogen ^a	IFA titer (geometric mean and range) on day:	
	0	28
R32TT(ADH)	<40	400 (200–800)
R32CG(ADH)	<40	400 (200–800)
R32CT(ADH)	ND ^b	ND
R32TA(ADH)	<40	1,270 (800–1,600)
R32TT(ADH) + R32CG(ADH) ^c	<40	200
R32TA(ADH) + R32CT(ADH) ^c	<40	800 (400–1,600)

^a Rabbits were immunized on days 0 and 14 with 100 µg of vaccines.

^b ND, Not determined.

^c Rabbits were immunized simultaneously on days 0 and 14 with 50 µg of each vaccine.

or CT ($P < 0.01$). Increasing the number of R32 molecules per molecule of the carrier (TT) from 6.6 to 10.8 did not result in better antibody response (data not shown). In an attempt to increase the response to the R32 component, two R32 conjugates differing in the carrier moiety were simultaneously administered at two sites. A combination of R32TA(ADH) and R32CT(ADH) was far more effective at engendering anti-R32 IgG antibody on day 28 than a mixture of R32CG(ADH) and R32TT(ADH) ($P < 0.05$).

It was somewhat surprising that a booster dose of vaccine given at day 14 did not routinely stimulate a further rise in anti-R32 antibody. In two instances [with conjugates R32CG(ADH) and R32TT(ADH) + R32CG(ADH)], the second dose had no apparent effect on antibody titer. In the other groups, anti-R32 antibody levels were increased from approximately threefold to sevenfold after a second immunization. A third dose of vaccine given 14 or 28 days (being either homologous or heterologous to the carrier protein) after boosting was ineffective at increasing the titer of anti-R32 antibody.

Sera from rabbits immunized with the conjugate vaccines also reacted with the native CSP from the sporozoites, as was shown in the indirect IFA assay, with endpoint titers ranging from 1:200 to 1:1,600 at 28 days after the primary immunization (Table 2). The results observed with the IFA assay correspond with the anti-R32 titers in the ELISA system in that high IFA titers were also observed in rabbits immunized with R32TA(ADH) and R32TA(ADH) + R32CT(ADH) conjugates.

The magnitude and kinetics of the anti-carrier antibody response differed from those observed for the R32 conjugate component (Table 1). After a single dose of vaccine, most animals manifested only a modest rise in anti-carrier antibody. In contrast, good levels of anti-R32 antibody were seen after only one immunization. A booster dose of vaccine resulted in a vigorous anti-carrier antibody response in most instances, i.e., a 60-fold rise for R32CT(ADH) and a 70-fold rise for R32CG(ADH).

DISCUSSION

Recent studies have conclusively shown that antibody directed against the NANP repeat of the *P. falciparum* CSP can block infection with viable sporozoites (2, 17, 25). Two NANP-containing vaccines, a TT-(NANP)₃ conjugate (10, 16) and a recombinant protein containing 30 NANP repeats (1), have been evaluated in humans. While safe, neither vaccine was able to consistently engender protective levels of antibody. Possible explanations for this modest immunogenicity include failure of the vaccine to elicit a vigorous

booster response, the relatively low molecular weight of the recombinant protein (<40,000), and suboptimal presentation of the NANP₃ moiety of the conjugate vaccine.

The covalent coupling of nonimmunogenic or weakly immunogenic antigens to carrier proteins has been shown to be a highly effective means by which to engender good levels of antibody to such antigens (13, 19). Several factors, including the method of conjugation, degree of substitution, and selection of the carrier protein, are known to affect the immunogenicity of conjugate vaccines. We have therefore covalently coupled R32, which by itself is poorly immunogenic, to various carrier proteins by using a technique previously used with excellent results (3, 6).

To date, TT has been the most extensively used carrier protein because of its long history as a safe and effective human vaccine. Several TT-bacterial polysaccharide (PS) conjugate vaccines have been found to be safe and immunogenic in humans (6, 19). However, several potential problems exist concerning the use of TT as a carrier for sporozoite antigens. First, most individuals have received multiple doses of TT as part of routine immunization programs, leading to preexisting antibody levels which can be quite high. Subsequent immunization with a TT conjugate, which would contain approximately 5 to 10 times more TT antigen than the recommended adult booster, can be expected to elicit an Arthus-type reaction (19, 21). Second, preexisting anti-carrier antibody may have a deleterious effect on the immune response to the conjugate via epitope-specific suppression (20). In contrast, Lise et al. (15) have reported that a priming dose of TT can moderately increase the immune response to a TT-NANP conjugate in mice. However, evidence exists to suggest that this would not be the case in humans. Schneerson et al. (19) found that while various PS-TT conjugates are capable of evoking a booster response in several animal species, they were able to elicit only a primary response in humans with preexisting tetanus antibody. Furthermore, we have found that the antibody response in humans to the PS components of a *Pseudomonas aeruginosa* PS-TT conjugate was inversely related to preexisting anti-tetanus antibody levels (6).

By synthesizing various R32 conjugates by identical means, we were able to study the role of the carrier protein in the anti-R32 immune response. We found that the protein used could have a profound effect upon the levels of anti-R32 antibody attained. Conjugates constructed with either CT or TA were significantly more immunogenic than those made with CG or TT. This difference is not simply due to antigen load, since the TT conjugate, which elicited only moderate levels of anti-R32 antibody, possessed the highest ratio of R32 to carrier protein of any conjugate. Neither was the immune response to the R32 moiety governed by the magnitude or the kinetics of the antibody response to the carrier protein. For example, maximal anti-R32 titers were attained after a single dose of R32CG(ADH) conjugate with only a modest rise in anti-CG antibody. A second dose of vaccine increased the anti-CG titer by more than 50-fold with no concomitant rise in anti-R32 titer. Similarly, good levels of anti-R32 antibody were observed after the first immunization with R32CT(ADH), but only a slight rise in anti-CT antibody levels was observed. These findings indicate that physiochemical properties of the conjugate, as related to the physical presentation of the R32 determinants, are of great importance in determining the magnitude of the anti-R32 antibody response. Therefore, the best conjugate would position the R32 moiety in such a manner as to ensure optimal recognition by the immune system, perhaps by being

in proximity to a T-cell epitope expressed by the carrier protein.

For the currently described conjugate vaccines to be effective at preventing malaria, it is essential that the anti-R32 antibody engendered recognize the native CSP expressed on the sporozoite surface. To determine whether this was the case, we reacted immune rabbit sera with *P. falciparum* sporozoites in an IFA assay. Although there was a correlation between ELISA and IFA titers, IFA titers were much lower than the ELISA titers. The discrepancy in absolute titer values may be a reflection of lower sensitivity of the IFA assays as compared with the ELISA system. Alternatively, the lower IFA titer may be an indication that not all anti-R32 antibody reacting in the ELISA system reacts with native CSP of the sporozoites.

We have shown that R32CT(ADH) and R32TA(ADH) conjugates are nontoxic, nonpyrogenic, and highly immunogenic in rabbits when administered with Al(OH)₃ as an adjuvant. On the basis of such promising preliminary results, these conjugate vaccines are currently being evaluated for safety and immunogenicity in phase I human volunteer trials.

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