Immunologic memory response induced by a meningococcal serogroup C conjugate vaccine using the P64k recombinant protein as carrier

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Abstract

In this study, we used an adoptive lymphocyte transfer experiment to evaluate the ability of the P64k recombinant protein to recruit T-helper activity and induce immunologic memory response to the polysaccharide moiety in a meningococcal serogroup C conjugate vaccine. Adoptive transfer of splenocytes from mice immunized with the glycoconjugate conferred antipolysaccharide immunologic memory to naive recipient mice. The observed anamnestic immune response was characterized by more rapid kinetics, isotype switching from IgM to IgG and higher antipolysaccharide antibody titers compared with those reached in groups transferred with splenocytes from plain polysaccharide or phosphate-immunized mice. The memory response generated was also long lasting. Sera from mice transferred with cells from conjugate-immunized mice were the only protective in the infant rat passive protection assay, and also showed higher bactericidal titers. We demonstrated that priming the mice immune system with the glycoconjugate using the P64k protein as carrier induced a memory response to the polysaccharide, promoting a switch of the T-cell-independent response to a T-cell dependent one.

Introduction

Several vaccination campaigns with plain meningococcal capsular polysaccharide vaccines have demonstrated the efficacy of these antigens to stop epidemics caused by serogroups A, C, Y and W135 (Artenstein *et al.*, 1970; Borrow, 2000). However, polysaccharide vaccines are not immunogenic in children younger than 2 years old and induce hyporesponse at these ages (Maslanka, 1998; Mac Lennan *et al.*, 1999). Furthermore, these vaccines also generate a short-lasting antibody response characterized by low bactericidal titers, which is associated with their ineffective protection against the disease (Cadoz, 1998). All these disadvantages were eliminated by chemical conjugation of capsular polysaccharides to carrier proteins (MacDonald *et al.*, 2000; Richmond *et al.*, 2001).

A previous study demonstrated an obligatory role for T-helper cells in the immune response to glycoconjugate vaccines *in vivo*, mediated through presentation of processed antigens in the context of class-II major histocompatibility complex (MCH-II) molecules on antigen-presenting cells to the T-cell receptors, by the interaction of the costimulatory molecules B7-1 and B7-2 on antigen-presenting cells with CD28 on T-helper cells, and by stimulation of CD40L on activated T-helper cells (Guttormsen *et al.*, 1999). Thus, conjugate vaccines allow the generation of a T-cell-dependent response to the polysaccharide moiety facilitated by the carrier protein – that is to say, a better quality of the antibody response and a long-lasting protection (Ahmad & Chapnick, 1999).

There is increasing concern about epitopic overload and hapten-specific carrier-induced suppression that could take place when the same protein carrier is used in subsequent vaccination routines, because there are a lot of polysaccharide antigens needed for vaccination of children and only a few carrier proteins have been assayed for this purpose (Schutze *et al.*, 1985; Dagan *et al.*, 1998; Fattom *et al.*, 1999). Proteins that have been used as carriers in meningococcal conjugate vaccines licensed to date include tetanus toxoide and the nontoxic mutant of the diphtheria toxin (CRM197). The three serogroup C conjugate meningococcal vaccines licensed so far are highly immunogenic and induce

memory responses (Richmond *et al.*, 1999a, b, 2000). The development of pneumococcal vaccines increased the need for novel carrier proteins, because they suppose the simultaneous administration of several antigens. The characterization of new carrier proteins able to confer T-cell-dependent characteristics to a bacterial polysaccharide seems to be a compulsory step for the achievement of a proper immune response against all the polysaccharide antigens needed for childhood vaccination.

The meningococcal P64k protein was identified (Silva, 1992), cloned and expressed in *Escherichia coli* after the isolation of their coding gene (*lpdA*) (Guillen, 1998). The recombinant protein was sequenced, described as a dihydrolipoamide dehydrogenase and its molecular three-dimensional structure was determined (Li de la Sierra *et al.*, 1997). An immunologic study demonstrated that P64k is well recognized by human sera from convalescents of meningococcal disease or vaccinated individuals immunized with the Cuban outer-membrane-based vaccine VA-MENGOC-BC® (Finlay Institute, Cuba)(Guillen, 1998). It was demonstrated that P64k is immunogenic in laboratory mice and that is expressed in the majority of the *Neisseria meningitidis* strains examined (Silva, 1999; González *et al.*, 2000b). The protein safety and immunogenicity was demonstrated in a phase I clinical trial in healthy volunteers (Perez, 2001). The high molecular mass (64 kDa) and immunogenicity of the P64k protein have made it a suitable carrier protein for weak immunogens, either covalently coupled (González *et al.*, 2000a) or in chimera constructs (Guillén, 1996; González, 1997).

A serogroup C polysaccharide-P64k conjugate (CCPS/P64k) was obtained in our laboratory by the reductive amination method and its immunogenicity was demonstrated in BALB/c mice and African green monkeys (Carmenate *et al.*, 2004, 2005). In the present work, we used an adoptive lymphocyte transfer experiment to evaluate the ability of P64k recombinant protein to recruit T-helper activity and induce immunologic memory response to the polysaccharide moiety.

Materials and methods

Materials

Native meningococcal CCPS was obtained from the serogroup C meningococcal strain [C11 American Type Culture Collection (ATCC), USA]. The molecular size of the native polysaccharide was $\geq$60 kDa and the degree of O-acetylation was 3.1 μmol mg^{-1}. This material was donated by the Finlay Institute, Havana, Cuba. The recombinant P64k protein used for conjugation was produced at the Centro de Ingeniería Genética y Biotecnología, Havana, Cuba.

CCPS-P64k conjugates

The conjugate used for immunization was obtained by the reductive amination method (Wessels *et al.*, 1990). Briefly, 50 mg of native CCPS were oxidized by incubation with 20 mg of $NaIO_4$ previously dissolved in 5 mL of acetate buffer (5%) (pH 5.5). The oxidation reaction proceeded for 30 min in the dark at 25 °C and was stopped by addition of ethylene glycol. Oxidized polysaccharide was dialyzed against double distilled water using a membrane (12 000–14 000 molecular weight cut-off; SpectraPor, Inc., Rancho Dominguez, USA) and dried by lyophylization. Purified, oxidized polysaccharide (10 mg) was coupled to the P64k protein (6.6 mg) by reductive amination with sodium cyanoborohydride. The conjugation reaction was monitored by gel filtration chromatography with a Bio-Gel A0.5 column (2.6 × 100 cm, Biorad, Hercules, CA). The reaction was considered to be complete when the magnitude of void volume-peak remained constant. The conjugate named CCPS-P64k was treated with sodium borohydride and incubated at room temperature for 60 min to reduce any remaining aldehyde, dialyzed against sterile water at 4 °C and lyophilized to dryness. The final protein : -polysaccharide ratio was 1 : 1 (mg : mg). The conjugate was assayed by nuclear magnetic resonance (NMR) for determination of the acetylation level and a high degree of acetylation was found in the carbon 7 (data not shown).

Immunization and adoptive transfer

Three groups (A, B and C) consisting of 25 female BALB/c mice each (8–9 weeks old) (CENPALAB, Havana, Cuba) were immunized intraperitoneally with the corresponding antigen in a final volume of 100 μL (Table 1). For groups A and B, aluminium phosphate (Adju-Phos®, Superfos, Vedbaek, Denmark) at 0.2 mg mL^{-1} was used as an adjuvant. Mice were immunized at days 0 and 14, and bled at days –1, 7, 14, 21 and 35. Mice were sacrificed on day 35 and their spleens were harvested. Single-cell suspension of whole spleen cells of each group at 2×10^8 splenocytes per transfer (200 μL) were transferred intravenously via the tail vein to nine groups consisting of 10 nonimmune, nonirradiated recipient mice each with similar characteristics to the donor mice (Table 1). A blood extraction was performed 24 h after the adoptive transfer. These groups were immunized 24 h after the adoptive transfer with the corresponding antigen and a second dose of plain CCPS was applied to all groups 42 days later. Bleedings were performed every 7 days until day 63.

Immunoassays

Anti-CCPS IgG titration

The standardized enzyme-linked immunosorbent assay (ELISA) protocol to measure anti-CCPS antibody responses

Table 1. Splenocytes donor groups A, B and C were immunized with two doses of the corresponding antigen 14 days apart using aluminum phosphate as adjuvant. They were sacrificed 3 weeks after the last dose and their spleens were harvested. Groups A1 to C3 were adoptively transferred with 2×10^8 splenocytes via the tail vein and immunized 24 h after with the corresponding antigen. Forty-two days after the adoptive transfer, a plain polysaccharide dose was applied to all transferred groups

Group	Adoptive transfer	First dose	Second dose
A	Nontransferred donor group	2.5 μg CCPS-P64k	2.5 μg CCPS-P64k
B	Nontransferred donor group	5 μg CCPS	5 μg CCPS
C	Nontransferred donor group	200 μL phosphate buffer	200 μL phosphate buffer
A1	Group A	2.5 μg CCPS-P64k	5 μg CCPS
A2	Group A	5 μg CCPS	5 μg CCPS
A3	Group A	200 μL phosphate buffer	5 μg CCPS
B1	Group B	2.5 μg CCPS-P64k	5 μg CCPS
B2	Group B	5 μg CCPS	5 μg CCPS
B3	Group B	200 μL phosphate buffer	5 μg CCPS
C1	Group C	2.5 μg CCPS-P64k	5 μg CCPS
C2	Group C	5 μg CCPS	5 μg CCPS
C3	Group C	200 μL phosphate buffer	5 μg CCPS

described elsewhere (Gheesling, 1994) was performed with some modifications. Briefly, 5 μg mL^{-1} of human methylated albumin plus 5 μg mL^{-1} of CCPS in carbonate buffer was used as coating antigen. The plates were incubated overnight at 4 °C. After they were washed with phosphate-buffered saline (PBS) containing 0.1% Brij 35 detergent, a blocking solution of PBS containing 1% bovine serum albumin (BSA) and 0.1% Brij 35 was added for 1 h at 37 °C. The serum samples were two-fold diluted and incubated overnight at 4 °C in the same buffer. After three washes, the plates were incubated with antimouse IgG-peroxidase (Sigma, St Louis, MO, USA) at 37 °C for 1 h. Finally, plates were washed once more and the substrate H_2O_2 (3%) and *o*-phenylenediamine (0.5 mg mL^{-1}) in citrate buffer 0.1 M (pH 5) was used to develop the chromogenic reaction that was chemically stopped after 15 min. The OD was read at 492 nm, in a plate reader.

Anti-CCPS immunoglobulin subclasses detection

For the anti-CCPS immunoglobulin subclasses measurement on days 7 and 14 after the adoptive transfer of the CCPS-P64k-immunized mice, the ELISA steps were performed as described before, except for the use of the Mouse Monoclonal Antibody Isotyping Reagents kit (Sigma) as secondary antibody and a streptavidin–peroxidase conjugate (Sigma) before developing the colored reaction. The increase factor was calculated by dividing the OD (492 nm) of each postimmune serum by the OD of the corresponding preimmune serum. An immunoglobulin IgG/IgM relationship was calculated.

Anti-P64k IgG titration

Antibody levels to P64k were determined as previously described (Perez, 2001). Briefly, plates were coated with pure recombinant P64k 1 μg mL^{-1} in carbonate buffer for 2 h at 37 °C. After three washes with PBS/0.05 % Tween 20 (BDH Inc., Toronto, Canada), 100 μL of serial dilutions of each serum were added to the plates, which were incubated for 2 h at 37 °C. The rest of the steps were performed as described for the antipolysaccharide response detection.

Individual serum titers were calculated for the anti-CCPS and the anti-P64k IgG response. Titer was considered as the reciprocal of the dilution at which the double of the OD (492 nm) value of the preimmune serum was obtained.

Bactericidal assay

The standardized bactericidal assay was performed in microtiter plates as described before (Maslanka, 1997) to evaluate the bactericidal activity of the sera of the CCPS-P64k-immunized mice 7 or 14 days after the immunization carried out following the adoptive transfer. Bacteria of the strain C11 ATCC were grown overnight in brain heart infusion (BHI) agar at 37 °C in a candle jar. To prepare the inoculum for the test, bacteria were passed to a second plate of BHI agar and were allowed to grow for other 4 h in the same atmosphere and temperature conditions. Two-fold dilutions of the sera previously inactivated for 30 min at 56 °C were tested with an inoculum of 80–100 CFU per well, in the presence of 25% of human sera lacking detectable intrinsic bactericidal activity used as external complement source. The reaction was incubated at 37 °C for 60 min and titers were determined as the reciprocal of the final serum dilution yielding $\geq$50% killing with respect to the control. Mean bactericidal titers of each group were calculated.

Infant rat passive protection assay

An efficacy assay was performed as described previously (Saukkonen, 1988) with some modifications. In this study,

the strain C11 ATCC was grown on BHI agar (Oxoid, Basingstoke, UK) supplemented with 7% (volume in volume (v/v)) defribinated goat blood (BHI–blood agar) and containing vancomycin (Oxoid) as selective supplement. After incubation overnight at 37 °C in 5% CO_2, bacteria were subcultured on a fresh BHI–blood agar plate and allowed to grow for 4 h under the same conditions. Then, bacteria were removed from the plate and adjusted to the appropriate concentration. The challenge was performed as follows: 5- to 6-day-old rat pups (outbred Wistar; CENPA-LAB) were randomly distributed in groups of six animals. Groups were treated intraperitoneally 1 h before challenge with test and control pooled antiserum, diluted 1 : 10 in 0.9 % saline to a final volume of 100 μL. The animals were challenged intraperitoneally 150 μL of *N. meningitidis* (*c.* 5×10^6 CFU) suspended in sterile PBS containing 3.3 mg iron dextran per mL. The development of bacteremia was monitored 4 h after the bacterial challenge when 10 μL of blood was taken from each rat and diluted into sterile saline. Serial dilutions from each sample were plated in duplicate onto BHI–blood agar plates. After 20 h of incubation at 37 °C in 5% CO_2, the CFU on each plate were counted. The results of blood cultures were transformed to logarithmic values to calculate the geometric mean value of the CFU in each group of six animals. Pools of sera from those groups of CCPS-P64k-immunized mice were assayed (groups A1, B1 and C1). Also, sera from group A2 were evaluated. A pool of group C3 was included as negative control and a pool of sera from group A was included as nontransferred control.

Statistical analysis

For purpose of analysis, the serum titers were logarithmically transformed. The significance of differences in antibody levels was assessed by using the Kruskal–Wallis nonparametric analysis followed by Dunn's test for multiple comparisons (INSTAT version 2.0 software; Graphpad Software, Inc., San Diego, CA). The comparison of bactericidal titers was performed following the same analysis. Statistical significance of protection assays was evaluated using a one-way analysis of variance followed by a Dunnet's post test, using GRAPHPAD PRISM version 4.00 for Windows (GraphPad Software). Data are expressed as mean ± standard errors. In all cases, a P value under 0.05 was considered statistically significant.

Results

To assess the induction of memory response to the polysaccharide moiety by the CCPS-P64k conjugate, donor BALB/c mice were primed with two doses of CCPS-P64k, plain CCPS or phosphate buffer administered 2 weeks apart. Three weeks after the last priming dose, the spleens were removed from the donor mice. The splenocytes (2×10^8) were transferred intravenously via the tail vein to nine groups of 10 nonimmune, nonirradiated mice (Table 1). Each of these groups was subsequently immunized with CCPS-P64k, plain CCPS or phosphate buffer to determine whether or not immunologic memory was transferred to the polysaccharide.

Anti-P64k IgG responses

The anti-P64k IgG responses were measured in those groups inoculated with the CCPS-P64k conjugate after the adoptive transfer of splenocytes from CCPS-P64k or CCPS-immunized animals (groups A1 and B1), and also in the group transferred with cells from nonimmunized control animals (group C1) (Fig. 1). Group A, previously immunized with the conjugate, was included in the comparison as nontransferred group.

Seven days after the CCPS-P64k dose applied following the adoptive transfer, the anti-P64k IgG showed by group A1 was significantly higher than the response observed for the group previously transferred with cells form nonimmunized control mice (group C1) and the nontransferred group (group A) ($P < 0.001$). Fourteen days after the immunization, the same difference was observed. The anti-P64k IgG response of the group A1 was higher that the observed for group B1 and it also was maintained 14 days afterwards ($P < 0.01$). No difference was detected between the response of the nontransferred group (group A) and the group transferred with splenocytes from nonimmunized control animals ($P > 0.05$).

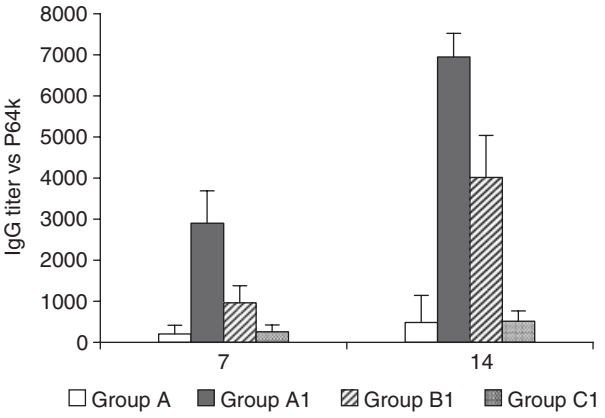

Fig 1. Anti-P64k immunoglobulin (IgG) responses 7 and 14 days after a single CCPS-P64k dose of those groups previously transferred with splenocytes from CCPS-P64k-immunized animals (group A1), CCPS-immunized mice (group B1) or nonimmunized control mice (group C1). Group A was included as a nontransferred control.

Anti-CCPS IgG responses

Groups immunized with the CCPS-P64k conjugate

To determine whether or not the P64k recombinant protein stimulates the induction of an immunologic memory response to the polysaccharide moiety, we analyzed the anti-CCPS IgG responses of those groups transferred either with splenocytes from CCPS-P64k, plain CCPS or nonimmunized control animals and further immunized with the glycoconjugate (groups A1, B1 and C1) (Fig. 2a). Group A, previously immunized with the CCPS-P64k conjugate, was included in the comparison as nontransferred group.

The group previously transferred with splenocytes from CCPS-P64k-immunized mice (group A1) showed a significantly higher response 7 days after immunization compared with the nontransferred group (group A) ($P < 0.001$), and also compared with the group previously transferred with cells from CCPS-immunized mice (group B1) ($P < 0.01$). Besides, a significantly higher response was observed in group A1 compared with the group transferred with splenocytes from nonimmunized control mice (group C1) ($P < 0.001$). Fourteen days after immunizations, the difference between groups A1 and A was still significant ($P < 0.01$), as was that between groups A1 and B1 ($P < 0.05$). In addition, a significant higher response was observed in group A1 compared with group C1 14 days after immunization ($P < 0.01$).

No statistical different responses were detected either 7 or 14 days after immunization between the group previously transferred with splenocytes from CCPS-immunized mice (group B1) and the nontransferred group (group A), or between the group A and the group transferred with cells from nonimmunized control mice (group C1) ($P > 0.05$).

Forty-two days after the adoptive transfer and immunization with the corresponding antigen, a plain CCPS dose was applied to all groups. The differences in the anti-CCPS IgG response were evaluated 7 and 14 days afterwards.

No statistical differences were detected 7 days after the plain polysaccharide boost between the group previously transferred with splenocytes from CCPS-P64k-immunized mice (group A1) and the group transferred with cells from CCPS-immunized mice (group B1) ($P > 0.05$). However, 14 days after the plain CCPS dose, the group A1 showed a higher response ($P < 0.05$). The same behavior was observed between the response of group A1 and that detected for the group previously transferred with splenocytes of nonimmunized control mice (group C1). No differences were detected between groups B1 and C1 either 7 or 14 days after the plain CCPS dose ($P > 0.05$).

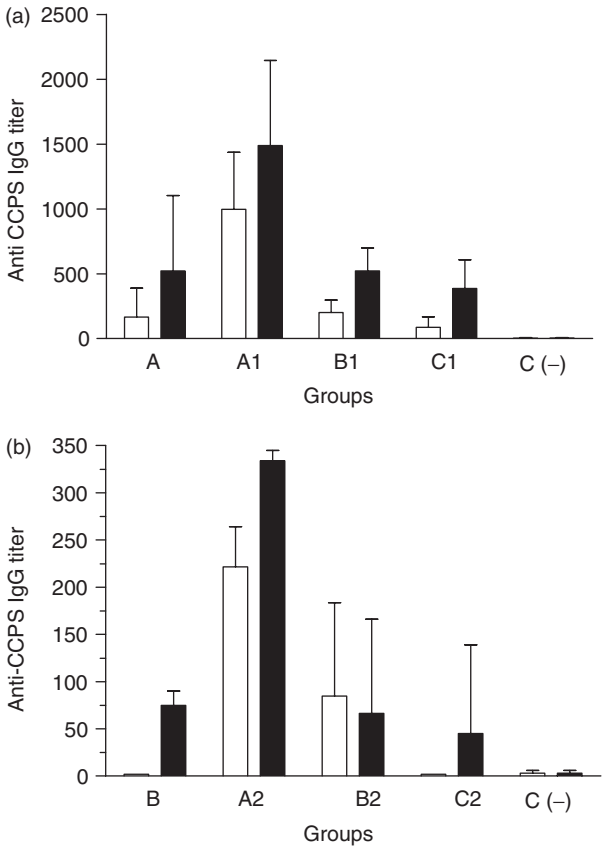

Fig. 2. Antipolysaccharide immunoglobulin (IgG) responses 7 (empty bars) and 14 days (filled bars) after immunization with the CCPS-P64k conjugate (a) and plain CCPS (b) of those groups previously transferred with splenocytes from CCPS-P64k (groups A1, A2), CCPS (groups B1, B2) or nonimmunized control mice (groups C1, C2). Groups A (immunized with the conjugate) and B (immunized with plain CCPS) were included as nontransferred control groups. In both cases, a negative control was also included.

Groups immunized with the plain CCPS

The anti-CCPS IgG responses of those groups transferred either with splenocytes from CCPS-P64k, CCPS or nonimmunized control animals and further immunized with plain CCPS (groups A2, B2 and C2) were compared to determine whether a conjugate dose was indispensable to recall the memory response generated in donor mice and transferred afterwards (Fig. 2b). Group B was included in the comparison as a nontransferred group. In this group, we did not find any evidence of the occurrence of hyporesponsiveness, because a second dose of the polysaccharide generated a higher anti-CCPS IgG response ($P < 0.05$).

The group transferred with splenocytes from CCPS-P64k-immunized mice (group A2) showed a significantly higher anti-CCPS IgG response compared with the nontransferred group (group B) 7 and 14 days after immunization with plain CCPS ($P < 0.001$). Compared with the group previously transferred with spleen cells from CCPS-

immunized mice (group B2), group A2 showed a higher response 7 days after immunization ($P < 0.05$). This difference increased 14 days after immunization ($P < 0.01$). In addition, a statistically higher response was detected in group A2 compared with the group previously transferred with splenocytes from nonimmunized control mice (group C2) even at 7 ($P < 0.001$) or 14 days after immunization ($P < 0.01$).

Although at 7 days after immunization the immune response of the group previously transferred with spleen cells from plain CCPS-immunized mice (group B2) was higher than that showed by the nontransferred group (group B) ($P < 0.01$), the difference disappeared by day 14 ($P > 0.05$). The anti-CCPS IgG response between group B2 and the group previously transferred with splenocytes from nonimmunized control mice (group C2) was similar 7 and 14 days after immunization ($P > 0.05$).

The IgG response of groups B2 and C2 was statistically different 7 days after immunization ($P < 0.05$), but the difference disappeared by day 14 ($P > 0.05$).

Forty-two days after the adoptive transfer and immunization with the corresponding antigen, a plain CCPS dose was applied to all groups. The differences in the anti-CCPS IgG response were evaluated 7 and 14 days afterwards, and a statistically higher response was detected in the group previously transferred with splenocytes from CCPS-P64k-immunized mice (group A2) compared with the group transferred with cells from CCPS-immunized mice (group B2) ($P < 0.05$). The same behavior was observed between the response of group A2 and that detected for the group previously transferred with splenocytes of nonimmunized control mice (group C2). No differences were detected between group B2 and C2 either 7 or 14 days after the plain CCPS dose ($P > 0.05$).

Groups immunized with phosphate buffer as negative controls

To evaluate the memory response induction or otherwise, and also to demonstrate whether or not this response was long lasting, we studied the anti-CCPS IgG response in those groups transferred with splenocytes from CCPS-P64k, CCPS or nonimmunized control animals and inoculated with phosphate buffer after the adoptive transfer (groups A3, B3 and C3) (Fig. 3). These comparisons also allow us to evaluate the quality of the adoptive transfer experiment, namely that no anti-CCPS IgG-producing plasma cell was transferred. Group C3 was the 'true' negative control, because it was transferred with splenocytes from nonimmunized control animals, and later inoculated with the same buffer. This group was not immunized with plain or conjugated CCPS until the plain CCPS dose applied 42 days after the adoptive transfer.

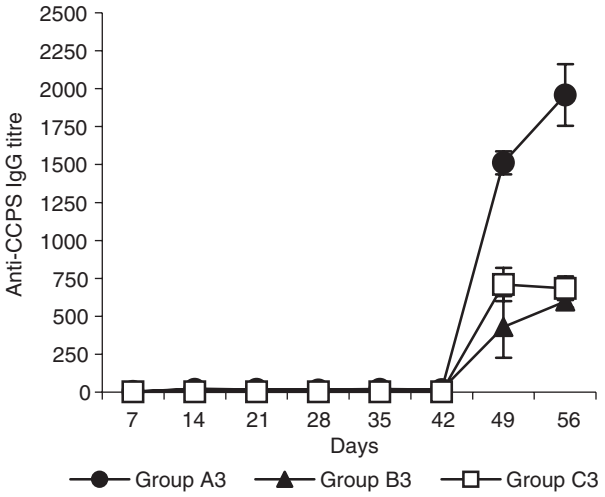

Fig. 3. Time course of the anti-CCPS immunoglobulin (IgG) responses in those groups inoculated with phosphate buffer and previously transferred with splenocytes from: CCPS-P64k (A3); CCPS (B3); or nonimmunized control mice (C3). A plain CCPS dose was applied for all groups on day 42. A typical booster response was observed for group A3.

No IgG response and no statistical differences were detected in any of the above-mentioned groups until the second dose was applied on day 42 ($P > 0.05$). Forty days after the adoptive transfer, a plain CCPS dose was applied to evaluate whether or not an immunologic memory response was induced, and also to evaluate the influence of the P64k in the immune response generated to the polysaccharide moiety.

Seven days after this plain CCPS dose was applied, a typical booster response was observed in group A3. The anti-CCPS IgG response of the group A3 was statistically higher than the observed response for the groups transferred with cells from CCPS (group B3) or nonimmunized control animals (group C3) ($P < 0.01$). This difference increased after 14 days ($P < 0.001$). The same behavior was observed between group A3 and the group transferred with splenocytes from nonimmunized control mice (group C3). No statistical differences were detected between groups B3 and C3 either 7 or 14 days after the plain CCPS dose ($P > 0.05$).

Anti-CCPS immunoglobulin subclasses pattern

To evaluate the ability of the P64k protein to change the anti-CCPS thymus-independent response to a thymus-dependent response, the anti-CCPS immunoglobulin pattern was studied in those groups transferred with splenocytes from CCPS-P64k, CCPS or nonimmunized control mice and afterward immunized with the CCPS-P64k conjugate (groups A1, B1 and C1) (Fig. 4). Group A, previously immunized with the conjugate, was included in the comparison as a nontransferred group.

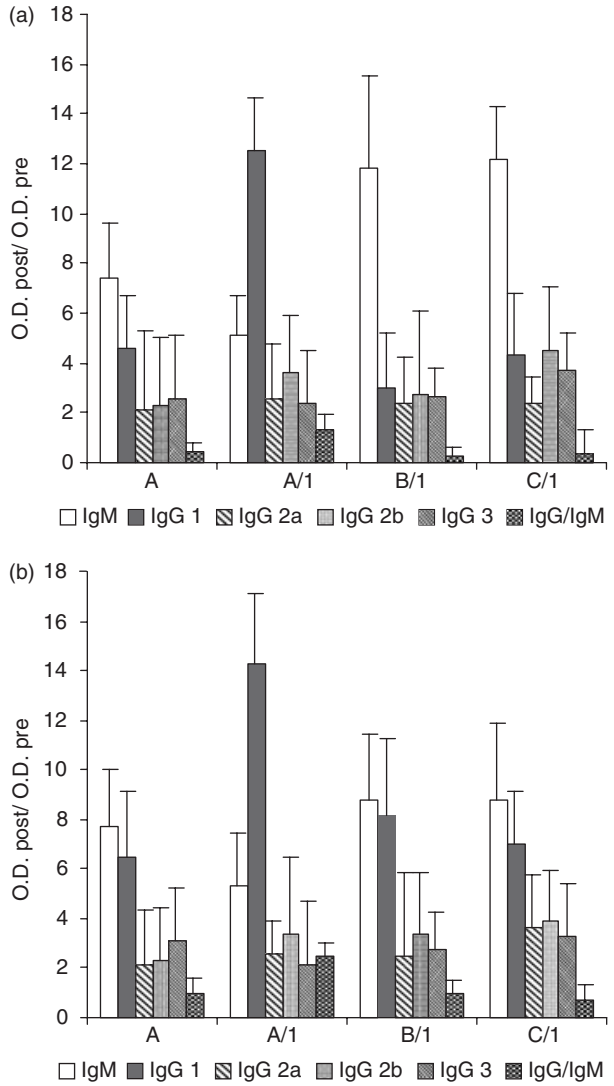

Fig. 4. Anti-CCPS antibody subclasses determination by enzyme-linked immunosorbent assay (ELISA). Sera from all groups immunized with the CCPS-P64k conjugate collected on days 7 (a) and 14 (b) after the immunization were evaluated. Group A: nontransferred; A1: transferred with splenocytes from CCPS-P64k-immunized mice; B1: transferred with splenocytes from CCPS-immunized mice; and C1: transferred with splenocytes from nonimmunized control mice.

Group A1 showed a higher anti-CCPS IgG1 response than the rest of the evaluated groups 7 ($P < 0.01$) and 14 ($P < 0.05$) days after immunization. No difference was detected among groups B1, C1 and A ($P > 0.05$). A smaller IgM response was detected in group A1 compared with group A ($P < 0.01$), and an even smaller one when compared with the rest of the groups ($P < 0.001$). The anti-CCPS levels of IgM, IgG2a, IgG2b and IgG3 were similar between all groups: no difference was detected ($P > 0.05$). When analyzing the ratio calculated by dividing total IgG by the IgM levels, group A1 showed a higher ratio at 7 and 14 days after immunization, meaning that total IgG response was higher than the IgM response compared with the rest of the groups. No differences were detected between the rest of the groups at 7 or 14 days after immunization ($P > 0.05$).

Table 2. Mean serum bactericidal activity on days 14 and 56 of all groups immunized with the CCPS-P64k conjugate after the adoptive transfer

Group	Day 14*	Day 56†
A	256	–
A1	640	1280
A2	512	1024
B1	156	312
C1	135	288
C3	$< 1:4$	$< 1:4$

*For groups from A1 to C3, the bleeding corresponds to 14 days after the first dose after the adoptive transfer.

†For groups from A1 to C3, the bleeding corresponds to 14 days after the plain polysaccharide dose applied on day 42 after the adoptive transfer.

Group A: nontransferred; A1: transferred with splenocytes from CCPS-P64k-immunized mice; B1: transferred with splenocytes from CCPS-immunized mice, C1: transferred with splenocytes from phosphate-buffered saline (PBS)-immunized mice; and C3: negative control, transferred with splenocytes from PBS-immunized mice and a further PBS dose. Group A2, immunized with plain CCPS after the adoptive transfer, was also included.

Serum functional activity

Serum bactericidal activity

The serum bactericidal activity of the CCPS-P64k-immunized mice was evaluated 14 days after the conjugate dose, and also after the plain CCPS dose applied 42 days after the adoptive transfer (Table 2). Group A, immunized with the conjugate, was included in the comparison as a nontransferred group. Group C3 was included as a negative control. To study the influence or otherwise of the conjugate state of the CCPS after the adoptive transfer, the bactericidal activity of group A2, immunized with plain CCPS and previously transferred with splenocytes from CCPS-P64k-immunized animals, was also evaluated.

Fourteen days after the conjugate dose, the mean bactericidal titer of the group previously transferred with splenocytes from CCPS-P64k-immunized mice (group A1) was four-fold higher than the mean titer of the group transferred with cells from CCPS-immunized mice (group B1), almost five-fold higher than the mean bactericidal titer of the group transferred with spleen cells from nonimmunized control mice (group C1) and the double of the mean titer of the nontransferred group (group A). A similar behavior was observed for group A2. Group C3 showed no bactericidal activity.

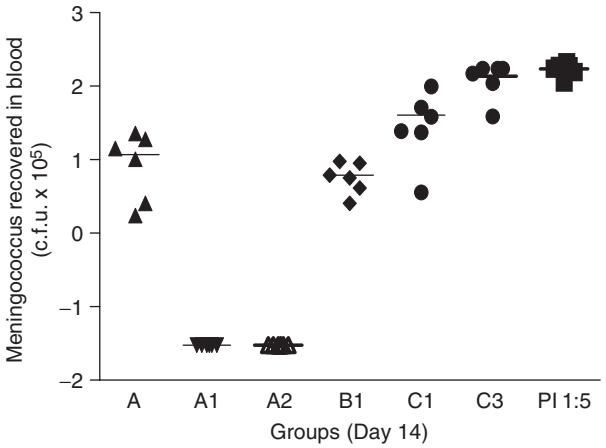

Fig. 5. Passive protection assay in the infant rat model. We compared the protection conferred by pools of the following mouse sera. Group A: nontransferred and immunized with the CCPS-P64k conjugate; A1: transferred with splenocytes from CCPS-P64k-immunized mice and further immunized with the CCPS-P64k conjugate; A2: transferred with splenocytes from CCPS-P64k-immunized mice and further immunized with plain CCPS; B1: transferred with splenocytes from CCPS-immunized mice and further immunized with the CCPS-P64k conjugate, C1: transferred with splenocytes from nonimmunized control mice and further immunized with the CCPS-P64k conjugate and C3: negative control, transferred with splenocytes from nonimmunized control mice and a further PBS dose. A 1 : 5 dilution of a preimmune serum was included (PI 1 : 5)

Fourteen days after the plain CCPS boost, the mean bactericidal titer of all groups increased two-fold, and the difference was maintained.

Passive protection in the infant rat passive protection assay

The *in vivo* ability of pools of sera obtained 14 days after immunization with the CCPS-P64k conjugate of those groups previously transferred with splenocytes from CCPS-P64k, CCPS or nonimmunized control mice (groups A1, B1 and C1) was assayed in the infant rat passive protection assay (Fig. 5). Group A, previously immunized with the conjugate, was included in the comparison as a nontransferred group. Group C3 was included as a negative control. A pool of preimmune sera diluted 1 : 5 in 0.9 % saline was included and the recovered meningococcal CFU were compared to those obtained in this group as a protection measurement.

The only groups where no meningococcal CFU were recovered 4 h after the bacterial challenge were groups A1 and A2, showing statistical differences with the rest of the groups ($P < 0.001$). No differences were detected among the rest of the groups and the preimmune sera pool ($P > 0.05$).

Discussion

Bacterial capsular polysaccharides are type II thymus-independent antigens, failing to induce protective memory responses (Cadoz, 1998). The antibody response to a polysaccharide alone is typically of the IgM isotype, and repeated immunizations do not result in increased levels of polysaccharide-specific antibodies. Covalent coupling of a polysaccharide to an immunogenic carrier protein promotes a switch in the anticarbohydrate response to a thymus-dependent one. This response is characterized by higher levels of polysaccharide-specific antibodies, rapid kinetics and isotype switching of the polysaccharide-specific antibodies (IgM to IgG) upon booster vaccination or subsequent encounter with the polysaccharide as presented in the bacteria (Garner & Pier, 1989). Memory of a T-cell-dependent antigen results in secondary responses to the antigen *in vivo* when the memory is recalled by booster vaccination or by encounter with the antigen as presented on the pathogenic organism.

Few proteins have been clinically evaluated and are available for use as carrier proteins. In the case of antimeningococcal serogroup C vaccines, only tetanus toxoid and diphtheria toxin or its genetic mutant CRM197 have been used, and the immunological memory induction to the polysaccharide moiety was demonstrated (Richmond *et al.*, 1999a, b, 2000, 2001). The use of these proteins as universal carriers is controversial, because they can induce a reduction of the response against several vaccines that are administered during infancy and share common protein epitopes by inducing epitopic overload and hapten-specific carrier-induced suppression (Schutze *et al.*, 1985; Dagan *et al.*, 1998; Fattom *et al.*, 1999). The characterization of new carrier proteins able to confer T-cell-dependent characteristics to a bacterial polysaccharide seems to be a compulsory step for the achievement of a proper immune response against all the polysaccharide antigens needed for childhood vaccination.

Many of the classic studies from the 1960s and 1970s on T-cell dependence and induction of immunologic memory for haptens conjugated to carrier proteins were performed by adoptive cell transfer (Mitchison, 1971; Katz & Benacerraf, 1972). Recently, an adoptive cell transfer model to evaluate the ability of a glycoconjugate conjugate vaccine to induce immunologic memory to the polysaccharide moiety was developed in nonirradiated mice (Guttormsen *et al.*, 1998). In the present work, we used an adoptive transfer experiment to demonstrate the ability of the P64k carrier protein to induce immunologic memory to the polysaccharide moiety of a meningococcal serogroup C glycoconjugate. We also used nonirradiated mice to eliminate the effect of irradiation in the activation of memory T cells after adoptive transfer (Kundig *et al.*, 1996).

Only the adoptive transfer of splenocytes from CCPS-P64k-immunized mice led to anti-CCPS typical booster responses in the recipient groups when a reencounter with the CCPS occurred, independently if it was plain or conjugated (groups A1 and A2). This anti-CCPS response was characterized by higher IgG titers with more rapid kinetics, a switch in the immunoglobulin subclass to a predominance of IgG instead of IgM, higher bactericidal titers and the ability to confer passive protection in the infant rat model. All these findings indicate that CCPS-specific memory cells were generated by two doses of the glycoconjugate in splenocytes donor mice.

The immune response generated in those groups immunized with the conjugate or plain polysaccharide and previously transferred with splenocytes from CCPS or nonimmunized control mice was identical to that primary response reached in nontransferred animals, showing that no memory response was induced by plain polysaccharide in splenocytes donor mice.

The memory response induced was maintained in time, as was demonstrated by the higher response observed in those groups transferred with splenocytes from CCPS-P64k-immunized mice after a plain CCPS dose applied 42 days after the adoptive transfer. This response also was a typical booster response, with more rapid kinetics compared with the response observed for the rest of the groups.

The measurement of the anti-P64k IgG titers also demonstrates the generation of an immunologic memory response, because a high response was observed in those groups transferred with cells from CCPS-P64k-immunized mice. The similar humoral response pattern to the carbohydrate moiety and the P64k carrier protein observed provides experimental data supporting the hypothesis that conjugation of CCPS to the P64k protein converts the polysaccharide from a T-cell-independent to a T-cell-dependent antigen *in vivo*.

Groups inoculated with phosphate buffer after the adoptive transfer of cells from CCPS-P64k, CCPS or nonimmunized control mice (groups A3, B3 and C3) also received a plain CCPS dose 42 days after the adoptive transfer acting as transference controls. As was demonstrated, no specific anti-CCPS IgG response was detectable on those groups until the immunization with the polysaccharide, showing that no anti-CCPS IgG-producing plasma cell was transferred. However, 7 days after this immunization, a significant higher response was detected only in the group transferred with cells from CCPS-P64k-immunized mice (group A3). This finding supports the hypothesis that long lasting anti-CCPS specific memory cells were induced only by the CCPS-P64k conjugate in donor mice, and that when transferred to nonimmune, nonirradiated recipient mice they were able to generate a higher anti-CCPS IgG response with fast kinetics when re-encountered with the polysaccharide. It was also demonstrated that no memory response was generated by plain CCPS immunization, because no difference was detected between those groups previously transferred with cells from CCPS (group B3) or nonimmunized control animals (group C3). The anti-CCPS IgG response observed in the group transferred with splenocytes from CCPS or nonimmunized control animals was a typical primary response: a lag phase of at least 7 days was detected.

Only group A1 showed a significant higher IgG1 response and a higher IgG : IgM ratio when compared with the rest of the groups immunized with the conjugate after the transference, indicating that only priming the splenocytes donor mice immune system with the conjugated CCPS allowed a switch in the polysaccharide T-cell-independent response to a T-cell-dependent response by the immune effect of the P64k carrier protein.

The memory response induction by the CCPS-P64k conjugate in donor mice was also demonstrated in functional assays, because higher bactericidal titers were measured only in those groups transferred with splenocytes from CCPS-P64k-immunized mice independently if they were afterwards vaccinated with the conjugated or plain CCPS.

In a previous work, the bactericidal activity of sera against the protein P64k was determined and very low titers (1 : 8, 1 : 16) were obtained using different meningococcal strains (Silva, 1992). In other work where sera elicited against the carrier protein was tested, no bactericidal activity was detected even when Z4181 strain was used, even though it expressed the P64k protein in whole-cell ELISA (Guillen, 1998). Because bactericidal activity is established as the best indicator of protection against serogroup C meningococci, we think that the contribution of P64k to the protection conferred by an antimeningococcal glycoconjugate is very poor, if existent, acting only as carrier protein.

In the infant rat model, the group passively immunized with sera from mice transferred with splenocytes from CCPS-P64k-immunized mice was much better protected than that immunized with cells from group A. Note that for passive protection experiments we used sera from day 14 after the first immunization. At this time, group A was only immunized once with the conjugate, whereas group A1 (although with only one dose also) had splenocytes transferred from group A that were previously activated twice. This result indicates that at least two doses of 2.5 μg of the conjugate are necessary to generate antimeningococcal protection in this model, as was previously demonstrated (Carmenate *et al.*, 2004).

We demonstrated that the conjugation of CCPS to P64k changed the immune response to the polysaccharide to a T-dependent one, allowing the generation of a memory response to the capsular polysaccharide, irrespective of whether or not the polysaccharide was conjugated in further

encounters. This memory response was characterized by more rapid antibody kinetics with higher antipolysaccharide titers, a switch in the antibody subclass from IgM to IgG predominance, and higher serum functional ability to eliminate the pathogen, as was evidenced *in vitro* in the bactericidal assay and *in vivo* in passive protection studies in infant rats.

Putting together all these results demonstrating the ability of P64k to induce memory responses against the polysaccharide moiety in glycoconjugate vaccines with the results obtained before in humans, where it was demonstrated the protein safety and immunogenicity (Perez, 2001), P64k seems to be an excellent carrier candidate to be used in all the polysaccharide antigens needed for vaccination of children, and thus contribute to eliminate the epitopic overload and hapten-specific carrier-induced suppression that could take place when the same protein carrier is used in subsequent vaccination routines.

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