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Long-lasting cellular immune response in babies, children, and pre-teenagers vaccinated with a proteoliposome based anti-meningococcal BC vaccine

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LARGA DURACIÓN DE LA RESPUESTA INMUNITARIA CELULAR INDUCIDA EN LACTANTES Y NIÑOS INMUNIZADOS CON VACUNA PROTEOLIPOSÓMICA ANTI-MENINGOCÓCICA BC

RESUMEN

VA-MENGOC-BC® es una vacuna contra los serogrupos B y C de *Neisseria meningitidis*. La respuesta humoral ha sido extensivamente evaluada pero no la respuesta celular. Estudios prospectivos y retrospectivos fueron realizados para especificar la inducción y duración de la respuesta inmunitaria. El estudio prospectivo fue llevado a cabo en 62 lactantes usando un test de hipersensibilidad retardada (DTH) antes de la primera (3,5 meses de edad), junto a la segunda (42 días después) y 28 días después de la segunda dosis. En los lactantes, la DTH fue negativa antes y 100% positiva después de la vacunación. El estudio retrospectivo incluyó 535 niños que habían sido vacunados entre 2 y 7 años antes. La positividad de la DTH fue de 100% en todos los grupos. En los niños vacunados 5 ó 7 años antes, las técnicas de linfoproliferación (LP) y las células secretoras de anticuerpos (ASC) fueron también determinadas. La LP fue positiva en el 26 y 34% antes de la dosis de refuerzo en los niños vacunados de 5 y 7 años, respectivamente y decreció posteriormente. Los ASC fueron negativos antes de la dosis de refuerzo y generalmente positivos 7 días después de ésta. No obstante, en los niños vacunados hacía 7 años el 12% tuvo una pequeña cantidad de ASC antes del refuerzo, los cuales pudieran estar relacionados con la alta frecuencia de circulación de *Neisseria* en la población o de microorganismos con reactividad cruzada. El mayor incremento en los ASC después del refuerzo (desde 0,73 hasta 166,24 x 10^6 PBMC) fue observado en aquellos negativos que tenían bajos números de ASC antes del refuerzo al compararlos con los totalmente negativos (desde 0 hasta 67,7 x 10^6 PBMC). Estos resultados muestran claramente la inducción de respuesta celular en lactantes, la persistencia de respuesta celular en los grupos vacunados hacía tiempo y la memoria de larga duración detectada por una tercera dosis.

PALABRAS CLAVE: Respuesta celular/ Vacuna anti-Meningocócica BC/ Proteoliposoma/ Memoria/ ELISPOT.

ABSTRACT

VA-MENGOC-BC™ is a vaccine against Neisseria meningitidis serogroups B and C. The humoral response has been extensively evaluated, but not the cellular one. Prospective and retrospective studies were conducted to specify inducement and duration of immune response. The prospective study was carried out in 62 nursing babies by using the delayed-type hypersensitivity (DTH) dermal test, before the first dose (3,5 months of age), at the second dose (42 days later), and 28 days after that second dose. In babies, DTH were negative before and positive in 100% cases after vaccination. The retrospective study included 535 children that had been vaccinated between 2 to 7 years earlier. The positivity of DTH was 100% in all groups. In children vaccinated 5 or 7 years earlier, lymphocyte proliferation (LP) and antibody secreting cell (ASC) tests were also determined. LP was positive in 26 and 34% before the booster dose the 5 and 7 years group, respectively, and decreased afterwards. ASCs were negative before the booster but it was generally positive 7 days later. Nevertheless, in children who had been vaccinated seven years earlier, 12% had a small amount of ASC before the booster, which may be related to the high frequency of Neisseria *circulation in the population, or to cross-reacting organisms. The highest increase in ASC after boosting (from 0.73 to 166.24 x 10^6 PBMC) was observed in those negatives that had low numbers of ASC before boosting, as compared to the absolutely negative ones (from 0 to 67.7 x 10^6 PBMC). Those results clearly show the induction of cellular response in nursing babies, the persistence of cellular response in the older vaccinated groups, and the long lasting memory as detected by a third dose.*

KEY WORDS: *Cellular response/ Anti-meningococcal BC vaccine/ Proteoliposome/ Memory/ ELISPOT.*

INTRODUCTION

VA-MENGOC-BC™ is the commercial trade mark of the Cuban vaccine against Neisseria menigitidis of serogroups B and C. This contains a defined amount of purified outer membrane proteins from serogroup B of *N. meningitidis* (CU385, B:4: P1,19, 15,L3,7,9) enriched with proteins from the high molecular weight protein complex (65-95 kD), and a controlled proportion of lipooligosaccharide and phospholipids to constitute an external membrane vesicle (EMV). In addition, the vaccine contains purified capsular polysaccharide of *N. meningitidis* serogroup C (C11, ATCC), both adsorbed on $Al(OH)_3$ gel (1,2). This adsorption significantly increased the bactericidal response to the outer membrane protein based vaccine (3). It was also safer and more immunogenic than without the aluminum adjuvant (4).

The pre-clinical and clinical (phase I and II) studies of VA-MENGOC-BC™ showed its safety and ability to induce antibodies against different pathogenic serotypes (5). A double blind placebo-vaccine trial was conducted in junior high school students (10-16 years old), with an efficacy of 83% (6). A second field trial was carried out in infants, children and young adults from 6 months to 24 years of age with an efficacy of 83-90% (7). This vaccine was included in the Cuban National Immunisation Scheme with the first dose starting at 3.5 months of age and the second dose at 5 months of age; a mass vaccination campaign was organised in 1989-90 for babies under 6 months. The overall efficacy of this massive campaign was estimated as 92.5% (2).

The humoral response induced by this vaccine is characterised by the induction of specific IgG and bactericidal antibodies against some of the most frequent *N. meningitidis* B serotype pathogens. After a 3rd dose, the concentration of these antibodies increased many times, showing an excellent specific anamnestic response, which explains the high efficacy of the vaccine (2).

Over 50 million doses of VA-MENGOC-BC™ have been administered in Cuba and in other countries, mainly in Latin America. Brazil has used the vaccine in more than 15 campaigns with high effectiveness (8). The Cuban vaccination campaign was followed by a fall in the morbidity and mortality rate caused by *N. meningitidis* B (9).

The most important finding of the Cuban vaccine trial was the demonstration that antibodies induced to non-capsular surface antigens can protect against meningococcal disease (4). The mechanism responsible for the development of natural immunity against meningococci remains unclear. Protection has been correlated with the presence of bactericidal antibodies (10), but this has been formally demonstrated only for the groups A and C polysaccharide vaccines. This pattern of response has been extended to include bactericidal antibodies against outer membrane protein based vaccines. The development of such vaccines was necessary because of the poor immunogenicity of serogroup B capsular polysaccharide, which shows a similar structure to sialic acid moieties present in gangliosides of all nucleated humans cells, but mainly in brain tissues (11), plus their sensitivity to neuraminidase which may induce immunotolerance (4). In addition, the manipulation of this polysaccharide or its potentiation by adjuvants may induce autoimmunity.

While the great majority of the studies have focused on the role of serum bactericidal activity in the host's defense against meningococcal disease, cellular responses induced by VA-MENGOC-BC™ have been less studied (12). Little is known about the importance of cell-mediated immune responses against *N. meningitidis* as compared to humoral responses. We have shown the existence of lymphocyte proliferative (LP) responses in vaccinated mice and in humans (13). Also, and even more important, is the presence of delayed-type hypersensitivity response (DTH) against protein antigens in vaccinated humans, which we have reported (14). DTH is an *in vivo* functional test that indicates the existence of a Th (T helper) 1, cellular immune response (15). In addition, specific LP has been widely accepted as a measure of T-cell activity.

To continue and complete the study on the Cuban vaccine, the present work was aimed: i) to determine the induction of cellular responses by VA-MENGOC-BC™ in nursing babies, ii) to specify the duration (memory) of cellular responses induced by VA-MENGOC-BC® in children, and iii) to compare DTH, LP and ASC induced by this vaccine.

MATERIAL AND METHODS

Subjects and immunisation. Nursing infants (3.5 months of age at the beginning of the study), children (from 2 to 6 years old) and pre-teenagers (from 11 to 13 years old) were included in the study. The babies all received the first dose of VA-MENGOC-BC™ as per the immunisation schedule at 3.5 months of age during this study, and the second dose 6 to 8 weeks later. The children had been vaccinated at ages ranging from 5 to 7 years during mass vaccination campaigns, and received a booster dose during this study.

Prospective evaluation. To evaluate the induction of cellular responses, a cutaneous DTH test was performed in 62 nursing babies before the vaccination scheme started, after the first dose (6 months of age, n = 50), and 28 days after the second dose (7 months of age, n = 20). The decrease in the

number of babies evaluated was due to their withdrawal (address change, did not come in the specific day, etc.) from the study.

Retrospective evaluation. To determine the duration of the induced cellular response, a group of 535 children or pre-teenagers vaccinated 2 to 7 years before the study were evaluated. Table I shows the distribution of the subjects according to the time post-vaccination. Dermal tests were carried out without exceptions in all subjects. In addition, in pre-teenagers (5 and 7 years after vaccination) two blood samples were taken for the evaluation of LP response (16) and ASC by the ELISPOT technique (17-19).

Table I
Distribution of subjects included in the retrospective study

Years after vaccination	*Number of subjects*
2	62
3	50
4	48
5	100
6	47
7	228

Delayed-type hypersensitivity testing. This test was carried out by skin multi-puncture, using EMV with 14 µg of proteins diluted in glycerol-PBS-phenol. The indurated area was delimited with a pen, copied onto paper and its diameter (mm) measured. Initially readings were taken at 4, 12, 24 and 48 hours, carefully evaluating all dermal reactions. These showed that the peak reactivity was at 48 hours, and all further tests were read at 48 hours only.

Purification of cells. Peripheral mononuclear cells (PBMC) used in the assay were obtained by a Ficoll-Hypaque cushion from 10 ml of blood at time zero (t = 0) and 7 days (t = 7) after a third dose of vaccine. The pre-teenagers 5 years after vaccination were divided into four groups, and blood samples were taken on days 0 and 7, 0 and 14, 0 and 21, and 0 and 28 to determine the kinetics of ASC and LP responses.

Lymphocyte proliferation. The PBMC were cultured in complete RPMI-1640 medium, supplemented with 10% foetal calf serum and antibiotics, challenged *in vitro* with 2, 5 and 10 µg/ml of proteins (from vesicles) during 5 days. The cultures were pulsed with [^{3}H] thymidine during the last 18 hours, then harvested and the incorporation into DNA determined in a β liquid scintillation counter. The mean counts per minute (cpm) of triplicate samples and the stimulation index (SI) were determined. SI >= 2 were considered positive.

ELISPOT assay for antibody secreting cells. The method used was based on those of Czerkinsky and Sedwick y cols. (20, 21). Briefly, individual wells of surfactant-free mixed cellulose ester membrane bottomed 96-well MultiScreem-HA (Millipore, MA) were filled with 100 µL of 10 µg/ml of protein antigens of EMV and incubated at 4°C over-night. This coating concentration was found optimal in a preliminary checkerboard titration experiment. After three washes with phosphate-buffered saline (PBS), the individual wells were filled with 150 µl of RPMI 1640 complete medium supplemented with 10% heat inactivated FCS, and the plates were incubated at 37°C for 30 min in a humidified atmosphere with 5% CO_2 in order to saturate remaining protein binding sites. Wells coated with albumin (5 µg x ml^{-1}) were prepared in the same way and were included in the assays for control purposes. To enumerate the total ASC irrespective of their specificity, the wells were coated with 100 µl of affinity purified goat antibodies directed against human IgG diluted to 5 µg/ml (found to be optimal in previous checkerboard titration experiments). The well contents were replaced with 100 µl of cell suspensions containing 10^5 or 10^6 PBMC/well in RPMI complete medium. Then, the plates were incubated without disturbance for 4 h at 37°C in 5% CO_2 in a humidified atmosphere. They were then rinsed three times with PBS and three times with PBS containing 0.05% Tween 20. Next, 100 µl of PBS-0.05% Tween 20 containing 1% BSA and a affinity-purified goat antibody to human IgG conjugated with horseradish peroxidase were added. All enzyme-conjugated antiglobulin reagents were purchased from Sigma, Mo, USA. Spots were developed with the substrate 3-amino-9 ethylcarbazole in 0.1 citrate pH 5 buffer. The spot numbers were counted in triplicate wells under low magnification (x 40). Subjects who showed a two-fold increase in the number of ASC after the booster dose and had a final number higher or equal than 2 per 10^6 PBMC were considered positive.

Ethical aspects. Because of the inclusion of children and young pre-teenagers in this study, an authorization by the National Group of Pediatrics, Health Authorities, and the Institutional Ethics Committee were necessary. The written consent of each parent or guardian was also obtained.

Statistical Analysis. The mean and standard deviations were calculated and Student's T test was applied to compare the results between experiments for DTH and ELISPOT assay, taking $p < 0.05$ as a significant difference.

RESULTS AND DISCUSSION

The evaluation of cellular immune responses is very important, since T cells are the principal orchestrators of any kind of immune response. Therefore, once we knew that VA-MENGOC-BC™ induced a T-dependent immune response, we wished to determine what kind of response was stimulated the vaccine was eliciting.

The well-proven safety record of this vaccine in babies prompted the present study. This was indispensable in order to know children's immune response to VA-MENGOC-BC™. This was especially important in Cuba, where this vaccine is included in the routine vaccination program of children. The first dose is administered to babies 3.5 months after birth, with a coverage of over 98% of the total population.

The absence of DTH in nursing babies before vaccination, the positive reactions seen after the first dose and their subsequent increase thereafter, emphasizes that VA-MENGOC-BC™ induces a strong cellular immune response, not only in adults where a preferential Th1 pattern was demonstrated (DTH, IFNg, IL-2 and IgG1) (26, 27, 28), but also in nursing infants (Fig. 1). No response was observed immediately after skin testing, or 4 and 12 h later, with the maximum induration appearing after 48 h. This indicates an absence of type I (immediate) or type III (Arthus reaction) hypersensitivities in response to EMV from *N. meningitidis* B, strongly suggesting that the dermal response is mainly a DTH phenomenon. In addition, no IgE anti-VME (the hallmark Th2 antibody) was detected in vaccinated sera (our unpublished results).

The T cell response, evaluated by functional tests (DTH), was positive in 100% of those tested, and maintained for 2 to 7 years, without significant differences ($p < 0.05$) between them (Fig. 2). These results indicate that the memory induced by the vaccine is long-lasting and may in turn be enhanced by natural boosting with *N. meningiti - dis* from the environment or by other cross-reacting organisms, as has been suggested by others (29).

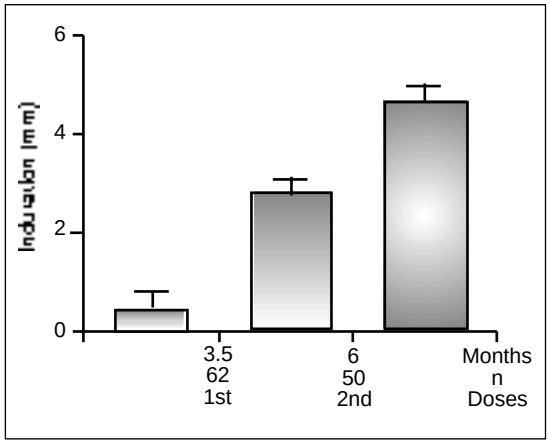

Figure 1. *Follow-up study of delayed-type hypersensitivity (DTH) against protein antigens of VA-MENGOC-BC, in nursing babies vaccinated with two doses (at 3.5 and 6 months of age). Significant increase in DTH response was observed after the first and the second dose ($p<0.05$).*

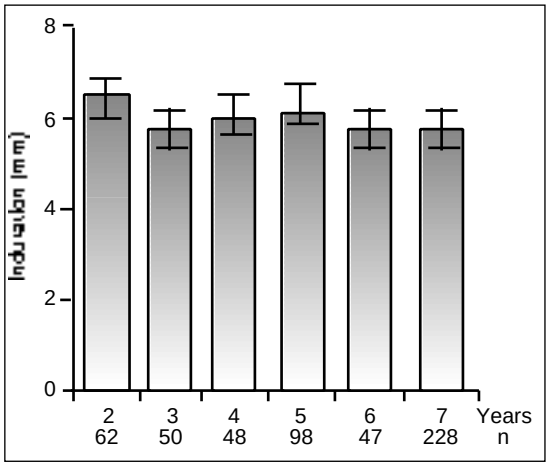

Figure 2. *Duration of delayed-type hypersensitivity response against protein antigens of VA-MENGOC-BC, in children and pre-Teenagers prime-vaccinated 2 to 7 years before. Significant differences between groups were not observed.*

For pre-teenagers vaccinated 5 years earlier, DTH was positive in 100% of subjects. T cell responses were also evaluated *in vitro* by LP at intervals after boosting, whereas the anti-EMV B cell responses were measured by ASC with ELISPOT at 7 days after a booster. Table II shows that whereas LP was positive in 26% before boosting, this dropped considerably after the booster dose. This drop could be due to the recruitment of specific T lymphocytes from the periphery to the immunisation site. Specific ASC were absent before boosting in pre-teenagers vaccinated 5 years earlier, and there was a positive conversion in 78,6% ($p < 0.05$) 7 days after boosting. At that time all the negative subjects seroconverted by ELISA assay (data not shown). The rest of the ELISPOT determinations (14 and 21 days after boosting) were negative. It has been reported that activated B cells, after proliferation and differentiation at regional lymph nodes, were detected in blood over a narrow window (5-12 days) and soon afterwards they were directed by interaction with homing receptors to immune response effector sites, mainly the lamina propria of the gut (30-32).

Table II
Comparison of positive Lymph Proliferation (LP), positive Delayed-Type Hypersensitivity (DTH) and Antibody Secreting Cells (ASC) of children vaccinated 5 or 7 years earlier. T0, time before third dose. T7, seven days after the challenge with third dose

Vaccinated, years ago	*% LP, T0*	*% DTH, T0*	*% ELISPOT (ASC x 10^6 PBMC)*	
			T0	*T7*
5 (Havana)	26	100	0 (0)	78.57 (13.5)
7 (Holguín)	34	100	88 (0)	97.73 (67.68)
			12* (0.73)	100 (166.24)
Total				98 (73.9)

* These 12% of low responder were really negative (<2 x 106 PBMC)

The results described above in children vaccinated five years earlier showed that LP dropped following boosting and ASC were only positive at 7 days after boosting. Therefore, the immune response in children vaccinated 7 years earlier was only determined before boosting by LP and DTH, and 7 days after boosting by ELISPOT. LP and DTH were positive in 34 and 100% of children, respectively. The B cell response (ELISPOT) was very low (negative) in 12% of children before the third dose, and became positive in 98% (98/100) 7 days after the third dose. It should be emphasised that the number of ASC in the population which gave a low but detectable response at time zero (0.73 x 10^6 PBMC), showed a greater increase ($p < 0.05$) after challenge (166.24 x 10^6 PBMC) than was seen in those which gave a completely negative reaction at time zero (67.68 x 10^6 PBMC) (Table II and Fig. 3).

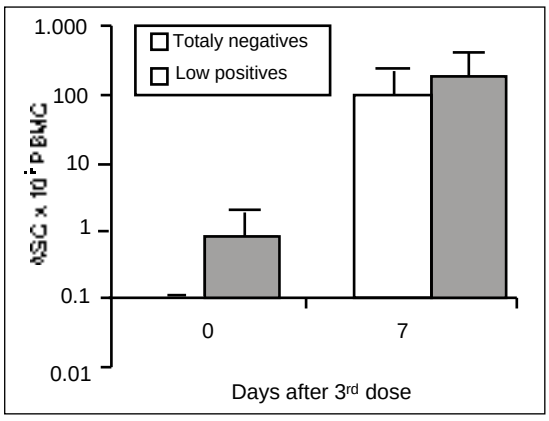

Figure 3. *Comparison between Low Positive (empty bar) and Totally Negative (shaded bar) ELISPOT from peripheral mononuclear cells (PBMC) before a booster with VA-MENGOC-BC, of pre-teenagers 7 years after prime vaccination. After 7 days, the spots were significantly different (p<0.05).*

Considering the above kinetic results from ELISPOT, that only showed positive values 7 days after boosting, the presence of ASC circulating in peripheral blood of 12% of the children could be due to a very recent natural or cross-reactive antigen restimulation.

Responses were higher in children from Holguín, boosted 7 years after vaccination, than in children boosted at 5 years, who came from Havana. Table II shows that both LP and ASC responses were both higher in the 7-year group. Holguín Province was the most affected by the *N. meningitidis* B outbreak that affected Cuba in 1983. Preliminary results (unpublished) of studies currently under way indicate that vaccination with VA-MENGOC-BC™ does not fully reduce the carrier rate, which could explain the higher results seen in children from Holguín. We are currently working on the monitoring of this hypothesis.

The high proportion of pre-teenagers with positive LP, all with positive skin tests, and the absence or low percentage of B cell responders in children vaccinated 5 and 7 years earlier, suggest that the long-lasting memory induced by VA-MENGOC-BC™ is mainly T cell-mediated. This is in agreement with recent data from our laboratories showing that VA-MENGOC-BC™ preferentially induces a Th1 pattern in adult humans (26, 27, 28). In addition, the main subclass in vaccinated people was IgG_1, which is also the predominant isotype after a booster dose (33). This has been reported to be an opsonic antibody (34) and this isotype is believed to indicate a Th1 response (35). We are also working to define the Th pattern induced by VA-MENGOC-BC® in nursing babies. It is important to note that bactericidal antibodies, meaning isotypes that fix complement, are Th1 dependent.

Our results show that VA-MENGOC-BC™ induces a T-cell immune response, not only in adults, but also in children and nursing babies. This response is long-lived, lasting at least 7 years after vaccination, and is fundamentally T cell-mediated. The cellular immune response stimulated may be related to the high protection induced by independent of the presence or absence of bactericidal antibodies and the integrity of the complement system.

Despite the proven protective efficacy of VA-MENGOC-BC™, its correlation with bactericidal antibodies is not as clear-cut as with polysaccharide vaccines. The test as currently performed is complex, and may not be sufficiently sensitive. It is also possible that other *in vivo* functions of antibodies such as opsonisation may be responsible for protection conferred by antibodies which for several reasons may not be bactericidal. Antibodies to outer membrane proteins elicited by the vaccine may be reacting with the OMPs on the bacterial surface at a distance from the membrane such that they are not bactericidal, but opsonic and protective. Sialylation of the cell surface can abort the assembly of the membrane attack complex (36) and complement-mediated lysis (37, 38). The $\alpha 2 \rightarrow 8$ polysialyl capsule of *N. meningitidis* B stoichiometrically interferes with complement activation (4, 39, 40). *N. meningitidis* B could, therefore, interfere both complement pathways.

For these reasons, the fact that bactericidal antibodies are correlated with protection against capsular based vaccines may not be applicable to OMP vaccines such as VA-MENGOC-BC™, at least against serogroup B, because protection against serogroup C is also induced by this vaccine. Higher titers of bactericidal antibodies against C than B serogroups were found in serum from vaccinated people (41).

In conclusion, VA-MENGOC-BC™ induces a functional (DTH) cellular response in nursing babies and this response is of long duration. DTH is also induced in vaccinated children. The persistence of specific T cells in the blood up to 7 years after vaccination and the antibody response to a booster dose suggest that this vaccine induces a long-lasting immune response and immunological memory and may explain the high protection induced by it.

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