Original

VOL. 21 / NÚM. 1 / ENERO-MARZO 2002
INMUNOLOGÍA, 2002; PP 3-9

Mucosal IgA anti-lipopolysaccharide antibodies induced by 638 oral live attenuated candidate vaccine

M. LASTRE, J. DEL CAMPO, B. CEDRÉ, T. VALMASEDA, L. GARCÍA, G. BRACHO, T. SERRANO, R. FANDO**, G. SIERRA, O. PÉREZ*

*Departments of Basic and Clinical Immunology and Enteropathogenic Bacteria, Finlay Institute. *Clinical Laboratory, Pedro Kouri Institute. **CENIC. Havana City, Cuba*

ANTICUERPOS IgA ANTI-LIPOPOLISACÁRIDO INDUCIDOS POR CANDIDATO VACUNAL ORAL 638 VIVO

RESUMEN

La cepa de Cólera atenuada 638 ha inducido una buena respuesta en modelos animales y en un estudio piloto humano ha probado ser segura e inmunogénica. Sin embargo, no ha sido evaluada la IgA específica en mucosas ni tampoco se ha comparado la respuesta inducida por la cepa 638 con aquélla inducida por la conocida cepa reactogénica JBK70. Por ello, fueron evaluadas las células secretoras de anticuerpos (ASC) anti-lipopolisacárido (LPS) sanguíneas y los anticuerpos anti-LPS en saliva como indicadores de respuestas mucosas de voluntarios inoculados con las cepas 638 o JBK70. Con vistas a determinar la producción local o no de la IgA específica, la cinética de los anticuerpos IgA anti-LPS séricos y salivares fueron comparados. La respuesta vibriocida sérica fue también medida. Tres grupos con 638 (10^9, 10^8 y 10^7 unidades formadoras de colonias, CFU), uno con JBK70 (10^9 CFU) y otro con placebo fueron enrolados. La respuesta sérica de ASC IgA^+ fue mayor que la de ASC IgG^+. La IgA anti-LPS en saliva tuvo valores máximos a los 9 días y decayó hasta valores negativos en el día 14 después de la inoculación. La IgA anti-LPS sérica permanece elevada entre los 7 y 28 días después de la inoculación lo que sugiere que la IgA en saliva es localmente y transitoriamente producida. La respuesta vibriocida sérica fue incrementada después de la inoculación. Respuestas similares fueron obtenidas con las cepas 638 y JBK70.

PALABRAS CLAVE: Vacunas/ Inmunidad de mucosas/ Células secretoras de anticuerpos/ IgA/ Saliva/ *V. cholerae*/ LPS.

ABSTRACT

The 638 attenuated cholera strain has induced a good response in animal models and has proven to be safe and immunogenic in a pilot human study. However, specific IgA at mucosal sites and comparison of the response induced by the 638 strain with that induced by the known reactogenic JBK70 strain were not evaluated. Therefore, blood anti-LPS antibody secreting cells (ASC) and salivary anti-lipopolysaccharide (LPS) antibodies were evaluated as indicators of mucosal responses of volunteers inoculated with 638 or JBK70 strains. In order to determine the local production of specific IgA detected in saliva, the kinetics of serum anti-LPS IgA antibodies and salivary IgA were compared. The serum vibriocidal responses was also measured. Three 638 groups (10^9, 10^8, and 10^7 colony forming units, CFU), one JBK70 (10^9 CFU), and a placebo group were included. The serum IgA^+ ASC response was higher than the IgG^+ ASC. Salivary anti-LPS IgA peaked at day 9 and declined to negative values at day 14 after inoculation. Serum anti-LPS IgA remained high between 7 to 28 days after inoculation which suggest that salivary IgA is locally and transiently produced. The vibriocidal response increased after inoculation. Similar responses were obtained with 638 and JBK70 strains.

KEY WORDS: *Vaccine/ Mucosal immunity/ Antibody secreting cells/ IgA / Saliva/* V. cholerae/ *LPS.*

INTRODUCTION

Most bacterial and viral infections gain access to the host via mucosal tissues (1-4). The mucosal surface forms a barrier that prevents microbes from the external environment reaching internal organs (5). Colonisation by *Vibrio cholerae* evokes a mucosal immune response consisting mainly of IgA anti-lipopolysaccharide (LPS) antibodies (6,7). Following oral immunisation, activated lymphocytes migrate from inductive sites to mucosal effector sites and can be detected in the blood as antibody secreting cells (ASC) or in the

saliva after they have become antibody-secreting plasma cells (8).

The oral live attenuated El Tor Candidate Vaccine 638 strain is safe and immunogenic in animals and in preliminary clinical trials (9-11). However, mucosal responses have not been evaluated. We report the study of the mucosal response as measured by blood anti-LPS ASC and specific antibodies in saliva, and the kinetics of the serum anti-LPS response and bactericidal activity. The response to strain JBK70 and a placebo were also evaluated.

MATERIAL AND METHODS

Study design and participants

A prospective, double-blind, placebo controlled multi-center study was carried out between 1998-1999. Sixty eight 18 to 40 year old healthy male volunteers without previous vaccination against cholera and living outside the endemic area were recruited. The study protocol and informed consent were approved by the Ethical Committee of the Finlay Institute, the Pedro Kouri Institute and CEDMED, Cuba. All volunteers gave prior written consent.

Vaccine strains

V. cholerae strain 638, a new CTX∨ -negative, hemagglutinin/protease-defective El Tor Ogawa candidate vaccine produced in the CENIC, Cuba (12,13) and the JKB70 reactogenic but protective strain (14) were used.

Strain inoculations

Sixty-eight volunteers were included. Twenty-nine received one oral 638 dose of 2.1×10^9 CFU (high), 6 received 1.8×10^8 CFU (medium), 7 received 4×10^7 CFU (low), and 18 received 0 CFU (placebo). Eight volunteers received one oral JBK70 dose of 10^9 CFU. The vaccine strains and the placebo were all administered in 30 ml of 2% sodium bicarbonate buffer. Volunteers ingested 120 ml of buffer 30 min before inoculation. Food and drink were withheld for 90 min. before and after dosing.

Samples

Heparinized venous blood samples (15 IU/mL) were collected before and 7 days after inoculation. Samples of stimulated parotid saliva were taken before and at 7, 8, 9, 10, and 14 days after inoculation. Saliva was inactivated at 56°C for 30 min, clarified by centrifugation at 9 000 x g for 10 min, and frozen (-20°C) until used.

Isolation of cells

Peripheral blood mononuclear cells (PBMC) were separated from blood by centrifugation over Histopaque 1.077, washed three times, and resuspended in RPMI-1640 supplemented with gentamicin 50 µg/ml, L-glutamine (2 mM), sodium-pyruvate (1 mM), Hepes (15 mM), and inactivated foetal calf serum (FCS) 10%. The viability of the isolated cells was µ 99% by trypan blue exclusion. All products used were from Sigma, Mo, USA.

ELISPOT assay for antibody secreting cells

The method used was based on that of Czerkinsky and Sedwick *et al.* (15,16). Briefly, individual wells of surfactant-free mixed cellulose ester membrane bottomed 96-well MultiScreem-HA (Millipore, MA) were filled with 100 µL of LPS from 638 strain at 25 µg/ml. This coating concentration was found optimal in a preliminary checkerboard titration experiment. After three washes with phosphate-buffered saline (PBS), remaining protein binding sites were blocked by filling the individual wells with 150 ≥l of RPMI 1.640 complete medium containing 10% inactivated FCS, and incubating at 37°C for 30 min in a humidified atmosphere with 5% CO_2. Controls coated with albumin (5 ≥g/ml) were also included. For enumeration of total ASC irrespective of their specificity, the wells were coated with 100 ≥l of affinity purified goat antibodies directed against human IgA or IgG at 5 mg/ml (found to be optimal in a previous checkerboard titration experiment). The well contents were replaced with 100 µl of cell suspensions containing 10^5, 2.5×10^5, 5×10^5 or 10^6 PBMC per well in RPMI complete medium, incubated without disturbance for 4 h at 37º C in 5% CO_2 in a humidified atmosphere. Plates were rinsed three times with PBS and three times with PBS containing 0.05% Tween 20, and 100 µl of PBS-0.05% Tween 20 containing 1% BSA and either a mixture of affinity-purified goat antibody to human IgA or 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 M citrate pH 5 buffer. The spot numbers were counted in triplicate wells under low magnification (x 40). Volunteers who showed a two-fold increase

in the number of ASC after inoculation and had a final number higher than 2 per 10^6 PBMC were considered positive.

ELISA antibodies

Anti-LPS IgA salivary antibodies were determined as previously described (17). Briefly, plates were coated with LPS at 25 ≥g/ml (100 ≥l) and incubated overnight at 4º C. The saliva was diluted 1:2 and was incubated 2 h at 37ºC. Peroxidase-labeled goat anti-human IgA was used at 1:1.000 for 1 h at 37º C. The results were considered positive if at least once after inoculation there was a twofold or greater increase in OD, and if the OD was higher than the cut-off for each plate. Secretory anti LPS IgA was detected using the system described above but including an anti-secretory component as a second antibody and a third conjugated antibody.

Serum IgG and IgA anti-LPS antibodies were determined by ELISA (11). The antibody titre was defined as the dilution of serum, calculated by interpolation, giving an absorbance value of 0.4 units above background.

Vibriocidal antibody assay

Vibriocidal antibody titres were determined in a microassay (18). The vibriocidal antibody titre was defined as the highest dilution of serum causing complete inhibition of bacterial growth as judged by colour comparison of the culture medium with a control without serum.

Statistics

Significance ($p<0.05$) was measured by the non parametric Kruskal-Wallis test.

RESULTS

Circulating antibody secreting cells

The anti-LPS ACS response following a single oral dose of vaccine was studied at 7 days, after which the response declined. The IgA^+ ASC response was significantly higher ($p<0.05$) than the IgG^+ ASC response. Volunteers inoculated with the three different doses of strain 638 all showed similar levels of specific IgA^+ ASC. The highest response observed with JBK70 strain was seen in only two individuals with high specific IgA responses (930 and 950 spots / 106 PBMC) (Fig. 1A). In contrast, IgG^+ ASC were not always present at the low and the middle doses, but were consistently seen

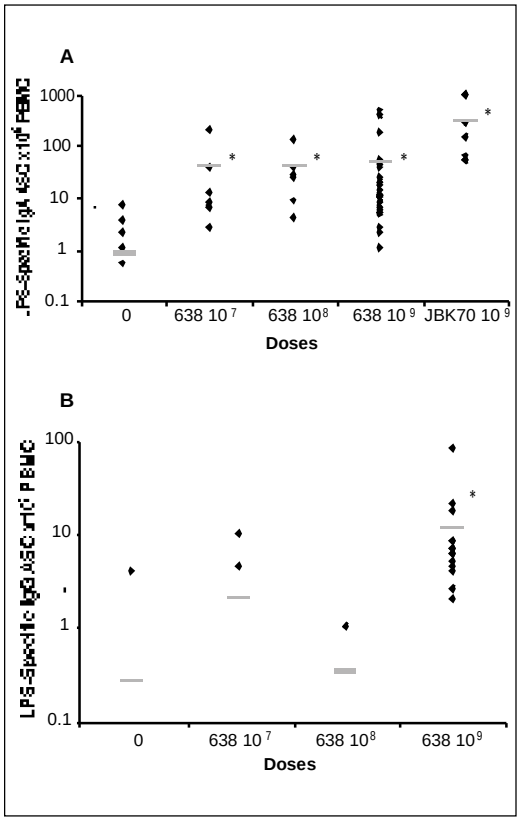

Figure 1. *Effect of 638 and JBK70 strains on the induction of specific ASC on PBMC. A, anti LPS IgA^+ASC and B, anti LPS IgG^+ASC. The presence of ASC was evaluated at 0 (data not shown; but all was negative) and 7 days after oral inoculation of different 638 doses (10^7, 10^8, and 10^9 UFC). Results are expressed as the logarithmic of the mean number of ASC per 10^6 PBMC, determined on triplicate wells for each individual. The line represents the mean values of each group. Volunteers who experienced a 2-fold increase in the number of ASC after inoculation and had a final number higher than 2 x 10^6 PBMC were considered positive. * Significant differences related to placebo group (0) p<0.05.*

($p<0.05$) with a dose of 10^9 CFU (Fig. 1B). The percentage of specific IgA positive subjects was high with all doses. Conversely, the percentage of specific IgG positive subjects was only significantly increased with a dose of 10^9 CFU of strain 638 dose, and was not seen in JBK70 inoculated subjects (Table II).

Kinetics of salivary antibody responses

Specific IgA in parotidal saliva began to increase at day 7 with the higher levels ($p<0.5$) at day 9 for the 10^9 dose and the two strains. The 10^9 dose also showed the highest OD response and also the

Table I
Seroconversion in volunteers orally inoculated with 638 and JBK70 attenuated *Vibrio cholerae* strains. The highest value is only represented

Dose	*n*	*Salivary IgA (%) ELISA**	*Vibriocidal Antibodies (%)***	*Serum IgA (%) ELISA***	*Sera IgG (%) ELISA***
638 High	29	27 (93.1)	24 (82.7)	26 (86.6)	21 (72.4)
638 Medium	6	5 (83.3)	5 (83.3)	4 (66.6)	4 (66.6)
638 Low	7	5 (87.5)	5 (71.4)	5 (71.4)	3 (42.8)
JBK70 High	8	7 (87.5)	7 (87.5)	7 (87.5)	2 (25)
Placebo	18	2 (11.1)	0 (0)	1 (5.5)	0 (0)

* maximum value at day 9 and ** maximum value at day 14 except for Vibriocida with low dose that was at 7 days.

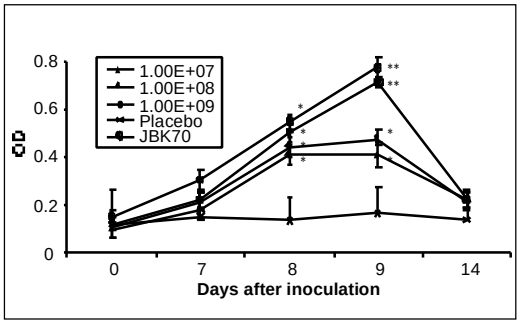

Figure 2. *Kinetics of anti-LPS IgA antibodies in saliva after 638 and JBK70 strains were inoculated. Parotid saliva was obtained at each time point represented from each volunteer inoculated with different 638 doses (10^7, 10^8, and 10^9 CFU) and JBK70 10^9 dose. The later was not evaluated at 14 days. The bars represent the mean values ± SEM of duplicate determination. *Significant differences related to placebo group (0) p<0.05.*

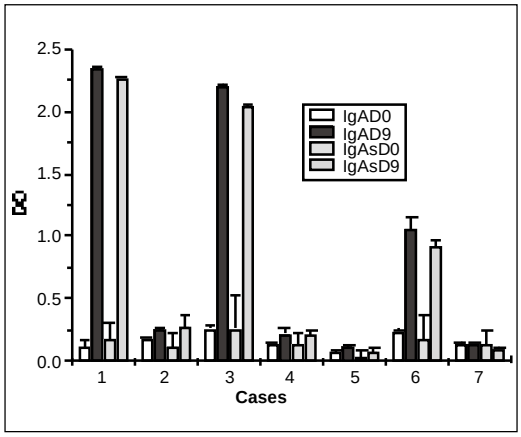

Figure 3. *Detection of secretor component on salival anti-LPS IgA. The parotid anti LPS IgA was measured before and after 638 strain (1, 3 and 6) or placebo (2, 4, 5 and 7) were inoculated using ELISA assay. The bars represent the mean values ± SEM of duplicate determination of specific IgA developed with conjugated anti IgA or anti secretor component. D0, day before and D9, 9 days after inoculation. *Significant differences p<0.05 vs T7, T14, and T0. **Significant differences p<0.05 vs all the evaluated times.*

highest percentage of seroconversion (Fig. 2 and Table I). In addition, all specific salivary IgA appears to be secretory IgA (Fig. 3) and not serum IgA (data not shown).

Kinetics of serum antibody responses

IgA and IgG anti-LPS antibodies were measured before and at weekly intervals after inoculation. In general, the specific IgA responses against both strains were stronger than the IgG response. However, the magnitude of the IgA response was not related to the vaccine dose. Conversely, an increased specific IgG response was observed in subjects given 10^9 CFU of strain 638. Both Ig classes remained high over the 4-week observation period (Fig. 4A and 4B). Only one subject of the placebo group seroconverted for serum IgA. The percentage of specific IgG seroconversion for strain 628 was dose dependant: 42.8, 66.6, and 72.4 at doses of 10^7, 10^8, and 10^9, respectively. In contrast, seroconversion to strain JBK70 was only seen in 25% of volunteers at the dose of 10^9. Seroconversion for both antibodies peaked between days 14 and 21 days (Table I).

Kinetics of vibriocidal responses

The seroconversion observed was very high in both strains at all doses, and no placebo subject was positive. The peak was at 14 days, after which titres started to decline in some groups (Fig. 5).

Table II
Anti LPS-specific Antibody Secreting Cells response in peripheral blood of volunteers following inoculation of 638 or JBK70 strains are represented

Dose	*n*	*IgA Positives (%)*	*Mean IgA$^+$ASC per 10^6 PBMC (range)*	*IgG Positives (%)*	*Mean IgG$^+$ASC per 10^6 PBMC (range)*
638 High	29	27 (93.1)	48.5 (0-475)	17 (58.62)	18 (0-155)
638 Medium	6	6 (100)	37.7 (40-128.5)	0 (0)	0.33 (0-1)
638 Low	7	6 (85.7)	37.1 (0-204)	2 (28.57)	2 (0-9.5)
JBK70 High	8	7 (87.5)	305 (0-950)	ND*	ND
Placebo	18	1 (5.5)	0.75 (0-6.5)	1** (7.14)	1.05 (0-3.75)

* ND, Not done and ** 14 volunteers were only evaluated.

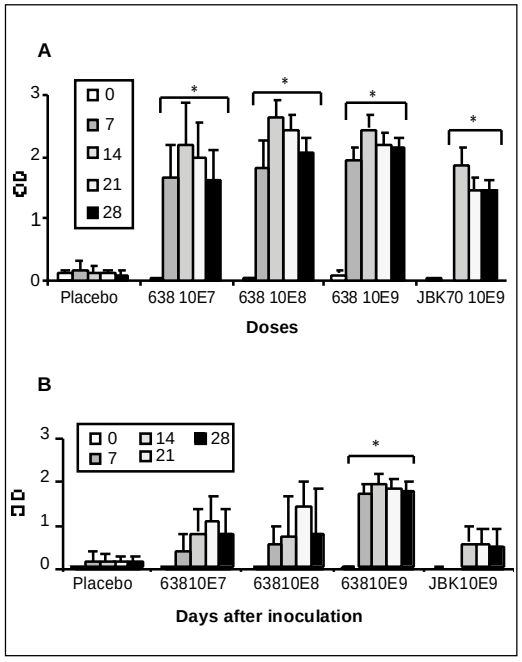

Figure 4. *Kinetic serum anti-LPS response. A, specific IgA and B, specific IgG. The bars represent the mean ± SE of duplicate determination. *Significant differences related to placebo group p<0.05.*

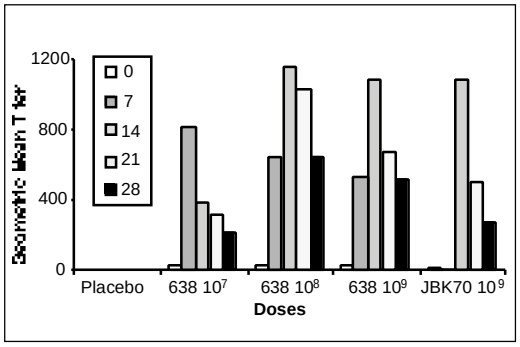

Figure 5. *Time behaviour of geometric mean titre of vibriocidal serum activity. JBK70 sera at 7 days was not evaluated.*

DISCUSSION

We found a preferential induction of IgA instead of IgG anti-LPS responses in all samples. Blood (ASC) and salivary anti-LPS IgA peaked at 7 and 9 days respectively and then declined. The salivary IgA had secretory component. In contrast, serum anti-LPS IgA remained high for several weeks. The responses induced by 10^9 CFU of strains 638 and JBK70 were similar.

Our finding of a substantial specific IgA$^+$ ASC response in PBMC and salivary IgA antibody suggests that the 638 strain efficiently elicits the right mucosal immune response. More IgA than IgG was induced. Long-lasting protection against *V. cholerae* infection is due to induction of high levels of secretory IgA antibodies and immunological memory for these antibodies in the intestine (19). Nevertheless, the high dose significantly increased the serum IgG$^+$ ASC response as well. The importance of the IgG induced is not clear yet, as IgG is not generally accepted as important in protection of the mucosa. Some authors think, however, that this antibody or some of its fractions may do so protect (20). In addition, the vibriocidal antibody response, due in part to IgG, is considered the best immunological correlate of protection against cholera (21). LPS is one of the most immunogenic cholera antigens. A as a T-independent antigen it may induce mainly IgM, the possible influence of which on vibriocidal activity should be investigated.

The activation/proliferation of the lymphocytes takes some days after leaving the peripheral lymphoid organs for the blood. For that reason, blood ASC are present between 5 to 12 days after antigen administration (22-24). In addition, immune networks in humans and circulating specific IgA$^+$ ASC

in blood appear to reflect recent or ongoing antigen exposure at the mucosal surface and the existence of a generalised mucosal immune network in humans (25,26). This circulation of lymphocytes may be exploited mainly in humans to evaluate the induction of mucosal response at Peyer's patches. It requires, however, that samples have to be taken at precise times during the recirculation of ASC in the blood or when they migrate through a specific receptor to the lamina propia and begin to produce antibodies some time later.

The appearance of antigen-specific IgA in saliva showed a kinetic response. This is in accordance with the transient ASC found in the blood with a peak at 7 days (27). The peak of specific IgA in saliva was two days later; and was also transient. It may be related to the migration of a few ASC to the parotid gland in comparison with the gut where the cholera colonisation begins and it may be that some antigens still remain there. In addition, high seroconversion rates in salivary antibodies were observed, which were similar or greater than the seroconversion of the other technique used. This specific IgA may come from local production or from the blood. The present data suggests that the former may be the case. First, all the salivary specific IgA obtained by masticatory stimulation from the parotid gland had the secretory component. Second, the highest value of specific IgA was detected at 14 days. Third, the presence of specific IgA in saliva was transient and picked at 9 days. Lastly, if the salivary IgA came from serum, it would have been stayed high for longer. Salivary IgA has not been consistently found by other authors, and this could be due to timing of the samples (weekly intervals) (28).

It has been demonstrated that the oral administration of one dose of a live attenuated strain could induce a solid and long protection against cholera (29,30). CVD 103 HgR strain (classic Inaba) has been extensively evaluated in volunteers and it has demonstrated to be well tolerated and immunogenic in industrialized countries (31,32), as well as in endemic areas (33,34). This strain is the only life attenuated oral vaccine commercially available at this moment. There are some strains of the El Tor biotype with similar development and results to be used as oral vaccine. Taylor et al. (35) reported the development and evaluation of three attenuated strains (Perú-3, Bah-3 and Bang-3). These strains were immunogenic in 93% of volunteers but provoked diarrhoea and other adverse effects in 40% of this population. Better results were obtained with the Perú-15 strain, which was less reactogenic (36). Nevertheless, these results should be confirmed with a higher number of volunteers or in a field trailt in a zone where the cholera is endemic (13).

JBK70 and CVD110, the first attenuated strains of El Tor biotype, provoked diarrhoea in 70% of inoculated volunteers (2 to 3 episodes by volunteer with 1 to 8 litres of diarrhoea as media) (37,38). Similar levels of reactogenicity showed the first of these strains in assays with Cuban volunteers (13).

The 638 strain has been extensively evaluated showing to be safe and immunogenic in Cuban volunteers as well as in endemic area (Ecuador). This strain is able to induce immune response against the main antigens of *V. cholerae* (39) and to be potentially protective in a challenge assay. All these results pointed out the possibility of 638 strain as a good candidate vaccine.

The present study clearly shows that strain 638 can induce ASC and also specific salivary IgA antibodies with, both of them with a characteristic kinetic. This response was in agreement with the serum and vibriocidal antibodies induced by this candidate vaccine.

ACKNOWLEDGEMENTS

We want to thank C Zayas, M Díaz, C Taboada for their excellent technical assistance; the nurses, auxiliary staff, and particularly the technician Felicita Nuñez for their help and taking the blood samples. We want also to thank to Prof. Carlos Hormaeche for his criticism and English corrections.

CORRESPONDENCE:
Miriam Lastre
Basic and Clinical Immunology Department
Finlay Institute
P.O. Box 16017, Havana, Cuba.
Phone: 53 (7) 218221. Fax: 53 (7) 286075.
e-mail:mlastre@finlay.edu.cu

References

1. Childers NK, Bruce MG, McGhee JR. Molecular mechanism of immunoglobulin A defence. Annu Rev Microbiol 1989; 43: 503-36.
2. Iscaki S, Bouvet J-P. Human secretory immunoglobulin A and its role in mucosal defence. Bull Inst Pasteur 1993; 91: 203-24.
3. Mulle CP. Mucosal vaccines: High-tech reagents for low-tech immunization. Immunol Today 1994; 15: 458-9.
4. Staats HF, Jackson RJ, Marinaro M, Takahashi KH, McGhee JR. Mucosal immunity to infection with implication for vaccine development. Curr Opin Immunol 1994; 6: 572-83.
5. Mims CA. The Pathogenesis of Infectious Disease, 3rd ed. London: Academic Press, 1987.
6. Cash RA, Music SI, Libonati JP, Craig JP, Pierce NF, Hornick RB. Response of man to infection with *Vibrio cholerae*. II Protection from illness afforded by previous

disease and vaccine. J Infect Dis 1974; 130: 325-33.
7. Svennerholm AM, Jertborn M, Gothefors L, Karim AM, Sack DA, Holgrem J. Mucosal antitoxic and antibacterial immunity after cholera disease and after immunization with a combinet B subunit-whole cell vaccine. J Infect Dis 1984; 149: 884-93.
8. Czerkinsky C, Quiding M, Eriksson K, Nordström I, Lakew M, Wenerås C, et al. Induction of specific immunity at mucosal surfaces: prospects for vaccine development. In: Advances in Mucosal Immunology. Edited by Mestecky J, et al., New York: Plenum Press, 1995: 1409-16.
9. García L, Oliva R, Cedré B, Valmaseda T, Garcia H, Talavera A, et al. Intraduodenal Inoculation of adult rabbits for evaluatiating the immunogenicity of genetically attenuated *Vibrio cholerae* strains. Laboratory Animal Science 1998; 48: 538-41.
10. Cedré B, García L, García H, Fariñas M, Talavera A, Infante JF. Intestinal colonization of the infant mouse model by attenuated and virulent *Vibrio cholerae* strains. Arch Med Res 1998; 29: 231-4.
11. Benitez J, Garcia L, Silva A, Garcia H, Fando R, Cedre B, et al. Preliminary Assessment of the Safety and Immunogenicity of a New CTX≡-Negative, Hemagglutinin/Protease-Defiective EL Tor Strain as a Cholera Vaccine Candidate. Infect Immun 1999; 67: 539-45.
12. Robert A, Silva AJ, Benites JA, Rodríguez BL, Fando R, Campos J, et al. Tanning a *Vibrio cholerae* EL tor candidate vaccine strain by disruption of its hemagglutinin/protease gene using a novel reporter enzyme: *Clostridium thermocellum* endogluconase A. Vaccine 1996; 14: 1517-22.
13. Benites JA, Silva AJ, Rodríguez BL, Fando R, Campos J, Robert A, et al. Genetic manipulation of *Vibrio cholerae* for vaccine development: construction of live attenuated EL Tor candidate vaccine strains. Arch Med Res 1996; 27: 275-83.
14. Levine MM, Kaper JB, Harrington D, Losonsky G, Grenn Morris J, Clements MI, et al. Volunteers studies of deletion mutants of *Vibrio cholerae* O1 prepared by recombinant techniques. Infect Immun 1988; 56: 161-7.
15. Czerkinsky CA. Solid-phase enzyme-linked immunospot (ELISPOT) assay for the enumeration of specific antibody-secreting cell. J Immunol Methods, 1983; 65: 109-21.
16. Sedwick JD, Holt PG. A solid-phase immunoenzymatic technique for the enumeration of specific antibody-secreting cells. J Immunol Methods. 1983; 57: 301-8.
17. Del Campo JM, Ochoa R, Lastre M, Bracho G, Taboada C, Díaz M, et al. ELISA de IgA anti-Lipopolisacarido de *Vibrio cholerae* en saliva. VacciMonitor 2001 (accepted).
18. Cedré Marrero B, García Sánchez HM, García Imía LG, Talavera Coronel A. Estandarización y Evaluación del Ensayo vibriocida modificado. Rev Cub Med Trop 1999; 51: 156-9.
19. Czerkinsky C, Prince SJ, Michalek SM, Jackson S, Russell MW, Moldoveanu Z, et al. IgA antibody-producing cells after antigen ingestion: evidence for a common mucosal immune system in humans. Pro Natl Acad Sci USA. 1987; 84: 2449-53.
20. Bouvet J-P, Fischetti VA. Diversity of Antibody-Mediated Immunity at the Mucosal Barrier. Infect Immun, 1999; 67: 2687-91.
21. Levine MM, Kaper MK. Live oral vaccines against cholera: an update. Vaccine, 1993; 11: 207-12.
22. Stevens RH, Saxon A. Immunoregulation in humans. Control of antitetanus antibody production after booster immunization. J Clin Invest, 1978; 62: 1154-60.
23. Stevens RH, Macy E, Morrow C, Saxon A. Characterization of a circulating subpopulation of spontaneous antitetanus antibody producing B cells following in vivo booster immunization. J Immunol 1979; 122: 2498-504.
24. Yarchoan R, Murphy BR, Strober W, Clements ML, Nelson DL. In vitro production of anti-influenza virus antibody after intranasal inoculation with cold-adapted influenza virus. J Immunol 1981; 127: 1958-65.
25. Kantele A, Arvilommi H, Jokinen I. Specific inmunoglobulin-secreting human blood cells after peroral vaccination against Salmonella typhi. J Infect Dis 1986; 153: 1126-32.
26. Lycke N, Lindholm L, Holmgren J. Cholera antibody production *in vitro* by peripheral blood lymphocytes following oral immunization of humans and mice. Clin Exp Immunol 1985; 62: 39-47.
27. Lastre M, Batista A, Sierra G, Pérez O. Cuantificación de Células Formadoras de IgG Totales y Específicas por ELISPOT en Balb/c durante la respuesta primaria se VA-MENGOC-BC®. VacciMonitor 1995; 7: 2-3.
28. Lycke N, Holmgren J. Long term cholera antitoxin memory in the gut can be triggered to antibody formation associated with protection within hours of an oral challenge immunization. Scand J Immunol 1987; 25: 407-12.
29. Levine MM. Immunity to cholera as evaluated in volunteer. In: Ouchterlony O, Holmgren J, eds. Cholera and related diarrheas. Basel: S. Karger 1980; 195.
30. Levine MM, Black RE, Clements ML, Nalin DR, Cisneros L, Finkelstein R. A Volunteer studies in development of vaccines against cholera and enterotoxigenic *Escherichia coli* a review. In Holme T, Holmgren J, Merson MH, Mollby R. (ed), Acute enteric infections in children. New prospects for treatment and prevention. Amsterdam: Elsevier / North-Holland Biomedical Press, 1981; 443-59.
31. Levine MM, Kaper JB, Herrington D. Safety, immunogenicity and efficacy of recombinant live oral cholera vaccines CVD 103 and CVD 103 HgR. Lancet, 1988; 2: 467-70.
32. Cryz SJ, Levine MM, Losonsky G, Kaper JB, Althaus B. Safety and immunogenicity of a booster dose of *Vibrio cholerae* CVD 103 HgR live oral cholera vaccine in Swiss adults. Infect Immun, 1992; 60: 3916-7.
33. Lagos R, Avendaño A, Prado V, Horwitz I, Wasserman S, Losonsky G, et al. Attenuated live cholera vaccine strain CVD 103-HgR Elicits significantly higher serum vibriocidal antibody titers in person of blood group O. Infect Immun 1995; 63: 707-9.
34. Gotuzzo E, Butron B, Seas C, Penny M, Ruiz R, Losonsky G, et al. Safety, immunogenicity and excretion pattern of single-dose live oral cholera vaccine CVD 103 HgR in Peruvian adults of high and low socioeconimic levels. Infect Immun, 1993; 61: 3994-7.
35. Taylor DN, Killen KP, Hack DC, Kenner JR, Coster TS, Beatle DT, et al. Development of a live, oral, attenuated vaccine against El Tor cholera. J Infect Dis 1994; 170: 1518-23.
36. Kener JR, Coster TS, Taylor DN, Troffa AF, Barrera-Oro M, Hyman T, et al. Peru-15, an improved live attenuated oral vaccine candidate for *Vibrio cholerae* O1. J Infect Dis 1995; 72: 1126-9.
37. Levine MM, Kaper JB, Herrington D, Losonsky G, Glenn Morris J, Clements MI, et al. Volunteer studies of deletion mutants of *Vibrio cholerae* O1 prepared by recombinant techniques. Infect Immun, 1988; 56: 161–7.
38. Tacket CO, Losonsky G, Nataro JP, Cryz SJ, Edelman R, Fasano A, et al. Safety and immunoge-nicity of live oral cholera vaccine candidate CVD110, a *DctxA Dzot Dace* derivative of El Tor Ogawa *Vibrio cholerae*. J Infect Dis 1993; 168: 1536–40.
39. Pérez JL, García L, Talavera A, Oliva R, Valmaseda T, Año G, et al. Passive protection of serum from volunteers inoculated with attenuated strain 638 of *Vibrio cholerae* O1 in animal models. Vaccine 2001; 19: 76–384.