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Antibody response on mice immunized by mucosal routes with formalin inactivated enteropathogenic *Escherichia coli* (EPEC) strains

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ABSTRACT. Secretory and systemic antibody response in mice against enteropathogenic *Escherichia coli* (EPEC) was evaluated. Groups of mice were immunized with formalin inactivated EPEC 0127:H6 strain by intranasal, peroral, intragastric and intrarectal route, with and without cholera toxin (CT) used as mucosal adjuvant. Mice immunized subcutaneously and a non treated control group were included. Other groups of mice were immunized intranasally with different EPEC strains and a non pathogenic *E. coli* K12 strain. Antibody response tested by ELISA assay showed that specific anti EPEC 0127:H6 antibodies were induced in serum by intranasal, subcutaneous and intragastric routes. A strong increase of antibody response against EPEC 0127:H6 strain was observed in saliva after intranasal delivery, while a lower response was detected by peroral and intrarectal immunization. Only the intranasal route increased IgA anti EPEC 0127:H6 antibody titers in feces. Specific and cross reactive antibodies to EPEC 0127:H6 were seen in mice immunized intranasally with different EPEC strains. Some control mice showed a background of anti EPEC 0127:H6 antibodies in feces. CT had a negative effect as adjuvant. We showed that nasal mucosa rendered the strongest antibody response in serum and secretions. These results might contribute to optimize the protective effect of enteric vaccines against infections associated to EPEC.

Key words: Mucosal immunization, secretory IgA, EPEC, formalin inactivated bacteria, ELISA assay, mice.

RESUMEN. La producción de anticuerpos en suero y secreciones fue evaluada en ratones inmunizados por vía peroral, intranasal, intragástrica e intrarrectal con la cepa EPEC 0127:H6 inactivada con formalina, con o sin toxina colérica (CT) usada como adyuvante. Se incluyó a un grupo de ratones inmunizados subcutáneamente con la cepa EPEC 0127:H6 y a un grupo control de ratones sin tratar. Otros grupos de ratones fueron inmunizados intranasalmente con diferentes cepas inactivadas de EPEC y con la cepa no patógena *E. coli* K12. La respuesta de anticuerpos cuantificada por la técnica de ELISA, mostró que las inmunizaciones por vía intranasal, subcutánea e intragástrica promovieron el aumento de anticuerpos séricos específicos anti-EPEC 0127:H6. Un incremento de anticuerpos en saliva fue promovido por la inmunización intranasal, y en menor grado, por la inmunización peroral e intrarrectal. Sólo la inmunización intranasal estimuló el aumento de anticuerpos IgA anti-EPEC 0127:H6 en heces. Se observó la presencia de anticuerpos específicos y de reactividad cruzada contra EPEC 0127:H6 en los ratones inmunizados con distintas cepas de EPEC y con *E. coli* K12. Algunos ratones del grupo control presentaron títulos de anticuerpos anti-EPEC 0127:H6 en heces. La toxina colérica tuvo un efecto negativo como adyuvante. Estos resultados podrían contribuir a optimizar el efecto protector de vacunas contra infecciones asociadas a EPEC.

Palabras clave: Inmunización en mucosas, IgA secretora, bacterias inactivadas con formalina, EPEC, ratones.

INTRODUCTION

Enteropathogenic *Escherichia coli* (EPEC) bacteria belong to the group of pathogens causing enteric diseases among pediatric population mainly in developing countries.[5] The pathogenesis of diarrhea associated to EPEC is based on the ability of this non invasive bacterium, to carry out intestinal colonization forming attaching and effacing (AE) lesions. Other colonization antigens such as bundle-forming pilus (BFP), intimin and others type III secreted proteins termed EspA, EspB, EspD and intimin receptor, Tir[25] also are involved.

Experimental studies in rabbits infected with rabbit enteropathogenic *Escherichia coli* (REPEC)[26] strain suggest that this pathogen promotes a local antibody response on account of binding onto intestinal Peyer's patches considered as inductive of mucosal associate lymphoid tissue (MALT).[16] On the other hand, clinical evidence showed that EPEC promotes IgA secretion in human colostrum[21] and IgG in serum[21,18] against colonization antigens.

Mucosal immune response to EPEC is being now extensively studied.[13] Some works have been focused on searching of virulence factors as putative targets for development of enteric vaccines[25,13] such as oral live *E. coli* recombinant strain as carrier of pilus subunit A (BfpA) from EPEC BFP.[22,24a]

At this time, strategies in vaccine development have been performed using live vaccines, highly efficient because resemble natural infection and therefore render a long lasting and vigorous immune response,[8] however

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these live vaccines may carry a risk of generate diseases, especially in immunodeficient individuals.[8] Another approach of vaccine preparation includes heat or formalin killed whole bacteria.[20] Most of non living whole cell vaccines are safer than live vaccines and because of their self adjuvant effect, mucosal adjuvants are not required.[8]

Several strategies of mucosal delivering have been tested in order to protect mucosal surfaces by eliciting secretory IgA involved in the initial inhibition of pathogen colonization.[2] Mucosal delivering of vaccines is particularly important because promotes *in situ* secretion of IgA antibodies at oral cavity, respiratory, gastrointestinal and urogenital tract covered by mucosal tissues belonging to MALT, which are main sites of entry and colonization for pathogens.[2] Since intestinal response is mandatory to face enteric infections caused by non invasive bacteria like EPEC, oral and intestinal mucosa have been chosen in experimental studies as routes of delivering of live[19,24] and non replicant vaccines based on EPEC bacteria.[19]

At present, intrarectal immunization mainly focus to boost an IgA secretion at colon, rectum and intestinal mucosa[11] but as nasal delivery, has not been proved yet as an alternative route of delivery for live or killed EPEC bacteria.

The nasal mucosa has been chosen as a non conventional route to evaluate immunogenic and pathogenic properties of live Enterotoxigenic *Escherichia coli* (ETEC) bacteria causing of traveler´s diarrhea.[4] Nasal delivery is a preferential route of immunization to non proliferant vaccines against respiratory diseases caused by pathogenic bacteria for instance *Bordetella pertussis*,[1] Group B streptococci[12] and *Streptococcus pneumoniae*.[14]

Considering the different strategies of mucosal delivery to assess the most efficient way of inducing mucosal antibody response, the work was focused to evaluate mucosal routes such as peroral, nasal, intragastric and intrarectal to elicit antibody response to formalin inactivated EPEC strains.

MATERIALS AND METHODS

Animals. Inbred female BALB/c mice, 8 to10 weeks old, were obtained from Bomholtgård-Breeding and Research Center, Ry, Denmark.

Bacterial strains. The follow EPEC bacterial strains included in this study were isolated from feces of Mexican children with diarrhea: *Escherichia coli* enteropathogenic (EPEC) 0127:H6 serotype (2348/69 prototype strain), EPEC 0111 serotype (B171 strain), EPEC 055:H6 serotype (065570/0 strain), EPEC 0R:H6 serotype (88255 strain), EPEC 086:H34 serotype (94859 strain) and EPEC 0142:H6 serotype (851/71) strain. A nonpathogenic *Escherichia coli* K12 control strain was included.

Preparation of formalinized bacteria. Suspensions of formalin-inactivated whole bacteria were prepared according to a method described before.[23] Briefly, cultures obtained after 12 h incubation at 37 ºC in Luria broth (Acumed, Remscheid, Germany) were washed three times by centrifugation at 11,300 x g for 10 min. at 4 ºC, after which the pellets were resuspended in sterile 0.01 M phosphate buffered saline (PBS), pH 7.4. Thereafter, the bacteria were incubated for 24 h in PBS containing 0.5% formaldehyde (Merck Sharp & Dohme AB Sollentuna, Sweden) with continuous shaking (Bio-Rad Laboratories, Hercules, CA, USA) at room temperature. Bacterial pellets were washed once and diluted in sterile PBS. The final bacterial suspensions were plated on blood agar to ensure sterility and bacterial inactivation, and stored at 4 ºC until used. The protein content was quantified by using bicinchoninic acid (BCA) protein assay reagent (Pierce Chemical Co., Rockford, IL, USA). Cholera toxin (CT) type Inaba 569B (Calbiochem Corp., La Jolla, CA, USA) were added to some of the preparations for mucosal immunizations.

Immunization. Groups of six mice were immunized four times at weekly intervals, as described before,[14] with the formalin-inactivated EPEC strain 0127:H6 with and without CT as mucosal adjuvant, by the intranasal, peroral, intragastric and intrarectal routes, or twice subcutaneously without CT on the days of the first and last mucosal immunization. Other groups were immunized four times intranasally at weekly intervals with one of the five formalin-inactivated EPEC strains 0111, 055:H6, OR:H6, O86:H34 or O142:H6, or the non-pathogenic *E. coli* strain K12, all without CT. Non-treated animals served as controls. For mucosal immunizations, each dose of bacterial antigen corresponded to 100 µg protein, with or without 5 µg CT. Each subcutaneous dose corresponded to 10 µg protein.

The intranasal immunizations were carried out with the mice held in a supine position with head down while 30 µl of the antigen suspension were delivered slowly onto the nares so that they could sniff it in. For peroral immunizations, the same volumes of antigen suspension were given slowly with a micropipette so that the mice could suck the fluid from the tip. The antigen suspension to be given intragastrically was mixed with 150 µl of 0.1 M $NaHCO_3$ (pH 8.1), making up 180 µl per dose, which was administered with a blunt steel feeding tube. For intrarectal immunizations, 30 µl of the antigen suspension were delivered by a feeding tube inserted via the anus with the tip approximately 3 cm from the anal opening.

Collection of samples. Saliva and feces were collected one week after the fourth mucosal dose, and extracts made as described previously.[14] Briefly, saliva was collected by inserting the tips of absorbent cylindrical wicks (Polyfiltronics Group Inc., Rockland, MA, USA) into the mouth of

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mice immediately after salivation was induced by a single intraperitoneal injection of 0.1 mg of pilocarpine-HCl (Sigma Chemical Co., St. Louis, MO, USA) in 200 µl PBS, and the net weight was recorded. Two wicks saturated with saliva were obtained from each mouse, frozen at –20 °C in 1.5-ml microcentrifuge tubes, and subsequently extracted with 400 µl of PBS with 0.05% Tween 20 and the following protease inhibitors: 0.2 mM 4-(2-aminoethyl)-benzenesulfonylfluoride (AEBSF) (Calbiochem), 1 µg of aprotinin (Sigma) per ml, 10 µM leupeptin (Sigma), and 3.25 µM bestatin (Boehringer Mannheim, Indianapolis, IN, USA).

After vortexing, the wicks were centrifuged at 2,000 x g for 15 min at 4 °C, and the resulting clear supernatant was collected and stored at –20 °C.

Three to five pieces of freshly voided feces were collected into 1.5 ml microcentrifuge tubes, frozen at –20 °C, and subsequently vacuum dried before their net dry weights were recorded. Extracts of feces were made by adding 20 µl PBS with 0.05% Tween 20 and protease inhibitors, per mg of dry feces, as described previously.[14] The fecal pellets were suspended by extensive vortexing, centrifuged at 2,000 x g 15 min at 4 °C, and the resulting clear supernatant was collected and stored at –20 °C.

Blood was obtained one week after the fourth intranasal dose by cardiac puncture during CO_2 anesthesia, and serum was separated and stored at –20 °C until analyzed.

Quantification of antibodies. Antigen-specific IgA antibodies in saliva and extracts of feces, and IgG antibodies in serum,[14,9] were analyzed by ELISA by using Nunc immunoplates (Maxisorp F96; A/S Nunc, Roskilde, Denmark). The plates were coated with 62.7 µg of protein per ml (100 µl per well) of inactivated bacterial suspensions diluted in 0.1 M carbonate-bicarbonate buffer at pH 9.6,[1] and incubated at room temperature until dryness.

Non-specific protein-binding sites were blocked with PBS containing 5% skim milk (Oxoid, Unipath, Ltd. Basingstoke, Hampshire, UK) in PBS by incubation for 1 h at 37°C and washed with PBS containing 0.05% Tween 20. Serum samples, extracts of saliva and feces, and standard solutions were applied to the ELISA plates (100 µl per well), serially diluted twofold in blocking solution, and incubated overnight at 4°C. The plates were then washed with PBS containing 0.05% Tween before being incubated for 1 h at room temperature with peroxidase conjugated goat anti-mouse IgG or anti-mouse IgA (both from Sigma). After washing, bound antibodies were detected with *o*-phenylendiamine (Sigma) in 0.05 M phosphate citrate buffer (pH 5.0). Optical densities were read at 492 nm with a Titrek Plus Multiscan MK II (Labsystems, Helsinki, Finland). Standard curves were generated, and antibody concentrations (in arbitrary units) in unknown samples were determined based on a defined pool of sera or secretions. The unknown samples were corrected for the weights of the original samples and for dilutions made during extraction from wicks and preparation for ELISA.

Statistical analysis. Differences of significance between groups of animals were determined by the two-tailed Mann-Whitney *U* test, using PRISM Software (GraphPad Software, San Diego, CA, USA).

RESULTS

Different routes of mucosal immunization promoted specific antibodies in serum and secretions. Table 1 depicts the results of antibody response in serum, feces and saliva from mice immunized by different routes of immunization by using formalin killed EPEC 0127:H6 strain.

Intranasal immunization of formalin inactivated EPEC 0127:H6 strain without CT induced at least 50 times higher median levels of specific IgG anti-EPEC 0127:H6 antibodies compared with the control group of mice ($P = 0.004$). The seric response induced by intranasal immunization with CT was also significant ($P = 0.004$) but less than without CT. By subcutaneous route, the specific IgG response was significant ($P = 0.004$) and higher than the intranasal route. Intragastric route without CT induced a significant increase of specific IgG anti EPEC 0127:H6 antibody titer ($P = 0.004$) , in contrast, intragastric immunization with CT, as well as peroral and intrarectal routes of immunization failed to elicit an increase of seric IgG anti EPEC 0127:H6 antibody concentration.

The intranasal immunization without CT increased 50 times more the median of IgA anti EPEC 0127:H7 antibody titer in saliva as compared with the median IgA concentration from control mice ($P = 0.004$). In contrast, intranasal immunization including CT induced a significant increase of median IgA title of only 5 times higher ($P = 0.004$) compared with non immunized. By peroral route of immunization including or not CT, a similar and significant increase of median IgA antibody titer in saliva was generated while on mice immunized by intrarectal route with PBS an increase of IgA titer in saliva was obtained ($P = 0.004$). On mice immunized by subcutaneous and intragastric via as well as intrarectal route with CT, no increase of IgA antibody titer in saliva was generated.

On feces, only intranasal immunization of EPEC 0127:H6 induced a significant increase ($P = 0.004$) of the median IgA antibody titer compared to control mice (Table 1). On mice immunized by intragastric and intrarectal immunization without CT, the increase of the median IgA titer in feces was not significant but was twice compared to control mice (Table 1). Some nonimmunize mice from control group showed higher or similar levels of secretory IgA anti EPEC 0127:H6 antibodies in feces than immunized mice.

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Intranasal immunization of mice with different formalin inactivated EPEC strains rendered specific antibodies in serum and secretions.

Intranasal immunization of five different EPEC strains and nonpathogenic *E. coli* K12 control strain promoted an increase of specific IgG antibody concentration in serum as compared with control mice (P = 0.004).

Except EPEC 086:H34 strain, all EPEC strains tested were be able to induce a significant increase (P = 0.004) of the specific IgA antibody titer in saliva. The significant increase of specific IgA antibody level on feces on mice immunized nasally was observed only with EPEC 055:H6 and EPEC O86:H34 strains (Table 2). A high background of secretory IgA anti EPEC 0127:H6 antibodies were found in some non immunized control mice.

Intranasal immunization of mice with different formalin inactivated EPEC strains rendered cross reactive antibodies in serum and secretions. The intranasal immunization of mice with five different EPEC strains and *E. coli* K12 non pathogenic strain promoted a significant increase (P = 0.004) of IgG titer anti-EPEC 0127:H6 antibodies in serum compared with levels of cross reactive anti EPEC 0127:H6 antibodies from non immunized control mice. Only EPEC OR:H6 elicited a cross reactivity IgA response in saliva and all EPEC strains failed to induce a significant increase of cross reactive IgA anti EPEC 0127:H6 antibodies in feces (Table 3).

DISCUSSION

The present study showed that immunization through the nasal mucosa produced the highest levels of antibodies in serum, saliva and feces when compared with other mucosal sites. These results suggest that nasal mucosa as inductive site of immunization,[27] to formalin killed EPEC 0127:H6, promoted effectively the priming, spreading and homing of immunocompetent cells to effector sites belonging to MALT[16] such as, salivary glands and intestinal lamina propria and to peripheral lymphoid organs of systemic compartment. Besides nasal mucosa, delivery by peroral route without CT elicited a significant increase of specific IgA anti EPEC 0127:H6 antibodies in saliva; this is probably due to the stimulation of lymphoid cells from nasopharyngeal nodules[27] which represent a relevant inductive site for IgA secretion at oral mucosa. Salivary glands represent an alternative site of lymphocyte stimulation leading of IgA secretion in saliva.[17]

Intrarectal mucosal has been recognized as a delivery site to induce IgA secretion at colon and rectum.[11] In this work intrarectal immunization with EPEC O127:H6 strain also produced and increase of specific antibody titer in saliva. When humans were intrarectally immunized with recombinant cholera toxin (CTB) subunit, an increase of antibody titers in saliva was also observed.[15]

Besides nasal and subcutaneous routes, immunization via intragastric route without CT promoted a remarkable

Table 1. Median values of anti-EPEC 0127:H6 antibody concentration on samples from mice immunized by different mucosal routes with formalin killed EPEC 0127:H6 strain with or without cholerae toxin as mucosal adjuvant. A group of mice immunized by subcutaneous route with the same killed strain was included. Results were compared with the control group of non immunized mice. Maximum and minimum ranges of antibody titers are also included in parentheses.

	Antibody concentration in:		
Route of immunization	Serum IgG kU/ml	Saliva IgA U/ml	Feces IgA U/g
Intranasal plus cholerae toxin[A]	122.4* (268-22)	100.1*,** (236.4-34.1)	642.8* (2231-504.3)
Intranasal[A]	195.1* (623-168)	960*,** (1778-758)	723.8* (1051-516)
Per oral plus cholerae toxin[A]	4.9 (30.4-1.7)	44* (97-19.5)	358.6 (512.5-141)
Per oral[A]	3.8 (8.8-1.9)	40.9* (76.3-30)	212.2 (280-105)
Intragastric plus cholerae toxin[B]	3.6** (11.1-3.0)	17.5 (49.2-9.1)	419.7 (1560-316)
Intragastric[A]	84.5*,** (958-6.9)	19.6 (28-9.5)	474.8 (814.4-286)
Intrarectal plus cholerae toxin[A]	7.4 (616.4-2.4)	11.6** (40-4.0)	336.6 (408-128.5)
Intrarectal[A]	5.2 (1318-0.9)	34.6*,** (73.5-18.2)	494.7 (1219-107)
Subcutaneous[A]	270.9* (497-144)	14.4 (51-10)	204.9 (445-150)
Control[B]	3.4 (4.2-2.5)	18.1 (31.6-10)	218 (1689-24.2)

[A] n = 6 mice [B] n = 5 mice

* Statistical significance *versus* control group according to Mann-Whitney U test at P < 0.005

** Statistical significance between groups at P < 0.005

increase of anti EPEC 0127:H6 IgG antibodies in serum maybe derived by antigenic molecules that reached blood stream as consequence of alterations on permeability of intestinal mucosa caused by bacterial components associated to whole killed EPEC vaccine.[10]

The negative antibody response in feces observed with any mucosal route of immunization other than the nasal via, could be due to immunological tolerance induced by the previous contact of mice with intestinal nonpathogenic *E. coli* strains from normal microbiota.[1a,7] This interpretation is supported by our finding of a high background of secretory antibodies in feces from some non immunized control mice (data not shown).

A mild adjuvant effect of CT was only observed in saliva from mice immunized perorally, but in most cases, a lower antibody response was observed on mice immunized by different mucosal routes with formalinized EPEC 0127:H6 strain containing CT. This negative effect of CT

Table 2. Median values of specific antibodies concentration samples from mice intranasally immunized with different EPEC strains and a non pathogenic *E. coli* K12 strain. Results were compared with the control group of non immunized mice. Maximum and minimum of antibody titers are shown in parentheses.

	Antibody concentration in:		
Group of mice	Serum IgG kU/ml	Saliva IgA U/ml	Feces IgA U/g
EPEC 0111[A]	6.2* (12.7-4.1)	103.8* (369.4-59.4)	< 2.5
Non immunized group[B]	0.13 (0.27-0.07)	30.6 (36-22)	< 2.5
EPEC 055:H6[A]	12.6* (19.6-6.2)	1186.8* (1606.5-805.2)	1114.8* (5405-695)
Non immunized group[B]	0.09 (0.12-0.05)	21.9 (39.4-16.7)	< 1.25
EPEC OR:H6[A]	10.7* (109.8-9.8)	749.2* (1490.1-104.8)	< 1.25
Non immunized group[B]	0.133 (0.17-0.09)	52.1 (55.4-34.3)	< 1.25
EPEC O86:H34[A]	3.0* (7.3-1.7)	28.7 (349.5-12.1)	1261* (2125.7-603.3)
Non immunized group[B]	0.08 (0.19-0.03)	19.7 (23.9-11.1)	< 2.5
EPEC 0142:H6[A]	12.6* (25-4)	211.4* (405.2-141.3)	< 2.5
Non immunized group[B]	0.080 (0.15-0.06)	44.2 (88.1-39.3)	< 2.5
E. coli K12[A]	26.0* (29.2-10.1)	646.5* (976.9-98.7)	< 1.25
Non immunized group[B]	0.27 (0.3-0.08)	49.7 (116.2-16.5)	< 1.25

[A] n = 6 mice [B] n = 5 mice
* Statistical significance *versus* control group at P<0.005.

Table 3. Median values of cross-reactive anti-EPEC 0127:H6 antibodies concentration on samples from mice intranasally immunized with different EPEC strains and a non pathogenic *E. coli* K12 strain. Results were compared with the control group of non immunized mice. Maximum and minimum of antibody titers are shown in parentheses.

	Antibody concentration in:		
Group of mice	Serum IgG kU/ml	Saliva IgA U/ml	Feces IgA U/g
EPEC 011[A]	16.5* (23.4-7.2)	57.2 (110.3-16.6)	468 (492-80.3)
EPEC 055:H6[A]	14* (54.1-5.5)	19.9 (24.4-9.7)	< 2.5
EPEC OR:H6[A]	76.3* (191.7-51.2)	158.4* (333.4-29.6)	278.9 (1543.2-174.1)
EPEC O86:H34[A]	40.3* (196.6-25.9)	35.5 (118.2-8.1)	< 1.25
EPEC 0142:H6[A]	59.2* (77.9-24.1)	49.3 (117.4-9.0)	< 2.5
E. coli K12[A]	25.9* (116.5-9.4)	29.7 (106.8-23.2)	< 2.5
Non immunized group[B]	1.7 (3.3-1.1)	34.3 (41.49-20.65)	734.3 (942.8-525.8)

[A] n = 6 mice [B] n = 5 mice
* Statistical significance *versus* control group at P < 0.005.

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has been also observed in mice after the coadministration of CT with whole cell vaccines from *Bordetella pertussis*[1] and group B streptococci.[12]

The evaluation of immune response following intranasal immunization in mice of formalinized EPEC strains and non pathogenic *E. coli* K12 control strain showed that all these inactivated bacteria possess a self adjuvant activity because they were able to induce an antibody response in serum and saliva without using CT as mucosal adjuvant.

The all five inactivated EPEC strains and even *E. coli* K12 control strain administered intranasally elicited a specific and cross-reactive antibody response to EPEC 0127:H6 in serum and saliva. We could evaluate different patterns of immunogenicity of each strain in serum and secretions by using intranasal immunization. These results match with other study which the nasal mucosa was proposed to evaluate the differences on immunogenicity of live enterotoxigenic *E. coli* (ETEC) strains.[4]

Some studies support the role of seric cross-reactive antibodies to enhance and strengthen the protective activity of specific antibodies,[6] on other hand, unwanted immunological effects such as autoimmune diseases derived of cross reacting antibodies to bacteria from normal flora and generated following the delivering of inactivated enteropathogenic bacteria through nasal mucosa, shall be taking in account[3] for future studies.

These results might contribute in future studies to optimize the protective effect of enteric vaccines against infections associated to EPEC highly frequent in developing countries.

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REFERENCES

1. Berstad, A.K.H., J. Holst, B. Mögster, I.L. Haugen & B. Haneberg. 1997. A nasal whole-cell pertussis vaccine can induce strong systemic and mucosal antibody responses which are not enhanced by cholera toxin. Vaccine. 15:1473-1478.

1a. Berg, R.D. & D.C. Savage. 1975. Immune responses of specific pathogen-free and gnotobiotic mice to antigens of indigenous and nonindigenous microorganisms. Infect. Immun. 11:320-329.

2. Brandtzaeg, P. 2003. Role of secretory antibodies in defence against infections. Int. J. Med. Microbiol. 293:3-15.

3. Brandwein, S.L., R.P. McCabe, Y. Cong, K.B. Waits, B.U. Ridwan, P.A. Dean, T. Ohkusa, E.H. Birkenmeier, J.P. Sundberg & Ch. Elson. 1997. Spontaneously colitic C3H/HeJBir mice demonstrate selective antibody reactivity to antigens of the enteric bacterial flora. J. Immunol. 159:44-52.

4. Byrd. W., S.R. Mog & F.J. Cassels. 2003. Pathogenicity and immune response measured in mice following intranasal challenge with Enterotoxigenic *Escherichia coli* strains H10407 and B7A. Infect. Immun. 71: 13-21.

5. Clarke, S.C., R.D. Haigh, P.P. Freestone & P.H. Williams. 2002. Enteropathogenic *Escherichia coli* infection: history and clinical aspects. Br. J. Biomed. Sci. 59:123-127.

6. Donnenberg, M.S., C.O. Tacket, G. Losonsky, G. Frankel, J.P. Nataro, G. Dougan & M.M. Levine. 1998. Effect of prior experimental human enteropathogenic *Escherichia coli* infection on illness following homologous and heterologous rechallenge. Infect. Immun. 66:52-58.

7. "Deleted in proof" 67: 5917-5924.

8. Haneberg, B., A.K.H. Berstad & J. Holst. 2001. Bacteria-derived particles as adjuvants for non-replicant nasal vaccines. Adv Drug Deliver Rev 51:143-147.

9. Haneberg, B., D. Kendall, H.M. Amerongen, F.M. Apter, J.P. Kraehenbuhl & M.R. Neutra. 1994. Induction of specific immunoglobulin A in the small intestine, colon-rectum, and vagina measured by a new method for collection of secretions from local mucosal surfaces. Infect. Immun. 62:15-23.

10. Higgins, L.M., G. Frankel, I. Connerton, N.S. Gonçalves, G. Dougan & T.T. MacDonald. 1999. Role of bacterial intimin in colonic hyperplasia and inflammation. Science. 285:588-591.

11. Hopkins, S., J.P. Krahenbuhl, F. Shödel, A. Potts, D. Peterson, P. De Grandi & D. Nardelli-Haefliger. 1995. A recombinant *Salmonella thyphimurium* vaccine induces local immunity by four different routes of immunization. Infect. Immun. 63:3279-3286.

12. Hordnes, K., T. Tynning, T.A. Brown, B. Haneberg & R. Jonsson. 1997. Nasal immunization with group B streptococci can induce high levels of specific IgA antibodies in cervicovaginal secretions of mice. Vaccine. 15:1244-1251.

13. Horne, C., B.A. Vallance, W. Deng & B.B. Finlay. 2002. Current progress in enteropathogenic and enterohemorrhagic *Escherichia coli* vaccines. Expert. Rev. Vaccines. 1:483-493.

14. Hvalbye, B.K.R., I. Aaberge, M. Løvik & B. Haneberg. 1999. Intranasal immunization with heat-inactivated *Streptococcus pneumoniae* protects mice against systemic pneumococcal infection. Infect. Immun. 67:4320-4325.

15. Kozlowski, P.A., S. Cu-Uvin, M.R. Neutra & T.P. Flanigan. 1997. Comparison of the oral, rectal, and vaginal immunization routes for induction of antibodies in rectal and genital tract secretions of women. Infect. Immun. 65:1387-1394.

16. Kraehenbuhl, J.P. & M. Neutra. 1992. Molecular and cellular basis of immune protection of mucosal surfaces. Physiol. Revs. 72:853-879.

17. Marcotte, H. & M.C. Lavoie. 1998. Oral microbiology and the role of salivary immunoglobulin A. Microbiol. Mol. Biol. Rev. 62:71-109.

18. Martínez, M.B., C.R. Taddei, A. Ruiz-Tagle, L.R. Trabulsi & J.A. Girón. 1999. Antibody response of children with enteropathogenic *Escherichia coli* infection to bundle-forming pilus and locus of enterocyte effacement encoded virulence determinants. J. Infect. Dis. 179:269-274.

19. Milon, A., J. Esslinger & R. Camguilhem. 1992. Oral vaccination of weaned rabbits against enteropathogenic *Escherichia coli*-like *E. coli* O103 infection: use of heterologous strains harboring lipopolysaccharide or adhesin of pathogenic strains. Infect. Immun. 60:2702-2709.

20. Pace, J.L., H.A. Rossi, V.M. Esposito, S.M. Frey, K.D. Tucker & R.I. Walker. 1998. Inactivated whole-cell bacterial vaccines: current status and novel strategies. Vaccine. 16:1563-1574.

21. Parissi-Crivelli, A., J.M. Parissi-Crivelli & J.A. Girón. 2000. Recognition of enteropathogenic *Escherichia coli* virulence determinants by human colostrum and serum antibodies. J. Clin. Microbiol. 38:2696-2700.

22. Quintana-Flores, V.M., R.C. Campos de Souza Fernandes, Z. Sousa de Macedo & E. Medina-Acosta. 2002. Expression and purification of the recombinant enteropathogenic *Escherichia coli* vaccine candidates BfpA and EspB. Protein Expr Purif. 25:16-22.
23. Roberts, J.A., M.B. Kaack, G. Baskin & S.B. Svenson. 1995. Vaccination with a formalin-killed P-fimbriated *E. coli* whole cell vaccine prevents renal scarring from pyelonephritis in the non-human primate. Vaccine. 13:11-16.
24. Robins-Browne, R.M., A.M. Tokhi, L.M. Adams & V. Bennett-Wood. 1994. Host specificity of enteropathogenic *Escherichia coli* from rabbits: lack of correlation between adherence *in vitro* and pathogenicity for laboratory animals. Infect. Immun. 62:3329-3336.
24a. Schriefer, A., J. R. Maltez., N. Silva., M. Y. Stoeckle., M. Barral-Netto & L.W. Riley. 1999. Expression of a pilin subunit BfpA of the bundle forming pilus of enteropathogenic *Escherichia coli* in an aroA live salmonella vaccine strain. Vaccine. 17:770-778.
25. Vallance, B.A., C. Chan, M.L. Robertson & B.B. Finlay. 2002. Enteropathogenic and enterohemorrhagic *Escherichia coli* infections: emerging themes in pathogenesis and prevention. Can. J. Gastroenterol. 16:771-778.
26. Von Moll, L.K. & J.R. Cantey. 1997. Peyer´s patch adherence of enteropathogenic *Escherichia coli* strains in rabbits. Infect. Immun. 65:3788-3793.
27. Wu, H-Y., H.H. Nguyen & M.W. Russell. 1997. Nasal lymphoid tissue (NALT) as a mucosal immune inductive site. Scand. J. Immunol. 46:506-513.

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