Effect of Intranasal Administration of *Lactobacillus fermentum* on the Respiratory Tract of Mice

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This paper studied the effect of intranasal inoculation of *Lactobacillus fermentum*, a microorganism belonging to the normal flora of the mouse pharynx, on the respiratory tract of mice. Optimal temporary colonization in different areas of the tract was obtained through administration of 4 times a dose of 5×10⁶ CFU. *L. fermentum* remained in the trachea and bronchial up to the 7th day after inoculation. Re-inoculation of lactobacilli on the 10th day produced a transient colonization of the respiratory tract. Histological modifications produced in the trachea were mainly observed as an increased lymphocyte population at sub-mucosa level on the 4th day after inoculation. There was an increased number of activated macrophages in cytological slides of lung tissues on days 2 and 4. Re-inoculation also produced stimulation of the G2 macrophages on days 12, 14 and 17. From a histological point of view there were no other important changes in the organs studied. These results suggest stimulation of the immune system, especially of that of the mucosal surfaces, after intranasal administration of *L. fermentum* in the experimental model employed. Stimulation was reflected in tracheal lymphocyte proliferation and increased lung macrophage population which have to be further studied in more detail.

Key words lactobacilli; respiratory tract; intranasal administration; histological changes

The indigenous microflora associated to the mucosa of the oropharynx is a stable ecosystem, which protects the host against pathogens which enter through the respiratory tract.\(^1\) The ecological balance can be modified by a number of factors, some of them depending on the host.\(^2\) From studies performed in animal models (*in vivo*), colonization of the microorganisms can be altered by: a) factors of the microorganisms themselves, such as their adhesion or colonization capability mediated by proteins, carbohydrates and adhesins; b) nutrient competition or production of antagonistic substances;\(^3\) c) host factors (immunosuppression, cancer, modifications of the mucosa by different treatments, *etc.*); and d) environmental factors (antibiotic therapy, chemotherapy, hormones, *etc.*). Microorganism adhesion to the epithelia is generally mediated by a complicated set of hydrophobic properties which interact on the surface, complemented by the presence or absence of specific receptors and appendices on the microbial cell surface.\(^4\)

In the last years, there has been an increased tendency in modern medicine to apply preventive treatments, using natural products like probiotics, defined by Havenaar\(^5\) as viable microorganisms administered to the host to modify the indigenous microflora and exert a beneficial effect on humans and animals. In a previous paper we studied qualitatively and quantitatively the microorganisms present in different areas of the respiratory tract of mice, their colonization kinetics in growing mice and the predominant species.\(^6\) A screening of the surface properties with respect to the adhesion capability and production of inhibitory substances was performed with the microorganisms isolated (data sent for publication). Microorganisms sharing one or more of these properties all belonged to the genus *Lactobacillus*.

As the final objective of our group is the design and formulation of a probiotic with live microorganisms, applied as aerosols or sprays, to prevent respiratory infections, an experimental model was developed that allowed us to study the colonization capability of microorganisms and the prevention of pathogens in the respiratory tract. The aim of the present work was to a) obtain mutants from the lactobacilli selected which are resistant to antibiotics commonly used, b) optimize the method and the way of inoculation of the microorganisms in the respiratory tract to get an optimal or a transitory colonization, c) study the kinetics of the colonization of the respiratory tract by *L. fermentum*, intranasally administered to mice, d) know the effect of *L. fermentum* re-inoculation by the same way, and e) evaluate the histological and cytological modifications produced by inoculations of lactobacilli.

MATERIALS AND METHODS

Microorganisms *L. fermentum* was isolated from the respiratory tract (pharynx) of adult Balb/c mice. Lactobacilli were identified by the methods described in Bergey's Manual of determinative bacteriology,\(^7\) standard techniques used in the laboratory, and API 50 CHL (Biomerieux-France). They were stored at −70 °C in milk-yeast extract (10% skim milk, 1% glucose, 0.5% yeast extract). *L. fermentum* mutants were obtained after determining their sensitivity to different antibiotics (performed by monodisc diffusion test and Minimal Inhibitory Concentration (MIC)). The antibiotics assayed were erythromycin, cephalixin and rifampin. Mutants were obtained after growing on MRSA agar\(^8\) with 100 μg/ml rifampin. *L. fermentum* RR (resistant to rifampin) maintained this characteristic over long periods of time, by storing it at −70 °C and subculturing it alternately in media with and without the antibiotic. The RR strain had the same phenotypic properties as the parental strain (tested with API System). The mutant strain showed the same properties as the wild type. Strains were subcultured three times in LAPtg broth\(^9\) at 37 °C, alternatively in media with and without the antibiotic, to obtain the suspensions for inoculation. Suspensions were centrifuged, washed with saline solution and resuspended to the desired concentration.

Animals Balb/c mice from the breeding stock colony

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kept in our laboratory were used throughout this study. The animals were housed in plastic cages and fed “ad libitum” keeping their environmental conditions constant. Each experimental assay was carried out with groups of 20 to 30 mice. A group of control animals of the same size was similarly fed with a solution without microorganisms (placebo). On day 0, 2, 4, 7, 12, 14 and 17, 3 to 4 animals from both treated and untreated groups were randomly sacrificed.

The experimental protocol for using animals was approved by the Ethical Committee for the use of animals at CERELA.

Mice were intranasally inoculated with 50 μl of L. fermentum in SS (1×10⁶ CFU/ml) every 12 h. The exposition time was around 1 min per animal, performing an intranasal inoculation of 10 μl each time. Inoculation was performed by using a firmegel-loading pipetter equipped with a nose tip and the suspension was sniffed by the animals in normal tidal breathing. To facilitate downward migration of the inoculum, mice were held in a vertical position for 2 min. Different groups of mice (3–4 animals each) were inoculated with 1, 2 or 4 doses of 1×10⁶ CFU/ml (50 μl each), administered every 12 h.

Other groups of mice were re-inoculated 10 d after the first inoculation with 4 doses (50 μl) of the same L. fermentum suspension in SS (1×10⁶ CFU/ml).

Mice were sacrificed by cervical dislocation on days 2, 4, and 7 after inoculation. The re-inoculated group was sacrificed 12, 14, and 17 d after inoculation. Nasal and pharynx instillations were obtained by washing each open cavity with 1 ml of peptone water and later scraping them gently with mini sterile cotton tips. Trachea, bronchia and lung were aseptically removed and homogenized in 1 ml peptone water with a Teflon pestle. The number of microorganisms was determined by the serial dilution method. Microorganisms were diluted in peptone water (1% peptone) and inoculated on MRS-rifampin (15 μg/ml) agar plates (Biokar Diagnostics-Beauvais-France). Plates were incubated at 37°C for 48–72 h in a micro aerobic environment. Lactobacilli resistant to rifampin were counted after that period.

The inoculation and sacrificed protocol is schematized as follows:

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Histopathological Studies From each experimental group, two mice were randomly selected for histological examination. Trachea, bronchia and lung were separated for further studies after cervical dislocation.

Cytological Studies The left lobe was used to perform lung cytological slides. Previous to light micrographs, the organ was immersed in saline solution, fixed in methanol for 3 min, and stained with the Romanovsky method (Giemsa stain-Merck), described by Grignaschi et al.¹⁰

Determination of the Percentage of Cells in the Cytological Slides The number of cells in the lung slides was counted based on the method employed for bone marrow counts, according to Grignaschi.¹⁰ A total of 500 cells was counted in different areas of the slide and according to the proportion of cells, the percentage was later determined.

Histological Technique The areas studied were: a) high trachea located in the neck basin near the thyroid gland, b) bronchia, where they divide into main bronchia and c) lungs (left and right lobe): terminal bronchiole and alveolar wall with their capillary and connective tissue to which the alveolar macrophages are associated. The samples were fixed with 10% paraformaldehyde for 24 h at room temperature, then embedded in paraffin for 24 h according to the method standardized at our laboratory. Afterwards, the samples were cut into sections of 5 μm thick, stained with Hematoxylin-Eosin,¹¹ and then processed for light microscopy (40X objective). In order to quantify the lymphocyte population an area of the epithelium, corresponding to 50 epithelial cells, was taken as a reference. This area was correlated to the number of lymphocytes present in the lamina propria area. With these data qualification and quantification of the lymphocytes present in the epithelial cells were carried out, as can be read in Fig 2.

Statistical Analysis The experiments were performed at least three times. The results obtained were used to calculate the mean and respective Standard Deviation (SD). Figures are expressed in the tables and graphs. Student’s t-test was used to determine the statistical significance of the differences between data.

RESULTS

Colonization Assays L. fermentum RR transiently colonized the respiratory tract of mice at different days after inoculation as shown in Fig. 1. These results were obtained after the 4 inoculation doses. No transient colonization was obtained after inoculation of 1 or 2 doses. Optimal administration was 4 doses. The number of microorganisms in nasal and pharynx instillations was higher than that in the lower respiratory tract (trachea, bronchia and lung). The number of lactobacilli was significantly lower on the 7th day. After re-inoculation performed on the 10th day, the number of lactobacilli increased in almost all tissues again as shown in Fig. 1. The control group of mice inoculated with saline only showed a very low number of lactobacilli in the upper respiratory tract. There was no isolation of microorganisms resistant to rifampin in LBS-rif, which indicates that the control mice did not harbor lactobacilli resistant to rifampin.

Histological and Cytological Modifications Lactobacillus administration produced cytological and histological modifications in the respiratory tract, which can be summarized as follows.

Histological Changes In the histological preparations of trachea, a steady increase in the number of lymphocytes in the lamina propria and submucosa was observed until day 7. After re-inoculation the number of lymphocytes stayed rather stable. The number of lymphocytes and epithelial cells counted in the histological sections is shown in Fig. 2. Histo-
Fig. 1. *L. fermentum* Colonization of the Respiratory Tract of Mice

Animals were intranasally inoculated with 4 times $5 \times 10^7$ CFU of a suspension of *L. fermentum*. The arrows indicate the 4 doses administered on day 0 and 1, and re-inoculations performed on day 10 and 11. The figures represent the means and standard deviation (SD) obtained from 3 to 4 mice. Control mice were inoculated with Saline Solution. Methods are described in the text.

Fig. 2. Quantification of Lymphocyte Population from Tracheal Lamina Propria of Mice

Animals were intranasally inoculated with 4 times $5 \times 10^7$ CFU of a suspension of *L. fermentum*. The arrows indicate the 4 doses administered on day 0 and 1, and re-inoculations performed on day 10 and 11. The figures represent the means and standard deviation (SD) of the results obtained from 3 to 4 mice. Control mice were inoculated with Saline Solution. The methods are described in the text.
logical sections on the 4th day after inoculation stained with Hematoxylin-Eosin are shown in Fig. 4 (normal mice are shown in Fig. 3). There were no qualitative morphological modifications of the epithelial, cartilage and muscular tissues, except the formation of edema produced as a consequence of the lymphocyte infiltration. No histological modification could be observed in the remaining part of the respiratory tract.

Cytological Changes  a) The lung alveolar macrophages observed on day 4 (Fig. 6) presented an increased number of lysosomes and an active nucleus and nucleolus. They returned to normal state on the 7th day after inoculation.

b) The majority of the lung macrophages emigrated first to the alveolar spaces and then to the bronchioles and to the pharynx by means of ciliary action and were later eliminated. Cell exchange is very fast, and a very high number of macrophages was eliminated within a few hours.\(^{12}\) The different degree of macrophage activation is shown in Figs. 6, 7 and 8, obtained from lung cytological slides, while normal non-activated macrophages are represented in Fig. 5. The proportion of these cells present in the lung cytological slides of mice on different days after *L. fermentum* inoculation is shown in Table 1A. The results demonstrate an increased number of activated macrophages on the 4th day after inoculation, decreasing to normal values on the 7th day. The lymphocytic population had decreased on the 2nd day, their values increasing on the 7th day. After re-inoculation performed on day 10, as observed in Table 1B, G2 macrophages remained constant on days 12, 14 and 17 without marked cytoplasmic changes. On day 17, the presence of macrophages with vacuolated cytoplasm increased (Fig. 8). The lymphocytic population after re-inoculation was similar to that of control animals (Table 1B). Data of control animals are given as a single value, because numbers were stable throughout the experiment (day 0 to day 17). Given numbers are the means of all values collected.

DISCUSSION

In the last years, modern medicine has focused on the use of more preventive rather than curative treatments, together with natural products. Some of these products are "probiotics,"\(^{59}\) a concept with a wide application in the digestive,
urogenital, or respiratory tract, both of human beings and animals. Recently, the old concept of using bacterial preparations with inhibitory activity against enteric pathogens has been rediscovered to protect pigs.\textsuperscript{13} This concept is known as bacterial interference,\textsuperscript{14} and has been applied to different ecosystems: gastrointestinal tract, nasopharynx and urinary tract. Protection can be exerted by several suggested mechanisms such as production of organic acids (lactic acid) and antagonistic metabolites (hydrogen peroxide\textsuperscript{15} and bacteriocins\textsuperscript{16}) that inhibit growth of pathogenic microorganisms through stimulation of the immune system,\textsuperscript{16,17} competition of nutrients\textsuperscript{18} or competitive exclusion.\textsuperscript{19}

Many researchers have reported intranasal administration of live pathogenic microorganisms or microorganism parts to study the physiopathogenesis of the illness\textsuperscript{20} or to study protection as a result of vaccination.\textsuperscript{21} Kawaguchi\textsuperscript{22} reports a study on the presence of microorganisms in the respiratory tract of chickens with special interest in lactobacilli. The idea of using probiotics has not been yet applied to or, at least, there are no reports available on their application in the respiratory tract. Therefore, one of the motivations for our research is to try to set up an experimental mouse model to study the effect of the "indigenous" microflora of the respiratory tract with special interest in the genus Lactobacillus. With this objective, based on the host-specificity phenomenon,\textsuperscript{23} a Lactobacillus strain isolated from the pharynx of adult mice was selected. This strain showed adhesive properties together with production of inhibitory substances,
and experiments with respect to colonization ability in an “in vivo” model were performed. Mutants resistant to rifampin were obtained in order to determine exactly the number of inoculated microorganisms able to survive in the respiratory tract, as well as their colonization ability. The mutant having the same homogeneous phenotypic and “in vitro” properties as the parental strain, was administered at different doses to several groups of mice. The preliminary study reported here shows that the lower doses (1 or 2 doses of $5 \times 10^7$ CFU) did not show colonization of the respiratory tract. The most effective dose was 2 intranasal inoculations of 50 $\mu l$ of *L. fermentum* in each nostril (4 doses altogether). This dose allowed a transient colonization, with lactobacilli present up to the 7th day. As one of the objectives of probiotic administration is the maintenance of a stable microorganism population through time, a set of re-inoculation experiments was performed, based on the fact that administration of 4 *L. fermentum* doses produced only a transient colonization up to 7 days. Re-inoculation with 4 doses on day 10, showed that lactobacilli were able to transiently colonize the tract for 7 more days. This fact is remarkable when studying the colonization ability of microorganisms from the respiratory tract, because, from microbial ecology studies, it is known that colonizing microorganisms are always in contact with the mucosal epithelia during growth. Colonization of the respiratory tract in newborns is stable throughout their whole life and thus occurrence of transitory colonization of the tract by an indigenous strain is remarkable, because of the possibilities that this phenomenon could generate. This is also important because the presence of certain “beneficial” microorganisms in a given tract could be modulated exogenously.

When studying the probiotic and colonization capability of microorganisms, another interesting aspect was the effects produced in the host by local administration. This made us study the respiratory tract of mice in order to know if there was an inflammatory response or cytological and/or histological modifications produced after intranasal *Lactobacillus* administration. The only modifications observed were an increased lymphocyte population in trabecula and a modification of the type and number of macrophages in lung cytological slides. The increased population of lymphocytes in the trabecular lamina propria on the 4th day after inoculation would indicate that lactobacilli are able to produce stimulation of the specific immune response, even though antibodies against lactobacilli or a different antigen were not assayed. This effect was also observed after oral administration of lactobacilli, both in fermented and non-fermented milk.

Experiments to determine what kind of lymphocytes increases their number are being developed. The lymphocyte population gradually decreased until reaching normal values after day 4. Re-inoculation neither produced an increase in the lymphocyte population, nor any other histological modifications. The higher number of activated macrophages on the 4th day after inoculation would indicate a stimulation of cells involved in the non-specific immune response. Activation of macrophages following oral administration of lactobacilli was previously reported in the peritoneal cavity. This type of population also appeared during some other situations reported in the uterus. The same cause can be the answer to the vaculated macrophages G2 seen after *Lactobacillus* re-inoculation. The appearance of macrophages but not of neutrophils indicates that *Lactobacillus* inoculation did not produce an inflammatory response, although an increased number of cells involved in the specific or non-specific immune response was present. Further studies are being developed that will explain the mechanisms involved in intranasal administration of *L. fermentum* and also if there is a specific immune response against lactobacilli. Electron microscopy will reveal modifications produced at a structural level.

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REFERENCES