4. Immunology and Vaccines

4.01 Technological innovation in the process of obtaining vaccine against bacillary hemoglobinuria

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Introduction: Toxoid-composed vaccines have a higher degree of purity, consequently inducing more specific immunological responses and less adverse reactions. Bacillary hemoglobinuria is caused by the toxin produced by *Clostridium haemolyticum*, a gram-positive, strict anaerobic bacterium, which is also spore-forming. Objectives: In this study, two processes of bacterial fermentation were evaluated, static cultivation and cultivation under stirring conditions (vibromixer and bubbling with N_2), for a highly pathogenic *C. haemolyticum* sample (ATCC no. 9650). **Methods:** The bacterial suspension titers (minimum lethal dose) showed no significant difference; however, the toxin produced by the fermentation process under stirring was 20 times greater than the one produced in static fermentation. A batch of toxoid was prepared from the toxin produced by fermentation under stirring conditions. Eight guinea pigs were vaccinated with 1/5 the bovine dose (1.0 ml) on days 0 and 21, and five guinea pigs were inoculated with the same volume of saline solution. After 14 days after the second dose, all animals were challenged by inoculation of *C. haemolyticum* spore suspensions with 100 LD50. **Results and Discussion:** The group of animals vaccinated with toxoid showed 100% protection to the challenge (8/8), while the control group showed 100% lethality, given the requirements of test. According to the results, the efficiency of the toxoid produced under stirring conditions was proven, inducing an immunological response above of the minimum required for vaccine approval, according to the Federal Code Regulations (9CFR113.107). The requirements for a veterinary vaccine are different from those for a human vaccine, which needs greater refinement. This study describes a differentiated methodology applied in the process of veterinary vaccine production, demonstrating that this toxoid reduces the need for downstream processes, which are responsible for 50-70% of the cost of the final product.

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4.02 Detection of antibodies (IgG) against hantavirus in human population of Amazon region and Brazilian southwest (rain florest), using recombinant antigen of Araraquara virus

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Introduction: The genus *Hantavirus* of the family *Bunyaviridae* includes a large number of rodent-borne viruses that are distributed worldwide. The occurrence is due mainly to ecological disturbances and it is transmitted to the humans through inhalation of virus particles contained in the excreta of wild rodents. Two different human diseases known to be caused by *Hantavirus* are hemorrhagic fever with renal syndrome (HFRS) and hantavirus cardiopulmonary syndrome (HCPS). The diagnosis of the infection is accomplished mainly by serology (detection of antibodies) against the *Sin nombre* and/or *Andes* virus. These viruses are found mainly in the US and in Latin America (except for Brazil). The nucleoprotein is the major antigenic protein of the hantavirus. The nucleoprotein is the most appropriate target for use in the diagnosis of hantavirus infection. For accurate diagnosis, early during the course of the disease, it is essential to show an ELISA reaction for Brazilian Hantavirus. **Objectives:** The main objective of this study was to use and to evaluate the recombinant protein (antigen) of the *Araraquara* virus expressed in *Escherichia coli* (kindly donated by Professor Luiz Tadeu Moraes Figueiredo - USP, and Professor Marcos Lázaro Moreli -UESC), in populations of Amazonia and rural workers of the state of Sao Paulo, who live in contact with wild rodents. **Methods:** ELISA, imunoblotting, focus reduction neutralization test (FRNT), and strip immunoblot assay (SIA). **Results and Discussion:** Serum samples from 1308 individuals were analyzed by ELISA for antibodies against *Hantavirus* (IgG), and they were later confirmed by Western blotting. The cross reaction between the *Araraquara* virus and other *Hantavirus* and *Arbovirus* also needs to be carefully studied. Of the incoming sera from the Amazonian area, in the years of 2003 and 2005, 59 (5%) positive sera were found. From the city of Machadinho do Oeste - RO, 633 sera were analyzed, where 20 were found to be positive (4.5%). On the Machado River (RO), 435 sera of the river-dwelling population were analyzed, where 39 (5%) positive sera were found. After the analysis was accomplished for 151 human sera coming from the Vale do Ribeira - SP, in 2007, and 84 from the Pontal do Paranapanema - SP, in 2008, 14 (9%) and 6 (7%) of the samples were observed to be positive, respectively. A possible explanation is that the incidence found is approximately 8% for antibodies of the IgG class for *Hantavirus* in the sub-tropical (Atlantic forest of the state of São Paulo) area and approximately 5% in the tropical area (western Amazônia in the state of Rondônia), an endemic among the rural workers that live together with wild rodents.

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4.03 Standardization of an in vitro test for determination of beta toxin titer of *Clostridium haemolyticum*

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Introduction: Clostridia are gram positive, spore-forming bacilli and anaerobic. *C. haemolyticum* causes bacillary hemoglobinuria in cattle and occasionally sheep. In bacillary hemoglobinuria, beta toxin destroys circulating red cells, resulting in the excretion of hemoglobin in the urine; simultaneously, there is the presence of blood in the intestine due to destruction of the endothelium capillary. **Objectives:** The aim of this study was the standardization of an *in vitro* test for determination of the beta toxin titer of *Clostridium haemolyticum* as an alternative to *in vivo* method for process control in the production of clostridial vaccines for veterinary use **Methods:** The strain of *Clostridium haemolyticum* ATCC 9650 was grown statically in medium recommended by WHO. The beta toxin was concentrated and purified by molecular ultrafiltration. Its toxicity was observed in Swiss mice weighing 18 to 22 g; the mice were inoculated with 0.5 mL, intraperitoneally. Cytotoxicity assays were performed according to ISO 10993-5. **Results and Discussion:** The beta toxin was highly toxic in vivo causing 100% mortality in calves in less than 24 h, confirming the in vitro tests where the L929 cells treated with beta toxin and quantified by the method of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction showed no viability in 24 h until a dilution of 1:64. The morphological changes in cells treated with beta toxin can be visually verified by optical microscopy when compared with control L929 cells.

4.04 Two loci interact with Slc11a1 gene to regulate the sensitivity to LPS-induced endotoxic shock

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Introduction: AIRmax (high inflammation) and AIRmin mice (low inflammation) differ in their resistance to LPS-induced endotoxic shock. *Slc11a1 R* and *S* alleles are involved in this phenotype. To study the *Slc11a1* gene interaction with acute inflammatory reaction loci (AIR-QTL), AIRmax*RR*, AIRmax*SS*, AIRmin*RR* and AIRmin*SS* sublines were produced. AIRmax*RR* mice are extremely susceptible to LPS-induced shock, while AIRmin*SS* are the most resistant. **Objectives:** The objective of this work was to identify loci that interact with *Slc11a1* alleles to modulate LPS shock. **Methods:** Mice were injected i.p. with 20 ug LPS and mRNA from bone marrow (BM), and liver cells were isolated. Global gene expression analysis was performed on Codelink bioarrays (36 k- genes) using RNA pools (n=4) of LPS-treated or control BM cells from AIRmax*RR*, AIRmax*SS*, AIRmin*RR* and AIRmin*SS* mice. Serum levels of inflammatory cytokines were determined by ELISA. In parallel, genome wide association (GWA) studies with SNPs (Illumina bead arrays) were performed to demonstrate LPS-resistance QTL in the F2 (AIRmax x AIRmin) population. **Results and Discussion:** The highest number of differentially expressed genes ($P<0.001$) after LPS injection was found in AIRmin*SS* mice. AIRmax*RR* had higher serum levels (2- to 5-fold) of inflammatory cytokines and higher expression of *Tnf*, *Il6* and *IL1b* genes in liver and BM cells. *Il10* expression was higher in AIRmin*RR* mice (2-fold) than in the other lines. GWA analysis revealed two significant QTL on chromosomes 4 and 11 (LOD=3.6) for resistance to LPS shock. These results suggest that *Slc11a1* alleles may interact with these loci to modulate the activation of inflammatory genes during LPS shock.

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4.05 Effect of temperature on the immunoreactivity of murine monoclonal antibodies against Shiga toxin (Stx1) from *Escherichia coli*

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Introduction: Shiga toxin-producing *Escherichia coli* (STEC) has been identified as an important food- and water-borne pathogen worldwide, causing from uncomplicated diarrhea up to hemolytic uremic syndrome. For STEC the production of enterotoxins (Shiga toxin 1 and/or 2) is considered the principal virulence factor and responsible for the serious complications. The Bacteriology Laboratory of the Butantan Institute has been dedicated to developing polyclonal and monoclonal antibodies (MAbs) against these toxins aiming to detect their expression in bacterial isolates. Murine MAbs against Shiga toxin 1(Stx1) have been developed and well characterized by L.B. Rocha. To expand the application of these MAbs antibodies as reagents in the detection of this toxin, these antibodies must be stable. **Objectives:** The aim of this study was to purify murine MAbs against Stx1 and to investigate the effects of temperature on the immunoreactivity of these MAbs. **Methods:** The hybridoma was cultured in RPMI supplemented with 10% fetal bovine serum. The supernatants (1.5 liter) were collected and filtered (0.45μm), and MAbs were purified using a Protein-A column. The MAbs were eluted with 0.1 M glycine-HCl, pH 3.0 and the pH was neutralized with 1 M Trisma base. Afterward, it was dialyzed against PBS, pH 7.4, overnight. The Pierce BCA assay kit was used for protein determinations and bovine serum albumin as the standard. The immunoreactivity of the MAbs was measured by ELISA. Briefly, 96-well ELISA plates were coated for 2 h at 37° C with Stx1 purified toxin at 1 μg/mL in PBS, pH 7.4. After blocking, the plates were incubated with MAbs dilutions. After incubation with HRP-goat anti-mouse IgG, the reaction was revealed by the addition of O-phenylenediamine (OPD) plus hydrogen peroxide. The reaction was stopped with 1M HCl. The absorbance was measured at 492 nm in a Multiskan EX ELISA reader. All samples were tested in duplicate. A heat inactivation curve was determined by the incubation of purified MAbs at 25, 37, 50, 60, 70, 80, 90 or 100° C for 10 min. Following the heat treatment, the MAbs were tested for immunoreactivity by ELISA. A time course for the loss of reactivity was determined by incubating the MAbs for 1, 2, 4, 5, 6, 8 or 10 min. **Results and Discussion:** A total of 4.6 mg of purified murine MAbs against Stx1 were obtained. Retention of immunoreactivity was observed up to 50° C. Heating at 80° C caused a total loss after one minute, and partial loss of immunoreactivity was observed between 60° C and 70° C. The evaluation of the stability of the murine MAbs available against Stx1 will contribute to the assurance of the methodology employed.

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4.06 Immunomodulatory effect of high-molecular weight components from *Ascaris suum* extract in TLR2- and TLR4-deficient mice

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Introduction: High MW components (PI) from *Ascaris suum* extract have immunosuppressive effects and down-modulate the ability of antigen-presenting cells (APCs) to activate OVA-specific T cells. APCs, as dendritic cells (DCs) when recognizing pathogens through distinct receptors, such as the toll like receptors (TLRs), acquire the capacity to induce a specific cellular response. **Objectives:** The aim of this study was to determine the involvement of TLR2 or 4 in the suppressive effect of PI on anti-OVA humoral and cellular responses. Furthermore, the role of these receptors in the ability of PI to down-modulate DC maturation induced by agonists of TLRs was investigated. **Methods:** For this, WT, TLR2$^{-/-}$ or TLR4$^{-/-}$ C57BL/6 mice were immunized with OVA (200 μg/animal) or OVA+PI (200 μg/each antigen/animal) in CFA. After 8 days, the mice were challenged with aggregated OVA in the footpad, and the DTH reaction measured after 24 h. All groups were also bled and anti-OVA IgG1 and IgG2a production evaluated by ELISA. The effect of PI on the expression of the molecules involved in the antigenic presentation was evaluated in cells obtained from these groups of mice immunized with OVA or OVA+PI 5 days before and stained with anti-MHC-II, anti-CD80 and CD86 mAbs labeled with FITC or PE by flow cytometry. In another experiment, immature DCs derived from WT, TLR2$^{-/-}$ or TLR4$^{-/-}$ mouse bone marrow were induced to differentiate in RPMI medium plus GM-CSF/IL-4. On day 7, these cells were incubated *in vitro* with LPS (1 μg/mL), PI (200 μg/mL), LPS+PI (1 μg+200 μg/mL), pam3 (3 μg/mL) or pam3+PI (3 μg+200 μg/mL), Poly I:C (10 μg/mL) or Poly I:C+PI (10 μg+200 μg/mL) for 18 h. Afterward, the supernatants were collected for cytokine detection by ELISA. **Results and Discussion:** PI was able to inhibit both anti-OVA DTH reaction and antibody production as well as in WT, TLR2$^{-/-}$ or TLR4$^{-/-}$ OVA+PI-immunized mice when compared with OVA-immunized groups. Lower expression of CD80, CD86 and MHC-II molecules was also seen in cells obtained from these groups of OVA+PI-immunized mice when compared with those observed in cells from OVA-immunized groups. High production of IL-12, IL-6 and IL-1 was obtained in cultures of DCs from WT, TLR4$^{-/-}$ or TLR2$^{-/-}$ mice stimulated with different TLR agonists. In contrast, PI down-modulated the secretion of these cytokines when added to the DC cultures stimulated with the TLR agonists. The results indicate that TLR2 and 4 are not involved in the suppressive effect of PI on DC activity.

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4.07 Population dynamics of myeloid-derived suppressor cells during the course of tumorigenesis in Airmax and Airmin mice treated with urethane

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Introduction: Predisposition to neoplastic process is due to both genetic and environmental factors that determine the resistance or susceptibility phenotypes. The suppressor activity of myeloid-derived suppressor cells (MDSC) during tumorigenesis is an important factor that determines the susceptibility phenotypes. These cells are produced in the bone marrow compartment and migrate to the tumorigenesis site or to the secondary organs, interfering with the antitumoral activity of the T cells. In mice, these cells can be identified by co-expression of the surface molecules that determine the $CD11b^{+}/Gr1^{+}$ phenotype. Resistance to tumorigenesis in mice genetically selected for high (AIRmax) or low (AIRmin) acute inflammatory reaction is influenced by regulatory genes, which determine respectively the resistance or susceptibility to urethane action. Moreover, these lines of mice show important differences concerning the bone marrow activity related to myeloid cell maturation. **Objectives:** The aim was to study in AIRmax and AIRmin mice the dynamics of MDSCs after lung tumorigenesis induced by urethane by analyzing myeloid and B and T cell populations in the lung, spleen and bone marrow tissues. **Methods:** Mice were treated by two i.p. injections of urethane (1000 mg/kg body weight) at a 48-h interval for tumorigenesis induction and observed for 200 days after treatment. The number of tumor lesions was determined by macroscopic observation and the cellularity was evaluated by both specific antibodies to GR1/CD11b (myeloid cells), CD4/CD8 (T cells) and B220 (B cells) molecules and morphology analysis. **Results and Discussion:** At 10 days of urethane treatment, we observed a significant increase in cell number in the lung parenchyma, especially in AIRmax, indicating an inflammatory process, followed by a decline cell number. Forty days after urethane injection, we observed in AIRmin mice, a second cell increase in the $CD11b^{+}/Gr1^{+}$ population consisting of macrophages and mature and immature neutrophils, which were revealed by different molecular expression. At the same time, the CD4 and CD8 T cell populations showed a significant decrease in the lung and spleen tissues. This cellular alteration is related to the high tumor multiplicity in AIRmin mice, indicating a possible modulation by MDSC on T cell action. These preliminary results indicate that MDSC playd a role in the susceptibility of the AIRmin line during tumorigenesis provoked by urethane.

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4.08 Biologic effects of *Propionibacterium acnes* on mouse peritoneal B1b lymphocytes

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Introduction: B1 lymphocytes differ from conventional B cells on the basis of their main localization on peritoneal and pleural cavities, surface phenotype and functional features, such as auto-renewing, natural IgM and auto-antibodies production and their role in immune response regulation by IL-10 synthesis. In the mouse peritoneal cavity, B1 lymphocytes are divided into B1a ($CD11b^+$ $CD5^+$), B1b ($CD11b^+$ $CD5^-$) and B1c ($CD11b^-$ $CD5^+$), but B1c are a differentiation stage of B1a and B1b. *In vitro*, B1b lymphocytes can be obtained from adherent cells of mouse peritoneal exudates, and, when re-cultured for 5 to 10 days, they differentiate into macrophage-like phagocytes. However, B1 response to antigens or adjuvants is still poorly understood. An important biological adjuvant is *Propionibacterium acnes* (*P. acnes*), whose effects, as killed bacterial suspension, include activation of macrophage phagocytic and tumoricidal activities, adjuvant effect on antibody response, resistance to infections and induction of pro-inflammatory cytokine synthesis. A bacterial compound of great importance is its soluble polysaccharide (PS), with effects similar to those of the whole bacterium. **Objectives:** Herein, we studied *P. acnes* and PS effects on peritoneal B1b cell functions, differentiated or not into phagocytes. **Methods:** Mice were treated with one intraperitoneal injection of saline (control), heat-killed *P. acnes* or PS. After 24 h, peritoneal exudate cells were analyzed by flow cytometry or cultured for 2 or 5 days to obtain a B1b lymphocyte-enriched population, and these were re-cultured for 24 h for phagocyte differentiation. Proliferation and cytokine release were evaluated in B1b cells and phagocytes, as well as phagocytic activity in phagocytes. **Results and Discussion:** We observed higher numbers of B1b and macrophages in the peritoneal cavity of *P. acnes*-treated mice, compared to the control group, as well as an *in vitro* increase in the percentage of phagocytic cells. We also found that B1b cells proliferated *in vitro*, on days 2 and 5. When re-cultured on 2nd day, *P. acnes* cells proliferated less than control, but, when re-cultured on the 5th day, the proliferation was higher in bacteria-treated group. Besides, there was a decrease in IL-10 levels in *P. acnes* and PS phagocyte supernatants, compared to saline, and increase in IL-12 in the *P. acnes* group. These data indicate that *P. acnes* can stimulate early differentiation of B1b cells into phagocytes, in just 24 h of re-cultivation, as shown by increased phagocytic cell percentage and IL-12 release. The polysaccharide compound does not seem to be responsible for this effect.

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4.09 Investigation of human CD59 orthologs in the *Schistosoma mansoni* genome

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Introduction: *S. mansoni* is the predominant parasite responsible for schistosomiasis, which affects 200 million individuals in several countries. The treatment is based on praziquantel, but chemotherapy does not prevent re-infection, emphasizing the need for a more effective approach. With the outcomes of the *S. mansoni* genome, it was possible to investigate *in silico* new vaccine candidates as human CD59 orthologs. CD59 is an important inhibitor of the membrane attack complex (MAC) by the complement system. **Objectives:** The aim was the investigation of human CD59 orthologs in the *S. mansoni* genome and characterization of one member of this family (Ly-6.1 gene). **Methods:** The alignment and the phylogenetic analysis of family members were performed. The mRNA expression levels of the genes across the life cycle stages were evaluated by real-time RT-PCR. The Ly-6.1 gene was cloned into the vector and transformed into *Escherichia coli*. The protein was purified by affinity chromatography, and polyclonal antibodies were obtained. Western blot and immunolocalization assays were performed to characterize the protein. Molecular shaving assays were carried out by incubating adult worms with the PiPLC enzyme that cleaves membrane-binding proteins by GPI anchor. **Results and Discussion:** In *S. mansoni,* the Ly-6 family is composed of six genes (Ly-6.1, Ly-6.2, Ly-6.3, Ly-6.4, Ly-6.5, Ly-6.6) with 25-30% identity to human CD59. They contain Upar/Ly-6 domains, signal peptides, transmembrane domains and GPI anchors. Most of them show increased gene expression in schistosomulum stage by real-time RT-PCR, except the Ly-6.3 gene, which shows increased expression in eggs and Ly-6.4 increased expression in adult worms. These results were corroborated by microarray analysis. One of the genes, Ly-6.1, was cloned and Western blot analysis revealed high expression levels of the Ly-6.1 protein in schistosomula, some in cercariae and adult worms and none in eggs and miracidia. The protein was immunolocalized to the tegument of 3-h, and 7- and 21day-old schistosomula by confocal microscopy. Localization of the Ly-6.5 protein was also determined on surface of adult worms. The characteristic of these proteins to be membrane binding by GPI-anchor was confirmed for Ly-6.1 and Ly-6.5 by the molecular shaving technique. In conclusion, most of the genes of the Ly-6 family are up-regulated in the schistosomulum stage, and the protein is associated with the tegument and possesses similarity with human CD59, which inhibits the complement system. These results indicate that the family should be investigated as potential vaccine candidates. Immunization and challenge assays, the characterization of other members of the family, and functional assays are underway.

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4.10 Selection of a family 1 PspA capable of inducing broad-ranging cross-reactivity by complement deposition and opsonophagocytosis by murine peritoneal cells

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Introduction: *S. pneumoniae* is a major cause of pneumonia, meningitis and sepsis. Among the vaccine candidates against this pathogen is pneumococcal surface protein A, an exposed and protective protein. Due to its structural diversity, an effective PspA-based vaccine should include at least one fragment from each of the two major families (1 and 2). Also, it has been shown that PspAs from different clades show variable degrees of cross-reactivity. In the present work, we investigated the level of cross-reaction among different PspA molecules within family 1, in order to determine the best candidate to be included in a PspA-based vaccine. Since PspA inhibits complement deposition onto pneumococci, therefore avoiding phagocytic clearance by the immune system, we evaluated the ability of the antibodies produced to abrogate PspA's function, enhancing complement deposition onto pneumococci and promoting opsonophagocytosis. **Objectives:** The aim of this study was to determine, from a panel of Brazilian pneumococcal isolates, which is able to induce the higher level of cross-reactivity within family 1. **Methods:** We have produced recombinant PspA fragments from 10 family 1 pneumococci (5 of each clade), containing the whole N-terminal half of the protein. These fragments were used to immunize BALB/c mice, and the sera were tested for their ability to recognize diverse pneumococcal strains bearing PspAs of clades 1 and 2 by Western blotting. The most cross-reactive antibodies were tested for their ability to enhance complement deposition on pneumococci. Also, the two antisera selected were tested for their ability to promote the opsonophagocytosis of different pneumococcal strains by peritoneal cells. **Results and Discussion:** The analysis of serum cross-reactivity among PspA fragments from clades 1 and 2 revealed a significant variation in the level of recognition. Four sera able to recognize bacteria from both clades were tested for their ability to increase complement deposition on the pneumococcal surface. Of these, two led to an increase in complement deposition on strains bearing PspAs from both clades, in FACS analysis. The opsnophagocytic assay indicated that both anti-PspA antisera tested were able to promote opsonophagocytosis, leading to a minimum reduction of 30% in the number of pneumococci recovered, suggesting a possible protective effect. We therefore suggest that the inclusion of either one of the two PspA fragments in a PspA-based anti-pneumococcal vaccine could induce broad protection against family 1 strains.

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4.11 Evaluation of DNA vaccines expressing N-terminal fragments of pneumococcal surface protein from clade 4 (PspA4) against an intranasal lethal challenge model in mice

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Introduction: *Streptococcus pneumoniae* causes serious diseases such as meningitis and pneumonia, besides common infections of the respiratory tract. The vaccine composed of different capsular polysaccharides (PS) purified from pneumococci has low efficacy in children and the elderly, besides not being able to induce immunological memory. Although the development of vaccines composed of PS conjugated to carrier proteins was an advance, their production cost is still a major barrier for their use by public health systems. A proposal to increase vaccine coverage at a low cost consists in the identification of an antigen common to the majority of strains. Several proteins have been investigated as alternatives for a cost-effective vaccine against *Streptococcus pneumoniae*. PspA (pneumococcal surface protein A) is one of the most promising candidate antigens. Our group has recently shown that PspA from clade 4 (PspA4) induces antibodies with broad cross-reactivity with pneumococcal isolates. **Objectives:** The proposal of the present work was to evaluate two different fragments of PspA4 - PspA4Pro encompasses the complete N-terminal alpha-helical region plus the proline-rich region and PspA4A has only the first half of the alpha-helical region - in an intranasal lethal challenge model as recombinant protein adjuvanted with alum or as DNA vaccine. **Methods:** BALB/c mice were immunized subcutaneously with the recombinant proteins using alum as adjuvant or intramuscularly with the DNA vaccines. Immunizations were performed in 3 doses with intervals of two weeks. The animals were bled after the last immunization for the analysis of serum antibodies by ELISA. The capacity of the antibodies to bind to intact pneumococci and to mediate deposition of C3 was evaluated by FACS using anti-mouse IgG antibodies or anti-mouse C3 antibodies conjugated with FITC, respectively. Mice were then challenged intranasally for the analysis of survival. **Results and Discussion:** High antibody concentrations were elicited by both PspA4A and PspA4Pro protein and DNA immunizations. Antiserum to PspA4A showed reduced capacity to bind and to mediate C3 deposition onto intact pneumococci as analyzed by FACS. Furthermore, binding of antibodies to PspA4Pro was not blocked by the addition of recombinant PspA4A. Only animals injected with PspA4Pro, either as recombinant protein or DNA vaccine, showed significant higher survival. Our results indicate that the response elicited against the complete alpha-helical region in PspA4Pro is essential for the immune response and for protection against pneumococcal infection.

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4.12 Peritoneal inoculation of pristane induces distinct leukocyte infiltration kinetics in experimental arthritis resistant (HIII) and susceptible (LIII) mice

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Introduction: Mice selected for low (LIII) or high (HIII) antibody production against *Salmonella* flagellar antigens are respectively susceptible and resistant to pristane-induced arthritis (PIA). In the first 2 weeks after pristane injection, more splenic cells from LIII mice produced IL-1β, TNF-α and IL-12, while more cells from HIII mice produced IL-4. IL-6, considered as an important cytokine in PIA pathogenesis, which is produced in similar high amounts by peritoneal cells of both lines 72 h post-pristane injection. These results suggest that the differences between HIII and LIII mice are expressed in the early phase of PIA induction, influencing the late-phase of arthritis development. However, little is known about the cellular and molecular events that occur in the peritoneal cavity during this phase. **Objectives:** The aim of this study was to evaluate the kinetics of the cell populations in the peritoneal cavity after pristane injection in HIII and LIII mice. **Methods:** HIII and LIII mice were i.p. injected with 0.5 mL pristane and evaluated after distinct time points (48 h; 4, 7, 17 and 32 days). Peritoneal cells were harvested and counted in Malassez hemocytometer chambers. Giemsa staining of cytospin cell preparations was used for morphological analysis and differential counts. **Results and Discussion:** Total lymphocyte numbers did not show significant alterations between lines or treatments. Low numbers of mast cells were detected in control HIII and LIII mice, but these cells were absent in the peritoneal exudates of the respective pristane-treated animals. Eosinophil numbers were slightly increased in both lines up to day 7. On the other hand, the kinetics of monocytes/macrophages and neutrophils was dramatically different in LIII as compared to HIII mice. The number of inflammatory infiltrate cells was highest in LIII mice at day 7, while HIII mice showed a modest increase in neutrophils and decreased numbers of macrophages. At day 32, inflammatory cell numbers had decreased in LIII mice, while in HIII neutrophils increased and macrophages returned to control levels. This differential pristane-induced inflammation kinetics may be related to the divergent cytokine profile observed in the spleen, although more data on the molecules expressed/secreted by these and other cell populations are needed to support this hypothesis.

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4.13 Adjuvant activity of *Salmonella* FliCi flagellin in the development of a subunit vaccine against leptospirosis

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Introduction: Leptospirosis is a global zoonotic disease caused by pathogenic leptospires that colonize the renal tubules of wild and domestic animals. Commercially available leptospiral vaccines, consisting of killed whole cells, suffer from several limitations such as short-term immunity and adverse reactions. The development of a subunit vaccine could be a promising strategy against this disease. Various outer membrane proteins (OMPs) have been evaluated as potential vaccine candidates; one of these, the *Leptospira* immunoglobulin-like protein A (LigA), was able to induce immunoprotection against leptospirosis. However, immunization with LigA did not confer sterilizing immunity. Adjuvants are necessary to increase the immunogenicity and efficacy of purified antigens. Flagellin, a highly conserved bacterial protein that elicits TLR5-dependent responses, has been successfully used as a vaccine adjuvant. **Objectives:** In the present study, we evaluated the adjuvant activity of *Salmonella* FliCi flagellin in the protective immunity of LigA and of six other novel recombinant leptospiral OMPs against lethal challenge with *L. interrogans* in hamsters. **Methods:** The recombinant 6xHis-tagged proteins expressed in *E.coli* were purified by nickel affinity chromatography. Native *S.Typhimurium* FliCi was purified from the attenuated SL3201 strain. Hamsters were immunized subcutaneously with a cocktail of six purified recombinant OMPs with or without LigA as well as in combination with FliCi or alum. All animals were bled to evaluate the antibody response against each antigen and the expression levels of Th1/Th2 cytokine mRNA in the peripheral blood mononuclear cells. **Results and Discussion:** Immunization of hamsters with LigA or LigA coadministered with OMPs cocktail, either with FliCi or alum, induced robust antibody responses against recombinant proteins, as detected by ELISA and immunoblot, and conferred imunoprotection after challenge (80-100%). Animals inoculated with OMPs cocktail with alum or FliCi survived (30-70%) after challenge. Control animals vaccinated with PBS with alum or FliCi died with symptoms of leptospirosis, and hamsters vaccinated with commercial vaccine survived (100%) after challenge. Moreover, only groups inoculated with commercial vaccine or LigA coadministered with OMPs cocktail and FliCi as adjuvant showed reduced bacterial load in kidneys (> 90% negative culture) with significant enhancement of both Th1 and Th2 cytokine levels. Taken together, the data of this study suggest a new formulation for the development of a subunit vaccine for leptospirosis.

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4.14 Treatment of experimental melanoma with imiquimod

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Introduction: Aldara® (imiquimod 5%, 1-(2-methylpropyl)-1H-imidazo[4,5-c]quinolin-4-amine) is an immunomodulator mostly used for the treatment of genital warts and actinic keratosis. It is an agonist of the toll-like receptor 7 and induces dentritic cell migration and maturation, thus upregulating some of the cytokines that mediate Th1 immunity. **Objectives:** The aim of this study was to evaluate tumor growth and metastasis formation and to describe the immune system activation following topical application of 5% imiquimod in an experimental murine melanoma model. **Methods:** The tumor was induced by injecting $5x10^4$ B16F10 melanoma cells subcutaneously in two months old males C57BL/6J mice. The experimental group was treated with 5% imiquimod applied topically once a day at the inoculation site, from day one until death. Weight and tumor volume (using a caliper) were evaluated every two days and the tumor volume was defined by the formula: (length) x $(width)^2$ x (π/6). In order to describe the development of the treatment and establishment of the tumor growth, 4 treated and 2 control animals were euthanized on days 11, 18, 26 and 33 and submitted to necropsy for macroscopic evaluation; samples of normal tissues and tumors were fixed in 10% formalin and embedded in paraffin for further histological (HE) and immunohistochemistry analysis (S-100 and HMB-45). Part of the tumors was separated for flow cytometry analysis to qualify and quantify cell death by apoptosis and/or necrosis using FITC-labeled caspase-3 and annexin V. **Results and Discussion:** We observed a significant reduction ($p<0.001$) in tumor volume in treated animals, as well as an increase in the spleen and lymph node sizes, suggesting a systemic activation of the immune system. There was some neovascularization observed upon necropsy, which was proportional to the size of the tumor, with evident fibrinous exudates all around. Flow cytometry revealed that the treated animals had less necrotic cells when compared to the control group ($p<0.001$). The treated animals also showed a higher percentage of apoptotic cells on days 11 and 18 ($p<0.01$) and of cells in late apoptosis on days 26 and 33 ($p<0.001$). The presence of dendritic cells in the lymph nodes was evident using S-100 antibody, revealing an increased number of dendritic cells in the treated animals in comparison to control group. Metastases were visualized by HMB-45 antibody, revealing an increased number of melanocytes in the lymph nodes and some internal organs of the control group, in a time-dependent fashion. Imiquimod 5% in this therapeutic protocol delayed melanoma development, probably through the stimulation of Th1-mediated immunity by inducing tumor necrosis and/or apoptosis.

4.15 Influence of rHsp65 passive administration on survival, antibody production and histopathological changes in aged H responder mice

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Introduction: The Hsp60 are phylogeneticaly conserved molecules involved in protein folding, refolding and translocation across cell membranes, having a major role in cell growth and differentiation and being associated with chronic-degenerative processes. **Objectives:** The present study evaluated the actions of *M. leprae* rHsp65 wild type [WT] and its mutant $K^{409}A$ in the aging process and immunoresponse of mice. **Methods:** Measures of mean survival time [MST], titration of IgG1 and IgG2a isotypes, avidity tests of anti-Hsp65 IgG antibodies and histopathological analyses were performed in mice genetically selected for High [H_{III}] and Low [L_{III}] antibody production, inoculated intraperitoneally at 120 [adult] or 270 [aged] days of life with 2.5 μg/mL (200 mL of PBS) of recombinant proteins **Results and Discussion:** There was a decrease of 42% of the MST in aged H_{III} females inoculated with the WT protein ($p \leq 0.01$) compared to control and mutant groups. Adult H_{III} females receiving the WT molecule showed also a reduction of MST ($p \leq 0.05$) in relation to control and $K^{409}A$-treated group, but the interval between the treatment and first death was fourteen times higher (247 days) in relation to the aged ones (18 days). WT molecule administration in aged L_{III} females resulted in increased ($p \leq 0.01$) MST compared to the control and $K^{409}A$ groups. Aged male mice from both lines showed no difference in their mean survival time. There were no marked changes in the production of IgG1 and IgG2a anti-Hsp in all groups, but the IgG avidity was lower ($p \leq 0.05$) in old H_{III} female treated with wild type protein. Despite the occurrence of hepatitis in all groups, perhaps a normal process in the senescence of these strains, the histopathological analyses showed a worse phenotype of nephritis and nephrosis, with the presence of inflammatory influx and intraluminal eosinophilic material in the kidneys of aged H_{III} females treated with WT protein. The results presented revealed the interference of WT rHsp65 in the immunity of aged females from the H_{III} line during senescence and are discussed and related to those obtained previously on the effect of rHsp65 in autoimmune processes.

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4.16 Comparison of detoxification process of tetanus toxin by addition of glycine or lysine

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Introduction: Tetanus toxin is produced at Butantan Institute by fermentation of *Clostridium tetani* that releases the toxin during the process into the culture medium. The toxin is recovered by tangential flow filtration, concentrated by molecular ultrafiltration (30KDa) and converted into tetanus anatoxin by the addition of formaldehyde, glycine and sodium bicarbonate and incubation at 37ºC for 30 days. Tetanus anatoxin is purified by chromatography and after approval in the quality control test is designated Tetanus anatoxin final bulk. This product is one component of the associated vaccines (dT, DT, DTP and DTP-Hib) and is used as antigen to produce anti-tetanus serum for human. **Objectives:** The goal of this study was to evaluate lysine instead glycine in the detoxification process of tetanus toxins, analyzing efficiency and reprodutibility. **Methods:** Nine consecutive batches of tetanus toxin were detoxified with formaldehyde and lysine, and another nine with formaldehyde and glycine. After the detoxification process, each of three batches of tetanus anatoxin detoxified using lysine were mixed and purified by gel filtration chromatography. The same procedure was performed with tetanus anatoxin using glycine. Before purification, a sample of tetanus anatoxin was submitted to flocculation limit test, protein nitrogen quantity and antigenic purity test. After purification, the following process controls were analyzed: flocculation limit test, protein nitrogen quantity, antigenic purity test, potency, electrophoretic profile (SDS-PAGE) and chromatographic profile by gel filtration chromatography with Superdex 200® (GE Healthcare) resin. **Results and Discussion:** The flocculation limit average of tetanus anatoxin using glycine in the detoxification process was 783.33 Lf/mL and with lysine 700 Lf/mL. In relation to the tetanus anatoxin final bulk, the average of flocculation limit was 2666 Lf/mL and 2133.33 Lf/mL using glycine and lysine, respectively. All results obtained in the antigenic purity were above 1000 Lf/mgPN, and potency was higher than 2 IU/mL, in accordance with national and WHO requirements. The electrophoretic profile showed one band of approximately 75 KDa to 250 KDa in samples using lysine in the detoxification process and one band of approximately 65 KDa to 250 KDa using glycine. It was observed that the chromatographic profile was very similar in all samples, demonstrating the reproducibility of the process. The detoxification process with lysine and glycine showed the same efficiency, and therefore, lysine can be used as an alternative in the production.

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4.17 Production of monoclonal antibody to protein L2 of bovine papillomavirus type 2

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Introduction: Bovine papillomatosis is an infectious disease of worldwide occurrence, without any effective control. Several attempts have been undertaken with prophylactic or therapeutic purposes, but without effective results. Our group is involved in a project of national relevance (Renorbio) whose purpose is to develop vaccine products aimed at bovine papillomavirus (BPV). This study is part of this initiative to establish a comprehensive quantitative and descriptive framework on the prevalence of BPV in the national herd. The production and characterization of monoclonal antibodies against L2 protein of BPV-2 is an essential tool for the development of diagnostic methods and vaccines against this virus. **Objectives:** The aim of this study was to produce monoclonal antibodies against recombinant L2 protein of BPV-2 using the technique of cell fusion and hybridoma production, in order to allow its application in the development of diagnostic methods and evaluation of the effectiveness of prophylactic and therapeutic vaccine tests. **Methods:** As antigen, we used the recombinant protein of BPV-2 L2 cloned into expression vector pGEX, transformed and expressed in BL21 *E. coli*. Affinity chromatography column was selected for purification with a yield of about 530 ng/ul. Immunization was performed with L2-2 suspensions injected intraperitoneally into Balb/C mice (4 weeks of age), in a protocol repeated in the 7th, 10th and 13th weeks of life. **Results and Discussion:** The immunization protocol did not induce unexpected reactions and may be continued in the production of monoclonal antibody.

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4.18 Recombinant SmNPP-5 induces antibodies that partially inhibit surface enzymatic activity but fail to protect against challenge with *Schistosoma mansoni*

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Introduction: Schistosomiasis affects 200 million individuals in several countries, including Brazil; its treatment is based on praziquantel, but chemotherapy does not prevent re-infection, emphasizing the need for a more effective approach. Recent proteomic characterization of the *S. mansoni* tegument, the major parasite-host interface, identified a putative nucleotide pyrophosphatase/phosphosdiesterase (SmNPP) 5, as a plasma membrane-associated protein. NPPs are ubiquitous membrane-associated or secreted ecto-enzymes that act by regulating the metabolism of extracellular nucleotides, consequently having a role in purinergic signaling, which affects diverse biological processes such as platelet aggregation, apoptosis, cell proliferation, differentiation and motility. **Objectives:** We evaluated the potential of this protein as a vaccine candidate. **Methods:** The gene was cloned by RT-PCR from *S. mansoni* RNA, heterologous expression was obtained in *E. coli,* and the protein was purified by nickel affinity chromatography. The protein was formulated with Freund's adjuvant to immunize mice subcutaneously. The immune response profile was characterized by ELISA and ELISPOT to measure the levels of antibody isotypes and cytokines, respectively. The protective potential of this protein was evaluated by the challenge of immunized animals with cercariae followed by perfusion 45 days later. **Results and Discussion:** The protein was expressed as inclusion bodies, solubilized with 8 M urea and purified under denaturing conditions. The refolding was performed by slow dialysis, but the protein precipitated. The protein surface-exposed localization at the parasite-host interface was confirmed by immunoblotting and immunolocalization; the antibodies induced by the immunization with recombinant protein were able to partially inhibit the enzymatic activity in *ex vivo* live adult worms (~60%). The humoral immune response was characterized by high levels of anti-SmNPP-5 IgG1 and low levels of IgG2 antibodies; the cytokine profile revealed low levels of IFN-g and high levels of IL-10 and IL-5, suggesting a more Th2 drift immune response. Despite the induction of a specific immune response, no reduction in worm burden was observed in the immunized animals. It is possible that it will be necessary to obtain the recombinant protein in its native form to exert a stronger specific immune response capable of complete inhibition of the worm enzyme, and thereby obtain better protection levels. To achieve this goal, we will try to express this protein in a eukaryotic expression system, *Pichia pastoris*, and different routes of immunization with different adjuvants must be tested as well.

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4.19 Comparative study between the flocculation limit technique and total combining power (TCP) for quality control of clostridial vaccines

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Introduction: Enterotoxemy is considered the disease most important for ruminant livestock in the world. Due to the characteristics of the development of the disease, vaccination is the preventive measure with the greatest impact in the control of this illness. In the last years, many scientists have been looking for the development of alternative *in vitro* methods with the objective of reduceing or eliminating the use of *in vivo* methods, which involves a series of bioethical questions. Besides, they show lower sensitivity and specificity and greater economic cost when compared with *in vitro* methods. **Objectives:** This work was carried out to assess and standardize the *in vitro* limit of flocculation test to be used in the quality control of vaccine against enterotoxemy. **Methods:** This study used experimental vaccines against enterotoxemy and toxicoid produced from the epsilon toxin of *Clostridium perfringens* type D, previously evaluated by serum neutralization in mice and the TCP test. The limit of the flocculation test was performed by mixing different amounts of antitoxin and a fixed amount of toxicoid in each tube. It was proposed that the first tube to flocculate would indicate unit of flocculation (Fl) of the sample tested. **Results and Discussion:** The main results indicated that the lower degree of antitoxin detectable both in rabbit and in sheep serum by the limit of flocculation technique is equivalent to 10 IU/mL. The TCP and limit of flocculation correlation ratio was 99.97%. In conclusion, the limit of flocculation method is suitable for replacing *in vivo* methods for the analysis of epsilon toxin. Nevertheless, this technique is less effective when used for the potency test of vaccines against enterotoxemy in substitution of serum neutralization in mice.

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4.20 Action of disintegrin in expression of pro-inflammatory mediators by endothelial cells

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Introduction: Studies involving SVMPs (snake venom metalloproteinases) carried out by our group have shown the interesting role of these toxins as a cell activator, triggering responses such as apoptosis and inflammation, where complex signaling cell pathways are involved. However, it is important to understand the involvement of different structural domains of SVMPs in this process. The disintegrin domain of class P-II SVMPs is an integrin ligand that interferes with responses to extracellular signals and cell migration. Thus, we aimed to examine the role of a recombinant disintegrin, cloned from *Bothrops Insularis* venom and combined with a fusion protein (glutatione-S-tranferase, GST), on the activation of pro-inflammatory genes in endothelial cells. **Objectives:** The specific aim was to characterize the effects of a disintegrin (GST-INS) on cell viability and adhesion of endothelial cells, checking the expression of genes involved in the process of inflammatory responses, transcribed after the stimulus. **Methods:** Initially we used a Detoxi Gel TH Endotoxin Removing Gel (polymyxin B) column to remove LPS from the recombinant samples. The efficiency of the treatment was verified by LAL and SDS-PAGE methods. In experiments of cell viability and cell adhesion to the substrate, the endothelial cells (HUVECs) were treated with 200 nM GST-INS, GST or cell culture medium (as a control) and evaluated by the MTT method. In experiments for gene expression, cDNAs were transcribed from mRNA obtained from the HUVECs treated as described below and the fold change in expression of inflammatory genes was detected by real-time PCR. **Results and Discussion:** The recombinant samples were completely free of endotoxins and suitable for use in cell cultures. The cells treated with GST-INS showed a significant decrease in adhesion to substrate after stimulus. Cell viability was preserved even after the cells were detached. Of the primers analyzed, IL-6 and MMP-10 showed a small decrease in their expression while I-CAM 1 showed an increase in its expression. Disintegrins from snake venoms play a role as antagonists of cell adhesion of endothelial cells and interfere with the expression of genes coding for pro-inflammatory mediators.

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4.21 Evaluation of serological method for potency testing of whole cell pertussis vaccine

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Introduction: The potency of whole cell pertussis (wP) vaccines for human use needs to be checked and must comply with the requirements of the Brazilian Pharmacopeia. The vaccines need to have a potency of at least 4 IU per dose, (with a lower limit of the 95 % confidence interval of at least 2 IU) as determined in the Kendrick test (MPT). In this test, the dose necessary to protect 50 % of mice (ED50) against the effect of a lethal dose of *B. pertussis* administered intracerebrally is compared with the dose of a reference vaccine needed to give the same protection. The test inflicts severe pain and distress to the animals, requires highly experienced operators, is sensitive to small technical changes resulting in a high variability and requires frequent repeats due to invalid test results. Therefore, there is an urgent need of a viable alternative to MPT. **Objectives:** The aim of this study was to investigate whether serological potency testing in guinea pigs, using an ELISA based on plates coated with wP bacteria, is a possible alternative to the current MPT to the pertussis component potency assessment in DTP vaccines. **Methods:** Sera from guinea pigs immunized with DTP vaccines that are normally used to determine diphtheria and tetanus potency were tested by a direct ELISA. Plates were coated with *B. pertussis* suspension at 0.8 OpU/mL (opacity units/mL). As reference, a serum of guinea pigs immunized with reference vaccine is used. The titer was determined by comparison of the sample response and the reference by the four parameter analysis method (sigmoidal curve) using Combistats. To compare MPT to ELISA, correlation statistical assay followed by a paired non parametric t-test was used. **Results and Discussion:** Comparing MPT and ELISA results, the high variability of MPT was evident, resulting in confidence intervals of up to 240 %. Observing both, even with quite fair correlation, possibly due to the MPT variability, the differences as determined by t-test are not that different (p=0.353). In conclusion, the ELISA assay to assess pertussis potency in DTP vaccines can be considered viable, especially due to its qualities, namely rapidity and reproducibility, and most importantly, due to the fact that its use would reduce animal suffering. However, it demands some refinement in order to achieve a higher correlation with MPT.

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4.22 Comparative analysis of acute inflammatory processes in the subcutaneous tissue and exudate of mice genetically selected for maximal or minimal acute inflammatory response

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Introduction: The inflammatory response is a physiological process that aims to restore homeostasis and tissue integrity after an injury, whether triggered by physical, chemical or biological agents. AIRmax and AIRmin mouse lines were selected according to their maximal or minimal inflammatory response to a subcutaneous injection of polyacrylamide beads (Biogel). The cellular influx and protein concentration in exudates were the inflammatory phenotypes measured 24 h after the Biogel injection. Despite the cellular and protein concentration being well established in the exudate, nothing is known about the inflammatory mechanisms acting in subcutaneous tissue. **Objectives:** Here, we investigated the cellular and molecular mechanisms involved in the acute inflammatory process in the subcutaneous tissue of AIRmax and AIRmin mice. **Methods:** AIRmax and AIRmin mice were injected with Biogel-P100 in the subcutaneous dorsal region; 48 h later, the local tissue was excised and the RNA was extracted. Real-time PCR analysis for several genes involved in the inflammatory response was performed. A histological study was also carried out. **Results and Discussion:** AIRmax and AIRmin mice differ in cell counts and protein concentration in inflammatory exudates, which are respectively about 25- and 2.5-fold higher in AIRmax than AIRmin mice, with neutrophils predominating. AIRmax animals also had a significantly ($P<0.05$) higher expression of *Il6* (5-fold), *Cxcl2* (19-fold) and *Mmp9* (21-fold) genes than AIRmin mice. These molecules are known to favor leukocyte accumulation at the inflammatory site as well as stimulating their motility through the blood vessels to reach the inflamed tissue. Together, these results show that the largest number of cells and protein concentration in exudates may result from an increased local expression of inflammatory genes overexpressed in AIRmax mice.

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4.23 Oral immunization for hepatitis B vaccine: the promising use of mesoporous SBA-15 silica adjuvant

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Introduction: Mesoporous SBA-15 silica is an inorganic material with ordered channels of uniform hexagonal nanostructured pores measuring approximately 10 nm in diameter. These particles of silicon oxide are able to interact with atoms, ions and molecules, and due to their physicochemical properties, this material shows great potential as vehicle/adjuvant. **Objectives:** The applicability of SBA-15 silica as an oral adjuvant in immunizations with the recombinant vaccine for hepatitis B that contains particles purified of the surface antigen [HBsAg] produced by the Butantan Institute was experimentally assayed. **Methods:** Female isogenic BALB/c mice, 8 to 12 weeks old, [n= 5 per group] received subcutaneously or orally, 0.5 μg of HBsAg adsorbed/encapsulated on SBA-15 or adsorbed on $Al(OH)_3$ in a final volume of 0.25 mL PBS, mixed in a ratio of 1:10 antigen:SBA-15 or 1:20 antigen:$Al(OH)_3$ [v/v]. The mixtures were kept at 4°C for 24 h before immunizations. To assess the secondary immune response, following the same procedures, a booster was administered 30 days after the first immunization. Individual serum and pooled fecal samples of each group were periodically collected for titration of specific antibodies by ELISA. **Results and Discussion:** Analysis of secretory IgA [s-IgA] showed that mice orally immunized with HBsAg adsorbed on SBA-15 had increased levels of specific antibodies at day 14 post first immunization [4.5 log_2] and 7 days after booster [5 log_2]. Animals immunized by the subcutaneous route had undetectable s-IgA levels. Specific serum IgA in HBsAg:SBA-15 orally immunized mice reached 5 log_2 at days 7 and 14 after booster. When $Al(OH)_3$ was used as adjuvant in subcutaneous immunizations, specific anti-HBsAg titers were detected only at day 7 post booster [4.5 log_2]. The overall analysis of the results indicates the promising use of SBA-15 silica as an adjuvant to be used in oral immunizations. Proteins usually fail to produce detectable mucosal immune response because nearly all antigens are degraded along the gastrointestinal tract. It is believed that this nanoparticle acts on the physical protection of antigens and protective epitopes, providing its slow release, and in the efficient activation of the immune system.

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4.24 Investigation of venom allergen-like proteins (VALs) from *Schistosoma mansoni*

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Introduction: In mining the database generated from the *S. mansoni* transcriptome using Gene Ontology categorization, potentially surface-exposed or exported proteins were identified. Among the antigens tested as DNA vaccines, a gene with similarity to venom-allergen-like proteins (SmVAL5) conferred a worm burden reduction of 31 %. Further searches for similar domains in the *S. mansoni* genomic databank revealed 29 paralogs from this new gene family. SmVALs are members of a protein superfamily containing a conserved SCP/TAPS (sperm-coating protein/Tpx-1/Ag5/PR-1/Sc7) domain, which may be important in host-pathogen interactions. **Objectives:** Based on the transcriptome profile, proteomics and microarray data available for these molecules, we selected four members (SmVALs 4, 5, 7 and 26) to be investigated as vaccine candidates. We also evaluated the allergenic potential of these proteins by exploiting the murine model for allergenic airway inflammation. **Methods:** Native or codon optimized versions of the selected SmVALs genes were expressed in *P. pastoris*, and the recombinant proteins purified by nickel affinity chromatography. To evaluate the protective potential of these molecules, C57BL/6 mice were inoculated subcutaneously (sc) with 3 doses of 25 µg rSmVALs formulated with Freund's adjuvant. Six weeks after challenge infection, worms were collected and counted from the hepatic portal vein. The immune response profile was characterized by ELISA and ELISPOT. To evaluate allergenic airway inflammation, BALB/c mice were sensitized by 3 doses (sc) of 10 µg rSmVALs formulated with alum, and challenged by 2 intranasal doses of 10 µg rSmVALs. One day after the last challenge, the cells present in the bronchoalveolar lavage (BAL) were analyzed and the supernatants collected for cytokine measurements. **Results and Discussion:** Preliminary immunization and challenge assays revealed that rSmVAL5 induced a 40% reduction in worm burden, confirming the previous protective potential for this molecule. The humoral immune response was characterized by high levels of IgG1 and low levels of IgG2 antibodies; the cytokine profile revealed low levels of IFN-g and high levels of IL-4, suggesting a more Th2-driven immune response. The allergenic airway inflammation data revealed that rSmVAL-4 caused eosinophil (45.9%) and macrophage (37.5%) recruitment into the lungs of mice after sensitization and challenge. This effect is characterized by a systemic increase in specific IgE (serum) and by a local augment of IL-5 (BAL). Our results suggest the potential of this class of molecules as vaccine candidates, but also point out that allergenic effects should be considered in the design of a schistosomiasis vaccine.

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4.25 Evaluation of anti-EspA and anti-EspB polyclonal sera for diagnosis of enteropathogenic and enterohemorrhagic *Escherichia coli*

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Introduction: Among the six categories of diarrheagenic *Escherichia coli*, enteropathogenic *E. coli* (EPEC) is responsible for 30-40% of diarrhea episodes, and enterohemorrhagic *E. coli* (EHEC) is the principal cause of hemolytic uremic syndrome. EPEC and EHEC have in common a pathogenicity island called locus for enterocyte effacement (LEE) which encodes the proteins involved in the type three secretion system (SST3). EspA and EspB proteins are secreted by SST3, and are therefore excellent targets for the diagnosis of EPEC and EHEC. **Objectives:** In this study, we evaluated the use of anti-EspA and anti-EspB polyclonal sera for diagnosis of EPEC and EHEC. **Methods:** Recombinant proteins EspA and EspB purified by affinity chromatography column containing nickel were used to immunize rabbits. 100 μg of purified EspA or EspB plus aluminum hydroxide were intramuscularly injected into New Zealand rabbits, and a booster dose was administered after 15 days of immunization. Serum anti-EspA and anti-EspB obtained were absorbed and used in immunoblotting assays to evaluate the expression of EspA and EspB. For this, isolates of EPEC and EHEC were cultivated in different media and collected at different stages of growth. Subsequently, ELISA and immuno-dot assays were used for detection of EspB using the polyclonal anti-EspB. **Results and Discussion:** High titers of antibodies against the proteins EspA and EspB were observed. By immunoblotting, the serum anti-EspA was specific only for strain E2348/69, precluding the evaluation of EspA expression in other isolates. Furthermore, serum anti-EspB recognized all EPEC and EHEC isolates tested. Most isolates tested secreted EspB in higher amounts when cultured in Dulbeco´s MEM (DMEM) than in other media. These results point out the use of anti-EspB serum as a tool for the detection of EHEC and EPEC; since EspA has antigenic polymorphism, serum obtained with the protein of EPEC E2348/69 was unable to recognize other strains of EPEC and EHEC. Regarding EspB secretion, the use of DMEM promoted its increase in bacterial logarithmic growth phase, and immuno-dot assays can be used for qualitative detection and ELISA for quantitative detection of EspB.

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4.26 Production and use of EspB monoclonal antibody for the diagnosis of enteropathogenic *Escherichia coli* and enterohemorrhagic *Escherichia coli*

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Introduction: EPEC and EHEC are two important diarrheagenic *Escherichia coli* pathotypes; they are responsible for high rates of morbidity and mortality worldwide. *E. coli* secreted protein B (EspB) is among the virulence factors involved in their pathogenesis and is therefore a potential target for detection. Diagnosis is an essential tool either for the treatment of disease or to prevent further outbreaks. In Brazil, there are no commercial kits for detection of EPEC and EHEC, and thus, their developments are extremely important. **Objectives:** The present study aimed at the production and use of anti-EspB monoclonal antibodies for the diagnosis of EPEC and EHEC. **Methods:** Balb/c mice were immunized with 20 μg of purified EspB. The mouse with the highest antibody titer was boosted with the same amount of purified EspB without adjuvant four days prior to cell fusion, and then sacrificed by cervical dislocation. Popliteal lymph nodes were removed aseptically and B lymphocytes were fused with myeloma cells in the presence of polyethylene glycol. Ten days after fusion, hybridomas were screened for antibody production by ELISA. The specificity of the hybridomas was evaluated by immuno-dot assay using supernatant of positive and negative bacterial isolates. Two hybridomas were selected and subjected to limited dilution, and the selected clone was cultivated on a large scale in order to collect the supernatant. The IgG isotype was characterized by ELISA and purified by affinity chromatography column containing protein A. The purified monoclonal antibody was titrated by ELISA using 1.5 μg/mL EspB, and the dissociation constant was calculated. After the characterization of the monoclonal antibody, detection of EspB was standardized by ELISA using supernatants of bacterial isolates cultivated in Dulbeco's MEM. **Results and Discussion:** We obtained more than 300 anti-EspB-secreting hybridomas, and by immuno-dot we observed a high specificity of the 4D9 clone. This clone was characterized as IgG2a and its dissociation constant was 2×10^{-9} M, and only 1 μg/ml was needed for detection of EspB in the supernatant of the isolates. These results indicate that this monoclonal anti-EspB is a promising tool for the detection of EPEC and EHEC.

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4.27 SBA-15 silica adjuvant and the expression of co–stimulatory molecules

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Introduction: Adjuvants are compounds that can enhance the immune response to vaccine antigens by amplifying any of the signals involved in the process for eliciting an efficient immune response. The achievement of protective immunity through vaccination includes the recognition of relevant epitopes and activation of the antigen–presenting cells [APCs] such as macrophages [Mf] and dendritic cells [DC], either by the vaccine antigens themselves and/or by the adjuvant's physicochemical nature. The ordered nanostructured silica SBA-15 is an adjuvant that due to its physical and structural properties is capable of carrying, protecting and delivering antigens [International Patent WO 07/030901]. **Objectives:** The main goal of the present study was to evaluate the capacity of SBA-15 in inducing the expression of MHC class II and co-stimulatory molecules during the immune response against human g–globulin [HGG]. **Methods:** Isogenic BALB/c mice were immunized by the subcutaneous route with 10 μg/animal of HGG adsorbed or not on SBA-15 or $Al(OH)_3$. At different times after immunization, the expression of CD11c, CD11b, CD4, CD8, B220, CD80, CD86, CD40 and MHC-II in the lymph node cells and purified $CD11c^+$ cells were analyzed by flow cytometry. **Results and Discussion:** In the SBA-15 immunized mice, there was a significant increase in the expression of CD40, CD80 and CD86 in lymph nodes and purified $CD11c^+$ cells when compared to $Al(OH)_3$; nevertheless, these nanoparticles did not modulate MHC-II expression. No difference in recruiting of CD11c, CD11b, CD4, CD8, B220 cells was observed. These results agree with the ability of SBA-15 to induce successful immunity, recruiting and activating specific immune cells to the site of immunization. Thus, this new adjuvant is a candidate for immune modulation and nanovaccine design.

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4.28 The human complement regulators FH and C4BP interact with the leptospiral surface protein LcpA

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Introduction: Leptospirosis, an emerging global infectious disease, is caused by spirochetes belonging to different pathogenic species of the genus *Leptospira*. After penetrating the host, *Leptospira* have the ability to spread and to trigger a specific immune response. Like other pathogens, they have evolved strategies to evade innate immune defense systems, thereby causing severe disease. One strategy adopted by pathogenic *Leptospira* to resist hosts' innate immunity is their potential to acquire fluid phase complement regulators on their surfaces, particularly those of the alternative and the classical complement pathways such as factor H (FH), and C4b-binding protein (C4BP). Recently, we have shown that C4BP bound to *Leptospira* retains its cofactor activity, indicating that acquisition of this complement regulator may contribute to leptospiral serum resistance. **Objectives:** In this study, we screened a number of putative leptospiral membrane proteins for their capacity to interact with human complement regulators. **Methods:** The genes coding for putative outer membrane proteins were cloned and the proteins were expressed in *E. coli*. The binding of the purified recombinant proteins to FH and C4BP was assessed by Western blot overlay and ELISA. Surface exposure was assessed by immunoelectron microscopy and also by a proteinase K accessibility assay. **Results and Discussion:** We found that a predicted membrane lipoprotein of 20 kDa, named LcpA (leptospiral complement regulator-acquiring protein A), bound to both C4BP and FH. LcpA was shown to be surface-exposed, and the gene coding for this 20-kDa lipoprotein is conserved among pathogenic leptospiral species. Moreover, *Leptospira* strains that resist, at least to a certain degree, complement-dependent killing by normal human serum express LcpA, whereas the serum-sensitive strain Patoc does not. To our knowledge, this is the first description of a *Leptospira* protein that binds both C4BP and FH.

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4.29 Inflammatory and genetic mechanisms involved in ear tissue regeneration in mice selected for high or low acute inflammatory response

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Introduction: Mice selected for high (AIRmax) or low (AIRmin) acute inflammatory response were used in tissue regeneration experiments. It was observed that AIRmax mice show faster ear tissue regeneration than do AIRmin mice, suggesting the involvement of common regulatory loci for both inflammation and ear tissue regeneration phenotypes. **Objectives:** In this study, we investigated some inflammatory phenotypes and global gene expression profiles in AIRmax and AIRmin mice during the initial phase of ear tissue regeneration. **Methods:** Histology, ear edema thickness and MPO levels were investigated. Global gene expression analysis was performed with CodeLink bioarrays in both control and experimental 48-h ear punched mice, and to validate this research real-time PCR was used. **Results and Discussion:** The histological analyses showed that AIRmax regeneration was not only complete (with no sign of the original opposing epithelial surfaces), but cartilage islands and sebaceous glands were formed in the middle of the regenerated area. AIRmin mice displayed some regeneration but never closure. Ear edema thickness and MPO levels were higher in AIRmax than AIRmin mice ($P < 0.001$). Global expression analysis showed 794 activated and 528 repressed genes in AIRmax, while 1086 activated and 1145 repressed genes were observed in AIRmin mice 48 h after injury. AIRmax and AIRmin mice showed up-regulated genes over-represented in inflammatory response, cell adhesion and chemotaxis biological themes (gene ontology). However, down-modulated genes were significantly over-represented for transportation in AIRmax and for taxis, muscle contraction and ubiquitin cycle in AIRmin mice. In the QTL regions previously detected on chromosome 1 differentially-expressed *Stat1, Casp8* and *Hspe1* genes were found, while *Lect1, Fndc3 and Egr3* were detected on chromosome 14. qPCR experiments showed high expression of *Il-1β* and *Il-8rb* in AIRmax and *Cxcl2*, *Tnfa*, *Tgfb1* and *Mmp9* in AIRmin mice. AIRmax and AIRmin mice displayed several (some on chromosomes 1 and 14) differentially-expressed inflammatory genes which could be involved in the acute inflammatory response and ear tissue regeneration phenotypes.

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4.30 Expression profile of inflammatory genes in mice selected for high acute inflammatory response, bearing distinct Slc11a1 alleles after pristane-induced arthritis protocol

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Introduction: Rheumatoid arthritis is a chronic inflammatory autoimmune disease, and the main symptoms are chronic synovitis, joint erosion and several immune abnormalities. Mice selected for maximal acute inflammatory reaction (AIRmax) show high susceptibility to pristane-induced arthritis (PIA). The *solute carrier family 11a member 1 (Slc11a1)* gene interacts with AIRmax genetic background and modulates this susceptibility. This gene is involved in the ion transport at the endosomes in macrophages and neutrophils, interfering in their activation. **Objectives:** The aim of this study was to investigate the gene expression levels of peritoneal macrophages from AIRmax^{RR} and AIRmax^{SS} during the early phase of PIA. **Methods:** Mice received 0.5 mL i.p. pristane injection, and the peritoneal macrophages were isolated at day seven. Macrophage mRNA transcript levels of several inflammatory related genes (*Il1b, Tnfa, Il6, Il8rb, Il18, Cxcl2*) and *Tgfb* were measured by quantitative real-time PCR. Nitric oxide (NO) production was detected in culture supernatants after 48 h LPS stimulation. **Results and Discussion:** Results showed that pristane treatment significantly decreased the total cell number in the AIRmax^{RR} and AIRmax^{SS} peritoneal cavity. Distinct *Tnfa*, *Il1b*, *Il6* and *Cxcl2* gene expressions between AIRmax^{RR} and AIRmax^{SS} macrophages were observed. *Tnfa* and *Il1b* expression was higher in AIRmax^{RR} than AIRmax^{SS} macrophages ($p<0.001$). However, *Il6* and *Cxcl2* RNA expression was lower in AIRmax^{RR} after pristane treatment (3-fold and 5-fold, respectively). *Il8rb, Il18* and *Tgfb* did not show significant differences among all the groups. Significant ($p<0.001$) NO production was observed in all mice only after LPS stimulation *in vitro*. These data suggest that *Slc11a1* alleles modulate inflammation-related gene expressions in peritoneal macrophages, which could influence susceptibility to pristane-induced arthritis in AIRmax mice.

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4.31 PAS-1 released by *Ascaris suum* in early larval stages suppresses LPS-induced acute inflammation

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Introduction: Helminth infections have been associated with an important immunomodulatory effect on the host immune system, impairing immune response to heterologous antigen, allografts, and infections. In our laboratory, we have demonstrated that the immunessuppression induced by *Ascaris suum* whole extract is due to a protein called PAS-1. **Objectives:** In this study, we evaluated the suppressive effect of PAS-1 secreted by the earlier larval stages of *Ascaris suum* cultured *in vitro*. The effect of PAS-1 on LPS-induced inflammation was compared to that with the *Ascaris suum* adult worm extract (ASC) and the body fluid (BF). **Methods:** Air pouches were induced on the shaved back of BALB/c mice with 2.5 mL of sterile air on days 0 and 3. The air pouches were then stimulated with 1 mg LPS alone or mixed with 300 mg PAS-1 obtained from the supernatant, whole extract (ASC) or adult worm body fluid (BF). Control groups were injected only with LPS. Three hours later, the exsudates were recovered after washing with PBS, and the magnitude of inflammation was evaluated by cell migration and cytokine levels. Pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) and regulatory cytokines (IL-10 and TGF-β) were measured by ELISA. **Results and Discussion:** Our results demonstrate that mice injected with PAS-1, ASC or BF showed a significant suppression on the LPS-stimulated leukocyte infiltrate (mononuclear cells and neutrophils) into air pouches and production of TNF-α, IL-1β and IL-6 in comparison with the control group that received only LPS. In contrast, PAS-1, ASC or BF induced high levels of IL-10 and TGF-β in relation to control mice. On the other hand, no significant differences were seen between the experimental groups (PAS-1, ASC or BF). These data demonstrate that *Ascaris suum* products, including PAS-1 released by earlier larval stages, modulate the LPS inflammation in air pouches by suppressing cell influx and pro-inflammatory cytokines. The mechanism evoked in this suppressive activity is mediated by the regulatory cytokines IL-10 and TGF-β.

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4.32 Investigation of skin carcinogenesis in mice selected for maximal or minimal acute inflammatory response, homozygous for R and S alleles of Slc11a1 gene

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Introduction: Mice selected for maximal (AIRmax) or minimal (AIRmin) acute inflammatory response differ in susceptibility to carcinogenesis. AIRmin mice are significantly more susceptible to skin carcinogenesis than AIRmax mice due their genetic background and a polymorphism of aryl hydrocarbon receptor (Ahr) gene. They also differ in susceptibility to *Salmonella enterica* serotype *Typhimurium* infection, arthritis and wound healing. The *Slc11a1* gene (formerly *Nramp1*) polymorphism modulates macrophage activity and the susceptibility to infections and autoimmune diseases. To study the interaction of resistant (R) or susceptible (S) *Slc11a1* alleles with acute inflammatory reaction loci found in AIRmax and AIRmin mice, homozygous sublines for these alleles were produced. These mice were designated AIRmax^{RR}, AIRmax^{SS}, AIRmin^{RR} and AIRmin^{SS}. **Objectives:** The objective of this study was to investigate the skin carcinogenesis induced by DMBA in AIRmax^{RR}, AIRmax^{SS}, AIRmin^{RR} and AIRmin^{SS}. **Methods:** To induce carcinogenesis, 50 μg DMBA diluted in 0.1 mL acetone were applied epicutaneously to the shaved dorsal skin of mice for five consecutive days. Gene expression of several inflammatory cytokines was detected in control and experimental mouse skin 48 h after the last DMBA application. This analysis was made by real-time PCR. **Results and Discussion:** All control mice had a basal expression of *Il6, Tnfa, Il1b* and *Cxcl2*. However, in DMBA-treated AIRmin^{RR} mice, mRNA levels of these cytokines were increased 9-, 19-, 30- and 215-fold, respectively. In AIRmin^{SS}, these mediators were increased 11-, 4-, 6- and 75-fold in relation to their controls. *Saa3* and *Tgfb1* expression showed no significant differences among all DMBA treated mice. AIRmin^{SS} differed significantly from the other sublines in some cytokines, but AIRmin^{RR} showed the highest number of differentially expressed genes ($P<0.001$) after DMBA application. Preliminary data showed higher susceptibility to skin carcinogenesis in AIRmin^{RR} than in AIRmin^{SS} mice. These results suggest the involvement of on *Slc11a1* gene polymorphism modulating gene expressions to confer higher susceptibility to skin carcinogenesis in AIRmin^{RR} mice.

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4.33 Study of adjuvants for mucosal and systemic vaccines composed of the antigen PspC (pneumococcal surface protein C) in pneumococcal nasal colonization and invasive challenge models in mice

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Introduction: *Streptococcus pneumoniae* (pneumococcus) is a gram-positive bacterium responsible for the majority of pneumonia cases around the world. Available vaccines are based on capsular polysaccharides, but the variability and low cross-reactivity displayed by them require the fermentation of different serotypes for the induction of broad-range coverage immunity. Protein antigens are interesting alternatives for the constitution of a low-cost formulation that can elicit immunity to the different serotypes. PspC is a virulence factor related to pneumococcal adhesion to respiratory epithelial cells and evasion from the immune system. It has already been shown that it is a good candidate for vaccine formulations. **Objectives:** The aim of the present work was to evaluate PspC (a large fragment composed of the N terminal domain plus proline-rich region) and $PspC_{104}$ (a fragment composed of the N-terminal domain, responsible for PspC binding to factor H), in combination with different adjuvants, as vaccines against nasopharyngeal colonization or invasive challenge models in mice. **Methods:** C57BL/6 or BALB/c mice were immunized with the proteins through the nasal route, using whole cell *Bordetella pertussis* vaccine (WCP) or DTP (diphtheria, tetanus and pertussis) vaccine as adjuvants. The formulations were also tested through the subcutaneous route. Induction of mucosal and systemic anti-PspC antibodies was evaluated by ELISA and mice were challenged with different pneumococcal strains. Cross-reactivity of PspC and $PspC_{104}$ sera among the different strains of pneumococcus was tested by Western-blotting. **Results and Discussion:** Nasopharyngeal colonization analysis of immunized mice showed that the vaccines containing PspC proteins and WCP were able to protect mice from pneumococcal carriage. The protection was most effective when the entire N-terminal region was used as antigen instead of the fragment comprising the first 104 amino acids, $PspC_{104}$. The best level of protection correlated with induction of high levels of anti–PspC antibodies in the sera and the secretion of IFN-γ and IL-17 after challenge. On the other hand, the nasal immunization with PspC or $PspC_{104}$ in combination with WCP did not protect mice against the challenge with the invasive strain 6303. Similarly, subcutaneous immunization with PspC in combination with WCP or DTP did not protect the animals against the invasive challenge. These results can be explained by the low reactivity of sera from the animals against the PspC expressed by the 6303 strain, revealed by Western-blotting.

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4.34 The phospholipases A_2 MjTX-I and CB isolated from *Bothrops moojeni* and *Crotalus durissus terrificus* snake venom, respectively, induce mast cell degranulation

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Introduction: Snake venom phospholipases A_2 (PLA_2s) are able to activate immunological responses with activation of leukocytes and release of inflammatory mediators. Among these cells are the mast cells (MC). These cells are central to innate immune responses by releasing a vast array of inflammatory mediators. The literature shows that phospholipases A_2 isolated from snake venoms induce mast cell degranulation; however, this is until now unclear. **Objectives:** The aim of this study was to compare the effects of the two PLA_2s MjTX-I a myotoxin isolated from *Bothrops moojeni* and CB, a neurotoxin from *Crotalus durissus terrificus* snake venom on mast cell viability and degranulation. **Methods:** PT18 lineage mast cells were cultured in tissue culture flasks with RPMI-1640, supplemented with fetal bovine serum (10%), gentamicin (40 mg/mL), L-glutamine (2 mM), 2-mercaptoethanol (2 mM) and IL-3 (5%), and maintained at 37ºC and 5% of CO_2. $2x10^5$ cells/well were incubated with different concentrations of MjTX-I, CB or Tyrode buffer (control) for 1 h at 37ºC and 5% of CO_2. Cell viability was evaluated by counting mast cells after staining with trypan blue solution (1:10). Mast cell degranulation was evaluated by measurement of β-hexosaminidase in the supernadant of cell cultures after incubation with non-cytotoxic concentrations of MjTX-I or CB or Tyrode buffer (control) for 1 h. **Results and Discussion:** Results showed that the viability of mast cells was significantly reduced after incubation of cells with higher concentration of both MjTX-I and CB (3.3 mM). Non-cytotoxic concentrations of MjTX-I (0.06 up to 0.6 mM) significantly increased β-hexosaminidase release in comparison with control. CB also caused a significant increase of β-hexosaminidase release from 0.3 up to 0.6 mM when compared with control. These results demonstrate that despite triggering distinct pathophysiological activities, both snake venom phospholipases A_2 were able to induce mast cell degranulation. The contribution of this action to the final biological effect of each phospholipase studied remains to be determined.

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4.35 Immune evasion of pathogenic *Leptospira*: human complement regulators factor H and C4b-binding protein interact with LigA and LigB proteins

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Introduction: Leptospirosis is a worldwide zoonosis and represents a serious health problem in urban areas of developing countries. Like other pathogens, leptospires have evolved strategies to evade the innate immune system. Binding of the regulators factor H (FH) and C4b-binding protein (C4BP) has been demonstrated for pathogenic strains of *Leptospira*. Leptospiral immunoglobulin-like (Lig) proteins belong to a family of surface-exposed determinants that have Ig-like repeat domains found in virulence factors such as intimin and invasin. The Lig proteins are expressed during host infection, but loss of protein expression occurs upon culture attenuation of pathogenic strains. Lig proteins can bind to a variety of extracellular matrix components, thereby mediating adhesion to host cells. Moreover, LigA has been shown to be the best vaccine candidate against leptospirosis. **Objectives:** Considering that during infection important virulence factors of many pathogens may interact with multiple host proteins, including coagulation cascade molecules, ECM components and complement regulators, we decided to detemine whether Lig proteins contribute to leptospiral immune evasion by interacting with host complement regulators. **Methods:** Both subfragments corresponding to the C- and the N-terminal portions of LigA and LigB genes were cloned and the proteins were expressed in *E. coli*. The binding of the purified recombinant proteins to FH and C4BP was assessed by Western blot overlay and ELISA. **Results and Discussion:** Both LigA and LigB bound serum FH and C4BP in a dose-dependent manner. To date, only two leptospiral proteins (LenA and LenB) have been described to interact with FH, and a single leptospiral protein has been shown to bind C4BP. Considering that leptospires are highly invasive microorganisms, there could be several other bacterial receptors for these host molecules. The identification of these receptors is of great importance, since they may represent targets for immune interference.

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4.36 Crotoxin isolated from *Crotalus durissus terrificus* venom induces mast cell degranulation in vitro

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Introduction: Crotoxin (CTX) is the main toxin from *Crotalus durissus terrificus* (Cdt) snake venom, and it is responsible for the toxicity observed in envenomation. CTX is a presynaptic neurotoxin formed by the complex of two different subunits: the non-toxic and non-hemolytic component, called crotapotin (crotoxin A, CA) and the hemolytic component, the phospholipase A2 (crotoxin B, CB, or Cdt PLA2). CA acts as an inhibitory "chaperone" preventing promiscuous interactions of the phospholipase with phospholipids in membrane surfaces other than its target membrane. CTX is related to important envenomation effects, such as neurological disorders, myotoxicity, and renal failure. Otherwise, the local edema, another feature of snake bite, has been associated with phospholipase A2 activity which induces an increase in microvascular permeability and plasma extravasation. Distinct components from the immune system may be involved in this initial inflammatory reaction, for example, mast cells. **Objectives:** The aim of this work was to evaluate the ability of CTX to induce mast cell degranulation *in vitro* and the capacity of the anti-crotalic horse serum to inhibit this toxin effect. **Methods: R**at basophilic leukemia (RBL) cell line was incubated for 1 h with different concentrations of CTX (8, 4 or 2 μg), and the ability of the toxin to induce cell degranulation was analyzed by the measurement of the β-hexosaminidase enzyme released in the culture supernatants. To evaluate the capacity of the anti-crotalic serum to neutralize the CTX effect, samples of the toxin (2 μg) were pre-incubated with different concentrations of the anti-crotalic serum (2, 10 or 20 μL) for 30 min and centrifuged, and the supernatants were incubated with RBL cells for 1 h. Afterward, the cellular supernatants were collected and used to estimate the β-hexosaminidase contents. All experimental conditions were tested in quadruplicate. **Results and Discussion:** The results show that CTX was able to induce *in vitro* mast cell degranulation with 48-50% of β-hexosaminidase release even using 8, 4 or 2 μg of the toxin. Furthermore, the anti-crotalic serum was efficient in neutralizing the CTX effect on mast cell degranulation at the proportions of 10 or 20 μL of serum/2 μg of the toxin. In contrast, the non-immunized horse serum did not interfere with the ability of the CTX to induce mast cell degranulation. In conclusion, the CTX exerted a direct effect on mast cell degranulation and the anti-crotalic serum was able to neutralize this toxin activity. Next, we will test if the recombinant single chain variable fragment (scFv) anti-crotoxin is able to inhibit CTX-induced mast cell degranulation.

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4.37 Immunization of mice with *Lactobacillus casei* expressing a β-intimin fragment reduces intestinal colonization by *Citrobacter rodentium*

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Introduction: Diarrheal diseases are common in childhood and can be of particular importance in developing countries, where they are responsible for around 17 % of total mortality in children. Among the pathogens responsible for these infections, enteropathogenic *Escherichia coli* (EPEC) are frequently isolated from infantile diarrhea. Intimin is a 94-kDa adhesin that participates in the local histopathological lesions (attaching and effacing – A/E-lesions), which are characteristic of EPEC infections and are also essential for virulence. A promising approach in vaccine development is the induction of mucosal and systemic immune responses against protective antigens delivered by lactic acid bacteria. **Objectives:** This work aimed to develop a mucosal vaccine against EPEC infections based on lactic acid bacteria. **Methods:** A fragment corresponding to the conserved plus the variable regions of beta-intimin (Int_{cv}) was constitutively expressed in *Lactobacillus casei.* Mice were immunized through the sublingual or oral route with the recombinant *L. casei* (*L. casei*-Int_{cv}), the respective control bacteria carrying the empty vector (*L. casei)* or saline. **Results and Discussion:** Immunization with *L. casei*-Int_{cv} through either route did not induce detectable levels of anti-Intimin IgA or IgG in feces or sera. On the other hand, a significant increase in specific secretion of IL-6 and IFN-gamma was observed in the spleen cells of mice immunized with *L. casei*-Int_{cv}, when compared to the respective control groups. Analysis of feces samples from mice challenged with *C. rodentium*, the mouse model for EPEC infections, showed an average reduction of 100X on colonization, for both groups that received *L. casei*-Int_{cv}. Time course evaluation showed that the reduction was already observed 4 days after infection and maintained for at least 10 days, the endpoint of the experiments. The results show that despite the absence of specific antibodies against intimin, the cytokine responses elicited by *L. casei*-Int_{cv} vaccines seem to correlate with protection against *C. rodentium* colonization.

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4.38 Evaluation of the production of antibodies against botulinum toxins type A and type B and of their neutralizing capacity

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Introduction: *Clostridium botulinum* produces toxins (BT) that have been classified into 7 serotypes (A – G), according to their immunological characteristics. Toxin type A, and sometimes type B, have been used to treat dystonia and various other muscle hyperactivity syndromes in humans. **Objectives:** The aim of this study was to evaluate the production of antibodies to BT type A and type B in hyperimmunizated horses, as well as their neutralization capacity, avidity and the IgG subclass pattern. Moreover, the results obtained in test bleeding and final product were compared. **Methods:** Horses were injected with BT-A and BT-B. These antigens were prepared containing about 0.7 mg/mL of protein. The test bleeding (type A and type B) was obtained and the total quantity of specific antibodies and their subclass was determined by ELISA, and antibody potency was measured by the serum neutralization assay in mice. The serum was purified and concentrated, and the total quantity of specific antibodies was performed by ELISA and their potency measured by the serum neutralization assay in mice. **Results and Discussion:** The total specific antibody titers to type A and type B in test bleeding were similar (1/1024000 to 1/2048000 and 1/512000 to 1/1024000, respectively). However, the potency of type A was 335.8 ± 67.2 IU/mL and the potency of type B was 53200.0 ± 6800.0 IU/mL. The potency of type B was significantly higher ($p<0.01$) than type A. The IgG subclass pattern was similar in the two types, with predominance of IgGT (1/256000 to type A and 1/512000 to type B) followed by IgGa (1/64000 to type A and type B). In the final product, the total specific antibody titers to serum type A and type B were also similar (1/1024000 to 1/2048000 both of them). The potency of serum type A was 414.70 ± 38.2 IU/mL and the potency of serum type B was 28660.0 ± 1337.0 IU/mL. The potency of serum type B (final product) was significantly higher ($p<0.05$) than serum type A. BT-A and BT-B apparently have the same capacity to stimulate antibody production; furthermore, BT-A and BT-B induce the same IgG subclass pattern of antibodies. However, the antibodies derived from BT-B horse immunization offer higher protection in the mouse model.

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4.39 Characterization of antibodies from horse serum producers, used against *Botulinum* toxins type A and type E

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Introduction: Botulinum toxins are considered the most potent lethal substance known to humans. Human botulism is caused by neurotoxins types A (BT-A), B (BT-B) and E (BT-E). To date, no commercial vaccines have been available, and treatment consists of antitoxic therapy prepared by the hyperimmunization of horses. **Objectives:** The aim of this study was to compare the antibody production to BT type A and type E in horse serum producers, as well as their neutralization capacity, avidity and IgG subclass pattern. **Methods:** Horses were injected with BT-A and BT-E. These antigens were prepared containing about 0.5 mg/mL protein. The serum (type A and type E) was obtained, and the total quantity of specific antibodies and their subclass were determined by ELISA; antibody potency was measured by serum neutralization assay in mice and their avidity was determined by modified ELISA. **Results and Discussion:** The total specific antibody titers in the group of all producer horses (AP) type A and type E were significantly different (1/1,024,000 and 1/256,000, respectively). In the group of best producers (BP), this difference was higher (1/2,048,000 to type A and 1/256,000 to type E). The potency to type A was 335.8 ± 67.2 IU/mL and to type E was 155.9 ± 45.7 IU/mL. This difference was significant ($p<0.01$). Moreover, avidity showed a significant difference when we compared samples from AP (82.3% ± 3.3 to type A and 81.4% ± 2.7 to type E) and BP (96.8% ± 2.8 to type A, $p<0.05$ and 102.3% ± 1.3 to type E, $p<0.01$). The IgG subclass pattern was similar to both types, with predominance of IgGT (1/256,000 to type A and 1/128,000 to type E) followed by IgGa (1/64,000 to type A and 1/16000 to type E). The BT-A had a higher capacity to stimulate antibody production than did BT-E; this difference was higher when we observed the best producers. Apparently, the antibodies of type A have higher capacity of neutralization; however, it could be due to their total quantity. The antibodies derived from the best producers showed higher avidity; furthermore, BT-A and BT-E induce the same IgG subclass pattern of antibodies.

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4.40 Phenotypic characterization of cells during *Lagochilascaris minor* helminth infection

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Introduction: *Lagochilascaris minor* is a helminth that usually affects the cephalic region. The mechanism of infection is still unknown. This infection is also reported in cats, and mice are considered intermediate hosts. We have recently shown that C57BL/6 are more susceptible to *L. minor* than BALB/c mice, having more intense lesions in the lungs, with a greater number of nodules. Both mouse strains have a mixed cytokine pattern. **Objectives:** The aim of this study was to characterize cell populations present in the peripheral blood and spleens and to investigate the presence of lung infiltrating cells during the infection of both BALB/c and C57BL/6 mice with *L. minor*. **Methods:** BALB/c and C57BL/6 mice were orally infected with 10^3 eggs of *L. minor* per animal. After 7, 35, 100 and 250 days of infection, groups of 5 mice were sacrificed. The same numbers of non-infected mice were used as controls. Splenocytes and PBMC were isolated and incubated with FITC-, PE- or PE-Cy5-conjugated antibodies to perform phenotyping of cells. Immunohistochemistry of spleen and lung frozen sections was performed to analyze cell populations and IFNγ production, *in situ*. **Results and Discussion:** There was an increase in B220+ cells in PBMC from infected BALB/c (27.2 ± 5.2 vs 41.8 ± 5.6%, $p < 0.001$) and infected C57BL/6 (32.5 ± 6.2 vs 56.1 ± 6.6%, $p < 0.001$) mice compared to controls, 15 days after infection, with an increase in these cells in the spleen during the whole infection. In addition, there was an increase in CD3+ and CD4+ cells in PBMC from 60 day-infected BALB/c mice with a decrease of these cells in the spleens of these mice compared to controls. We detected more CD4+CD25+ cells in PBMC from infected BALB/c mice (2.8 ± 0.4%) compared to infected C57BL/6 (1.3 ± 0.5%), 60 days post-infection ($p < 0.001$). On the other hand, infected C57BL/6 mice showed a higher percentage of CD4+CD25+Foxp3+ spleen cells compared to controls (1.4 ± 0.4 vs 4.9 ± 2.1%), 7 days after infection ($p < 0.05$). Both infected BALB/c and C57BL/6 mice showed an increase in MHC IAIE+ cells that can be correlated to an increase of the F4/80+ macrophage cell population in the spleen in the late period of infection (250 days). CD3+ and CD19+ cells were detected by immunohistochemistry, in the lung of infected C57BL/6 and BALB/c mice. IFNγ+ cells were also detected in the lungs of *L. minor* infected mice but not in controls, suggesting the presence of an inflammatory process. Our results suggest the participation of different T, B and macrophage cell populations in the immune response to *L. minor* infection in attempt to eliminate or control the parasite.

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4.41 Evaluation of the *Slc11a1* gene's role in the activation of macrophages stimulated by thioglycollate or *Mycobacterium bovis* BCG

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Introduction: Mouse lines were genetically selected for maximal and minimal acute inflammatory response (AIR). AIRmax mice are more resistant than AIRmin when infected with *S. typhimurium*. *Slc11a1* (formerly *Nramp1*) gene is involved in resistance to this infection as well as to the control of BCG infection; it interferes with macrophage activation, oxidative burst, inflammatory cytokine production, and nitric oxide (NO) and hydrogen peroxide (H_2O_2) secretion. Mouse lines homozygous for resistance and susceptibility *Slc11a1* gene alleles ($AIRmax^{RR}$, $AIRmax^{SS}$, $AIRmin^{RR}$ and $AIRmin^{SS}$) were produced in our laboratory. These lines show differences in inflammatory capacity, tissue regeneration and carcinogenesis response, phenotypes which are influenced by macrophage (MΦ) activation. **Objectives:** The aim of this work was to evaluate the effect of *SLC11A1* gene polymorphism in the activation of MΦ by thioglycollate (TG) or by *M. bovis* BCG. Systemic infection, cell migration, NO and H_2O_2 secretion and cytokine production were analyzed comparatively in these selected mice. **Methods:** Mice were inoculated ip with PBS (control), TG (48 h) or BCG (14 days). The peritoneal cells were collected and placed in culture. After 2 h, the non adherent cells were discarded and the adherent MΦ stimulated or not with LPS were used in all experiments. Spleens were homogenized and cultured in specific BCG medium and the bacterial colonies were counted. Cytokine levels were determined in culture supernatants by ELISA. **Results and Discussion:** The total numbers of resident cells in the peritoneal cavity of $AIRmax^{RR}$, $AIRmin^{RR}$ and $AIRmax^{SS}$ were similar but higher than in $AIRmin^{SS}$ mice. TG promoted cell migration and induced low secretion of NO by MΦ in all lines. When LPS was added to the MΦ cultures, higher NO secretion was observed in $AIRmax^{RR}$ followed by $AIRmin^{RR}$, $AIRmax^{SS}$, and $AIRmin^{SS}$. Analyzing the systemic BCG infection by counting the bacterial colonies in the spleen, $AIRmax^{RR}$ mice were the most resistant to infection followed by $AIRmin^{RR}$, $AIRmax^{SS}$, and finally $AIRmin^{SS}$ which does not control BCG growth. Cell migration to the peritoneum was induced by BCG ip infection in all lines, especially in $AIRmax^{SS}$. Higher cytokine production (IL-1β, TNF-α, IL-6 and IL-12p40) by peritoneal MΦ was observed in $AIRmax^{RR}$ than in the other lines which produced basal levels of these cytokines. On the other hand high levels of NO and H_2O_2 were released by $AIRmin^{SS}$ cells. Taken together, our results show that *SLC11A1* ^{RR} Mf is efficiently activated to kill the bacteria and secrete cytokines, and indicate that NO and H_2O_2 play a limited role in the protection against BCG infection.

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4.42 Semliki Forest virus (SFV) as a vector for anti-rabies immunization

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Introduction: The non-replicative SFV system is characterized by the production, in a single round of infection, of a great amount of recombinant mRNA in infected cells, leading to a high expression of the recombinant protein. We have evaluated the antigenicity and immunogenicity of the rabies virus glycoprotein (RVGP) produced *in vivo* by the utilization of a recombinant SFV-RVGP as a vector for vaccination. The SFV system shows several advantages for RVGP presentation to organisms, such as apoptosis-mediated cross-priming, capability to produce high amounts of the antigen, no integration into the genome and stimulation of both humoral and cellular immune responses. **Objectives:** The aim of this study was to describe the characteristics of the immune response directed against the RVGP delivered by a recombinant SFV system (SFV-RVGP). **Methods:** SFV-RVGP suicidal particles were obtained by co-electroporation of expression and helper plasmids in BHK-21 cells. These particles were further titrated by qRT-PCR and used for the immunization of Balb/c mice in a protocol with one immunization and three booster injections within 14 days. Antibodies against RVGP were evaluated by ELISA (Platellia), and neutralizing antibodies by the FAVN method. Cellular immune response was evaluated by cytokine expression profile (ELISA) and by intracellular cytokine staining after splenocyte stimulation with RVGP. **Results and Discussion:** We found that immunization with SFV-RVGP was able to induce the production of satisfactory amounts (> 0.5 IU/mL) of anti-RVGP antibodies after three immunizations and 21 days, attaining very similar levels of positive control group vaccinated with commercial anti-rabies vaccine. The first results on cellular immune response pointed to a preferential proliferation of CD8+ lymphocytes in mice immunized with SFV-RVGP as compared to mice immunized with anti-rabies vaccine.

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4.43 Expression of α2,6-sialyltransferase genes and enzyme activity in hybridomas producing murine anaphylactic and non-anaphylactic IgG1 antibodies

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Introduction: The *N*-linked glycans in the Fc domain of IgG are indispensable for its interaction with Fcγ receptors on effector cells as described for antibody dependent cell-mediated cytotoxicity (ADCC). Recently, we demonstrated that the sialic acid content in the *N*-linked carbohydrate chain of the Fc region of murine IgG1 monoclonal antibodies is essential for their ability to develop anaphylaxis. Therefore, the evaluation of the expression of the sialyltransferase coding genes during the synthesis of anaphylactic and the non-anaphylactic IgG1 antibodies by the hybridomas should show a quantitative difference in the expression of these genes by B cells. **Objectives:** We tested this hypothesis by examining the expression of the sialyltransferase- coding genes and the enzymatic activity of these enzymes in hybridomas that secret the anaphylactic (U7.6 clone) and the non-anaphylactic (H5 clone) IgG1. In addition, we evaluated if the *in vitro* sialylation of the non-anaphylactic IgG1 antibody allows this molecule to induce *in vivo* anaphylactic reaction **Methods:** The expression of the *ST3Gal II-V, ST6GalNac I-IV* and *ST8Sial I-V* genes was analyzed by real-time-PCR in hybridomas secreting theses two types of IgG1 antibodies. Sialyltransferase activity was measured by specific *Sambucus nigra*-binding-ELISA. In the terminal sialylation *in vitro* assay, the non-anaphylactic IgG1 antibody was incubated with sialic acid and the sialyltransferases obtained from the U7.6 hybridoma. After incubation, the IgG1 antibody was purified by *Sambucus nigra* affinity chromatography and its biological activity and the antigenic affinity were tested by PCA reaction and ELISA, respectively. **Results and Discussion:** The expression of *ST3Gal III* and *IV, ST8 Sial III* and *ST6GalNac I*, *II* and *IV* was higher in hybridomas secreting the anaphylactic antibody rather than the non-anaphylactic IgG1. α2,6-ST activity was significantly higher in the anaphylactic IgG1 producing clone when compared to the non-anaphylactic one (1.2 versus 0.5 D.O/mg of protein, $p < 0.001$). These results showed an association between sialyltransferase gene expression and the sialylation grade of the glycan chain *N*-attached to the murine IgG1 antibodies. In addition, we observed that *in vitro* sialylation of the non-anaphylactic IgG1 leads to its ability to induce anaphylactic reaction. The present study explored the influence of the sialic acid residues on the anaphylactic activity of the IgG1 antibodies.

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4.44 Influence of inflammatory response in the determination of ear tissue regeneration in mice

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Introduction: The biological response to skin injury can be subdivided into two distinct categories, regenerative and non-regenerative types of wound healing. Homozygous AIRmax and AIRmin sublines for *Slc11a1 S* alleles, produced by genotype-assisted breeding, differ in their ability to close ear holes completely. AIRmax^{SS} mice showed faster ear tissue regeneration while AIRmin^{SS} mice did not show regeneration after ear punch. **Objectives:** Our aim in this work was to evaluate the influence of the inflammatory response in the determination of distinct phenotypes. **Methods:** Two-millimeter ear holes were punches in mice of each subline and the inflammatory reaction was characterized by measuring histomorphometric analysis, ear thickness and MPO activity. Gene expression profile analysis during the inflammatory stage of regeneration and wound healing was performed by quantitative PCR experiments. **Results and Discussion:** The local inflammatory response was subtly more intense in AIRmin^{SS} than AIRmax^{SS} mice 48 h after ear punch, which was demonstrated by histomorphometric analysis and elevated MPO levels, suggesting the predominance of neutrophils in the ear of AIRmin^{SS} animals. During this period, the expression profile of pro-inflammatory genes showed high transcripts levels of *Il6, Il1b, Tnfa, Cxcl2, Ccl2, Itgb2, Vegfa* and *Mmp9* in both lines, although slightly but significantly higher levels (>3-fold) were observed in AIRmin^{SS} mice ($P<0.05$). An increase in ear thickness was observed in AIRmax^{SS} compared to AIRmin^{SS} mice. The superior initial inflammatory response in the inflamed ear tissue with higher RNA expression of pro-inflammatory cytokines could inhibit epimorphic regeneration in AIRmin^{SS} mice. These results suggest that the degree of inflammatory response in the early events after injury modulates the quality of regeneration or wound healing.

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4.45 Reactivity of serum IgA and IgG anti-rotavirus serotype G3 in ELISA and immunoblotting assay

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Introduction: Rotavirus is well established as the major etiological agent of diarrhea worldwide. The role of serum antibodies in the immune protection against natural infection is not fully understood. Some studies have correlated serum antibodies against rotavirus in adults with protection and lower probability of infection and illness. Anti-rotavirus IgA and IgG are candidate markers for rotavirus immunity. **Objectives:** Our aim was to analyze the reactivity of anti-rotavirus IgA and IgG antibodies in human serum samples from healthy adult blood donors in immunoblotting (IB) assays. **Methods:** ELISA was carried out for detection of IgA and IgG in fifty serum samples using antigens from the SA-11 rotavirus strain propagated in MA-104 cells. Mock-infected cell preparations were used as control. Individual samples were then titrated including a pool of 50 samples as positive control in every assay. ELISA titer was determined as the reciprocal of the dilution giving an absorbance value of 0.5. The final titer was reported as a percentage, considering the pool as 100%. Rotavirus G3 antigen fractionated by gel electrophoresis (SDS-PAGE) was transferred to a nitrocellulose membrane by a semi-dry system. After transfer, the membrane was cut into 4-mm strips. Each strip was blocked for two hours with buffer containing skim milk; the serum sample was added at the appropriate dilutions and incubated overnight. Afterward, the strips were washed and treated with anti-IgA conjugates labeled with alkaline phosphatase at 1:200 dilution or anti-IgG at 1:2000 dilution. After two hours incubation and a cycle of washes, the strips were revealed with NBT/BCIP (5-bromo-4cloro-3-indolyl phosphate/nitro-blue tetrazolium). The reaction was then stopped with water. **Results and Discussion:** The serum samples recognized protein bands in a range from 80 to 20 KDa.The samples with higher IgG ELISA titers showed stronger bands compared to the ones with high IgA titers. Samples with similar IgG and IgA ELISA titers and samples with higher titers of IgG compared to IgA showed a stronger and more diverse IgG recognition. In some samples, we observed a very different recognition pattern when IgA and IgG were detected. The population studied has levels of anti-rotavirus G3 IgG and IgA antibodies varying over a wide range, perhaps due to different levels of exposure to the virus. The differences between the recognition patterns of IgA and IgG by the same sample suggest that the two antibody isotypes may originate by independent mechanisms.

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4.46 Effectiveness of the Brazilian influenza vaccination policy, a systematic review

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Introduction: Since 1999, Brazil has undertaken annual influenza vaccine campaigns targeting the elderly population, other groups vulnerable to influenza complications and health professionals. Flu vaccine has been provided, free of charge, in national campaigns, which usually have been held in late April/early May. In 1999, the campaign targeted the population over 65 years of age. Since 2000, it has targeted the population over 60. Influenza vaccination campaigns have been regarded as largely successful, reaching high vaccine coverage among the elderly, the largest group included in the initiative. According to Ministry of Health (MOH) figures, coverage has reached 70% of the target among the elderly population in all years, except 2000. MOH does not present figures on vaccine coverage in other groups targeted in the flu vaccine campaigns (health care workers, transplant patients, people living with immune deficiencies, and other groups of high risk patients for influenza complications), because of the alleged lack of denominators for the coverage determination. In spite of such apparent success, little is known about the effectiveness of the initiative. **Objectives:** The aim of this study was to assess the effectiveness of the Brazilian influenza vaccine policy. **Methods:** We conducted a systematic review of the literature to evaluate the effectiveness of the initiative. We used the keywords influenza, vaccine, Brazil and effectiveness, to search the main databases. **Results and Discussion:** We found 380 articles that matched our search criteria. We then analyzed the list, excluding the ones that were published before the beginning of the Brazilian influenza vaccination campaigns (1999), those that were not actually related to Brazil (some referred to the Brazil influenza B viral subtype), and a few others that did not address the issues of interest. A total of 51 papers were selected as potentially relevant. Of these, thirty-one studies met our inclusion and exclusion criteria. Influenza vaccine coverage among the elderly is high, although not as high as presented by the official figures. Effectiveness estimates are scarce. The majority of them come from ecologic studies which show a modest reduction in mortality and hospital admissions due to influenza-related causes. Such reduction is not evident in northern and northeastern states of Brazil, a finding that is probably related to the different seasonal pattern of influenza in equatorial and tropical regions. Brazilian epidemiologists still owe the society better designed studies addressing the effectiveness of influenza vaccine campaigns.

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4.47 Cloning, expression and characterization of *Leptospira interrogans* serovar copenhageni LIC13435 gene

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Introduction: Leptospirosis is a re-emergent zoonosis characterized by an acute febrile and systemic illness in humans caused by pathogenic spirochetes belonging to the genus *Lepstospira*. It is known that leptospirosis is one of the most common zoonotic infections in the world. In Brazil, leptospirosis is an important economic and public health problem. The complete genomic sequence of *Leptospira interrogans* offered a new strategy for the identification of new antigens that could be vaccine candidates, since environmental control measurements are difficult to implement and because there is no available vaccine for human use. **Objectives:** Secreted and surface exposed molecules are potential targets for inducing protective immune responses in the host. Thus, we selected the gene LIC13435 coding for a predicted outer membrane protein for biological characterization. **Methods:** The sequence of LIC13435 gene was selected from the genome of *Leptospira interrogans* serovar Copenhageni using bioinformatics tools. The sequence was amplified by PCR and the expression of the recombinant protein was tested in *Escherichia coli* strains. Purification of the recombinant protein was done by metal affinity chromatography. The antisera were produced by intraperitoneal immunization of BALB/C mice. **Results and Discussion:** Circular dichroism characterized the secondary structure as being mainly composed of α helix. ELISA test indicated that the recombinant protein is very immunogenic. PCR revealed that the LIC13435 gene is conserved among several serovars of leptospira. Indeed, orthologs of this gene can be identified in sequenced genomes of pathogenic leptospiras but not in the saprophytic *L. biflexa*. Preliminary challenge assay against *Leptospira* in hamster indicates that LIC13435 is not protective. Further characterizations are underway.

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4.48 Pilot scale production of yeast-derived recombinant hepatitis B vaccine containing S, pre-S1 and pre-S2 antigens

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Introduction: Hepatitis B is still among the major health threats, accounting for more than 500,000 deaths/year from an estimated 350 million individual sufferers due to complications of chronic HBV infection. Current single antigen vaccines that are produced by recombinant DNA technology are highly effective, but 5-10% or more of healthy immunocompetent subjects do not mount an antihepatitis B surface antibody protective response, while others respond poorly (hyporesponders). Instituto Butantan has produced the recombinant hepatitis B vaccine since 1998, using *Hansenula polymorpha* to express the virus surface S-antigen, HBsAg. In order to enhance the immunogenicity relative to that of conventional yeast-derived vaccines, a new construction containing pre-S1, pre-S2 and S surface components was tested. **Objectives:** The aim of this work was to standardize, on a pilot scale, the production of three consecutive lots of a new recombinant hepatitis B vaccine containing the pre-S1, pre-S2, and S antigenic components. **Methods:** The production process began with the fermentation of producing strain, in a 60-L fermentor, followed by cellular disintegration and several purification steps, including chemical precipitation, salt gradient and gel filtration. The fermentation stages were evaluated for biomass, absence of contamination and expression of the S-antigen, by passive hemagglutination assay (PHA). All purification steps were monitored for total and specific proteins, respectively by OD_{280} and PHA. The final product was submitted to quality control, including Lowry method for protein content, purity evaluation by SDS-PAGE and immunobloting for antigen specific, using anti-HBsAg anti-sera, anti-*Hansenula polymorpha* anti-sera, and pre-S1 and pre-S2 Mabs. **Results and Discussion:** The fermentation process of the new strain was slightly different from that of the process of the yeast expressing only HBsAg, with similar productivity index of 0.80 g/L/H on average, after 144 hour of fermentation. The expression of the antigen tested by PHA was satisfactory with 360 UHA/mL on average. After the purification processes, the final product met the requirements of regulators regarding the purity (>95%) and specificity of antigens. The yield was 100,000 doses per fermentation process (25 μg of HBsAg per dose) and 0.4 g of purified antigens per kg of fermented material. The results showed consistency among the lots produced, giving support to process scale-up.

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4.49 The adjuvant effect of DTP_{low} vaccine in combination with the pneumococcal surface protein A (PspA) antigen: proposal of a new vaccine against *Streptococcus pneumoniae*

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Introduction: *Streptococcus pneumoniae* (pneumococcus) is one of the major agents of respiratory acute diseases, accounting for about 1 million deaths per year, worldwide. PspA is a surface-exposed pneumococcal antigen, shown to elicit protection against animal models of pneumococcal infections, in different vaccine formulations. Due to its variability, PspA is classified into 6 different clades. We have previously shown that the whole cell pertussis vaccine (wP) and the new generation wP vaccine that contains low LPS (wP_{low}) are both able to act as adjuvants, when administered to mice in combination with PspA through the nasal route. Both formulations induce high titers of anti-PspA antibodies and protection against models of colonization and invasive disease in mice. **Objectives:** Here, we studied the role of the toll-like receptor 4 (TLR4), the cellular receptor for LPS, in the adjuvant activity elicited by both vaccines. In addition, we tested the adjuvant effect of the wP_{low} formulated in the DTP_{low} vaccine (Diphtheria – Tetanus –Pertussis) in combination with PspA through the subcutaneous route. **Methods:** C3H/HeJ mice (TLR4-) and the parental C3H/HePas mice were vaccinated through the nasal route with PspA from clade 5 (PspA5) in combination with wP or LPS and then submitted to a respiratory pneumococcal lethal challenge. BALB/c mice were vaccinated through the subcutaneous route with PspA5 combined with DT or DTP_{low} and were also submitted to respiratory lethal challenges with two different pneumococcal strains. In both cases, survival was monitored for 10 days. **Results and Discussion:** Co-administration of PspA5 with wP induced higher levels of anti-PspA5 antibodies in C3H/HeJ and C3H/HePas mice. On the other hand, the combination of PspA5 with *B. pertussis* LPS elicited similar levels of anti-PspA5 antibodies when compared to the protein alone. These results indicate that TLR4 expression is not essential and that other *B. pertussis* components besides LPS are important for the adjuvant effect observed. A single subcutaneous administration of PspA5-DTP_{low} in BALB/c mice also elicited significantly higher levels of anti-PspA5 antibodies when compared to PspA5 alone. This formulation elicited 100% protection against a pneumococcal strain that expresses PspA5 and 66% protection against a strain that expresses PspA from clade 2. Our results indicate that a combined vaccine composed of DTP_{low} and PspA has the potential to induce broad-coverage protection against pneumococcal infections.

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4.50 Cleaning validation of molecular ultrafiltration system used in diphtheria toxoid purification process

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Introduction: The cleaning validation of equipment applied in pharmaceutical production process is an integral part of Good Manufacturing Practices (GMP) meeting the national requirements (ANVISA) and WHO recommendations. Cleaning validation procedures are carried out to assure that residues are within acceptable limits after the cleaning process. Diphtheria toxoid, obtained through the detoxification of diphtheria toxin, is purified in the Center for Purification of Bacterial Products at Butantan Institute. In order to obtain a product with high purity the diphtheria toxoid is purified in two steps: the first consists in diafiltration and concentration by molecular ultrafiltration system using Pellicon® equipment, and in the second step the product is precipitated with ammonium sulfate and then diafiltered and concentrated using the same equipment. The purified product after approval in quality control test is denominated Diphtheria Toxoid Final Bulk, which is a component of combined vaccines: dT, DT, DTP and DTP-Hib. **Objectives:** The aim of this study was to validate the cleaning procedures of molecular ultrafiltration system providing documented evidence that the process is consistent. **Methods:** Three consecutive applications of the cleaning process in the molecular ultrafiltration system were perfomed after diafiltration and concentration of diphtheria toxoid. The cleaning process of equipment was conducted using sodium hydroxide, phosphate buffer, pH 6.4, purified water and formaldehyde solution. Samples of critical steps of the cleaning procedure were collected and submitted to the following tests: Bioburden test, pH, conductivity, total organic carbon analysis (TOC), protein determination by Lowry method and residual formaldehyde test. **Results and Discussion:** The results of Bioburden in three tests before and after using equipment were 0 (zero) CFU/50 mL, showing that the cleaning procedures were efficient. The averages of TOC in initial and final samples were 653.67 ppm and 344.00 ppm respectively, indicating a considerable reduction of total organic carbon. All results obtained in other tests were according to the established limits. The cleaning procedure of the molecular ultrafiltration system was demonstrated to be effective and reproducible, allowing the validation of the method. This cleaning validation process will be implemented for other equipment used in the diphtheria toxoid purification process at Butantan Institute.

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