ABSTRACT

On May 14, 1796 Edward Jener an english country doctor from Gloucestershire, the given world's first vaccine to a child of eight named James Phipps who was ill with smallpox and thus was born the first human vaccine and the first effective immunization.

Pasteur's first major breakthrough in the field of immunization took place in 1879 and is linked to avian cholera, he observe that repeated passages the bacteria lose their pathogenicity in birds and inoculating these cultures created immunity against wild bacteria. Studies as subsequently developed by Pasteur in vaccinology field were based on this principle.

Currently, vaccination is practiced worldwide for a variety of ailments preventing 2.5 million deaths annually in all age groups, but still has more than 1.3 million newborn deaths, especially in poor areas of the Earth. Today, more than 80 candidate vaccines are in the last phase of clinical trials and about 30 of them are designed to provide significant protection against diseases for which a vaccine not yet registered, such as Dengue and malaria.

Rabies is a serious zoonosis, spread on all continents except Antarctica, being encountered in over 150 countries and territories. In the world die annually from rabies more than 55,000 people and 40% are children under 15. The main source of illness in 99% of cases of human rabies is dog. Annually, over 15 million people receive special treatment after being bitten and this prevents another 327,000 deaths.

From Pasteur to the present rabies vaccine production technologies have evolved tremendously with the benefits of biotechnology development. For rabies immunization of domestic animals is used inactivated vaccines. These vaccines have different degrees of purity and may occur after vaccination neurological accidents wich are caused by the presence of whole viral particle, or incomplete inactivation or adjuvants used.

To overcome these drawbacks have been researched and have been tested subunit vaccines, why not use whole viral particles and vaccines that use only the viral genome and

recombinant vaccines. All these new types of vaccines have been shown to be at least level research, an alternative to conventional vaccines.

Moreover, the global economic crisis sluggish for some time is needed vaccines have a low production cost as yet to solve old problems with accidents after vaccination. To achieve this wish we resorted to the production and testing of vaccines vector obtained by recombinant DNA technology.

Adenoviruses have some important properties for their use in gene therapy, mainly due to very broad host spectrum, being able to infect a wide range of cell types, does not integrate into the genome of infected cells. For these reasons adenoviruses have been used to transfer genes "*in vivo*" were developed adenoviral vectors that incorporates various different genes of interest (B-gal, OTC, cytokines, and so on).

Animal adenoviruses are unable to propagate in human cells that have been tested, revealing that these types of adenoviruses are not complementary, thus eliminating the risk of recombination and propagation "*in vivo*".

Most often, in research are used standardized trunks of canine origin type CAV-2, such as Manhattan or A26/61 (ATCC VR-800). In general, heterologous DNA sequence that will be attached to the CAV-2 can be represented by: gene encoding the protein product, which encodes a mutant genes of cellular proteins, enzymes, derived blood lymphocytes, interleukins, growth factors, neurotransmitters or their precursors and various antigenic peptide. The main goal is to obtain recombinant pharmaceutical compounds may be formulated for administration by topical, oral or parenteral.

In this paper are presented scientific research that consisted of providing an alternative to classical rabies vaccines namely the development and construction of a rabies vaccine produced by recombinant DNA technology using the canine adenovirus type 2 vector and transgene of interest glycoprotein G as virus rabies. This innovative product has been tested in three species of domestic animals namely domestic cats in Landrace-Large White-Pietrain hybrid pigs, and rent horses and the results were encouraging.

PhD thesis titled "***Research on the use of CAV-2 adenoviral vector in immunoprophylaxis of infectious diseases***" covers 237 pages and according to the regulations in force at present it consists of two main parts, namely: the first part entitled "***Stage actual***

knowledge" that includes 48 pages, 10 tables and seven figures and the second entitled "***Personal contributions***", spread over 137 pages, 46 tables and 73 figures.

"***The current state of knowledge***" includes three chapters where are succinctly information in the literature regarding the subject thesis and were subsequently used for the interpretation and comparison of data obtained in the "***Personal contributions***".

The first chapter, entitled "***Aspects literature on canine adenovirus type 2 (CAV-2)***" is divided into five chapters and summarizes the literature on adenovirus capsid structure of canine CAV-2, viral genome organization and methods of networking with the host cell. This chapter deals with the use of CAV-2 as a vector in vaccinology and gene therapy.

Chapter entitled "***Rabies virus and rabies immunoprophylaxis animal***" in his first chapter deals with the etiology of rabies, describing aspects of viral particle morphology, chemical composition, its viral proteins and genome organization as taxonomic classification according to the International Committee of Viral Taxonomy (ICTV) in 2009. The last chapter deals with the rabies immunoprophylaxis, starting with the pioneering period in rabies immunization when, in 1884, Pasteur with E. Roux and Chamberland Ch developed the first vaccine against rabies in animals, using a strain of rabies virus from cow and attenuated pathogenicity and human rabies immunoprophylaxis was born Monday, July 6, 1885, when the Pasteur came a child of nine, in Alsace, named Joseph Meister, who had been bitten by a rabid dog on July 4th.

Then synthesized information on rabies immunization in the contemporary period, from the first generation of vaccines prepared from nervous tissue of adult animals, the preparations embryonated eggs or baby animal nervous tissue, continuing then with rabies vaccines produced in cell culture and ending with the third generation of vaccines against rabies consisting of vaccines produced in cell culture heteroploide Vero line type. The last part of this chapter deals with current prospects in rabies immunization, producing vaccines aimed at low-cost but that will not cause accidents after vaccination such as vaccines using whole viral particle.

After the first two chapters were presented bibliographic information on vaccine vector (CAV-2) and the gene of interest (glycoprotein G of rabies virus), in Chapter III, entitled "***Recombinant DNA Technology***" is presented the technology has been used for the production of rabies vaccine vector. For starters was mentioned some elements used in the technology and

then was presented the stages of development of a product used by this technique.

The "***Personal contributions***" begins with Chapter IV entitled "***Design and construction of a new rabies vaccine based on recombinant adenoviral vector CAV-2***". In this chapter we used the choice, production and purification of recombinant antigens with potential vaccine against rabies, which led to the construction and production of adenoviral CAV-2vectors, able to express new immunogenic antigens.

This goal was achieved in several distinct phases using molecular biology techniques specific recombinant DNA technology. Originally vector was constructed using molecular cloning and homologous recombination was done in order to obtain a plasmid containing adenoviral canine type 2 vector, which is integrated transgene of interest (rabies glycoprotein G / GFP) with conversion kit DHCα Invitrogen product. The transfection was successful introduction of recombinant CAV-G vector in DK-cré canine cell line using two different transfection kits namely Lipofectamine from Invitrogen and jetPrime from PolyPlus Transfection. For each transfection kit were used two concentrations of DNA (3µg and 5µg) and observed after 48-72 hours, using fluorescence microscope Olympus U-RFL-T, the occurrence of transient GFP expression. After 48 hours of transfection, using classical optical microscope, we have seen the appearance of characteristic cytopathic effect in adenoviral infections. Between the two transfection methods used have not noticed major differences in terms of effect citopatogen, but differences in the intensity of ECP were observed between the two concentrations of DNA used for transfection.

After transfection of DKcré canine cell line was characterized recombinant vaccine vector, after which he moved to his production in bioreactor systems.

After amplification process of CAV-G/GFP recombinant vector was purify the particles obtained simultaneously eliminating artifacts appeared and performed a stability test vectors obtained using CsCl method. Since purification method has some drawbacks, dialysis was performed of vector volume obtained after purification with CsCl, using PD10 columns from GE Healthcare Amersham and was recovered a quantity of 1.5 ml of virus CAV-G purified and dialyzed.

Titer of infectious particles suspended in TCID50%/ml was 10 x $10^{8.6}$ after use mathematical formula developed in 1939 by Reed and Muench. After the titrage by RT PCR was

found that 2 ml of virus represents a concentration the infectious particles $10^{10.4}$. If we consider the dilution factor, molecular weight of 2.3 x 10^7 Da and that of adenovirus proteins account for 87% of the total mass of CAV-2 virus, was obtained at a concentration of recombinant virus DO_{260} CAV-G of 1.1 x 10^{12}, amount corresponding wild strain of CAV-2.

In Chapter V entitled "***Testing rabies vaccine using live CAV-2 vector replicative and non-replicative in domestic cats***" experimental evaluation was performed immunogenic properties of interest induced by glycoprotein G of rabies virus from domestic cats and check the answer given intensity body to vector inoculated animals tested (CAV replicative and non-replicative).

In the experiment we used 20 domestic cats (11 males and nine females that are between one and four years of age, with a body weight ranging from one kilogram up to four kilograms) were distributed in 10 groups with two cats / group. Eight groups of cats were vaccinated with CAV-G vector vaccine (groups 1-4 received a dose of CAV-G^+ concentrations from 10^4TCID_{50}/ml to 10^7TCID_{50}/ml and groups 5-8 received a dose of CAV-G^0 concentrations from the 10^5TCID_{50}/ml to 10^8TCID_{50}/ml), group 9 (cats 17 and 18) were immunized with inactivated rabies vaccine from Intervet Nobivac Rabies and the last group (cats 19 and 20) have not been vaccinated against rabies. 200 days after the first vaccination was used in the first eight groups revaccination cats (cats 1-16) using the same type of vaccine and the same dosage.

Serological examination results using Platelia Rabies II ELISA after the first rabies vaccination at the end of the experiment are protected against wild virus only cats in groups vaccinated with the highest dose of vector CAV-G replicative and non-replicative namely cats 7 and 8 who received a concentration of 10^7TCID_{50}/ml CAV-G^+ and cats 15 and 16 that received a concentration of CAV-G^0 10^8TCID_{50}/ml vector. By comparison, it appears that specific antibody response in S_8 is more intense in the group of domestic cats vaccinated with commercial product Rabies Nobivac than domestic feline groups vaccinated with CAV-2 vector.

Serological examination results using ELISA anti-CAV-2 showed the higher titers in animals immunized with the highest concentrations of vaccine vector, which shows that the vector used remained stable after intramuscular administration to domestic cats.

After conducting viral neutralization test for CAV-2, there is a stronger response in cats vaccinated with vaccine vector defective for replication and one explanation would be related to

higher concentrations of vector R^0 (10^8TCID$_{50}$/ml than 10^7TCID$_{50}$/ml).

The results obtained after the booster vaccination at 200 days showed that, overall, revaccination with the same vaccine vector, with the same dosage, leads to a stronger immunization compared with a single administration and may represent in future a viable solution for rabies immunization of domestic feline, current results are promising.

In Chapter VI entitled "***Testing CAV-2 adenoviral vector in equine rabies immunoprophylaxis***" was tested the effectiveness of rabies vaccine produced by recombinant DNA technology in equine animals raised under their rent.

In this experiment we used 11 equine (six males and five females) aged between one and 10 years, were divided into three groups: group I (horses 1-5) received rabies vaccine replicative recombinant CAV-G at a dose of 4 ml, which has a concentration of 10^9TCID$_{50}$/ml and receiving two separate points cervical musculature, group II (horse 11) served as negative control in the experiment and group III (horses 7-11) was immunized with commercial preparation Nobivac from Intervet. Samples were collected weekly for seven weeks after the last harvest was performed on day 112 from the beginning of the experiment.

Following dosing of biochemical markers (AST, ALT, serum albumin, creatinine, total protein) for 14 days from the beginning of the experiment, it was found that CAV-G vaccine vector that has a concentration of 10^9TCID$_{50}$/ml, when administered intramuscularly in equine what are aged between 1-10 years do not show cytotoxic activity.

According to the results obtained from the use of Platelia Rabies II ELISA on serum samples from 112 days (S_{16}) after administration of the two types of rabies vaccines, none of the tested animals developed a specific humoral response to a level that would provide protection against wild virus (0.5 IU/ml) however observed that horses vaccinated with CAV-G^+ immune preparation have specific seroconversion rate much higher than those immunized with commercial preparation Nobivac Rabies.

A complementary method for Platelia Rabies II ELISA usum verinarum reaction used in this experiment was that viral neutralization test was performed in the Laboratory of rabies and wildlife from Nancy (France), which is collaborating center of the World Health Organization providing scientific expertise on rabies control methods in animals. This laboratory it is the French national reference laboratory for rabies and OIE reference laboratory for rabies and

actively participates in the development and standardization of diagnosis.

In case of using viral neutralization test that deliver occurrence of rabies protection (0.5UI/ml) from day 28 of the experiment but falls below the protection at the end of the experiment. If we compare the two types of vaccines used we can say that the group of horses receiving $10^9 TCID_{50}$/ml of CAV-G develops a stronger response than the product Nobivac Rabies vaccine group, as confirmed from the use of Platelia ELISA.

In order to evaluate the activity of cellular immunity effectors was appealed to a quantitative serological method that detects levels of serum IFNγ equine was rabies vaccinated using two types of immunological prepared, knowing that IFNγ are markers of cellular immunity. But the results showed that serum levels of IFNγ remains constant during researches, he restricted limits ranging from 1 pg/ml to 1000 pg/ml. So, we can say from the present experiment that does not change the level of IFNγ after inoculation of inactivated rabies vaccine or a vaccine produced by recombinant DNA technology and using canine adenovirus type 2 (CAV-2) as a vector.

Was assessed quality of specific humoral response anti-CAV 2 using virus neutralization test to show anti-adenoviral kinetic response that was developed equine group vaccinated with this vector and confirmed or refuted the efficacy of such a vector when it is used equine. The results obtained showed an ascending kinetics for anti-CAV 2 starting with day 14 post-administration, demonstrating that penetrated into the recipient vector that was injected where the message has multiplied and genetically, but this in latter was subliminal intensity.

Following research in this experiment, vaccination of horses with a commercial product as is the case Nobivac Rabies preparation or with either CAV-G vector vaccine will not protect against field virus following a single administration, but CAV-2 vector can be used successfully in other biomedical applications as witnessed by the high titers obtained from virus-neutralization test.

The most comprehensive research paper is presented in Chapter VII entitled "***Testing CAV-2 adenoviral vector in swine rabies immunoprophylaxis*** ", which was verified immunogenicity and quality of humoral and cellular immune response following administration of CAV-2 adenoviral vector vaccine that expressing glycoprotein G of rabies virus in pigs.

In this experiment we used 11 crossbred Landrace-Large White-Pietrain pigs, (six males

and five females, aged eight weeks), who were divided into three groups as follows: group I (pigs 1-5) was immunized with rabies vaccine replicative recombinant CAV-G at a dose of 2 ml, with a concentration of 10^8TCID$_{50}$/ml, which was administered intramuscular in two separate points into the buttocks muscles, group II (pigs 6-10) was immunized with inactivated rabies vaccine Nobivac Rabies trade at a dose of 1 ml, with a concentration of 2 IU/ml, which was administered intramuscular into the buttock muscle in a single point (T^+) and group III (pig 11) was used as a negative control. Sampling was done weekly for 56 days (S_0-S_8).

Following assessment and dosimetry of clinical, hematological and biochemical parameters can say that the intramuscular administration of vector 10^8TCID$_{50}$/ml CAV-G hybrids Landrace-Large White-Pietrain pigs, not show cytotoxic activities against them, particularly important in further research.

In the first part of this chapter has conducted a quantitative assessment of rabies humoral response by Platelia Rabies II ELISA and virus neutralization reaction. Results provided by Platelia Rabies II ELISA showed that pigs were vaccinated with rabies vaccine had in S_8 recombinant replicative the highest rate of seroconversion (2-4 IU/ml), while pigs were immunized against rabies using inactivated commercial vaccine resulted in S_8 specific humoral immune response unsatisfactory and incomparably lower (0.25-0.125 IU/ml).

Serum neutralization test results conducted in the laboratory of Nancy (France) were similar to those obtained ELISA Platelia Rabies II, with only a slightly reduced amplitude, neutralization test is, however, a method that is more precise quantification than ELISA where use DO$_{450}$ values read at the end of the reaction.

Next, to identify, quantify and interpret anti-CAV2 virus neutralization test was used to give specific results and high sensitivity. Intensity of antibody responses anti-CAV-2 is directly proportional to the intensity of rabies antibody levels throughout the experiment.

Because CAV-2 vector is stable and does not disintegrate in the host organism, there is a specific humoral response in the host that results in synthesis of antibodies specific for the vector and transgene of interest, which protects rabies in animals dosed . Reveals that there is no anti-CAV-2 in sera collected from pigs vaccinated group Nobivac Rabies or witness indicates that pigs subjected to experimental canine adenovirus are not carriers or other type of adenovirus that could give cross-reactions.

The last part of this chapter has made an assessment and quantification of cell-mediated immune response by ELISpot test and the use Luminex platform.

ELISpot assay was validated by reference to intrinsic factors and was performed on day 14, 21, 28 and 42 of the present experiment. It is worth mentioning that when using the ELISpot technique using some cell stimulants that are compatible with the structure of the antigen used (CAV-G vaccine and inactivated vaccine Nobivac Rabies). Thus, the two vaccines there is a homolog of glycoprotein G level with the same antigenic structure, being common to both products used. Classics stimulants in such a test are represented by specific antigenic peptides, but because the transgene of interest -glycoprotein G of rabies virus is very long (~ 500 aa.) and its homologous peptides should be many, their use almost impossible.

Also, if we refer to immunological epitopes, there is research on pigs were used in this experiment (we used pigs from an farm, animals with origin not known, are not special lines, "*pathogen free*" or inbred varying degrees). Had epitopes characterized this type of pork then you could purchase individual peptides to it, but as they are not then not achieved this goal.

An alternative to the use of peptides in antigenic stimulation is the use of vector. Once immunized animal, its T cells will recognize antigen initially a consecutive stimulation "*in vitro*" in ELISpot assay.

After testing by ELISpot method hybrids Landrace-Large White-Pietrain pigs subject experiment gave satisfactory results, which shows an increase in the level of IFNγ, so a response from specific cell-mediated immunity.

The cytokines in pigs play an important role in modulating physiological and immunological processes to maintain homeostasis. For this purpose we used the determination of cytokines by Luminex technology to see if there are variations of these cytokines after administration of the two vaccines or kinetics of these compounds differs depending on the type of vaccine administrated . Thus, was therefore conducted dosage IFNγ, IL10 , IL4, IL6 and IL8 using serum collected weekly from pigs subjected to experiment.

If we analyze the overall level of cytokines in the group of pigs that were inoculated with 2 ml of vector CAV-G, which has a concentration of $10^8 TCID_{50}$/ml, it appears that during the experiment the IFNγ, IL10 and IL4 of kept relatively constant. If IL6 observed high levels on day 0 after which it gradually decreases to reach a constant level on days 28 and 42 of the

experiment. Similarly if there is an increase IL8 above average at day 14 after which levels gradually decrease.

A constant level of cytokines registered in the group of pigs vaccinated with CAV-G vector is explained by the fact that the mechanism which causes rabies specific antibodies is different vector entering directly into the host cell where genetically send the message that will generate antibody synthesis.

If we analyze cytokine levels in the group of pigs immunized with Nobivac preparation throughout the experiment we can see that the cytokine dosage is increased at day 14 compared with rest periods analyzed.

If this immunogenic product should be determined strong rabies response that ensures protection against wild virus, then surely cytokine levels were much higher and some clear trend, but without installing protective immunity is found only higher levels at 14 days post-administration which then decreases. Cytokine determination by comparing the rabies antibody response when the same trend is observed with a weak peak, amplitude free in day 14 followed by a downward trend until the end of the experiment.

As with any work of nature scientifically, the last chapter is "***Conclusions and Recommendations***" which summarized a total of 24 conclusions and recommendations that result from this work.

Keywords: **CAV-2, rabies, vaccine, DNA recombinated technology, immunity.**