GENERATING THE FIRST ANTIBODY FOR AN ENZYME IMMUNOASSAY DEDICATED TO SERUM PROGESTERONE DETERMINATION. EVOLUTIONS OF TOTAL PROTEINS, ALBUMINS, GAMMA GLOBULINS AND HAEMATHOLOGICAL PARAMETERS SUBSEQUENT TO RABBITS IMMUNIZATION USING Progesterone 3-(O-carboxy-methyl) oxyme (SIGMA)

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Summary

The immunosorbent assays, or assays using antibodies as specific bounding reagents are usual methods in research labs (Tetin and Stroupe, 2004). In an ELISA, the antibody which binds to the antigen (the primary antibody) should be from a different species to the conjugated (secondary) antibody used in the next step of the assay. In the present work are described the results obtained in the process of generating the first antibody (directed against progesterone) on rabbit, as an element of the sandwich-type immunoassay we do intend to develop for serum progesterone assessment. Two groups consisting of three female rabbits each were immunized against progesterone BSA oxyme, using two different protocols, based on the same schedule: first administration represented by antigen and Freund`s Complete Adjuvant and four boosters, represented by antigen and Freund`s Incomplete Adjuvant. Total proteins, albumins and gammaglobulins were assayed for immune response characterization. The initial immunization was followed by an increase of total protein levels and of gammaglobulins (significant for both G1 and G2), accompanied by a decrease of albumins. Consequent boosters, all values returned near basic levels, for all investigated parameters (excepting gammaglobulins which remained high), in both groups. For both groups, total protein, gammaglobulins, albumins and hematological picture ranged in normal field during entire duration of study. Variations of total protein, gammaglobulins, albumins and hematological picture subsequent to four immunizations could represent a mark of immune response in rabbit.

The immune assays do represent –in theory and practice- a firm soil, the intimate description of entire process allowing peculiar approaches, regarding the objectives to be followed.

The immunosorbent assays, or assays using antibodies as specific bounding reagents are usual methods in research labs (Tetin and Stroupe, 2004).

ELISA (enzyme linked immunosorbent assay) combines antibodies specificity with the sensitivity of simple enzyme reactions, using antibodies or antigens bound to an easy quantifiable enzyme.

In an ELISA, the antibody which binds to the antigen (the primary antibody) should be from a different species to the conjugated (secondary) antibody used in the next step of the assay.

One of the most used immunoassay is the sandwich type, using two antibodies. The reaction is used for assaying antigen concentration in the unknown sample. It is a rapid and accurate reaction, and if exists a standard purified antigen, the reaction is able to determine the absolute antigen quantity in the sample. The sandwich reaction needs two antibodies which do not bind the same epitope. This aspect can be achieved using even two monoclonal antibodies, or a mixture of purified polyclonal antibodies. In this purpose, one of the antibodies will be purified and than bind to the solid phase. Than the antigen is added, allowing the formation of antigen-antibody complex. The unbound elements are discarded through simple washing and than the marked antibody is added; the last antibody will couple the antigen too, completing the sandwich. The reaction is quantified through measuring the volume of the second antibody bind to the antigen, reaction which is assayed through a colorimetric substrate. The major advantage of this technique is that the antigen should not be purified before using, thus generating exact results.

The major problem of any enzyme immunoassay is represented by obtaining the first and the second antibody, existing a wide variety of immunization schedules. in the next step of the assay.

Essential factors to be considered in the preparation of an immunization are represented by selection of an adjuvant, the route and volume of injection, and the immunization schedule.

Adjuvants are used to enhance the immune response. The ideal adjuvant can be characterized as a substance which stimulates high and sustainable antibody titres (even with small quantities of antigen), is efficient in a variety of species, applicable to a broad range of antigens, is easily and reproducibly prepared in an injection mixture, is easily injectable, is effective in a small number of injections, has low toxicity for the immunized subject, and is not harmful to the investigator.

In the present work are described the results obtained in the process of generating the first antibody (directed against progesterone) on rabbit, as an element of the sandwich-type immunoassay we do intend to develop for serum progesterone assessment.

Materials and methods

There were used three female rabbits (common breed) for group 1 (A, B and C) and also three for group 2 (females D, E and F), separately housed, fed at libitum. The females were dewormed using Dimerasol and were vaccinated against myxomatosis and haemorhagical diseas, 60 days before first immunization. Females were injected with *Progesterone 3-(O-Carboxymethyl)-oxime BSA fluorescein isothiocyanate conjugate* (SIGMA Aldrich P8779), mixed with Freund`s

Complete Adjuvant (FCA) for the first shot, than with Incomplete Freund`s Incomplete Adjuvant (FCI) for boosters, as mentioned in table 1. As long as progesterone molecule is no table to generate alone an immune response, it was necessary its binding to a carrier protein, represented in our case by BSA (bovine serum albumin).

In rabbit, the antigen and adjuvant volume administered suncutaneously in a single place should not excede 0.25 ml, being allowed no more than four sites for injections (IACUC GUIDELINES, 2002).

For immunization of female in group 2 we have used the protocole recommended by Szafranska et al (2002), as long as in group 1, we used the schedule and doses which we considered to be optimal.

Table 1.

Immunization schedule in rabbit

	12th of June 2006	10th of July 2006	24th of July 2006	7th of August 2006	21st of August 06
Group 1	100 µg + FCA	50 µg + FCI	50 µg + FCI	50 µg + FCI	50 µg + FCI
Group 2	300 µg + FCA	150 µg + FCI	150 µg + FCI	150 µg + FCI	150 µg + FCI

For immunization of female in group 2 we have used the protocole recommended by Szafranska et al (2002), as long as in group 1, the schedule and doses wich we considered to be optimal. Blood was sampled two weeks before first immunization (considered as initial value, for control – sample 1), after the first immunization (antigen plus FCA – sample 2), after first booster (antigen plus FCI – sample 3), and at the last booster (antigen plus FCI – sample 4). Total proteins, albumins and globulins were assayed through Beckman capillary electrophoresis, on Paragon CZE 2000, performed at “Bioclinica Laboratories” Timisoara, and expressed in g/l. Haematological profile was determined on Vet Screen, assays being performed in the clinical laboratory of the Faculty of Veterinary Medicine Timisoara.

Immunized females were closely observed for potential side effects of adjuvants, concerning general status and local reactions.

Results and discussions

All the results are included in table 2 and 3. For the studied parameters, referece values were obtained from BSAVA Manual for Exotic Pets – fourth edition, (2002). Subsequent to first immunization (antigen plus FCA), a rise in total proteins was registered in both groups, but all registered values ranged in physiological limits.

Table 2.

Average values for total proteins, albumins and gamma globulins in rabbits

	Total proteins (g/l)				Albumins (g/l)				Gamma globulins (g/l)			
	S1	S2	S3	S4	S1	S2	S3	S4	S1	S2	S3	S4
Group 1	61.53	64.46	61.36	62.33	44.46	46.23	43.33	43.16	6.1	6.63	6.4	6.56
Group 2	62.2	64.2	62.83	63.2	44.83	44.4	43.9	44.06	5.26	5.83	6.16	5.83

Table 3.

Average values of WBC and lymphocytes in rabbits

	WBC (m/mm^3)						Lymphocytes (%)					
	Normal range	S1	S2	S3	S4	Normal range	S1	S2	S3	S4
Group 1	4.3 – 10.6	6.0	5.77	6.75	6.22	22 - 63	50.3	57.9	54.7	55.3
Group 2	4.3 – 10.6	7.37	7.50	7.21	7.18	22 - 63	46.9	47.0	45.8	42.2

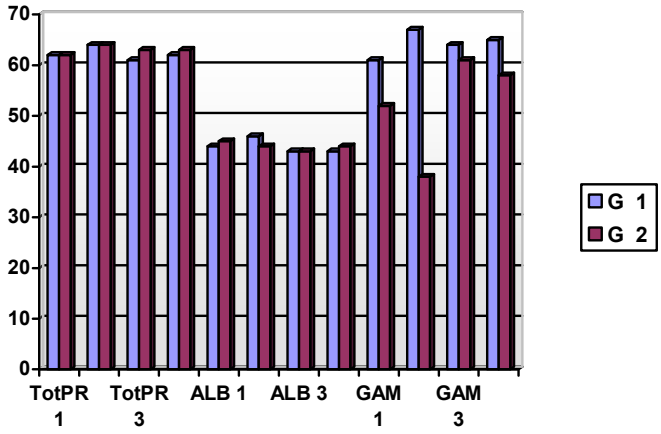
As long as subsequent to all boosters the total protein average levels decreased, we do hypotesised that Complete Freund`s Adjuvant induced the rise of total proteins. After the final booster, total protein levels remained above the initially values, although in normal limits. It has to be mentioned the significant rise of total protein level in G 1 ($p<0.05$), compared to G 2, representing an interesting fact, as long as in G1, the antigen dose was three times less than in G2. As long as the initially rise was followed by a decrease in both groups, it might be supposed that Freund`s Complete Adjuvant composition would decisively influence the level of serum total proteins. The suspension of *Mycobacterium tuberculosis* might be responsible for attracting macrophages and other cells to the injection site, thus enhancing the immune response (Stills and Baily, 1991).

The boosting protocol can have a decisive influence on the result of the immunization. The time between two immunization steps can influence both the induction of B memory cells and the class switch of B cells (from IgM to other antibody classes and subclasses). Specific recommendations for the interval between primary and booster immunizations are usually not cited. In general, a booster can be considered after the antibody titre has plateau or begun to decline. If the first immunization is performed without a depot-forming adjuvant, the antibody titre will usually peak 2 to 3 weeks after immunization.

When a depot-forming adjuvant is used, a booster injection can follow at least 4 weeks after the first immunization.

Graph. 1.

Average values of total proteins (TotPR g/l), albumins (ALB g/l) and gammaglobulins (GAM g/l), for all samplings (1, 2, 3 and 4), for G1 and G2 groups. In order to obtain an homogeny image, gammaglobulin values were multiplied by ten (e.g. instead of 6.1 g/l, was considered 61 g/l)



The long immunization schemes, with repeated boosting, not only result in the production of antibodies with increased affinity for the antigen of interest, but also in the production of more antibodies specific for contaminants in the antigen preparation. Such multispecific antisera require absorption before they are monospecific, a process with some inherent difficulties.

Primary injections with very low amounts of antigen (picograms) are not recommended, since this does not stimulate the immunologic memory sufficiently, and might induce tolerance to the antigen. Frequent immunisations with relatively low amounts of antigen can be counterproductive. In addition,

animal welfare argues against such schedules. However, low amounts of antigen

for booster immunisation may help raise the average affinity of the antibodies subsequently produced (Marlis Leenars et al., 1999).

In our work, gammaglobulins levels followed a similar pattern to that one registered for total proteins. In both G1 and G2, differences were significant ($p<0.05$) compared to initially values, and continue to rise after the first booster, too. Even after the final booster, gammaglobulins level remained high compared to initially values, in both groups.

Albumins followed a different pattern as reported by Bjorneboe and Jarnum (1981), who reported a fall of albumin subsequent to hyperimmunization in rabbits. In group 1, albumin level rised after first immunization than slightly decreased, whereas in group 2, their average level remained almost the same during entire period of observation.

Graph 1 allows an assembly image of all registered changes during experimental period, in both groups.

Supposing we could identify any change in blood picture, we also assayed haematological parameters, but all investigated parameters ranged in normal limits. As an example we put together average values of WBC (white blood cells) and lymphocytes in table 3, in order to emphasize that none significant change did not appear. In some cases, as a response to foreign antigen, reactive lymphocytes account for a substantial proportion of the total lymphocytes present in blood, but absolute lymphocitosis is uncommon. Vaccine reaction in young dogs is an instance in which reactive lymphocitosis may be encountered (Meyer and Harvey, 2004).

Conclusions

The initial immunization was followed by an increase of total protein levels and of gammaglobulins (significant for both G1 and G2), accompanied by a decrease of albumins.

Consequent boosters, all values returned near basic levels, for all investigated parameters (excepting gammaglobulins which remained high), in both groups.

For bought of the groups, total protein, gammaglobulins, albumins and hematological picture ranged in normal field during entire duration of study.

Variations of total protein, gammaglobulins, albumins and hematological picture subsequent to four immunizations could represent a mark of immune response in rabbit.

Acknowledgments

The results presented in this paper were obtained through CNCSIS grant nr. 996/2006

The following colleagues had a great contribution in solving all problems generated during animal housing (Bogdan Tau and George Aldea), immunization (Ianos Degi, Gabriel Otava and Ilinca Frunză), blood sampling (Doru Morar and Aurel Sala) and haematological exams (Doru Morar).

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