DEVELOPING AN ENZYME IMMUNOASSAY FOR SERUM PROGESTERONE DETERMINATION. EVOLUTIONS OF TOTAL PROTEINS, ALBUMINS, GAMMA GLOBULINS AND HAEMATHOLOGICAL PARAMETERS SUBSEQUENT TO SHEEP IMMUNIZATION USING RABBIT IgG AGAROSE (SIGMA)

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Summary

Serum progesterone level represents a crucial marker for reproduction, indicating pregnancy, infertility or other reproductive pathological conditions. There are several ways for progesterone assaying, between them immunoenzyme assay being the most appropriate, because fulfils sensitivity, expenses and equipment requirements. In this paper we are describing the results obtained in the process of generating the second antibody on sheep, useful for developing our progesterone immunoassay. Two groups consisting of three ewes each were immunized against rabbit gammaglobuline, 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 (significant for G1) and of gammaglobulins, accompanied by a decrease of albumins. Consequent boosters, all values returned near basic levels, for all investigated parameters, in both groups. For both 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 sheep.

Serum progesterone level represents a crucial marker for reproduction, indicating pregnancy, infertility or other reproductive pathological conditions. Nowadays no infertility or theriogenology diagnosis can not be defined without considering progesterone values.

There are several ways for progesterone assaying, between them immunoenzyme assay being the most appropriate, because fulfils sensitivity, expenses and equipment requirements.

Due to increasing relevance of progesterone assay for describing clinical aspects of fertility and infertility in domestic animals (Arthur et al., 1989; Hafez, 1993) or in research (Isobe and Nakao, 2003) , most researching labs are producing their own components in order to run their own immune assays for

progesterone (P4) determination. In practice are wide spreaded antibody binding (direct or indirectly enzyme immuno assays), or the second antibody technique (the so called sandwich reactions) on solid support.

The wide accepted way of producing antibodies is through repeated immunizations, resulting serum polyclonal antibodies (Ziecik et al., 1979; Szafranska and Ziecik, 1989). The repeated immunization technique allows obtaining polyclonal antisera (Szafranska et al., 2002), raised against some steroids and proteal antigens such as 17beta estradiol, estrone, testosterone, androstedione, cortisol, corticosterone, LH or pregnancy associated proteins (PAG). All serum described in the last quoted paper are suitable to be used in various techniques as radioimmunoassay, enzyme immunoassay or Western blot.

The advantages of polyclonal antibodies are:

Often they do recognize more epitopes, making them more tolerant to alterations in antigen's nature. The polyclonal antibodies do often represent the solution for detecting the denatured proteins. Polyclonal antibodies can be raised in rabbit, goat, sheep, donkey, chickens, allowing a wide variety for experimental design.

In this paper we are describing the results obtained in the process of generating the second antibody on sheep, useful for developing our progesterone immunoassay.

Materials and methods

The sheep were housed together, fed ad libitum. The two groups consisted of three sheep each: G 1 (sheep A, B and C) and G 2 (sheep D, E and F). Sheep were dewormed and injected against anthrax and anaerobioses with 90 days prior to first immunization. For each antigen does exist an optimal dose, the so called "immunological window" (Eide and Engh, 1995). If the doses are too low or too high, immunotolerance or immunosuppression could be induced. The antigen has to be administered in the appropriate place, in order to reach the nearest limphnode, or the spleen.

It was used Rabbit IgG – Agarose Purified Immunoglobulin (SIGMA A2909), together with Freund`s Adjuvant Complete (FCA), SIGMA F5881 for the first immunization, or with Freund`s Adjuvant Incomplete (FCI), SIGMA F5506, for the next four boosters. The mixture was intradermal administered, in 10-12 different injection sites, 4 centimeter apart one of each other, on the lateral side of sheep's neck, previously shaved and disinfected. The second booster followed first administration after 30 days, the other boosters coming at 15 days interval (table 1).

Table 1.

Sheep immunization using Rabbit IgG – Agarose Purified Immunoglobulin

	12th of June 2006	10th of July 2006	24th of July 2006	7th of August 2006	21st of August 2006
Group 1	250 µg + FCA	100 µg + FCI	100 µg + FCI	100 µg + FCI	100 µg + FCI
Group 2	1000 µg + FCA	500 µg + FCI	500 µg + FCI	500 µg + FCI	500 µg + FCI

In group 2 we have used the doses recommended by Szafranska et al (2002), and in group 1, the doses 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.

The potentially side effects of immunization were monitored through daily observation, based on the exam of general status, appetite and site of injection.

Results and discussions

The obtained results are depicted in table 2 and 3. The reference values for the investigated parameters were obtained from Ghergariu et al. (2000). Regarding changes of total protein levels, it can be noticed a marked increase after first immunization, in both groups, although the registered values remained in the physiological range.

This rise is significant for G1 ($p<0.05$), compared to G2, for the average values registered after first immunization, related to initially average values. It is a really interesting finding, considering the fact that in G2 antigen was in a four time higher concentration compared to G1. After that moment, it is obvious the decrease of the mentioned parameter, finally reaching values nearby the starting points. We can speculate that using Freund`s Complete Adjuvant determined the rise of total protein, mainly because the booster did not sustained the initial rise, but was followed by a slight decrease. An explanation could be based on the fact that intradermal administration generates an additional depot effect, due to the prolonged contact between antigen and macrophages, through this amplifying the immune response (Broderson, 1989).

In a similar manner evolved the gammaglobulin levels, indicating a possible immune response, although the values ranged in physiological domain. The gammaglobulins raised too after first immunization (but no significant difference

was registered among groups) and decreased subsequent all boosters, sustaining the idea of FCA effect on these parameters. The rise in gammaglobulin levels accompanies a potential immune response, as pointed out by Netto et al. (2004), and Daramola et al. (2005). Also the pattern of albumin decrease pleads for a possible immune response as a consequence of immunizations. It is widely accepted that the immune response is generally accompanied by a rise of gammaglobulins and a concomitant albumin decrease.

Graph 1 offers a complete and eloquent image of all these changes.

At the beginning of our work we considered that we could emphasize any potential change in the hematological picture, but all investigated parameters ranged in the physiological domain. We considered that an example could be offered by average values of WBC and lymphocytes for both groups (table 3). Although increased proliferation of lymphocytes is common in lymph nodes during response to foreign antigens, evidence of this reaction is often not present in blood (Meyer and Harvey, 2004).

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

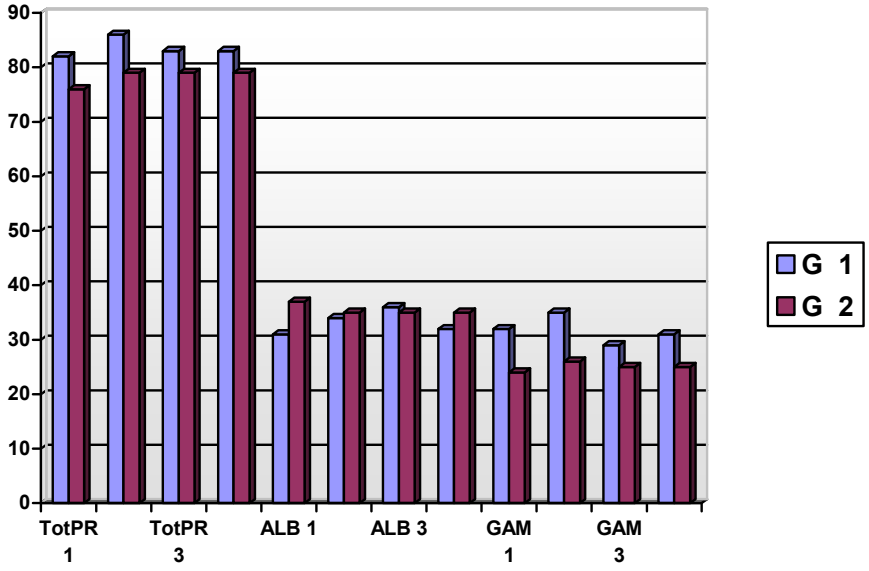

Table 2.

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

	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	82.1	86.1	83.06	83.06	30.9	33.8	36.03	32.2	32.6	34.73	28.6	31.6
Group 2	76.03	78.9	79.3	78.8	36.6	35.3	35.16	35.06	24.3	26.06	25.33	25.1

Table 3.

Average values of WBC and lymphocytes in sheep

	WBC (m/mm^3)						Lymphocytes (%)					
	Normal range	S1	S2	S3	S4	Normal range	S1	S2	S3	S4		
Group 1	4 - 12	4.61	4.75	4.51	4.12	40 - 78	51.8	56.8	60.0	64.2		
Group 2		6.58	6.02	6.41	6.82		49.9	52.6	58.7	54.9		

Conclusions

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

Consequent boosters, all values returned near basic levels, for all investigated parameters, in both groups.

For both 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 sheep.

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