CZU 619:616.98:578.822.2-085.371:636.7

ENHANCED RESPONSES IN ANTIGEN PRIMED DOGS AFTER THE ADMINISTRATION OF A COMPLEX MICROBIAL IMMUNOSTIMULATING COMPOUND

Florinel BRUDASCA, Carmen Dana SANDRU, Constantin CERBU, Aurel VASIU, Marina SPINU
University of Agricultural Sciences and Veterinary Medicine, Cluj-Napoca, Romania

Abstract. The research aimed to investigate the efficacy of antiparvoviral monovalent vaccine for puppies treated with a complex poly-bacterial compound. The experiment was carried out on 17 Labrador Retriever puppies, from 41 to 63 days of age. The two groups (experimental and control) were subjected to: a) a poly-bacterial preparation and antiparvoviral vaccine (n = 12) and b) to antiparvoviral vaccine (n = 5), respectively. One ml of a poly-bacterial preparation was administered on days 0, 2, 4, 6, while the antiparvoviral vaccine Duramune KF11 was injected on day 8 of the experiment. Phagocytic activity and total lysozyme concentrations were measured on days 0, 8 and 22 of the experiment. The global immunostimulating effects of the poly-bacterial preparation on the innate immune system of vaccinated puppies increased when compared to those of the control variant.

Key words: Puppies; Vaccine; Poly-bacterial preparation; Immune indicators; Phagocytosis; Lysozyme.

INTRODUCTION

Immune modulating products enhance the immune responses to antigen priming as well as the protective capacity of the organism against diseases and stress in both farmed and companion animal species. The most proficient vaccines fail to induce immunity against microbes in case of an immunologically week host. Immunostimulating compounds not only increase the immune response, but also facilitate the interaction of the immune effectors in expressing an appropriate reaction (Roitt, I. 1991; Sigal, L.H., Ron, Y. 1994; Stites, P.D., Terr, A.I. 1991).

The canine parvovirus infection is one of the most important diseases in puppies brought up in kennels, sometimes in spite of the previous vaccinations of the mother or the involved individuals. Optimizing the immunization protocols represents an aim in order to eradicate the disease and maintain healthy the kennel's sanitary status.

The investigations were carried out to establish the possible positive influence of using a poly-bacterial immunostimulating compound on the results of antiparvoviral vaccination, by monitoring humoral and cell-mediated innate immune functions.

MATERIALS AND METHODS

The poly-bacterial preparation tested in this experiment, commercialized for its immune modulating capacity, consisted of a mixture of 13 Gram-positive and Gram-negative suspended rods and cocci, inactivated by heat, partially lysed by bile (1%), diluted in saline (0.875%). The standard concentration of this mixture was of 48 million inactivated germs/ml of the product. The active composition consisted of: *Staphylococcus aureus* 6 million/ml, *Streptococcus pyogenes* 3 million/ml, *Streptococcus viridans* 3 million/ml, *Streptococcus faecalis* 11 million/ml, *Streptococcus pneumoniae* 6 million/ml, *Escherichia coli* 10 million/ml, *Klebsiella pneumoniae* 6 million/ml, *Neisseria catarrhalis* 3 million/ml, preserver phenol (max 0.25%). It was included into the pharmaceutical group of immune modulators.

Experimental design. Labrador Retriever puppies, aged of 41 days (n=17) were divided in two groups as follows:

a) group I, poly-bacterial preparation treated, vaccinated, n = 12;

b) group II, untreated, vaccinated, n = 5.

The animals were fed and watered *ad libitum*, under the same maintenance conditions. The investigations were carried out till the animals reached the age of 63 days.

The experimental protocol included for group I, unlike for group II, intramuscular poly-bacterial preparation injections (1 ml) on days 0, 2, 4, 6 after the beginning of the trial (aged of 41, 43, 45 and 47

days). Both groups were subjected to administration of a modified live antiparvoviral vaccine, according to the instructions, on day 8 of the experiment (49 days of age) and blood samplings on days 0, 8 and 22 (41, 49 and 63 days of age). Blood was taken from the brachial vein, 0.5 ml for heparin (50 IU/ml) to monitor the phagocytosis and 1.5 ml for serum, on special clotting gel, to monitor lysozyme activity.

The experimental protocol was drawn in table 1.

Phagocytic activity. The carbon particle clearance test was used to monitor the *in vitro* non-specific cell mediated activity, reducing the blood amounts to minimum aliquots necessary to perform the test (micromethod) (Ghergariu, S. et al. 2000; Rapuntean, Gh. et al. 1994; Dumitru, C. et al. 1996). For that purpose, 0.5 ml aliquots of heparinized blood were co-incubated at 37^0C with 1.5 µl of a supernatant of India ink, in sterile tubes. At 0, 30 and 60 minutes of incubation, 150 µl of the mixture were transferred to 3 ml of saline. Finally, all the tubes containing saline + blood + India ink were centrifuged 10 minutes at 1500 rpm.

Table 1. *Experimental protocol*

Group	Age (days)	Immune stimulation	Day of the experiment	Procedure
I	41		0	Blood sampling
	41	1st	0	Immune stimulation
	43	2nd	2	Immune stimulation
	45	3rd	4	Immune stimulation
	47	4th	6	Immune stimulation
	49	-	8	Blood sampling
	49	-	8	Vaccination
	14 days break			
	63	-	22	Blood sampling
II	41	-	0	Blood sampling
	41	-	-	-
	43	-	-	-
	45	-	-	-
	47	-	-	-
	49	-	8	Blood sampling
	49	-	8	Vaccination
	14 days break			
	63	-	22	Blood sampling

Supernatant optical densities, rendering 0,20 and 40 minutes of incubation for each sample, were red in 96 well plates, using a multichannel spectrophotometer (SUMAL PE2, Karl Zeiss, Jena), at 535 nm wavelength (d=0.5 cm). Phagocytosis was estimated as the reciprocal of the regression slope of the optical densities that were read (Vasiu, C. et al. 1995). Phagocytic activity index was calculated as the difference between the natural logarithms for the 0-20·min and 20-40·min intervals.

The agar gel diffusion test against *Micrococcus lysodeicticus* was used to assess the lytic capacity of serum lysozyme in the puppies during the experiment (Ghergariu, S. et al. 2000; Rapuntean, Gh. et al. 1994). A standard curve built with pure lysozyme (Sigma Aldrich) was used to quantify the amounts of lysozyme in the puppy sera in micrograms/ml.

Mean values, standard deviations and statistical significance of the differences were obtained using the Excel program.

RESULTS AND DISCUSSIONS

Phagocytic function is one of the most important mechanisms within the "first line of defense" against microbes, being performed by neutrophils as small phagocytes, and also by monocytes, as macrophages (Stites, P.D. and Terr, A.I. 1991). Their activation is being triggered by the presence of particulate antigens, which are subsequently engulfed and destroyed. The oxidative burst plays an important role in this process **(**Sigal, L.H., Ron, Y. 1994).

The nervous system, due to the presence of specific receptors on the cells of the immune system, can respond to the secretion of neurohormones. Thus, the nervous system exerts a modulatory action upon the immune response. This could happen by a direct hypothalamic or cortico-hypothalamic

stimulation, where bacteria or bacterial products could intervene, by neural and humoral pathways and also by hypothalamus-pituitary-adrenal axis. The immune response triggering and enhancement by the nervous system are essential, since it could favorably influence the outcome of infectious diseases (Baciu, I. 1992; Baciu, I. et al. 2003).

Age is an important factor influencing the non-specific immunity. In an experiment aimed at monitoring the neutrophil level in puppies on the first day of their life, it was indicated that it was about three times higher than the one of lymphocytes. There was a change in the proportion of these leukocyte populations, with a decrease of neutrophils and increase of lymphocytes, the latter becoming dominant as numbers. By the end of the first month of life, neutrophils level could be compared to the one in adults. The phagocytic activity of neutrophils for the same period was somewhat, but non-significantly higher, in puppies. The experiment indicated that the puppies with very low levels of maternal antibodies developed by the age of two weeks a specific immune response to a parvovirus antigen. This development was continuous, differing from the one of the adults till at least 3 months of age (Toman, M. et al. 2002).

Attempts to stimulate the phagocytosis along with the antibody response and cell mediated adaptive immunity were done. The use of glucan jointly with anti-parvovirus vaccination led to significantly increased phagocytic ability, metabolic and chemotactic activity of polymorphonuclear leukocytes and also improved the blastogenic response of lymphocytes. Similarly, the antibodies against canine parvovirus infection were statistically significantly increased by day 28 after vaccine and glucan administration, as compared to control group (Haladová, E. et al. 2011).

Bacteria were also used to stimulate the immune system. A diet supplemented with 5×10^8 colony forming units (cfu)/d of probiotic *Enterococcus faecium* (SF68) from weaning to 1 y of age, led to higher fecal IgA and distemper vaccine–specific circulating IgG and IgA. The percentage of mature B cells was also increased in puppies fed with the probiotic when compared to the control group (Benyacoub, J. et al. 2003).

Other treatments, such as chemotherapy, could change the phagocytic function, impeding the immune response against bacteria, in the case of chronic disease. Thus, induction chemotherapy diminished the percentage of neutrophils capable of oxidative burst in dogs with lymphoma, but phagocytosis improved over time (LeBlanc, A.K. et al. 2015).

Phagocytic activity was presented for the stimulated group in figure 1 and for the control group in figure 2. Average phagocytic indices were presented for the two periods, from 0 to 20 and from 20 to 40 min for each sampling.

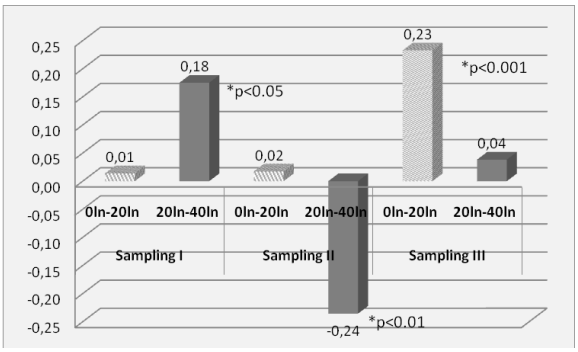


Figure 1. *Mean values of phagocytic activity in the immune stimulated group (ln)*

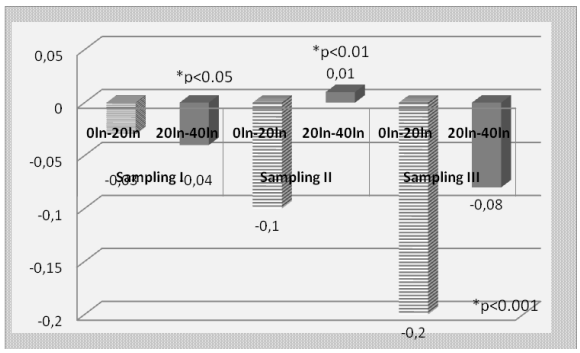
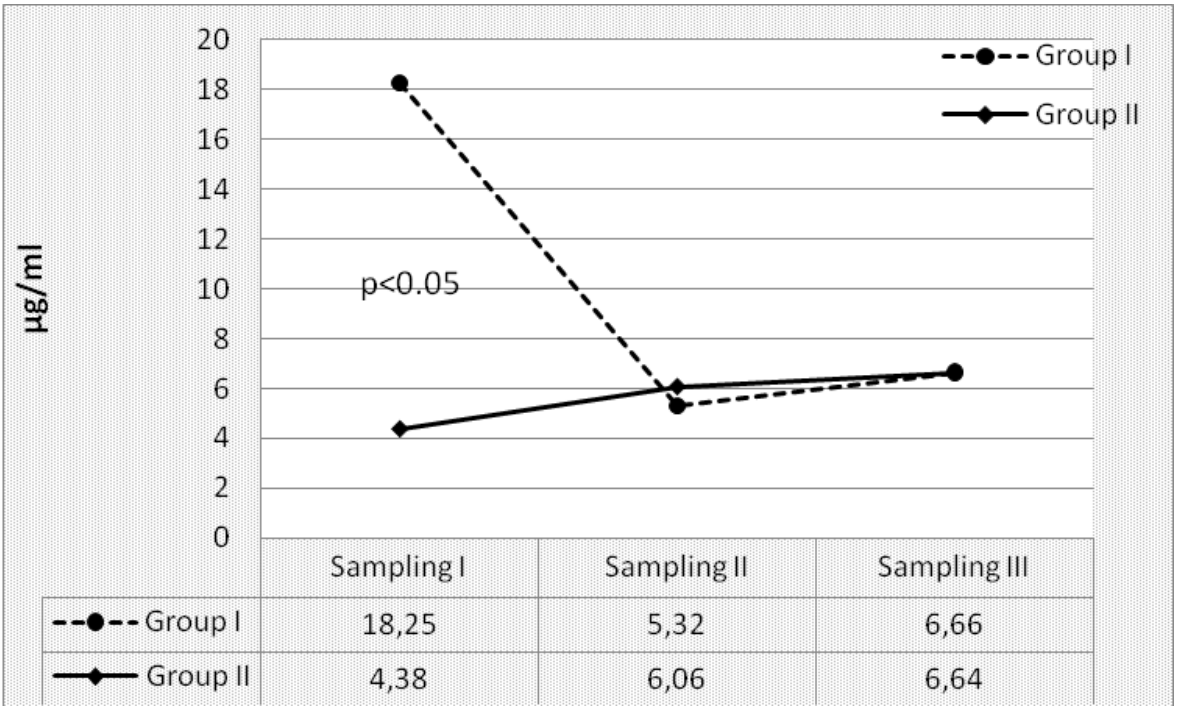


Figure 2. *Mean values of the phagocytic activity in the control group (ln)*

As shown, the phagocytic activity is in the positive field, with an increase towards the end of the experiment in the stimulated group, as opposed to the control group, where the majority of the indices are negative. The enhanced phagocytic activity of the poly-bacterial preparation was obvious. The comparison for the first reading period, third sampling, between the two groups indicated a high statistically significant difference ($p<0.001$). The differences for the first and second sampling, second period (20 to 40 min) were also statistically supported ($p<0.05$-0.01). Although the puppies at birth can respond to external stimuli, by augmenting the non-specific cell mediated activity, the poly-bacterial preparation could be of help in increasing the post-vaccinal immunity and lowering the caseload in parvovirus infection (Haladová, E. et al. 2011).

The lysozyme (N-acetyl-muramyl-hydrolase) is involved in the lysis of the bacterial wall especially in Gram positive cocci (Sigal, L.H., Ron, Y. 1994). Alterations of lysozyme concentrations were found during the development of infectious diseases (Babíčková, K. et al. 2009). Figure 3 presents the mean values of the lysozyme concentrations in the experimental groups. The value obtained at the first sampling was the highest and decreased towards the second sampling in the immune stimulated group, with no influence on the health status of animals. There was a second increase at the third sampling. Compared to the experimental group, the increase in lysozyme concentrations was gradual by the end of the experiment, but the levels were lower than in the control group. The differences in the dynamics of lysozyme concentrations were not supported statistically, except for the first sampling. There were no significant differences at the end of the experiment between the groups.



	Sampling I	Sampling II	Sampling III
Group I	18,25	5,32	6,66
Group II	4,38	6,06	6,64

Figure 3. *Lysozyme concentration in the experimental groups (mean values)*

One of the purposes of poly-bacterial preparation treatment preceding vaccination in puppies was to increase the basic non-specific immune response, in order to enhance protection against parvovirus infection. Most probably, the enzyme levels decreased since it was sensitive to the manipulation/ vaccination stresses, and these exerted a more pronounced influence than the antigen priming. The initial increased value could be the result of a different microbism of the environment, with a heavier load in the experimental group. Since the poly-bacterial preparation treatment did not significantly influence this parameter, probably there was a different pathway and other mechanisms were involved in ensuring the health of animals.

CONCLUSIONS

1. Phagocytosis was positively influenced by the poly-bacterial preparation treatment and antigen priming, suggesting an increase in the effectiveness of cell mediated non-specific immunity and a possible use of the procedure to improve vaccinal status of the puppies.

2. Lysozyme concentrations were negatively influenced by the combined poly-bacterial preparation and vaccination procedure, indicating supplementary pathways in the increased resistance against parvovirus infection.

REFERENCES

1. BABÍČKOVÁ, K., HYRŠL, P., TOLAROVÁ, S. (2009). Antibacterial activity of lysozyme and complement in

canine blood sera. In: 3rd EVIW European Veterinary Immunology Workshop. Berlin. Available: http://www.sci.muni.cz/ofiz/wp-content/uploads/2013/06/Berlin_2009.pdf

2. BACIU, I. (1992). The role of nervous mechanisms in the immune response. In: Revue Roumaine de Physiologie, vol. 29(1-2), pp. 5-11.

3. BACIU, I., HRISCU, M., SAULEA, G. (2003). Hypothalamic mechanisms of immunity. In: International journal of neuroscience, vol. 113(2), pp. 259-277. ISSN 0020-7454.

4. BENYACOUB, J., CZARNECKI-MAULDEN, G.L., CAVADINI, C., SAUTHIER, T., ANDERSON, R.E., SCHIFFRIN, E. J., von der WEID, T. (2003). Supplementation of Food with *Enterococcus faecium* (SF68) Stimulates immune functions in young dogs. In: *Journal of Nutrition,* vol. 133(4), pp. 1158-1162. *ISSN* 0022-3166.

5. DUMITRU, C., SPÎNU, Marina, VASIU, C., BRUDAŞCĂ, Gh., BOLDIZSAR, E., DOBREAN, V., FĂRTAN, S. (1996). Modificări ale activității fagocitare şi sintezei de lizozim în urma administrării extractului de Calendula officinalis la puii de găină. In: Actualități în patologia animalelor domestice: simp., 7-8 nov., Cluj, vol. XXII, p. 183.

6. GHERGARIU, S., POP, A., KADAR, L., SPÎNU, Marina (2000). Manual de laborator clinic veterinar. Bucureşti: All. 426 p. ISBN 973-684-112-X.

7. HALADOVÁ, E., MOJŽIŠOVÁ, J. , SMRČO, P., ONDREJKOVÁ, A., VOJTEK, B., PROKEŠ, M., PETROVOVÁ, E. (2011). Immunomodulatory effect of glucan on specific and nonspecific immunity after vaccination in puppies. In: Acta Vet Hungarica, vol. 59(1), pp. 77-86. DOI: 10.1556/AVet.59.2011.1.7

8. LeBLANC, A.K., LeBLANC, C.J., ROHRBACH, B.W., KANIA, S.A. (2015). Serial evaluation of neutrophil function in tumour-bearing dogs undergoing chemotherapy. In: Veterinary and Comparative Oncology, vol. 13(1), pp. 20-27. ISSN 1476-5829.

9. Manual of Small Animal Disease (1988). New York: Churchill Livingstone. 444 p. (pp. 49-61). ISBN 0443085080.

10. RĂPUNTEAN, Gh., SPÎNU, Marina, SPÎNU, O. (1994). Lucrări practice de imunologie. Cluj-Napoca: Agronomia.

11. ROITT, I. (1991). Essential Immunology. Oxford: Blackwell Sci. Publ.

12. SIGAL, L.H., RON, Y. (1994). Immunology and Inflammation. Basic Mechanisms and Clinical Consequences. McGraw-Hill. Inc.

13. STITES, P.D., TERR, A.I. (1991). Basic and Clinical Immunology. 8th ed. 500 p. ISBN 978-0838505618.

14. TOMAN, M., FALDYNA, M., KNOTIGOVA, P., POKOROVA, D., SINKORA, J. (2002). Postnatal development of leukocyte subset composition and activity in dogs. In: Veterinary Immunology and Immunopathology, vol. 87(3-4), pp. 321-326. ISSN 0165-2427.

15. VASIU, C., BOLDIZSAR, E., BRUDAŞCĂ, GH.F., DUMITRU, C., SPÎNU, Marina, DOBREAN, V., BOGDAN, Gh. (1995). Investigații privind modificările efectorilor imuni celulari la bovine serologic pozitive pentru leucoză enzootică. In: Actualități în patologia animalelor domestice: simp., 11-12 oct., Cluj-Napoca, vol. XXI, pp. 67-88.

Data prezentării articolului: 23.04.2017
Data acceptării articolului: 17.05.2017