Original paper

BASIC BLOOD ANALYSIS OF RABBITS IMMUNIZED WITH VACCINE AGAINST MYXOMATOSIS

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Abstract

The aim of this preliminary study was to assess the immune response of rabbits triggered by vaccination against myxomatosis. In experiments, 14 New Zealand White rabbits (7 does – D and 7 bucks – B at the age of 1 to 3 years) were used. Samples of rabbit peripheral blood (PB) were collected from *a. auricularis centralis* to heparinised tubes 2 weeks before and 4 days after the subcutaneous injection (0.5 mL) of vaccine against myxomatosis (Pharmavac MXT). Mononuclear cells from peripheral blood (PBMCs) were isolated using Ficoll centrifugation. Isolated PBMCs were then frozen and stored at -192 °C. For phenotyping, the frozen cells were thawed and stained with the following anti-rabbit monoclonal antibodies (mAbs): anti-IgM (NRBM, IgG1), anti-CD4 (RTH1A, IgG1), anti-CD8 (ISC27A, IgG2a), anti-pan T2 (RTH21A, IgG1) and anti-CD45 (L12/201, IgG1). As the secondary immunoreagent, fluorescein isothiocyanate (FITC) or R-phycoerythrin (R-PE) labelled anti-mouse conjugates of appropriate subisotypes were used. We found significantly ($P<0.05$) increased percentage of either T-cells (does D5 and D7, and bucks B5, B6 and B7), or B-cells (bucks B2 and B7) in the rabbit peripheral blood. In conclusion, fast and adequate immune response to antigen (vaccine against myxomatosis) was indicated by the increase in T lymphocyte subsets 4 days after immunization. Thus, rabbit does (D5 and D7) and bucks (B5, B6 and B7) might be selected to create F1 generation for the future experiments.

Key words: *immune response, lymphocyte subsets, rabbit*

Introduction

Rabbit (*Oryctolagus cuniculus*) is one of the animal species often used as an experimental model in human and veterinary research. The rabbit model served for the study of infectious diseases such as syphilis (Gamboa and Miller, 1984), tuberculosis (Dannenberg, 1991), human T lymphotropic virus-I (Sawasdikosol et al., 1993) and human immunodeficiency virus (Filice et al., 1988). Rabbit is also useful for studies of various non-infectious diseases such as atherosclerosis (Jayo et al., 1994) or eye disorders (Peiffer et al., 1994) and is still the animal of choice for production of many polyclonal antibodies (Mage, 1998). These antibodies are typically produced by inoculation of a suitable mammal, such as a mouse, rabbit or goat. An antigen injected into the mammal triggers an immune response.

The three key interacting elements in immune responses are antigen-presenting cells (APCs), thymus-derived lymphocytes (T cells), and bone-marrow derived lymphocytes (B cells). T cells emerge from the thymus as $CD4^+$ or $CD8^+$ cells. The former are involved in a helper function to B cells, in cell-mediated immune responses in lymphokine secretion. The latter are specialised for cytotoxic killing of other cells, particularly virus-infected cells or, at least in experimental circumstances, tumour cells (Nossal, 1997). B cells are responsible for antibody formation (Nossal et al., 1968). The early IgM response of B cells may be triggered directly by antigen in a T-cell-independent manner, but most long-lasting immune responses, involving IgG, IgA, or IgE antibodies, need the help of activated T cells (Miller, 1972).

Determination of lymphocyte subset distribution in the peripheral blood is a routine part of laboratory tests in human patients suspected on immunodeficiency. At present, flow cytometry is also becoming a useful tool in veterinary medicine, particularly in small animal practice. The knowledge of physiological values is necessary for recognition of changes in lymphocyte subset distribution (Faldyna et al., 2001).

Objective of this preliminary study was to assess the immune response of rabbits triggered by vaccination against myxomatosis.

Materials and methods

Adult (1 - 3 years old) and clinically healthy rabbits of New Zealand White (NZW) line (n = 14; 7 does – D and 7 bucks – B) reared in a partially air-conditioned hall of a local rabbit farm at RIAP Nitra were used in the experiments. The animals were housed in individual cages, under a constant photoperiod of 14 h of day light. Temperature and humidity in the building were recorded continuously by means of a thermograph positioned at the same level as the cages (average relative humidity and temperature during the year was maintained at 60 ± 5% and 17 ± 3°C). The rabbits were fed *ad libitum* with a commercial diet (KV; TEKRO Nitra, s.r.o.) and water was provided *ad libitum* with nipple drinkers. The treatment of the animals was approved by the Ministry of Agriculture and Rural Development of the Slovak Republic, no. SK P 28004 and Ro 1488/06-221/3a.

Samples of rabbit peripheral blood (PB) were collected from *a. auricularis centralis* to heparinised tubes 2 weeks before and 4 days after the subcutaneous injection (0.5 mL) of vaccine against myxomatosis (Pharmavac MXT; Pharmagal Bio, Slovak Republic). One dose of vaccine contained live attenuated virus of myxomatosis min. 10^3 $TCID_{50}$. Mononuclear cells from peripheral blood (PBMCs) were isolated using Ficoll centrifugation according to the original protocol: Isolation of mononuclear cells from human peripheral blood by density gradient centrifugation (Miltenyi Biotec, 2008). Isolated PBMCs were then frozen as follows. Cells were resuspended in 1.5 mL of the appropriate 4 °C cooled RPMI 1640 medium (Gibco, Life Technologies, Grand Island NY, USA) containing L-glutamine, 10% FCS and 10% dimethyl sulfoxide (DMSO). Once aliquoted, cryovials were placed on ice and then transferred into a "Mr. Frosty" freezing container (Thermo Scientific Nalgene, Rochester, NY, USA), and stored at -80 °C for 24h. Cryovials were then transferred into liquid nitrogen for long-term storage at -192 °C. For phenotyping, the frozen cells were then thawed as follows. Five mL of RPMI 1640 medium containing 10% FCS, warmed to temperature at 37 °C, was aliquoted into 50 mL centrifuge tubes. No more than 2 cryovials were thawed at the same time. The cryovials were thawed in a 37 °C water bath until the cell suspension was almost completely melted. Two mL of the appropriate medium was slowly added to thawed cells and then slowly transferred drop by drop to the tubes containing corresponding medium with simultaneous

mixing of the cell suspension. Then another 20 mL of appropriate medium was added to the tubes. The tubes were two times centrifuged at 488x g for 10 min in order to remove DMSO from the cell suspension and then the cells were resuspended in 2 mL of the Dulbecco's PBS without Ca and Mg (PAA Laboratories GmbH, Pasching, Austria). Cell count was evaluated using the Bürker-Türk counting chamber (Brand, Germany).

Frozen-thawed cells were divided into prepared tubes and stained with different clones of anti-rabbit monoclonal antibodies: anti-IgM (NRBM; Bio-Rad AbD SerotecGmbH, Germany), anti-CD4 (RTH1A; WSU, Pullman, WA), anti-CD8 (ISC27A; WSU, Pullman, WA), anti-pan T2 (pT2; RTH21A; WSU, Pullman, WA) and anti-CD45 (L12/201; Bio-Rad AbD SerotecGmbH, Germany) according to the producer's manual. As the secondary immunoreagent, FITC or R-PE labelled anti-mouse conjugates of appropriate subisotypes (eBioscience, Austria) were used. To assess contamination of the lymphocyte gate by other cell types, the cross-reactive FITC labelled mAb against human CD14 antigen (TUK4; Bio-Rad AbD SerotecGmbH, Germany) was used. In each sample, 10000 - 50000 cells were measured using flow cytometer FACS Calibur (Becton Dickinson, Mountain View, CA). 7-AAD Staining Solution (BD Biosciences, USA) was used to exclude dead cells from analysis. The common leukocyte antigen CD45 and CD14 expression was used for the ''lymphogate'' set up and lymphocyte purity determination as described by Jeklova et al. (2007). Results obtained for the other surface markers were recalculated to 100 % of $CD45^+$and $CD14^-$ cells in the''lymphogate''.

Observed results were evaluated statistically using χ^2-test in SigmaPlot software (Systat Software Inc., Germany) and expressed as the means ± standard error of means (SEM). P-values at $P<0.05$ were considered as statistically significant.

Results and discussion

In this study, we evaluated the changes in lymphocyte subsets of rabbit peripheral blood collected from seven does (D1-7) and seven bucks (B1-7) before and after immunization with vaccine against myxomatosis. After immunization, significant decrease ($P<0.05$) in the percentage of B lymphocytes (IgM^+ cells) was observed in almost all rabbit does (except in D1; Table 1). On the other hand, the percentage of T lymphocytes subsets increased significantly ($P<0.05$) in does D5 ($pT2^+$, $CD4^+$ and $CD8^+$ cells) and D7 ($pT2^+$ and $CD8^+$ cells), what was reflected in higher pT2/IgM ratio value for doe D5 (Table 1). Higher percentage of $CD8^+$ cells ($P<0.05$) was also found in doe D6 after immunization (Table 1). We noticed significantly decreased ($P<0.05$) value of CD4/CD8 ratio only in doe D3 (Table 1). There were no changes in the percentage of double positive lymphocytes ($CD4^+CD8^+$) in any doe.

On the contrary, inconsistent values in the percentage of B lymphocytes were obtained in rabbit bucks after immunization. Significant increase ($P<0.05$) of IgM^+ cells were found in bucks B2 and B7, whereas decreased ($P<0.05$) percentage of IgM^+ cells had bucks B3 and B4 (Table 2). The percentage of T lymphocytes subsets increased significantly ($P<0.05$) in bucks B5 ($pT2^+$, $CD4^+$ and $CD8^+$ cells), B6 ($pT2^+$ and $CD4^+$ cells) and B7 ($CD4^+$ and $CD8^+$ cells) after immunization (Table 2). On the other hand, buck B1 had lower ($P<0.05$) percentage of $pT2^+$ cells (Table 2). We observed higher ($P<0.05$) pT2/IgM ratio value only in buck B4 (Table 2). Similarly, no changes in the percentage of double positive lymphocytes ($CD4^+CD8^+$) or CD4/CD8 ratio values were found in any buck after immunization.

Table 1. *Lymphocytes subsets in peripheral blood of rabbit does before and after immunization*

Sample	Blood collection	pT2 (%)	CD4 (%)	CD8 (%)	$CD4^+CD8^+$ (%)	IgM (%)	pT2/IgM (ratio)	CD4/CD8 (ratio)
D1	Before IM	26.96	22.33	4.20	0.60	25.73	1.05	5.32
	After IM	21.66	15.38	4.40	0.71	17.74	1.22	3.49
D2	Before IM	40.19	36.52	4.57	0.89	28.50^a	1.41	8.00
	After IM	33.68	27.08	4.59	0.58	15.01^b	2.24	5.90
D3	Before IM	34.57	29.15	2.50	0.21	30.51^a	1.13	11.66^a
	After IM	25.58	19.01	3.94	0.21	15.23^b	1.68	4.83^b
D4	Before IM	56.53	22.85	27.22	2.05	24.17^a	2.34	0.84
	After IM	57.26	22.51	30.33	1.83	14.05^b	4.08	0.74
D5	Before IM	32.67^a	27.14^a	6.08^a	0.79	41.16^a	0.79^a	4.46
	After IM	55.38^b	37.53^b	14.48^b	0.81	14.43^b	3.84^b	2.59
D6	Before IM	47.07	30.13	11.51^a	1.73	31.48^a	1.50	2.62
	After IM	38.51	24.10	40.39^b	1.36	16.01^b	2.41	0.60
D7	Before IM	29.67^a	31.07	2.61^a	0.87	35.72^a	0.83	11.90
	After IM	43.29^b	40.57	5.88^b	1.17	15.16^b	2.86	6.90
Average values	Before IM	38.24	28.45	8.38	1.02	31.04	1.29	6.40
	After IM	39.33	26.59	14.86	0.95	15.37	2.62	3.58

IM – immunization; Results are expressed as means ± SEM; [a] vs [b] within the same column and sample were statistically significant at $P<0.05$.

Table 2. *Lymphocytes subsets in peripheral blood of rabbit bucks before and after immunization*

Sample	Blood collection	pT2 (%)	CD4 (%)	CD8 (%)	$CD4^+CD8^+$ (%)	IgM (%)	pT2/IgM (ratio)	CD4/CD8 (ratio)
B1	Before IM	46.45^a	34.11	5.48	0.93	21.37	2.17	6.22
	After IM	30.5^b	23.35	5.28	0.53	17.35	1.76	4.43
B2	Before IM	31.98	29.28	5.75	0.44	21.62^a	1.48	5.10
	After IM	27.87	21.58	7.25	0.38	31.55^b	0.88	2.98
B3	Before IM	39.58	28.42	4.91	0.53	28.27^a	1.40	5.79
	After IM	30.04	23.51	8.27	0.25	14.68^b	2.05	2.84
B4	Before IM	42.85	32.82	5.39	1.45	28.23^a	1.52^a	6.09
	After IM	45.89	39.73	6.80	1.51	2.11^b	21.75^b	5.85
B5	Before IM	29.43^a	27.51^a	4.07^a	1.05	29.38	1.00	6.76
	After IM	60.77^b	49.80^b	8.51^b	1.44	27.59	2.20	5.85
B6	Before IM	43.72^a	32.98^a	5.23	0.95	38.53	1.13	6.31
	After IM	64.74^b	53.75^b	8.60	2.26	30.29	2.14	6.25
B7	Before IM	38.43	31.91^a	3.40^a	1.09	13.18^a	2.92	9.40
	After IM	49.65	48.18^b	8.62^b	2.25	27.18^b	1.83	5.59
Average values	Before IM	38.92	31.00	4.89	0.92	25.79	1.66	6.52
	After IM	44.21	37.13	7.61	1.23	21.53	4.66	4.83

IM – immunization; Results are expressed as means ± SEM; [a] vs [b] within the same column and sample were statistically significant at $P<0.05$.

The average percentages of specific lymphocyte subsets (pT^+, $CD4^+$, $CD8^+$, $CD4^+CD8^+$ and IgM^+) in peripheral blood of rabbits before immunization (Table 1 and 2) are similar to those observed by Jeklova et al. (2007) (40.1%, 29.4%, 10.4%, 2.0% and 41.9%, respectively), thus confirming the normal health status of rabbits used for experiment. Although these authors found more than 10% higher value of B-cells in comparison to our results (Tables 1 and 2) this could be due to the use of CD79α antibody for enumeration of B-cells. However, the anti-rabbit IgM mAb has been already successfully used as B-cell marker also in other studies (Vajdy et al., 1998; Lanning et al., 2000; Tokarz-Deptula and Deptula, 2005).

According to observed results after immunization, we found significantly ($P<0.05$) increased percentage of either T-cells (does D5 and D7, and bucks B5, B6 and B7), or B-cells (bucks B2 and B7) in the rabbit peripheral blood (Tables 1 and 2). An increase in lymphocyte concentration is usually a sign of a viral infection, in this case caused by the attenuated virus of myxomatosis. Adequate immune response in observed rabbits might be indicated by the increased percentage of $CD4^+$ (D5, B5, B6 and B7) or $CD8^+$ (D5, D7, B5 and B7) cells. Although, both $CD4^+$ and $CD8^+$ lymphocytes are very important in the reaction cascade of immune response, they differ in their function. The most important distinction is that $CD4^+$ cells see antigenic peptides in association with MHC class II molecules whereas $CD8^+$ cells react with peptide plus MHC class I (Nossal, 1997). $CD4^+$ T cells, when activated, develop into T cells secreting a large variety of cytokines (Kelso et al., 1991). However, as the immune response matures, there are many instances where either a T helper (Th)-1 response or a (Th)-2 responses become dominant (Mosmann and Coffman, 1989). The Th-1 response leads to inflammatory phenomena and the Th-2 response to antibody formation, including IgG1 and IgE formation (Finkelman et al., 1990).

Conclusion

In conclusion, fast and adequate immune response to antigen (vaccine against myxomatosis) was indicated by the increase in T lymphocyte subsets 4 days after immunization. Thus, rabbit does (D5 and D7) and bucks (B5, B6 and B7) might be selected to create F1 generation for the future experiments.

Acknowledgments

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