NOTE *Internal Medicine*

Relationship between Immune Function and Serum Vitamin A in Japanese Black Beef Cattle

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ABSTRACT. This study evaluated the relationship between the serum vitamin A level and immune function in seventy non-diseased Japanese Black (JB) cattle during their fattening stages. The animals were divided into two groups, a Low Vitamin A (VA) group (N=9) with below 30 IU/d*l* of serum VA and a Control group (N=61). Blood samples were collected for biochemical analysis and examination of the leukocyte population and cytokine mRNA expression. The numbers of $CD3^+WC1^+$ T cells and MHC class-II^+CD14^- B cells were significantly lower in the Low VA group than in the Control group ($P<0.05$). The IFN-γ/IL-4 rate was significantly lower in the Low VA group, while IL-4 was higher and IFN-γ was lower in the Low group compared with the Control group. This study indicated that immune function imbalance was present in JB cattle with low serum VA levels during the fattening stage.

KEY WORDS: immune function, Japanese Black, vitamin A.

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Japanese Black (JB) cattle are well known as fattening steers. To produce valuable meat for the Japanese market, high carbohydrate and low vitamin A (VA) diets are acceptable for feeding to JB cattle during their fattening stages [18]. Adachi *et al.* [1] reported that the serum level of VA in JB cattle should be decreased from the level of 106 ± 35 IU/d*l* before the fattening stage and maintained at about 30 ± 9 IU/d*l* between months 20 and 24. Because VA affects the development and function of adipose tissues, and a low VA status increases fat deposition in some tissues of animals [5].

Although decreased VA in the diets is acceptable, signs of VA deficiency in cattle include by poor weight gain, ataxia, convulsions, night blindness and total blindness. Animals sometimes die in cases of serious VA deficiency [3, 6]. Infectious diseases, such as infectious diarrhea and pneumonia, are lethal to JB cattle with low levels of VA [2]. VA is known to be an indispensable substance for protection against infections, and VA deficiency is an immunodeficiency disorder characterized by widespread alterations in immune function, such as changes in lymphocyte subpopulations or altered T and B cell function [4, 8].

Most JB beef cattle are allowed to have VA deficiency during the feeding stage and therefore these animals have a high risk of occurrence of infection in this period. Understanding the relationship between immune condition and the serum VA level in non-infected JB cattle is valuable and important for the proper health care of these animals. This investigation evaluated immune condition in JB fattening cattle before and after 20 months of age.

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Seventy non-diseased JB cattle 18 to 21 month old from 14 farms in Yamagata and Miyagi Prefectures were used in the present study. These cattle were divided 2 groups, a Low VA group (N=9) with below 30 IU/d*l* of serum VA, as described previously [1], and a Control group (N=61). Blood samples were collected for biochemical analysis and examination of the leukocyte population and cytokine mRNA expression.

Blood samples were collected into tubes with heparin or dipotassium-EDTA as anticoagulants and without an anticoagulant. Serum was separated by centrifugation of tubes without an anticoagulant and was stored at –30°C until analysis. Serum samples collected by tubes with no anticoagulant were used to measure the serum total cholesterol (TC; enzyme assay), γ-glutamyl transpeptitase (GGT; enzyme assay) and blood urea nitrogen (BUN; urease indophenol method). The serum VA concentration was measured by high performance liquid chromatography [7]. White blood cell counts (WBC) were determined with a blood cell counter (PC607, ERMA, Germany).

Freshly isolated leukocytes were subjected to flow cytometry analysis as described previously [17]. Two m*l* of blood samples in tubes containing dipotassium-EDTA were mixed with 4 m*l* of 0.83% ammonium chloride solution to lyse the red blood cells, and the leukocytes were then separated. The lineage-specific monoclonal antibodies (VMRD, Pullman, WA, U.S.A.) used were MMIA (pan T lymphocyte, CD3 antigen), CACT183A (T-helper lymphocyte, CD4 antigen), BAT82A (T-cytotoxic/suppressing lymphocyte, CD8 antigen), CACTB32A (gamma delta T lymphocyte/WC1-N3), CAT82A (monocyte/B lymphocyte, Class II major histocompatibility complex antigen) and MY-4 (monocyte, CD14 antigen; Coulter Immunology, Hialeah,

Florida, U.S.A.). After washing with PBS, the cells were incubated with fluorescein isothiocyanate (FITC) labeled anti-mouse IgM and phycoerythrin (PE) labeled anti-mouse IgG1 (ICN Biomedicals, Inc., Costa Mesa, CA, U.S.A.) at 4°C for 30 min. Following the second incubation, the samples were twice washed with PBS, and data from 1×10^6 cells were analyzed for each sample analyzed with the Lysis software and a FACScan flow cytometer (BD Biosciences Immunocytometry Systems, San Jose, CA, U.S.A.). Data from 10000 events per sample were analyzed using software (Cell Quest; BD).

For analysis cytokine mRNA expression, heparinised blood was used. Initially 2×10^6 PBMCs in a total volume of 1 m*l* of 10% FCS-RPMI medium were placed in a 24-well plate and stimulated with 5 μg/m*l* of phytohemagglutinin (PHA; Sigma-Aldrich CO., St. Louis, U.S.A.) for 12 hr at 37°C. After incubation, PBMCS pellets were collected, the supernatants were removed and the cells were resuspended using TRIzol reagent (Invitrogen, Carlsbad, CA, U.S.A.) to collect RNA in the cells.

Real-time PCR was used to measure the mRNA levels of cytokines as previously described [17, 19]. Two micrograms of total RNA from each sample were used for synthesis of first-strand cDNA with oligo-dT primers (Invitrogen, Carlsbad, CA, U.S.A.) and Superscript II Reverse Transcripts (Invitrogen, Carlsbad, CA, U.S.A.) according to the manufacturer's protocols. Real-time PCR was performed with SYBR Green Master Mix on an ABI prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA, U.S.A.). The target cDNA sequence was specifically amplified using previously designed primers (IL-1, IL-4, IL-6, IFN-γ) [19]. The melting curve was determined for each PCR product. The comparative threshold cycle number ($2^{-\Delta\Delta Ct}$) method was used after a validation experiment demonstrated that the efficiencies of the target and reference (β-actin) were approximately equal. Ct values define the threshold cycle of PCR, at which amplified products were detected. The results are presented as ΔCt values, where ΔCt is the difference in the threshold cycles for the target and β-actin as an internal control. Fold changes in expression for the two groups ($\Delta\Delta$Ct) were calculated using the formula $2^{-\Delta\Delta Ct}$ as previously described [15].

Statistical analysis was performed using a repeated measures Student's *t*-test for each parameter in the 2 groups, and values of $p<0.05$ were regarded as significant. *F* tests (2 sided test) were performed to evaluate the normal distribution of each parameter in the 2 groups. The mean values and standard errors of the clinical and laboratory data were calculated, and the correlation between each parameter (leukocyte numbers, cytokines levels and biochemical data) in all the cows was examined using Kendall's test.

There was a significantly lower serum VA level in the Low VA group compared with the Control group. However there were no significant differences in other parameters in the biochemical analysis (Table 1). In the present study, there were no significant differences in any parameters among the herds of cattle.

The immune analysis data is shown in Table 2. The number of $CD3^+$ T cells was lower in the Low VA group than in the Control group, and a significant difference was observed in the number of $CD3^+WC1^+$ T cells. There was a significantly lower number of MHC class-II^+CD14^- B cells in the Low VA group than in the Control group. No significant differences in other leucocytes were observed between the 2 groups.

The levels of IL-4 and IL-6 were higher in the Low VA group than in the Control group; however, the IFN-γ level of the Low VA group, and the decrease in the IFN-γ/IL-4 rate was significant (Table 2).

There were significant relationships between the serum VA levels and $CD3^+WC1^+$ T cell number and the VA levels and MHC class-II^+CD14^- B cell number (Fig. 1).

VA deficiency impairs both innate immunity and adaptive immune response to infection, resulting in impaired ability to counteract extracellular pathogens [24]. The present study indicated that the immune condition of JB cattle during the feeding stage is compromised by following VA deficiency. Although marked differences were not observed in the biochemical data between the two groups, lower numbers of T and B cells were indicated. The present study suggested that suppression of immune response might also be observed in VA deficient JB cattle because lower numbers of lymphocytes have frequently been recognized as being a high risk factor for depression of immune function in animals [21].

VA deficiency reduces a type 1 cytokine response, and exogenously provided retinoids can induce a type 2 cytokine response. Previous studies using models of pathogen-challenged rodents have indicated that VA deficiency induces a dominant Th1 response that interferes with development of a protective humoral response [13]. These researchers proposed several potential mechanisms to account for these

Table 1. Biochemical data for Japanese Black cattle fed either a low vitamin A or control diet

Category	Low VA group	Control group	*F* value
Vitamin A (IU/dL)	22.3 ± 1.6	58.1 ± 3.2	* p<0.001
TC (mg/dL)	125.3 ± 6.5	139.1 ± 4.3	0.193
GGT (IU/dL)	38.0 ± 6.0	33.4 ± 1.8	0.526
BUN (mg/dL)	18.4 ± 0.6	17.1 ± 0.4	0.111
WBC ($\times 10^2/\mu$L)	104.2 ± 12.7	102.1 ± 5.2	1.812

Values are presented as means ± S.E.
* Significant difference between the two groups (P<0.05).

Table 2. Leukocyte populations and cytokine mRNA levels for Japanese Black cattle fed either a low vitamin A or control diet

Category	Low VA group	Control group	F value
$CD3^+WC1^-$ ($\times 10^2/\mu L$)	11.2 ± 1.3	13.2 ± 0.9	0.311
$CD3^+WC1^+$ ($\times 10^2/\mu L$)	3.1 ± 0.4	4.3 ± 0.4	* 0.103
$CD3^+$ ($\times 10^2/\mu L$)	14.4 ± 1.1	17.4 ± 1.1	0.052
$CD4^+$ ($\times 10^2/\mu L$)	5.6 ± 0.6	6.8 ± 0.5	0.060
$CD8^+$ ($\times 10^2/\mu L$)	3.6 ± 0.5	3.7 ± 0.3	0.525
$CD4^+/CD8^+$	1.7 ± 0.2	2.0 ± 0.1	0.513
MHC class-II^+CD14^- ($\times 10^2/\mu L$)	8.4 ± 0.8	11.2 ± 0.7	* 0.099
$CD14^+$ ($\times 10^2/\mu L$)	6.5 ± 0.9	7.5 ± 0.5	0.499
IL-1	1.7 ± 0.4	2.0 ± 0.3	0.163
IL-4	1.6 ± 1.0	0.8 ± 0.4	1.773
IL-6	4.7 ± 2.8	2.8 ± 0.7	0.081
IFN-γ	2.2 ± 0.8	3.3 ± 1.2	0.008
IFN-γ/IL-4	13.9 ± 8.1	41.7 ± 10.1	* p<0.001

Values are presented as means ± S.E.
* Significant differences between the two groups (P<0.05).

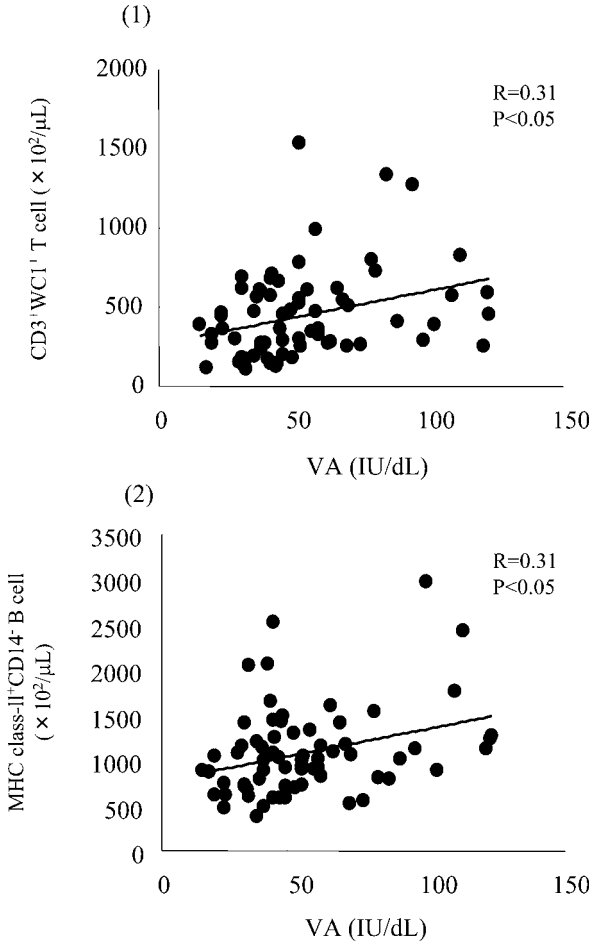

Fig. 1. Relationship between the serum VA levels and lymphocytes numbers in all cattle. (1) Serum VA levels versus $CD3^+WC\text{-}1^+$ T cells numbers, (2) serum VA levels versus MHC class-II^+CD14^- B cell numbers.

observations, including direct down regulation of T cell IFN-γ synthesis, direct promotion of Th2-cell differentiation and/or alteration of accessory or antigen presenting cell function toward a Th2-inducing phenotype [8]. The results of the present study indicated that there was a Th1 to Th2 shift following suppression of the IFN-γ level and improvement of the IL-4 level in the T cells of the Low VA group. This finding is similar to that in a previous report [16] and suggests that cellular immune function is depressed in the JB cattle with VA deficiency.

In experimental VA deficient mice, the percentage of $CD4^+$ T cells positive for IFN-γ and IL-2 were lower than the percentages of control mice [22]. We observed lower numbers of $CD4^+$ T and γδ T cells in VA deficient JB cattle. Normally, VA deficiency for more than four months is needed for feeding in JB cattle [2], and therefore it is possible that more serious depression of Th1 cells could develop in cattle around 20 months of age when they are subjected to long periods of VA deficiency. The reason for the significantly decreased γδ T cell number in the Low VA group was unclear in the present study, but the differences of T cell populations might have been affected by the decrease in the IFN-γ/IL-4 rate in the Low VA group because $CD4^+$ T and γδ T cells are lymphocytes that produce IFN-γ [14, 20].

Previous studies have shown that retinoic acid (RA) can enhance direct action on B cell populations [9]. RA can enhance antibody responses by a direct effect upon B cells, and modulate cytokine receptors to extend to B cells [23]. In the present study, we observed a significantly lower peripheral B cell number in the Low VA group, and low levels of RA seemed to decrease the number of activated B cells in the Low VA group.

Although non-diseased JB cattle were observed in the present study, there were cattle with seriously low levels of serum VA. A previous study has reported that the cause of VA deficiency in JB cattle is not only VA insufficiency in the feeding content but also the stress experienced by each animal [2]. The causes of lower levels of serum VA were

not clear in the present study, but it is possible that several stresses could have induced VA deficiency in the Low VA group cattle. In dairy cows, it is well known that a higher incidence of new intramammary infections occurs during the early milking period in cows fed a VA deficient diet [11, 12]. We suggest that VA deficiency leads to a high risk of the incidence of infection in beef cattle. Caution may be required in the care of JB cattle with serum VA levels are under 30 IU/d*l* during fattening stages. In dairy cows, β-carotene increase blood and milk phagocyte killing ability [11] as well as peripheral blood lymphocyte proliferation [12]. Feeding of the dietary beta-carotene heightens cell-mediated and humoral immune responses in animals [10], and thus, we suggest that maintenance of the level of VA is required to prevent infectious diseases in cattle with serious VA deficiency.

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EFERENCES

1. Adachi, K., Kawano, H., Tsuno, K., Nomura, Y., Yamamoto, N., Arikawa, A., Tsuji, A., Adachi, M., Onumaru, T. and Ohwada, K. 1999. Relationship between Serum Biochemical Values and Marbling Scores in Japanese Black Steers. *J. Vet. Med. Sci.* **61**: 961–964.
2. Adachi, K., Fukumoto, K., Nomura, Y., Katsura, N., Arikawa, A., Tsuji, A. and Onimaru, T. 1998. Significant decrease of serum vitamin A level in Japanese Black beef steers after introduction to a farm. *J. Vet. Med. Sci.* **60**: 101–102.
3. Adachi, K., Kawano, H., Tsuno, K., Nomura, Y., Katsura, N., Arikawa, Tsuji, A. and Onimaru, T. 1997. Values of the serum components in Japanese black beef steers at farms with high productivity and low frequencies of disease and death in Miyazaki Prefecture. *J. Vet. Med. Sci.* **59**: 873–877.
4. Blomhoff, H.K. 2004. Vitamin A regulates proliferation and apoptosis of human T- and B-cells. *Biochem. Soc. Trans.* **32**: 982–984.
5. Bonet, M.L., Ribot, J., Felipe, F. and Palou, A. 2003. Vitamin A and the regulation of fat reserves. *Cell Mol. Life Sci.* **60**: 1311–1321.
6. Booth, A., Reid, M. and Clark, T. 1987. HypoVitaminosis A in feedlot cattle. *J. Am. Vet. Med. Assoc.* **190**: 1305–1308.
7. Bowman, T. A., Goonewardene, I. M., Pasatiempo, A.M.G., Ross, A. C. and Taylor, C. E. 1990. Vitamin A deficiency decreases natural killer cell activity and interferon production in rats. *J. Nutr.* **120**: 1264–1273.
8. Cantorna, M.T., Nashold, F.E. and Hayes, C.E. 1994. In Vitamin A deficiency multiple mechanisms establish a regulatory T helper cell imbalance with excess Th1 and insufficient Th2 function. *J. Immunol.* **152**: 1515–1522.
9. Chen, Q. and Ross, A.C. 2007. Retinoic acid promotes mouse splenic B cell surface IgG expression and maturation stimulated by CD40 and IL-4. *Cell Immunol.* **249**: 37–45.
10. Chew, B.P., Park, J.S., Wong, T.S., Kim, H.W., Weng, B.B., Byrne, K.M., Hayek, M.G. and Reinhart, G.A. 2000. Dietary beta-carotene stimulates cell-mediated and humoral immune response in dogs. *J. Nutr.* **130**: 1910–1913.
11. Daniel, L.R., Chew, B.P., Tanaka, T.S. and Tjoelker, L.W. 1991. Beta-carotene and Vitamin A effects on bovine phagocyte function in vitro during the periparturn period. *J. Dairy Sci.* **74**: 124–131.
12. Daniel, L.R., Chew, B.P. Tanaka, T.S. and Tjoelker, L.W. 1991. In vitro effects of β-carotene and Vitamin A on peripartum bovine peripheral blood mononuclear cell proliferation. *J. Dairy Sci.* **74**: 911–915.
13. Dawson, H. D., Collins, G., Pyle, R., Key, M., Weeraratna, A., Deep-Dixit, V., Nadal, C.N. and Taub, D.D. 2006. Direct and indirect effects of retinoic acid on human Th2 cytokine and chemokine expression by human T lymphocytes. *BMC Immunol.* **7**: 1–15.
14. Glimcher, L.H. and Murphy, K.M. 2000. Lineage commitment in the immune system: the T helper lymphocyte grows up. *Genes Dev.* **14**: 1693–1711.
15. Livak, K.J. and Schmittgen, T.D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. **25**: 402–408.
16. Ma, Y., Chen, Q. and Ross, A.C. 2005. Retinoic acid and polyriboinosinic: polyribocytidylic acid stimulate robust antitetanus antibody production while differentially regulating type 1/type 2 cytokines and lymphocyte populations. *J. Immunol.* **174**: 7961–7969.
17. Ohtsuka, H., Watanabe, C., Kohiruimaki, M., Ando, T., Watanabe, D., Masui, M., Hayashi, T., Abe, R., Koiwa, M., Sato, S. and Kawamura, S. 2006. Comparison of two different nutritive conditions against the changes in peripheral blood mononuclear cells of periparturient dairy cows. *J. Vet. Med. Sci.* **68**: 1161–1166.
18. Oka, A., Maruo, Y., Miki, T., Yamasaki, T. and Saito, T.1998. Influence of vitamin A on the quality of beef from the Tajima strain of Japanese Black cattle. *Meat Sci.* **48**: 159–167.
19. Riollet, C., Rainard, P. and Poutrel, B. 2001. Cell subpopulations and cytokine expression in cow milk in response to chronic *Staphylococcus aureus* infection. *J. Dairy Sci*. **84**: 1077–1084.
20. Rogers, A.N., Vanburen, D.G., Hedblom, E.E., Tilahun, M.E., Telfer, J.C. and Baldwin, C.L. 2005. Gammadelta T cell function varies with the expressed WC1 coreceptor. *J. Immunol.* **174**: 3386–3393.
21. Stephensen, C.B. 2001. Vitamin A, infection, and immune function. *Annu. Rev. Nutr.* **21**: 167–192.
22. Stephensen, C.B., Jiang, X. and Freytag, T. 2004. Vitamin A deficiency increases the in vivo development of IL-10-positive Th2 cells and decreases development of Th1 cells in mice. *J. Nutr.* **134**: 2660–2666.
23. Wei, D., Yang, Y. and Wang, W. 2007. The expression of retinoic acid receptors in lymph nodes of young children and the effect of all-trans-retinoic acid on the B cells from lymph nodes. *J. Clin. Immunol.* **27**: 88–94.
24. Wintergerst, E.S., Maggini, S. and Hornig, D.H. 2007. Contribution of selected vitamins and trace elements to immune function. *Ann. Nutr. Metab.* **51**: 301–323.