Variations of Immunoglobulins in Colostrum and Immune Milk as Affected by Antigen Releasing Devices*

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ABSTRACT : This work was conducted to examine the variation of immunoglobulins (Igs) in serum, immune milk, normal milk and colostrum upon implantation of a new Antigen Releasing Device (ARD). The core of each ARD housed an immunostimulating complex (ISCOM) that was made of adjuvant Quil A and type XIII lipase from a *Pseudomonas* sp. Each ARD was coated with polylactic acid, known as polylactide, that controls antigen release. Twenty lactating Chinese Holstein cows were divided into 2 groups (n = 10): test group and control group. All cows in the test group were implanted with a single injection in the right iliac lymph node with 3 types of ARDs, which were designed to release the antigens at d 0, 14 and 28 post-implantation. Blood and milk samples were collected from both groups, and colostrum samples were also collected from other post-partum cows in the same farm. Concentrations of IgG_1, IgA and IgM in whey and serum were measured by sandwich ELISA. The results showed that the IgG_1, IgA and IgM concentrations in serum and whey from the test group were higher than from the control group. Among the three Igs measured, the IgG_1 concentration in serum was significantly higher at d 40 after ARD implantation, and the IgG_1 concentration in whey peaked at d 9, 17 and 30, which corresponded with release of the antigen. Based on Pearson's correlation between Ig concentration and production parameters, IgA concentration in normal milk was positively correlated with lactation period, which reflected IgA changes during the lactation period in immune milk. In colostrum, IgG_1, IgA and IgM decreased abruptly from d 0 to 3, and then decreased slightly. In conclusion, serum IgG_1 concentration can be affected by controlled release of the ARD, while whey IgA levels are primarily affected by lactation period. These results may be useful in future studies designed to regulate concentrations of Igs in immune milk. (**Key Words :** Immunoglobulin, Immune Milk, Colostrum, Antigen Releasing Device)

INTRODUCTION

Immunoglobulins (Igs) are gamma globulin proteins that are found in blood and other body fluids of vertebrates, and are used by the immune system to identify and neutralize foreign objects, such as bacteria and viruses (Korhonen, 2000). For cattle, Igs are grouped into four isotypes, IgG (IgG_1 and IgG_2), IgA, IgM and IgE, based on the heavy chain they possess. Each of these Igs plays different roles and helps direct the appropriate immune response to different types of foreign objects (Butler et al.,

1983; Korhonen, 2000). IgG, IgA and IgM are present in high levels in milk, especially in colostrum. Among these Igs, IgG_1 is the most common and abundant, and the milk concentrations of IgG_1, IgA and IgM are highly variable (Grosvenor et al., 1993). One of the main natural functions of Igs is to passively immunize or protect the calf during the early vulnerable stages of life (Bush et al., 1980).

Specific immune milk obtained from cows that are immunized with various intestinal bacterial or viral antigens can prevent the attachment of pathogens to the epithelial lining, which is a critical step in the initiation of infection (Mehra et al., 2006). Hyperimmune colostral or milk preparations directed towards specific diseases may promote human health in the future, when consumed as part of a health-promoting diet or as supplement to medical treatment regimes (Ashraf et al., 2001; Marnila et al., 2003). Bovine Igs in colostrum and milk have the potential to be used as an immunological supplement in infant formula and other hyperimmune food (Lo and Kleiman, 1996; Tawfeek

et al., 2003). Thus, strategies to increase milk concentrations of Igs are highly sought after.

Numerous researchers have attempted to increase the production of hyperimmune milk or milk levels of Igs. Recently, we have reported a new method using implantation of an Antigen Release Device (ARD) to increase the concentration of specific antibodies against a common antigen (lipoprotein lipase) in serum and whey (Liu et al., 2009). The ARD is a controllable type of antigen transfer system to stimulate the animal to produce Igs (Pearse and Drane, 2004). The core of this ARD is an immunostimulating complex (ISCOM)-based vaccine. The ARD device is coated with different concentrations of biodegradable and biocompatible polylactic acid, also known as polylactide, which can control antigen release. Thus, the ARD should release the antigen gradually and have the same effect as several vaccinations. The objectives of the study were to i) determine the concentrations of Igs in serum and whey after implanting of the ARD, ii) detect the correlation between concentrations of Igs and production parameters, and iii) compare the concentrations of Igs in colostrum and in normal milk.

MATERIALS AND METHODS

Cows and ARD implantation

Twenty healthy second-lactation Chinese Holstein cows in mid-lactation were divided into two groups (test group and control group, n = 10) according to milk yield (26.0±3.5 kg) and lactation period (114±34 d). All cows were housed under the same conditions in Cangdafu farm located in northern China, and fed a TMR ration three times daily.

Immune milk was produced by implanting the ARDs provided by Agri-BIOTECH (Perth, Western Australia). Each capsule of ARD contained 0.76 mg of *Pseudomonas* lipase and 0.25 mg of Quil A. Three types of ARDs were implanted into each cow of the test group. ARD_1 (2.3 mm in diameter and 6.2 mm in length) was not coated with polylactide and thus released its antigen immediately. ARD_2 and ARD_3 (both are 5.0×10.0 mm) were coated with different concentrations of polylactide and released their antigen at 14 and 28 d, respectively, after implantation. The immunostimulating complex (ISCOM) in the core of the ARDs was made of adjuvant Quil A and Type VIII lipase from a *Pseudomonas* sp. (Sigma, St. Louis, MO). Each cow of the test group was implanted with the three types of ARDs at the same time in the right iliac lymph node using an implantation gun (Synovex; Fort Dodge, Baulkham Hills, Australia) with a single injection.

Sample collection

The test period lasted for 40 d. Milk production was recorded every 10 d for each cow. Milk samples were collected at d 0, 5, 7, 9, 15, 17, 20, 26, 30 and 40 after ARD implantation. For each animal, duplicate milk samples were collected at 09:00, 16:00 and 22:00 on each sampling day using a robotic system, and mixed in a ratio of 4:3:3 for morning, afternoon, and evening milk by volume. The first aliquot of milk sample (50 ml) from each milking was preserved with bronopol-B2 and subsequently analyzed for fat, protein, lactose, dry matter and somatic cell count (SCC) using a MilkoScan Minor machine (MilkoScan 4000, Foss Electric, Hillerod, Denmark). The second aliquot of milk sample (30 ml) from each individual cow was centrifuged at 1,500 rpm for 15 min at 4°C to remove the fat (Legend Mach 1.6/R, Sorvall, Germany). Then, 5 μl of rennin (200 mg ml^{-1}, Sigma) was added to 10 ml of the centrifuged milk. The milk samples were kept at 37°C for 2 h and then were centrifuged at 5,000 rpm for 30 min. The whey samples, collected from the supernatant, were frozen at -80°C. Simultaneously, colostrum samples were collected every day for the first 7 days after parturition from 27 gravid Chinese Holstein cows on the same farm.

Blood samples were taken from the caudal vein of each animal in evacuated tubes containing EDTA (1.8 mg ml^{-1} of blood) at d 0, 5, 11, 15, 20, 30 and 40 after implantation, and then centrifuged at 3,000 rpm for 30 min to separate serum. The serum samples were frozen at -80°C for further analysis.

Determination of Igs concentrations in whey and serum

Concentrations of IgG_1, IgA and IgM were measured by sandwich enzyme-linked immunosorbent assay (ELISA) using the Bovine IgG_1/IgA/IgM ELISA Quantitation Kit (Bethyl Laboratories, Inc., Montgomery, TX). The serum samples were diluted to 1:5,000, 1:1,000 and 1:1,000, whereas the whey samples were diluted to 1:1,000, 1:500 and 1:1,000 for IgG_1, IgA and IgM assays, respectively. The final absorbance of the samples was measured at 450 nm, using an ELISA plate-reader (Infinite F200; Tecan, Switzerland). The quantification procedures were performed according to the manufacturer's instruction. Assay precision was defined by determining intra-assay and inter-assay variations. The intra-assay and inter-assay coefficients of variation were 8.9% and 10.3%, respectively.

Statistical analysis

The data was analyzed using the MIXED model of SAS 9.0 (SAS Institute, Cary, NC, USA). The statistical model included cows (individual animals) as a random effect and period and treatment as fixed effects, and significance was assumed at $p<0.05$. The correlation coefficients among indices were analyzed by Pearson's correlation coefficient, and significance was assumed at $p<0.05$. The data on the immunoglobulin concentrations of colostrum was analyzed using Duncan's multiple range tests and significance was

Table 1. The concentrations of IgG_1/IgA/IgM in serum and whey (mg ml^{-1})

Samples	Items	Treatment			ANOVA ($p<0.05$)		
		Test	Control	SEM	Treatment	Period	Treatment×period
Serum	IgG_1	3.186	2.344	0.113	NS	0.0014	NS
	IgA	0.133	0.121	0.022	NS	0.0003	NS
	IgM	2.864	2.454	0.409	NS	0.0007	NS
Whey	IgG_1	0.435	0.311	0.109	NS	NS	0.6248
	IgA	0.159	0.131	0.026	NS	<0.0001	0.0213
	IgM	0.039	0.040	0.006	NS	NS	0.0900

Test = Test group, implanted with 3 types of ARD (n = 10); Control = Control group, without ARD implantation (n = 10).
NS = Not significant ($p>0.05$).

accepted at $p<0.05$.

RESULTS AND DISCUSSION

Variation of Igs concentrations in serum and whey after ARD implantation

The IgG_1/IgA/IgM production in serum and whey at different days after ARD implantation is shown in Table 1. In serum, the concentrations of IgG_1, IgA and IgM increased after ARD implantation, but not significantly. The concentrations of IgM and IgA varied significantly ($p<0.05$) along with lactation period. Concentration of IgG_1 was maintained at a relatively low level during the first 15 d, then increased significantly ($p<0.05$) to peak at d 20, and remained at that level for the following 20 d. In particular, concentration of serum IgG_1 in the test group was significantly ($p<0.05$) higher than in the control group at d 40 (Figure 1), 12 d after the release of ARD_3.

In whey, the concentrations of Igs also increased after ARD implantation, but not significantly (Figure 2). Concentration of IgA in whey decreased slowly until d 15, then increased sharply, and finally decreased again;

concentration of IgM showed no significant ($p>0.05$) changes during the test period. For IgG_1, the concentration began to ascend at d 0 after ARD implantation, and then peaked at d 9, 17, 30, which was consistent with the release of the three ARDs. ARD_1, ARD_2 and ARD_3 released their antigens at d 0, 14 and 28, and the concentration of IgG_1 increased and persisted for 10 d, 2 d and 1 d, respectively, before dropping gradually. These results clearly demonstrated the stimulatory effect of the antigen on IgG_1 production.

ISCOM antigens in the core of the ARD can stimulate humoral response and production of Igs, of which IgG_1 is the main antibody involved in the immune response (Caffin et al., 1983). The three releases of ISCOM in the body of the cow were equivalent to primary, secondary, and booster vaccinations, respectively. In dairy cows, almost all milk IgG_1 comes from the blood, while milk IgA and IgM comes from both the blood and synthesis by plasmocytes in the mammary gland (Butler, 1998). IgG_1 is typically transferred selectively from serum into colostrum or milk by a neonatal Fc receptor (FcRn)-mediated mechanism in the mammary gland secretory epithelium (Kolb, 2002; Vaccaro et al.,
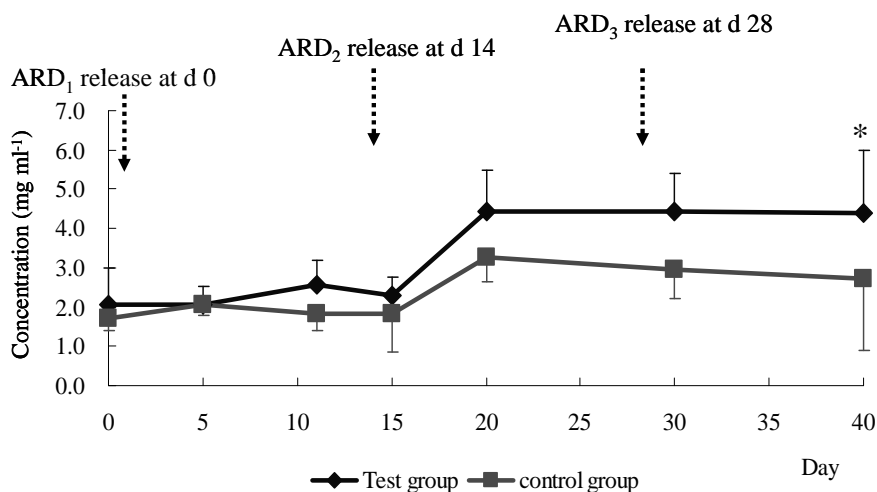

Figure 1. The variations of IgG_1 concentration in serum. The values are noted as mean±se. * Denotes levels that are significantly different from those of the control group at the same day ($p<0.05$). Test group, implanted with 3 types of ARD (n = 10); control group, without ARD implantation (n = 10).
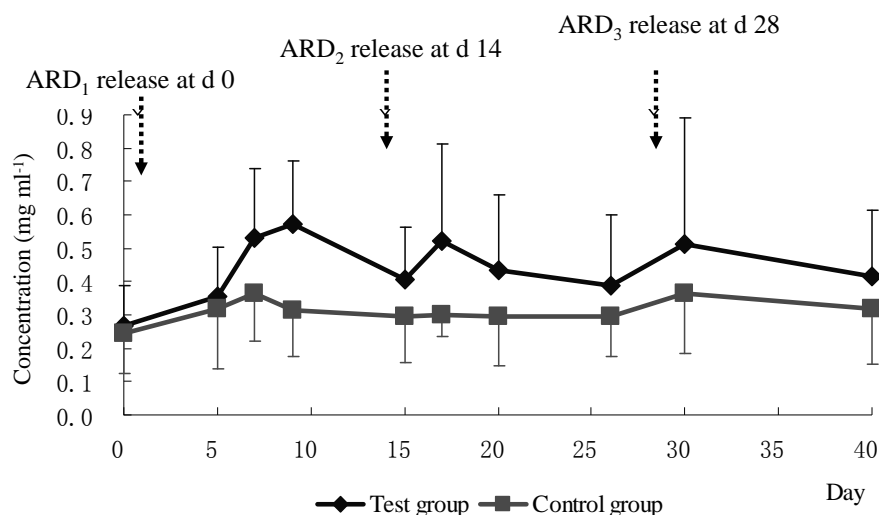
Figure 2. The variations of IgG_1 concentration in whey. The values are noted as mean±se. Test group, implanted with 3 types of ARD (n = 10); control group, without ARD implantation (n = 10).

2005; Anderson et al., 2006). Therefore, the regularity patterns of IgG_1 concentrations, but not IgA and IgM, were consistent with the releases of the three ARDs.

In this study, concentrations of IgG_1, IgA, and IgM in the serum and the whey were not affected significantly by the ARD implantation, but the serum IgG_1 concentration significantly ($p<0.05$) increased 12 d after the third release of the ARD. The delayed increase in concentration of serum IgG_1 suggests that the level of the ISCOM vaccine was inadequate to stimulate a quick immune response. In our previous study, Liu et al. (2008) revealed that specific IgG titer and IgG mass in serum and whey increased significantly ($p<0.05$) in cows implanted with the same ARD, and the specific IgG titer corresponded well with the releases of the antigen from 3 types of ARD. Cheng et al. (2008) indicated that the implantation of the same ARD did not significantly increase the concentration of lactoferrin in the serum and milk throughout the whole experiment period except on two occasions. The levels of lactoferrin in the milk and serum significantly increased on d 7 and 11 after implantation. In addition, Claerebout and Vercruysse (2000) found that the level of IgG_1 against gastrointestinal

nematodes (GIN) appeared to be dependent on levels of antigenic stimulation. Therefore, a higher level of ISCOM vaccine or prolonged immune program may be required to significantly increase the concentrations of Igs in milk.

Correlation between milk Igs concentrations and production parameters

In this study, we found that the lactation period also affected the concentrations of Igs after the ARD stimulus. To assess whether other production parameters can affect the concentrations of Igs in milk in normal dairy cows, we used the Pearson's correlation coefficient in comparing the differences between production parameters of dairy cows (including lactation period, dry matter, milk yield, fat, protein, lactose, SCC) and concentrations of Igs in milk (Table 2). There was no significant correlation between the concentrations of Igs and production parameters except for IgA in the whey. The whey concentration of IgA had a significant ($p<0.05$) correlation with lactation period with a Pearson's correlation coefficient of 0.76, which corresponded to the above-mentioned results in immune milk and is consistent with results from Korhonen et al.

Table 2. Pearson's correlation coefficients between concentrations of Igs and production parameters of dairy cow

Igs		Lactation period	Milk yield	Milk fat	Milk protein	Milk lactose	Dry matter	SCS
Serum	IgG_1	0.339	-0.256	0.064	0.073	-0.009	0.072	0.303
	IgA	-0.229	-0.223	-0.106	0.103	0.552	0.055	-0.268
	IgM	-0.498	-0.198	0.079	0.092	0.170	0.132	-0.348
Whey	IgG_1	-0.390	0.098	0.311	0.354	-0.328	0.303	-0.276
	IgA	0.76*	0.641	0.165	0.084	0.026	0.177	0.338
	IgM	-0.073	0.411	0.336	0.332	0.259	0.337	-0.174

The data were obtained from milk and serum samples of all the cows in both the test and control groups (n = 20) at d 0 before the ARD implantation.
The data marked with an asterisk (*) indicate significant correlation ($p<0.05$).
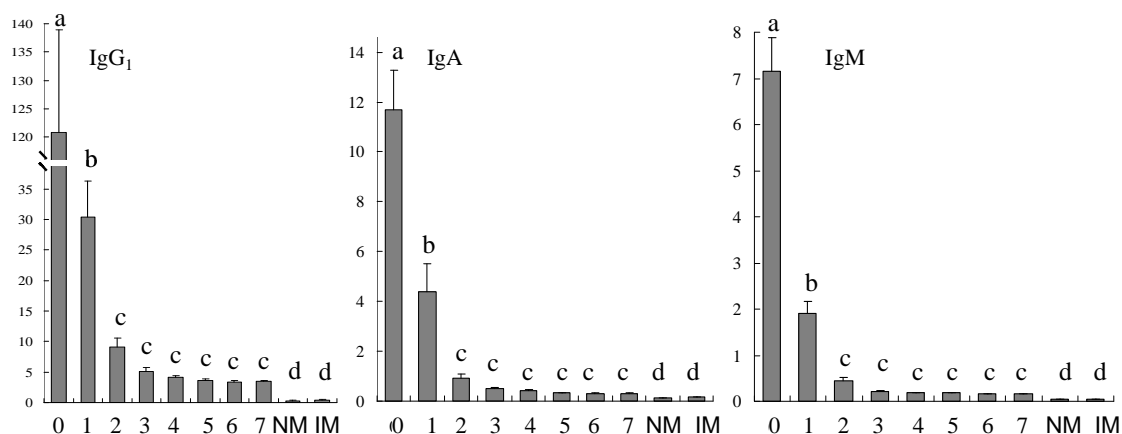Somatic cell score (SCS) was calculated from the formula: $\log_2 (SCC/100,000)+3$.

Figure 3. Variations of IgG_1/IgA/IgM concentrations from colostrum to normal milk (mg ml^{-1}). In abscissa, 0 to 7 refer to d 0 to 7. NM, normal milk from dairy cows (n = 10) without ARD implantation; IM, immune milk from the test group cows (n = 10). Different letters (a, b, c, d) indicate significant differences at p<0.05.

(2000). However, from an analysis of 299 Chinese Holstein cows, Liu et al. (2009) noted that milk IgG_1 concentration was significantly correlated with lactation number, stage of lactation, daily milk production and SCC. Sanchez et al. (2004) also revealed a correlation between lactation period and milk IgG concentration. Other factors, such as maternal age, parity, breed, nutritional status, premature parturition, premature lactation, and time after parturition, were also shown to affect the levels of Igs (McFadden et al., 1997). These conflicting results may arise from small sample numbers, selective transport process in the mammary gland, or varied milk production (Caffin et al., 1983).

Variation of Igs concentrations in colostrum

Figure 3 shows the variation of IgG_1, IgA and IgM in the colostrum sampled from the other 27 gravid dairy cows on the same farm. During the first 3 d, the concentrations of colostral IgG_1, IgA, and IgM all decreased significantly (p<0.05), from 120.81±94.38 to 9.09±8.03 mg ml^{-1}, from 11.69±8.31 to 0.91±0.88 mg ml^{-1}, and from 7.16±0.45 to 0.45±0.37 mg ml^{-1}, respectively. During the remaining 5 d of sampling, the concentrations of IgG_1, IgA and IgM also decreased, but not significantly. However, concentrations of IgG_1, IgA and IgM in normal and immune milk were significantly (p<0.05) lower than in the colostrum samples. Our results are, in general, consistent with previous reports (Quigley et al., 1994; Butler, 1998; Levieux and Ollier 1999), except for a higher colostral IgG_1 concentration (120.81±94.38 mg ml^{-1}) than that (59.8±28.5 mg ml^{-1}) noted by Levieux and Ollier (1999) who used a single radial immunodiffusion assay at d 0. Presumably, this discrepancy can be attributed to the different sampling period and/or the method used. Nevertheless, IgG_1 had the highest concentration compared to other Igs in the colostral samples (Figure 3). As the most abundant Ig in colostrum, the

concentration of IgG was approximately 50 to 100 times higher than in normal milk (Mero et al., 1997).

CONCLUSION

The present study showed that there were more Igs in colostrum than normal milk, and lactation period could affect the milk IgA concentration. Serum IgG_1 concentration only began to increase significantly after the third ARD release, suggesting that an ISCOM vaccine dose higher than the one used is needed to significantly increase milk concentrations of Igs. The results can be used to design future studies to determine the appropriate doses and/or the number of vaccine releases.

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