

Level of nutrition and age at weaning: effects on humoral immunity in young calves

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Thirty-two calves were studied to determine the effects of level of nutrition (400 and 1000 g (air-dry matter) milk substitute per d) and age at weaning (5, 9 and 13 weeks) on humoral immune responses measured in serum and bronchoalveolar washings (BAW). All calves were immunized with Keyhole limpet haemocyanin (KLH) at 3 weeks of age, and with horse erythrocytes (HRBC) 1 d after weaning. Feeding the higher level of nutrition compared with the lower level decreased anti-HRBC titres and serum IgG₂ and IgA responses to KLH ($P < 0.05$). Weaning at 5 compared with 9 weeks of age decreased serum anti-HRBC responses ($P < 0.05$), but weaning age had no effect on anti-KLH responses ($P > 0.05$). Feeding the higher level of nutrition increased total protein ($P < 0.05$) and IgG₂ concentrations ($P < 0.01$) in BAW. The results showed that variation in husbandry conditions that is within conventional limits affects humoral immune responses in young, artificially-reared calves.

Level of nutrition: Weaning: Immune function: Calves.

Husbandry practices used for the rearing of bucket-fed calves employ a variety of pre-weaning feeding levels and ages of weaning (Roy, 1980; Webster, 1984). Both of these factors have been shown to influence calf health. Feeding increased levels of milk substitute (MS) to calves before weaning resulted in decreased disease incidence (Williams *et al.* 1980) while weaning (cessation of feeding liquid MS) has been associated with increased incidence and severity of disease (Roy *et al.* 1971; Jenny *et al.* 1981).

The effects of weaning on disease incidence have been considered to be mediated partly by stress-induced effects on the immune system (Kelley, 1980; Griffin, 1989). Moreover, weaning of young calves involves a profound nutritional change along with behavioural stress. Adaptation to nutritional change results in metabolic and hormonal changes which alter immune responsiveness (Keusch *et al.* 1983; Stinnett, 1983). The intake of solid food by young calves varies with the intake of liquid feed, age of calf and nature of solid food. Furthermore, adaptation to the post-weaning diet may be affected by the pre-weaning diet. Typically, in calves weaned at 5 weeks of age, solid food intakes alone will supply maintenance requirements only. However, in calves weaned at 9 and 13 weeks of age the intakes may be adequate to provide maintenance and growth rates of 500–750 g/d.

This experiment was designed to determine, in calves, the effects of three ages of weaning, 5 and 9 and 13 weeks, and two levels of pre-weaning nutrition on humoral immune responses to antigens given either 2 weeks before weaning or 1 d after weaning.

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MATERIALS AND METHODS

Experimental design

Two consecutive blocks of sixteen calves were kept in individual pens. There were twenty-four Friesian (castrated male) and eight Hereford \times Friesian (four castrated male and four female) calves. The calves were allocated to four treatments on the basis of similar breed, sex and live weight at 7 d of age. There were eight calves per treatment, with four calves per treatment per block. Within a block, the treatments and immunizations (except horse erythrocytes (HRBC)) were given and the measurements were made on the same day to each calf.

All calves were given, by oesophageal intubation, 2 litres mixed-source colostrum by 6 h of age. Thereafter, additional colostrum was given by mouth. The calves had a mean serum IgG₁ concentration of 6.1 (SEM 1.61) mg/ml at 7 d of age, indicating satisfactory absorption of colostrum immunoglobulin (Roy, 1990). In three of the treatments, calves were given 400 g air-dry matter (ADM) MS per day, divided equally between two feeds, until weaning at 5, 9 and 13 weeks of age (treatments 5L, 9L and 13L respectively). In the fourth treatment calves were given 1000 g MS/d until weaning at 9 weeks of age (treatment 9H). The MS (Volac Easi-Mix, Instant High Fat Milk Replacer; Volac Ltd, Royston, Hertfordshire) was a skimmed milk-based diet containing 254 g crude protein (CP; N \times 6.25)/kg ADM and an estimated 20 MJ metabolizable energy (ME)/kg ADM. All calves from 7 d of age were offered *ad lib.* water, hay and calf-weaner concentrate diet (Calfwean Quickettes; BOCM Silcock, Basingstoke, Alton, Hampshire) containing 185 g CP/kg ADM and an estimated 10.5 MJ ME/kg ADM. Live-weight gains and intakes of concentrate diet were recorded.

Immunizations

All calves were immunized with 1 mg Keyhole limpet haemocyanin (KLH; Calbiochem, San Diego, California, USA) in alum, given subcutaneously at 3 weeks of age (Pollock *et al.* 1991). The calves were immunized with HRBC 1 d after weaning. For this, HRBC were washed three times in phosphate-buffered saline (9 g NaCl/l; PBS) and 1 ml of a suspension of HRBC in PBS (50 g HRBC/l) was injected intravenously. Serum samples were collected at 1, 3 and 5 weeks of age and then weekly to 16 weeks of age.

Collection of bronchoalveolar washings (BAW)

BAW were collected from all calves at 7, 11 and 15 weeks of age. For this the calves were sedated using intramuscular injections of 0.2 mg xylazine/kg body weight (Rompun; Bayer, Bury St. Edmunds, Suffolk) and placed in sternal recumbency. A sterile polyethylene cannula was then passed into the lung via an endotracheal tube and 100 ml sterile PBS was introduced using a syringe. The PBS was immediately withdrawn using gentle negative pressure. BAW with blood contamination, as detected by Multistix (Miles, Elkhart, Indiana, USA), were excluded from further analyses. Protein concentrations were measured by the Folin phenol method (Lowry *et al.* 1951) using standard curves derived from bovine serum albumin (BSA; Sigma, Poole, Dorset).

Antibody determinations

Anti-KLH IgM, IgG₁, IgG₂ and IgA antibody responses were measured in sera and BAW by indirect ELISA (Pollock *et al.* 1991). Monoclonal antibodies (MAb) specific for class and subclass of bovine immunoglobulins were kindly provided by Dr F. Westenbrink, Lelystad, The Netherlands (van Zaane & Ijzerman, 1984).

Anti-HRBC antibody responses were measured by direct haemagglutination. Starting from a 1:50 dilution, serum samples in duplicate, were double diluted in 50 μ l PBS across microtitre plates with round-bottomed wells (Flow, Rickmansworth, Herts.). A suspension of 5 g HRBC/l (50 μ l) was then added to each well. The plates were incubated for 1 h at 37° and then overnight at 4°. Titres were expressed as the \log_2 of the reciprocal of the highest dilution giving complete agglutination, a half-point being added if the next highest dilution showed partial agglutination.

Measurement of total immunoglobulin concentrations

Total concentrations of IgM, IgG₁, IgG₂ and IgA were measured by sandwich ELISA. Optimal concentrations of all reagents were determined by chequer-board titrations. ELISA plates (Flow, Rickmansworth, Herts.) were coated overnight at 37° with anti-immunoglobulin subclass-specific MAb in 0.05-M-carbonate buffer (pH 9.6; 100 μ l/well). The plates were then washed three times with PBS containing 0.5 ml Tween 20/l (PBST) and dilutions of test sera, BAW or purified immunoglobulin standards were added to duplicate wells. The immunoglobulin standards were prepared from serum or colostrum whey by gel filtration, ion-exchange chromatography and affinity chromatography following published methodologies (Butler & Maxwell, 1972; Collard *et al.* 1984; Nielsen & Duncan, 1985). Purity of the preparations was confirmed by ELISA, immunoelectrophoresis and polyacrylamide gel electrophoresis (SDS-PAGE). After incubation for 2 h at 37° the plates were washed as above and a rabbit antiserum known to bind to all bovine immunoglobulin subclasses (Pollock, 1990) was added in PBST containing 40 ml horse serum/l (PBST/H). The plates were again washed after 1 h incubation at 37° and goat anti-rabbit IgG conjugated to alkaline phosphatase (EC 3.1.3.1; Sigma) was added for 1 h at 37°. After washing, Sigma alkaline phosphatase substrate (S-104) was added in glycine buffer (pH 10.4) and absorbance (*A*) at 405 nm (*A*₄₀₅) was determined using an ELISA reader (Titertek) after colour development. Immunoglobulin concentrations in unknown samples were determined by comparison of *A*₄₀₅ with standard curves produced using purified immunoglobulins of known concentrations.

Statistical analysis

The method of least squares was used to fit general linear models for repeated measures. Analyses were made using a computer with Statistical Analysis System (SAS) GLM procedure software (SAS/Stat. Version 6.03, SAS Institute, Cary, NC, USA). Multivariate analyses of variance were used to determine statistically significant interactions and main effects. When significant interactions were identified, univariate analyses of variance were used to determine any significant within-time, between-treatment effects. Time effects and overall treatment effects were not reported as they were not considered appropriate given the experimental design. For treatments within age, SEM were calculated by univariate analyses of variance for each age.

One calf on treatment 5L developed urolithiasis. The results for this calf were tested for significance as outliers using the maximum normal residual test (Snedecor & Cochran, 1980). The growth rate and feed intakes for that calf were significant outliers and were excluded from the analyses ($P < 0.05$). The immunological measurements for that calf were not significant outliers ($P > 0.05$). Analyses of the immunological data were therefore made including and excluding results for that calf. As the means for treatment 5L were similar in both cases and there were no differences in the significance of effects, immunological data are presented including results from that calf.

Table 1. *Effects of age at weaning and level of nutrition on growth rates (g/d) and total intakes of crude protein (CP; N × 6.25; kg) and metabolizable energy (ME; MJ) in calves at 5, 9, 13 and 17 weeks of age*

Age at weaning (weeks)...	5	9	9	13	
Nutritional level...	Low	Low	High	Low	
Treatment group...	5L	9L	9H	13L	
n...	7	8	8	8	SEM*
9 d					
Live wt (kg)	39.6	39.6	38.0	39.6	1.46
5 weeks					
Growth rate†	446 ^a	478 ^a	736 ^b	438 ^a	34.6
Total CP	5.53 ^a	5.32 ^a	6.30 ^b	5.01 ^a	0.242
Total ME	386 ^a	373 ^a	464 ^b	355 ^a	13.9
9 weeks					
Growth rate	538 ^a	557 ^a	804 ^b	603 ^a	25.9
Total CP	14.7 ^a	15.1 ^a	17.2 ^b	14.0 ^a	0.59
Total ME	913 ^a	997 ^a	1244 ^b	933 ^a	34.1
13 weeks					
Growth rate	678	670	837	664	56.6
Total CP	29.7	28.9	30.7	28.4	1.27
Total ME	1782	1793	2022	1823	73.5
17 weeks					
Growth rate	692 ^a	700 ^a	834 ^b	768 ^{a,b}	27.1
Total CP	45.0	44.6	48.5	45.0	1.40
Total ME	2668 ^a	2697 ^a	3048 ^b	2784 ^{a,b}	80.9

^{a,b} Means with unlike superscript letters within a row were significantly different ($P < 0.05$).

* SEM was based on eight calves per treatment.

† SEM for age within calves 27.7 g/d.

RESULTS

Growth rates and intakes of calves

There was a significant age × treatment interaction for growth rates ($P < 0.001$; Table 1). The 9H calves had greater growth rates and total intakes of CP and ME than 5L, 9L and 13L calves, significantly so at 5 and 9 weeks of age ($P < 0.05$).

Serum concentrations of immunoglobulins

There were no significant age × treatment interactions and no significant effects of treatments within ages for serum concentrations of IgA, IgG₁, IgG₂ and IgM. Results for calves at 1, 5, 9 and 13 weeks of age are shown in Table 2.

Serum anti-KLH antibody responses

For IgG₂ and IgA responses there were significant age × treatment interactions ($P < 0.05$; Fig. 1). Calves given treatments 5L, 9L and 13L had similar responses at all ages, which suggested that age at weaning had no significant effect on these responses. At most ages calves given the high (9H) compared with the low (9L) level of nutrition had significantly lower IgG₂ and IgA responses ($P < 0.05$). There were similar trends for the effects of treatments on IgG₁ responses ($P > 0.05$). There were no apparent effects of treatments on IgM responses.

Serum anti-HRBC haemagglutination responses

Calves were immunized with HRBC 1 d after weaning and, therefore, any effects of age at weaning were confounded with age at immunization. However, there was a significant time

Table 2. *Effects of age at weaning and level of nutrition on serial serum concentrations (mg/ml) of immunoglobulins (Ig) in calves*

Age at weaning (weeks)...		5	9	9	13	
Nutritional level...		Low	Low	High	Low	
Treatment group...		5L	9L	9H	13L	
n...		8	8	8	8	
Age (weeks)*	Ig					SEM
1	A	0.10	0.14	0.11	0.09	0.015
	G ₁	6.33	7.65	5.66	4.82	1.613
	G ₂	1.96	1.65	1.57	1.68	0.288
	M	0.81	0.97	0.86	0.86	0.187
5	A	0.06	0.03	0.03	0.04	0.011
	G ₁	5.04	2.96	3.71	3.63	0.693
	G ₂	1.34	0.52	0.62	0.67	0.276
	M	0.47	0.50	0.31	0.42	0.131
9	A	0.07	0.09	0.04	0.03	0.017
	G ₁	4.33	5.98	4.05	4.56	1.107
	G ₂	0.87	0.55	0.88	0.46	0.212
	M	0.55	0.39	0.34	0.48	0.114
13	A	0.05	0.02	0.03	0.02	0.012
	G ₁	2.86	2.02	1.87	3.11	0.417
	G ₂	1.19	1.26	1.14	1.02	0.308
	M	3.18	0.68	0.66	0.91	0.694

* SEM for age within calves were 0.035, 0.069, 0.073 and 0.071 for IgA, IgG₁, IgG₂ and IgM respectively.

post-immunization (PI) × treatment interaction ($P < 0.01$; Fig. 2). At 1 week PI, in calves given the low level of nutrition, weaning and immunization at 5 (5L) compared with 9 (9L) weeks of age gave significantly lower titres ($P < 0.05$), and compared with 13 (13L) weeks of age tended to give lower titres ($P > 0.05$). The results for calves weaned at 9 weeks of age (9H and 9L) showed that level of nutrition affected haemagglutination titres. Calves given the high (9H) compared with the low (9L) level of nutrition had lower titres, significantly so at 1 week PI ($P < 0.05$).

Total immunoglobulins and anti-KLH antibodies in BAW

The mean volume of BAW recovered from 100 ml infusions was 53.2 ml. There were no significant age × treatment interactions and no significant treatment-within-age effects for the volume of BAW recovered ($P > 0.05$). It had been intended to circumvent any effects of variable dilution of bronchoalveolar fluids in BAW by relating immunoglobulin concentrations to total protein concentrations (Daniele, 1988). However, this was not indicated because of a significant age × treatment interaction for total protein concentration in the BAW ($P < 0.05$; Table 3). At 7 weeks of age, 9H calves had significantly greater total protein concentrations than all other calves ($P < 0.05$). Therefore, given that the volume of recovered BAW was similar across treatments, the unmodified concentrations of total immunoglobulins and anti-KLH antibodies for each treatment were compared.

For total immunoglobulin in BAW the greatest concentrations were for IgG₁. There were no significant age × treatment interactions and no significant treatment effects of IgG₁, IgM and IgA ($P > 0.05$). However, there was a significant age × treatment interaction for IgG₂ concentrations. At 7 weeks of age calves given the high (9H) compared with the low (5L and 9L) plane of nutrition had significantly greater BAW IgG₂ concentrations ($P < 0.01$).

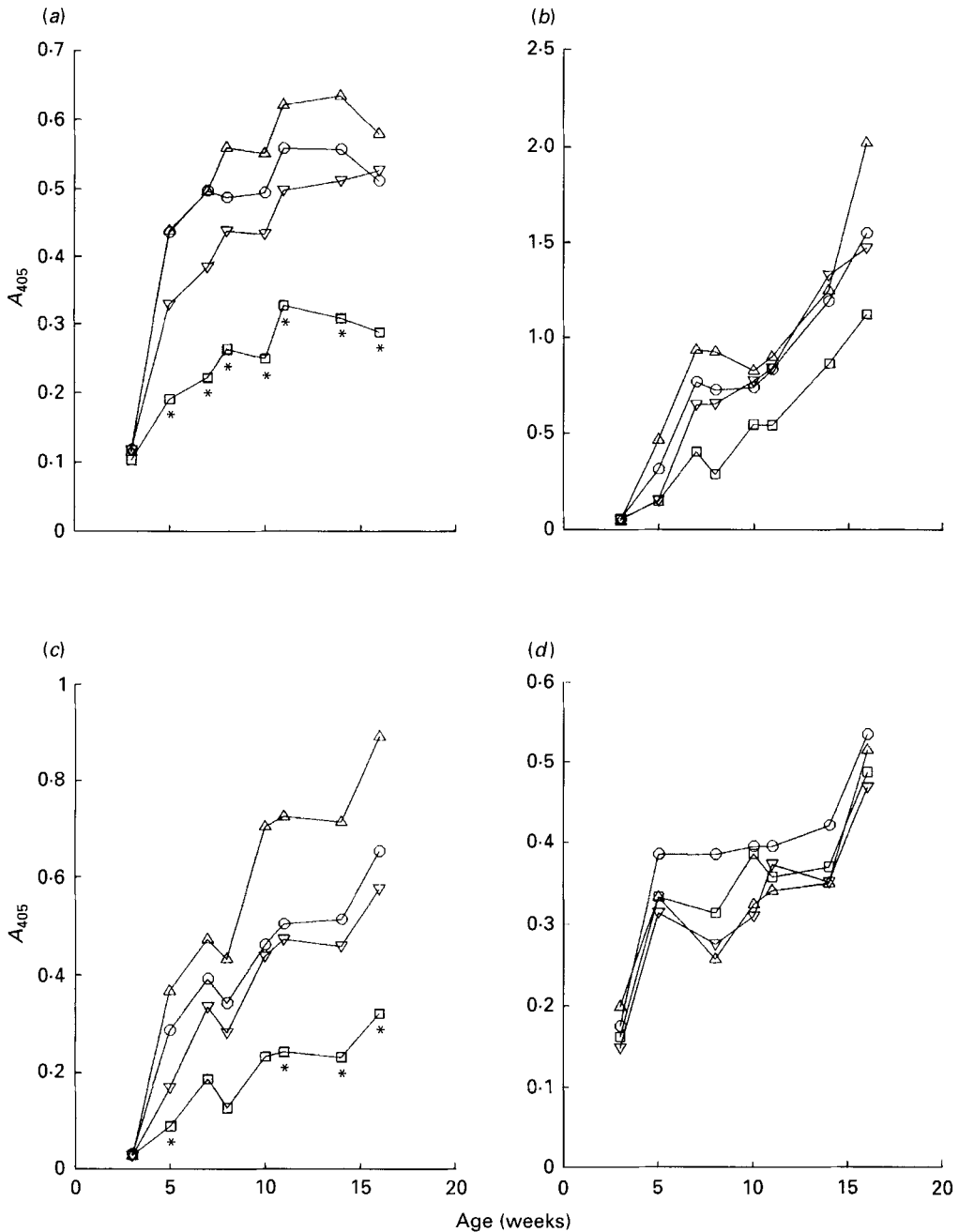


Fig. 1. Effects of age at weaning and level of nutrition on serial serum anti-keyhole limpet haemocyanin (KLH) antibody responses (mean A_{405}) in calves immunized with KLH at 3 weeks of age. Calves were given 400 g air-dry matter (ADM) milk substitute (MS) per d until weaned at 5 weeks (5L; ○), 9 weeks (9L; △) or 13 weeks (13L; ▽), or 1000 g ADM MS per d until weaned at 9 weeks (9H; □).

* At these times, 9H calves were significantly different from 9L calves, $P < 0.05$. SEM for age within calves were 0.0366, 0.1029, 0.0578 and 0.0285 for (a) IgA, (b) IgG₁, (c) IgG₂ and (d) IgM respectively.

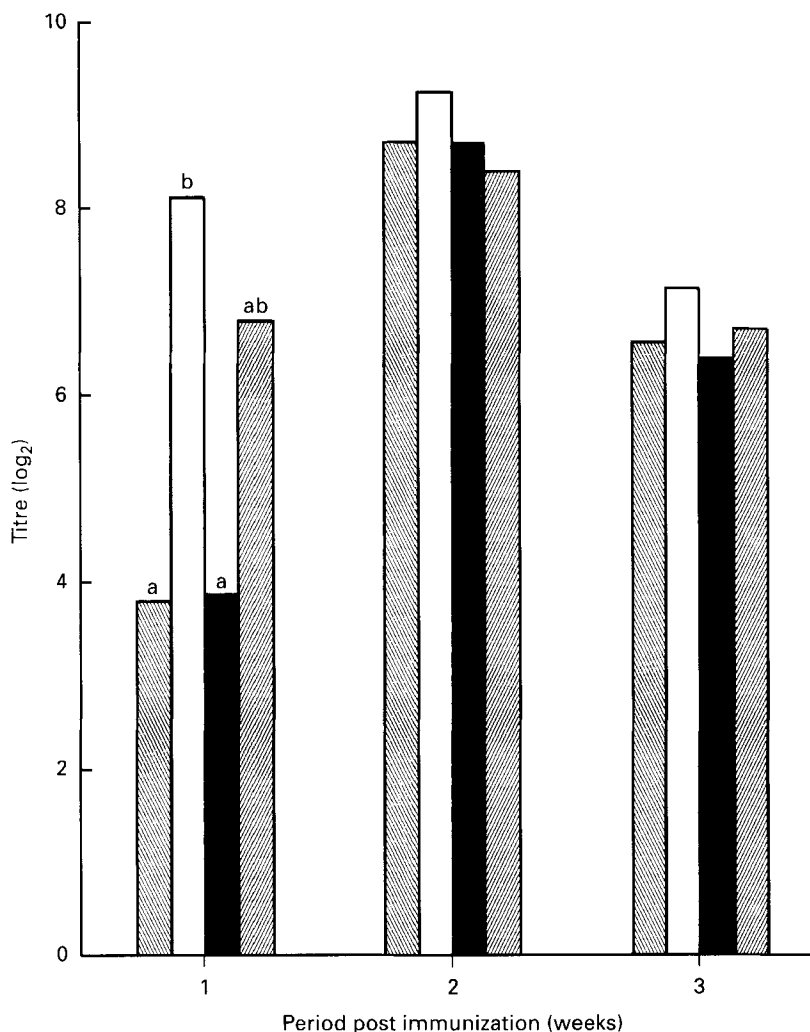


Fig. 2. Effects of age at weaning and level of nutrition on serial serum direct haemagglutination titres (\log_2 from initial dilution of 1:50) in response to immunization of calves with horse erythrocytes 1 d after weaning. Calves were given 400 g air-dry matter (ADM) milk substitute (MS) per d until weaned at 5 weeks (▨), 9 weeks (□) or 13 weeks (▩), or 1000 g ADM MS per d until weaned at 9 weeks of age (■).

a, b Means with unlike letters were significantly different ($P < 0.05$). SEM = 1.291, 0.344 and 0.365 (1, 2 and 3 weeks post-immunization respectively).

The anti-KLH antibody response in BAW appeared to consist mainly of IgA and IgG₁. There were no significant age × treatment interactions and no significant effects of treatment within age on anti-KLH antibodies in BAW (data not shown).

DISCUSSION

The results of this experiment show that age at weaning and level of nutrition affect humoral immune responses of young calves reared artificially on milk substitute given twice daily in buckets. The conditions of weaning and the levels of nutrition were selected to be

Table 3. *Effects of dietary and weaning treatments on total protein and immunoglobulin (Ig) concentrations (mg/ml) in serial bronchoalveolar washings*

Age at weaning (weeks)...		5	9	9	13		
Nutritional level...		Low	Low	High	Low		
Treatment group...		5L	9L	9H	13L		
n...		8	8	8	8	SEM	
Protein concentration*							
Age (weeks)	7	0.222 ^a	0.259 ^a	0.555 ^b	0.310 ^a	0.0721	
	11	0.362	0.298	0.271	0.250	0.0587	
	15	0.439	0.554	0.634	0.309	0.2350	
Ig concentration†							
Age (weeks)	7	A	0.012	0.014	0.037	0.021	0.0092
		G ₁	0.292	0.426	0.830	0.431	0.2282
		G ₂	0.086 ^a	0.128 ^a	0.437 ^b	0.216 ^{a, b}	0.1035
		M	0.018	0.015	0.025	0.021	0.0054
	11	A	0.117	0.062	0.063	0.067	0.0175
		G ₁	0.338	0.234	0.160	0.187	0.0829
		G ₂	0.146	0.162	0.100	0.119	0.0516
		M	0.021	0.018	0.017	0.019	0.0042
	15	A	0.066	0.065	0.058	0.073	0.0078
		G ₁	0.242	0.537	0.809	0.226	0.4148
		G ₂	0.132	0.248	1.269	0.085	0.6226
		M	0.027	0.025	0.024	0.027	0.0066

^{a, b} Means with unlike superscript letters within a row were significantly different ($P < 0.05$).

* Significant age \times treatment interaction (Multivariate ANOVA), $P < 0.05$. SEM for age within calves = 0.161.

† Significant age \times treatment interaction for IgG₂ (Multivariate ANOVA), $P < 0.01$. SEM for age within calves were 0.0113, 0.296, 0.389 and 0.0048 for IgA, IgG₁, IgG₂ and IgM respectively.

representative of practical farming conditions in the British Isles. Caution is required when comparing these results with those from other studies which have often involved extremes of stress and/or nutrition.

Under the current experimental conditions the high (9H) compared with the low (9L) level of nutrition caused decreased serum antibody responses to KLH and decreased anti-HRBC titres. Previously in cattle, lower levels of nutrition have been associated with lower serum antibody responses (Fiske & Adams, 1985; Griebel *et al.* 1987). The apparent contradiction between those reports and the results reported here was probably due, at least in part, to the levels of nutrition investigated. Fiske & Adams (1985) used a diet which resulted in neonatal calves losing 0.52 kg/d and Griebel *et al.* (1987) gave neonatal calves a diet providing 50% of maintenance requirements. In the present study the two diets provided at least maintenance requirements: the low level of nutrition approximated to at least maintenance requirements and the high level of nutrition approximated to twice maintenance requirements (Agricultural Research Council, 1980).

Interestingly, while serum antibody responses were affected by the nutritional treatments in the present study, there were no effects on total serum immunoglobulin concentrations. A similar situation has been reported in man, where undernourishment affected specific antibody responses but not total immunoglobulin concentrations (Law *et al.* 1973).

Previous studies in calves (Pollock *et al.* 1992) have shown that weaning at either 5 or 6 weeks of age increased serum antibody responses to KLH given at weaning but not to KLH given 2 weeks before weaning. In the present study serum antibody responses to HRBC given the day after weaning were lower in calves weaned at 5, compared with 9

weeks of age. However, this effect may be mainly one of age. Previous work (Pollock, 1990) found that calves weaned at 5 weeks of age produced similar anti-HRBC responses to unweaned calves of similar age. Gwazdauskas *et al.* (1978) found in 6–8-month-old calves that weaning lowered serum antibody responses to foreign RBC. The differences between, on the one hand, the results of Gwazdauskas *et al.* (1978) and, on the other hand, those of the present study and Pollock *et al.* (1992) may be caused by the differences in ages and the methods of feeding between these studies.

In this experiment BAW were collected from calves sedated with xylazine. Trials of an alternative technique using non-sedated calves (Fogarty *et al.* 1983) resulted in apparent severe distress for some of the small, young calves, so that a standardized use of xylazine in all calves was considered preferable. Total protein concentrations, which were assumed to be constant in bronchoalveolar fluids, have been used to correct immunoglobulin concentrations in BAW for differences in dilution of bronchoalveolar fluids by lavaging fluid (Daniele, 1988). The finding that experimental treatment may affect total protein concentrations, with elevated levels in BAW from calves on a high level of nutrition (treatment 9H), suggested that such an approach would be invalid in this study.

The present results and those of Pollock *et al.* (1992, 1993) indicate that weaning and nutrition influenced serum anti-KLH and anti-HRBC antibody responses but that circulating B-cell concentrations and total serum immunoglobulin concentrations were unaffected. The lack of effects on B-cell numbers, as described by Pollock (1990), and the absence of effects of weaning 2 weeks after immunization on anti-KLH antibody responses suggested that differences in antibody responses might be mediated by alterations in antigen presentation and/or helper-cell function. It was also possible that the maturation of B-cells to plasma cells and/or the antibody-producing capacity of individual plasma cells were affected. However, Kenney *et al.* (1968) reported that alterations in antibody responses in undernourished rats were not due to altered antibody production by individual cells. The fact that total serum immunoglobulin concentrations were unchanged by the weaning/nutrition treatments suggested that the effects on antibody responses were not due to changes in the catabolism of immunoglobulins. In man the half-life of γ -globulin was not affected by protein–energy malnutrition (Cohen & Hansen, 1962).

While the precise mechanisms leading to the effects noted in the present study and that of Pollock *et al.* (1992) are unclear, it is interesting to note that weaning and increasing nutrition had opposite effects on antibody responses. In young calves weaning is associated with behavioural stress (Griffin, 1989) and also with profound nutritional change (Martin, 1984). The present results and those of Pollock *et al.* (1993) suggest that the nutritional change that occurs when young calves are weaned is important in modifying immune responses and, thus, may affect disease susceptibility.

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