Immunology 1985 **56** 605

The effect of intraperitoneal and intramammary immunization of sheep on the numbers of antibody-containing cells in the mammary gland, and antibody titres in blood serum and mammary secretions

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Accepted for publication 6 August 1985

Summary. The contribution of gut-associated lymphoid tissue (GALT) to the local response in the mammary gland is well documented in laboratory animals and has been evaluated in this study in ruminants.

Ewes were immunized intraperitoneally (IP) with antigen in Freund's complete adjuvant (FCA), a procedure which stimulates the production of antibodies of the IgA class in the intestine, and challenged intramammarily (IMam) either during colostrum formation or mammary gland involution. Despite a substantial IgA antibody-containing cell (ACC) response in the intestine in IP immunized sheep, there was no evidence to suggest a relocation of IgA-specific ACC to the mammary gland. There was, however, an IgA antibody response in mammary secretion of IP immunized animals, regardless of whether the mammary gland was locally immunized, but the origin of this antibody is unclear. IP/IMam immunized sheep did have an enhanced antigen-specific ACC response of the IgG1 isotype in locally immunized glands, but whether these cells were of GALT or systemic origin is also unclear.

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Abbreviations: ACC, antibody-containing cells; AOCC, anti-ovalbumin-containing cells; ELISA, enzyme-linked immunosorbent assay; FCA, Freund's complete adjuvant; GALT, gut-associated lymphoid tissue; HSA, human serum albumin; ID, intraduodenal; IMam, intramammary; IP, intraperitoneal; OA, ovalbumin; SE, standard error of the mean.

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INTRODUCTION

The concept of a common mucosal immune system linking mucosal surfaces with the gut-associated lymphoid tissue (GALT) has been shown to exist in a number of species (Bienenstock & Befus, 1980), and the translocation of IgA precursor cells from GALT to other submucosae has been confirmed in laboratory animals (Rudzik *et al.*, 1975; Roux *et al.*, 1977; McDermott & Bienenstock, 1979; Weisz-Carrington *et al.*, 1979; Jackson *et al.*, 1981). Scicchitano, Husband & Clancy (1984) have demonstrated that this phenomenon occurs in the respiratory tract of sheep, and have shown that IgA-specific antibody-containing cells (ACC) observed in the trachea after intestinal immunization were predominantly of intestinal origin.

Contrary to the situation in most other species, IgG derived from serum is the predominant immunoglobulin in secretions of the ruminant mammary gland, and there is relatively little IgA present (Lascelles & McDowell, 1974). Although preliminary studies by Lascelles, Beh & Husband (1981) indicated that prior intestinal immunization of sheep resulted in enhanced local production of antibody in milk following intra-

mammary challenge, more recent findings from this laboratory showed that, following intestinal priming, there was no translocation of IgA precursors cells to the non-lactating ovine mammary gland (Sheldrake, Husband & Watson, 1985a). However, since studies in rodents have demonstrated that relocation of gut-derived IgA precursors to the mammary gland is under hormonal control and only occurs in association with lactation (Roux *et al.*, 1977; Weisz-Carrington *et al.*, 1979), the present investigation was undertaken to study this phenomenon in sheep at stages of lactation when IgA cell migration to the gland should be optimal (colostrum formation and mammary gland involution). This approach was used to determine whether the poor IgA plasma cell response previously reported (Sheldrake *et al.*, 1985a) was related to the stage of lactation, or whether in this species the mammary gland does not form part of the IgA-dominated common mucosal system.

In addition, since circulating IgA molecules in ruminants are predominantly dimeric and of gut origin (Heremans, 1974; Beh, Watson & Lascelles, 1974), and are selectively transported into ovine milk (Sheldrake *et al.*, 1984; Sheldrake, Scicchitano & Husband, 1985b), it was of interest to determine whether this is reflected in the appearance of specific IgA antibody in milk after intestinal immunization, as this phenomenon is potentially an additional dimension of the common mucosal system.

In order to examine these possibilities, ewes were primed intraperitoneally (IP) with antigen in Freund's complete adjuvant (FCA), a procedure which stimulates the appearance of IgA-specific ACC in GALT (Beh, Husband & Lascelles, 1979; Husband, Beh & Lascelles, 1979), and challenged intramammarily (IMam) with antigen in saline. The concentration and isotype of specific antibody were determined in serum and mammary secretion collected at intervals after immunization, and at slaughter various tissues were collected to determine the number of antigen-specific IgA-ACC.

MATERIALS AND METHODS

Animals

Forty adult Merino × Border Leicester ewes were used in this series of experiments and were allotted to two groups of either ewes with colostrum-secreting glands or ewes with involuting mammary glands.

Table 1. Immunization protocol

Treatment group	Day 0	Day 7	Day 14	Abbreviation used in text
1	—	—	Killed	Control
2	—	IMam*	Killed	-/IMam
3	IP	—	Killed	IP/-
4	IP†	IMam,ID‡	Killed	IP/IMam/ID
5	IP	IMam	Killed	IP/IMam

* IMam, intramammary immunization (OA in saline in right gland; *B. abortus* in saline in left gland).

† IP, intraperitoneal immunization (OA and *B. abortus* in FCA).

‡ ID, intraduodenal immunization (OA and *B. abortus* in saline).

Immunizations

Two antigens were used: ovalbumin (OA) (grade III, Sigma, St Louis, MO), and washed, killed *Brucella abortus* cells (Commonwealth Serum Laboratories, Melbourne, Australia).

Ewes were immunized according to the protocol in Table 1. For intraperitoneal (IP) injection, 50 mg OA and 5×10^9 killed *B. abortus* cells were prepared in 5 ml phosphate-buffered (pH 7·2) saline (PBS) emulsified in 5 ml Freund's complete adjuvant and injected in two equal doses via the lower left and right ventral quadrants of the abdominal region into the peritoneal cavity. For intraduodenal (ID) immunizations, the duodenum was exposed by a small laparotomy and 50 ml of a solution containing a mixture of 500 mg OA and 5×10^{11} killed *B. abortus* cells injected directly into the lumen using a 25-gauge needle.

For intramammary infusion, a 2·0 ml volume of OA in sterile PBS (2·5 mg/ml) was infused into the right mammary gland sinus via the teat canal, and 2×10^9 *B. abortus* cells in the same volume of sterile PBS similarly infused into the left mammary gland. *B. abortus* was administered to the contralateral gland in ewes given IMam immunization as a control for non-specific effects of immunization.

All ewes were killed on Day 14, and tissue was collected and processed for fluorescent histology as described previously (Sheldrake *et al.*, 1985a). For ewes with colostrum-secreting glands, Day 0 was chosen as approximately 17 days prior to the expected date of lambing, based on mating records. None of these ewes had lambed at the time of slaughter. For ewes with involuting glands, Day 0 was chosen as 8 weeks *post partum* and lambs were weaned 5 days later.

Immunofluorescent histology
Immunofluorescent staining of wax-embedded sections was conducted, and the population of anti-OA ACC (AOCC) and their isotype distribution determined using the double fluorochrome labelling techniques described previously (Sheldrake *et al.* 1985a).

Anti-OA ELISA assay
Serum samples were collected from all ewes on Days 0, 7 and 14, and mammary secretions were collected from both glands on Day 14, just prior to slaughter. Samples were stored at −20°. Prior to assay, samples of all mammary secretions were centrifuged at 3000 *g* for 10 min at 4° and fat was removed.

The assay was conducted according to the procedures of Engvall & Perlmann (1972). Briefly, monospecific rabbit anti-ovine IgG1, IgG2, IgM and IgA were prepared (Husband, 1978) and optimal dilutions determined by titrating standard sera positive and negative for anti-OA antibody. The optimal dilution of serum and mammary secretions for assay were similarly determined (see captions to Figs 1–4). Goat anti-rabbit sera conjugated with alkaline phosphatase (Miles Laboratories, Rehovot, Israel) was used at a dilution of 1:2000.

All samples were assayed in triplicate. On each plate, standard positive and negative reference sera were included and between-plate variation corrected using linear regression analysis of expected and observed absorbance readings to enable comparisons to be made between samples assayed on different plates. Optical densities were read at 405 nm using a Titertek Multiscan (Flow Laboratories, Irvine, Ayrshire, U.K.), and means of the triplicate determinations calculated. In order to determine the specificity of the assay, increasing concentrations of OA and human serum albumin (HSA) were added to known positive anti-OA sera and, after standing at room temperature for 60 min, anti-OA assays were performed. The results indicated that the assay was specific for OA with respect to all isotypes, in that addition of HSA did not affect the anti-OA titre, whereas addition of OA resulted in a dose-dependent diminuition in titre due to absorption of specific antibody.

Statistical analyses
Immunohistology data. The results for each treatment group are expressed as the mean ± standard error of the mean (SE). Statistical comparisons between means were made using Student's *t*-test. A $\log_{10}$ transformation was made of the data expressed as cells per 50 high power fields (magnification × 500) before statistical analyses were performed.

ELISA data. Means ± SE of OA antibody ELISA units are expressed in histogram format (Figs 1–4). Statistical comparisons between means were made using Student's *t*-test.

RESULTS

AOCC distribution in mammary and intestinal tissues

The results in Table 2 for ewes with colostrum-secreting glands show that for IP/IMam and IP/IMam/ID groups there was an increase in the number of AOCC present in the right mammary gland (chal-

Table 2. AOCC in mammary and gut tissue of ewes with colostrum-secreting glands

	Left* mammary gland	Left* lymph node	Right* mammary gland	Right* lymph node	Jejunum†
IP/IMam	9±4	251±165	47±14	1317±730	48±27
IP/IMam/ID	3±1	1270±610	186±132	1201±574	54±19
IP/-	0±0	98±19	0±0	82±28	13±3
-/IMam	0±0	0±0	0±0	0±0	8±3
Control	0±0	0±0	0±0	0±0	5±1

* AOCC/50 high power fields (× 500).
† AOCC/cm jejunum.
Values are presented as means ± standard errors of observations from four sheep.

lenged locally with OA) compared with the control group. There was no significant difference in the numbers of AOCC between IP/IMam and IP/IMam/ID groups, and for statistical comparisons with the control group these data were pooled. The results indicate a significant increase in AOCC ($P<0{\cdot}01$), compared with the control group. For IP/-, -/IMam groups, there was no significant difference in the AOCC response when compared to the control group. There was a small AOCC response in the left mammary gland (challenged with *B. abortus*) of the IP/IMam and IP/IMam/ID groups, but negligible responses in this gland in all other groups. A similar trend was also evident for AOCC in both the left and right supramammary lymph nodes.

In the ewes with involuting glands (Table 3), the

Table 3. AOCC in mammary and gut tissue of ewes with involuting glands

	Left* mammary gland	Left* lymph node	Right* mammary gland	Right* lymph node	Jejunum†
IP/IMam	131±65	395±181	298±146	776±219	56±16
IP/IMam/ID	101±31	349±156	221±114	883±233	37±6
IP/-	23±8	10±5	1±1	2±1	66±30
-/IMam	0±1	1±1	0±1	1±1	30±14
Control	0±0	1±1	1±1	0±0	10±3

* AOCC/50 high power fields (×500).
† AOCC/cm jejunum.
Values presented as means±standard errors of observations from four sheep.

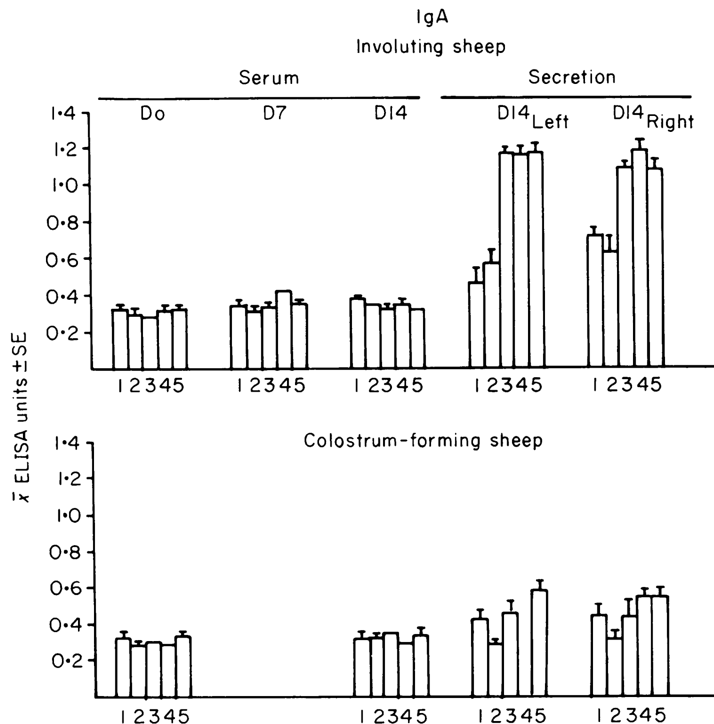

Figure 1. IgA-specific anti-OA antibody measured by ELISA for serum and mammary secretions from ewes with colostrum-secreting glands or ewes with involuting glands. Values represent mean ELISA units±standard error of the mean for serum and secretion samples diluted 1/10 prior to assay. Numbers refer to immunization regimes as outlined in Table 1.

results were essentially the same as for ewes with colostrum-secreting glands, with a significant increase in the number of AOCC in the right (OA-immunized) mammary gland of ewes in the IP/IMam and IP/IMam/ID groups compared with the control group ($P<0{\cdot}001$). Relatively more AOCC were found in the left mammary glands of ewes with involuting glands than for left glands of ewes with colostrum-secreting glands.

Approximately 90% of AOCC in mammary gland and supramammary lymph node tissue of both ewes with colostrum-secreting glands and ewes with involuting glands were of the IgG1 isotype, and AOCC-producing antibody of the IgA isotype in OA-immunized glands was rare.

Tissues collected from the jejunum of ewes which had been immunized either IP/-, IP/IMam or IP/IMam/ID had significantly elevated numbers of AOCC present in the intestinal lamina propria ($P<0{\cdot}05$) compared with intestinal tissues of the control group. Contrary to the results for mammary tissues, the majority (>90%) of AOCC in the jejunum of these groups were producing antibody of the IgA isotype.

Anti-OA antibody in mammary secretions and serum

The results in Figs 1–4 show the isotype distribution of anti-OA antibody in serum (Days 0, 7 and 14) and mammary secretion (Day 14; left and right glands) for ewes with involuting and colostrum-forming glands. It should be noted that comparisons between isotypes are not valid because of the differences in relative binding efficiencies of the various anti-isotype antisera. Comparisons between samples within each isotype should take into account the pre-assay dilution factor, which was uniform for all samples within each isotype-specific assay (see figure captions) except for IgG1 in which colostrum samples were diluted 1/10,000, whereas involution secretions and sera were only diluted 1/500.

In Fig. 1, the results show that there was no significant increase in IgA antibody levels in serum following immunization, irrespective of the immuniza-

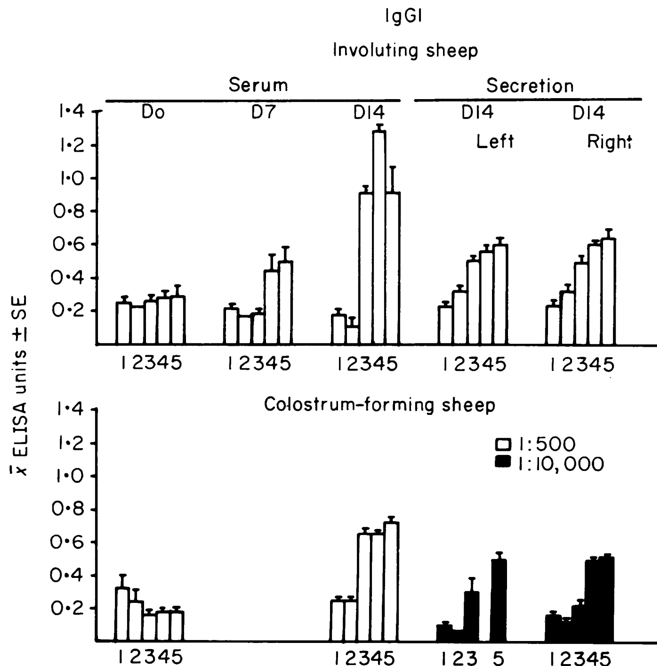

Figure 2. IgG1-specific anti-OA antibody measured by ELISA for serum and mammary secretions from ewes with colostrum-secreting glands or ewes with involuting glands. Values represent mean ELISA units ± standard error of the mean for serum, and involution secretion samples diluted 1/500 and colostrum samples diluted 1/10,000 prior to assay. Numbers refer to immunization regimes as outlined in Table 1.

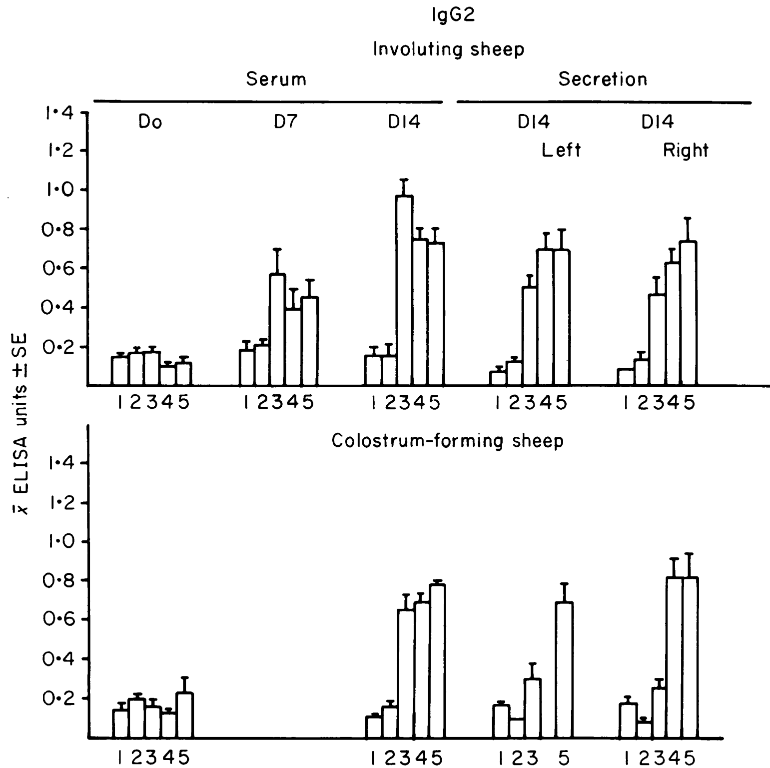

Figure 3. IgG2-specific anti-OA antibody measured by ELISA for serum and mammary secretions from ewes with colostrum-secreting glands or ewes with involuting glands. Values represent mean ELISA units ± standard error of the mean for serum and secretion samples diluted 1/500 prior to assay. Numbers refer to immunization regimes as outlined in Table 1.

tion regimen. However, in secretions of ewes with involuting glands, there was a significant increase beyond the control value for IP/IMam, IP/IMam/ID and IP/- groups ($P < 0{\cdot}001$) in both unimmunized and locally immunized glands. In colostrum formation, this trend was much less noticeable and the response was not significantly greater than the unimmunized control group.

For IgG1 (Fig. 2), serum levels of the IP immunized groups (IP/IMam, IP-IMam/ID, IP/-) were significantly elevated by Day 14 in ewes at all stages of lactation ($P < 0{\cdot}01$). A similar trend was evident for secretions of both the left and right glands of ewes with involuting glands, with the most noticeable effect being in the IP/IMam and IP/IMam/ID groups, that is, those groups receiving both an IP and an IMam immunization. A similar result was observed for colostrum, but only the IP/IMam and IP/IMam/ID groups were significantly greater than the control ($P < 0{\cdot}001$). It should be noted that colostral levels were much greater than serum levels after the dilution rate prior to assay is taken into account.

The results for IgG2 (Fig. 3) were similar to IgG1 (Fig. 2), whereas for IgM (Fig 4) there was a generally poor anti-OA antibody response, although levels in colostrum for the IP/IMam group were significantly greater than the control group ($P < 0{\cdot}02$).

The data in Table 4 show IgA-specific anti-OA antibody levels expressed as a secretion:serum ratio. Any values greater than unity reflect enhanced local antibody, arising either by local production or selective transport. The striking feature of these data is the high ratios obtained for IgA antibody in mammary secretion of ewes with involuting glands given IP immunization, regardless of the subsequent local immunization regime (Table 4). In ewes with colostrum-secreting glands (Table 4), an enhanced local antibody response was not as pronounced but there was some evidence for elevated ratios, particularly in sheep given IP immunization followed by IMam or IMam/ID immunization.

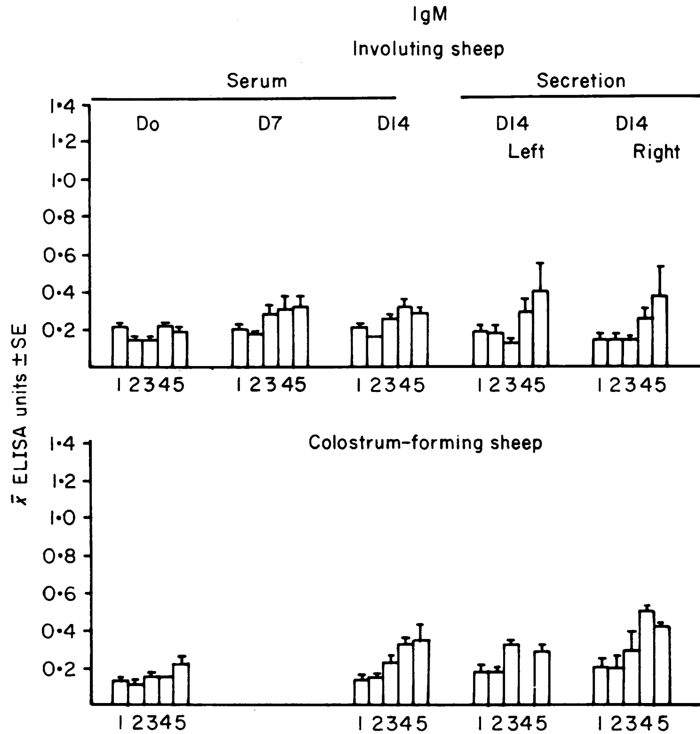

Figure 4. IgM-specific anti-OA antibody measured by ELISA for serum and mammary secretions from ewes with colostrum-secreting glands or ewes with involuting glands. Values represent mean ELISA units ± standard error of the mean for serum and secretion samples diluted 1/100 prior to assay. Numbers refer to immunization regimes as outlined in Table 1.

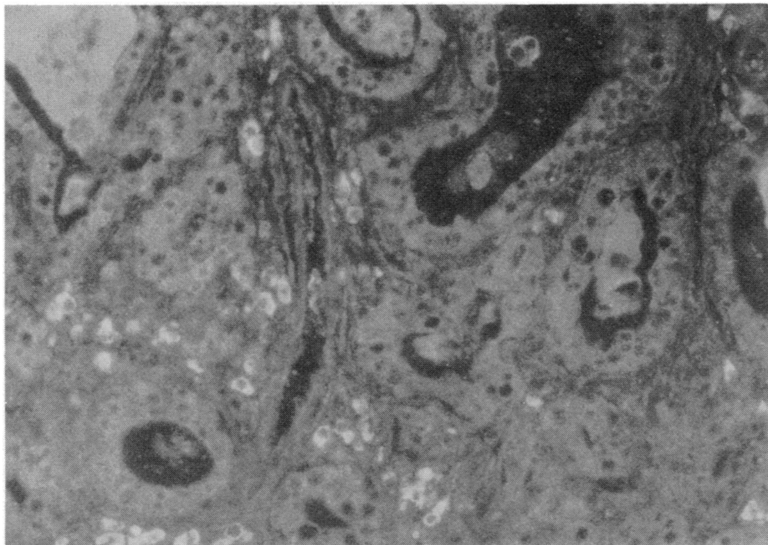

Figure 5. Anti-ovalbumin-containing cells detected by immunofluorescence in the interalveolar area of an involuting ovine mammary gland following IP/IMam immunization (magnification × 150).

Table 4. Secretion: serum ratios of IgA-specific anti-OA antibody on Day 14

	Control	-/IMam	IP/-	IP/IMam/ID	IP/IMam
Involution secretion					
Left mammary gland	1·24	1·67	3·51	3·32	3·62
Right mammary gland	1·48	1·79	3·33	3·47	3·40
Colostrum					
Left mammary gland	1·27	0·81	1·28	—	1·72
Right mammary gland	1·31	0·93	1·22	1·86	1·63

DISCUSSION

These experiments confirm that IP injection of antigen in FCA leads to an IgA-specific AOCC response in the jejunum (Tables 2 and 3) as previously described (Beh *et al.*, 1979; Husband *et al.*, 1979). IP immunization, followed by local presentation of antigen to the mammary gland, resulted in an enhanced number of AOCC in the gland to which the relevant antigen was administered compared with IP or IMam immunization alone, although these cells were predominantly of the IgG1 isotype (Tables 2 and 3). This indicates that the IgA-specific AOCC precursors generated by IP immunization failed to populate those glands locally immunized with OA, and this trend was consistent for ewes at both stages of lactation and similar to previous findings for non-lactating ewes (Sheldrake *et al.*, 1985a). Although this immunization regimen did enhance the IgG1-specific AOCC response in mammary tissue, cells of this isotype could have been of either systemic or GALT origin because, in addition to stimulating an intestinal response, IP immunization also stimulated a systemic IgG response. This is reflected in the elevated serum IgG antibody levels in any of the groups receiving IP immunization (Fig. 2).

These findings are in contrast to those of Scicchitano *et al.* (1984) who, using IP immunization followed by intratracheal immunization, detected an enhanced IgA-specific AOCC response in the respiratory tract mucosa of sheep, and this was shown to depend on the contribution of cellular precursors from GALT. Thus, the concept of a common mucosal immune system linking GALT and other mucosal sites by the relocation of IgA plasma cell precursors (Bienenstock & Befus, 1980) does not appear to apply in the ovine mammary gland, despite its existence with respect to the respiratory tract in this species. This could not be accounted for by the stage of lactation since, in the present study and the previous report (Sheldrake *et al.*, 1985a), ewes were examined at various stages of lactation and the same lack of IgA-ACC response in the mammary gland following concomitant IP and IMam immunization was observed.

It was interesting to note, however, that the levels of IgA antibody in involution secretion for groups receiving an IP immunization were significantly greater than the control group (Fig. 1), and the secretion: serum ratios for these ewes were also elevated (Table 4). This could be accounted for either by local production of antibody, or by its selective transport from serum. In view of the almost complete absence of IgA-specific AOCC in mammary tissues of these animals, local production seems unlikely.

It has been demonstrated previously that the bulk of circulating molecular IgA in ruminants is dimeric and derived from plasma cells in the intestine (Heremans, 1974; Beh *et al.*, 1974), and that serum-derived IgA is rapidly and selectively transported into mammary secretion of ruminants (Sheldrake *et al.*, 1984, 1985b). Thus, the IgA antibody observed in secretion in IP immunized sheep in this study could be derived from the antigen-specific plasma cells resident in the intestinal lamina propria following IP immunization, the bulk of which were producing IgA antibody (Tables 2 and 3).

However, there are several problems with this interpretation. First, IgA-specific anti-OA antibody was detected in the serum of all groups, and there was no evidence for elevated levels in the IP immunized ewes since serum levels in these sheep were similar to the unimmunized controls. These low levels of IgA antibody detected in serum may represent naturally occurring antibody, but, if this were the case, it is difficult to explain on the basis of serum origin the presence of elevated IgA antibody in secretion of immunized groups.

The low serum IgA antibody levels could simply

reflect background effects resulting from non-specific binding in the ELISA assay, and there may in fact have been no serum antibody of the IgA class. In view of the low dilution (1:10) required to detect IgA antibodies, it would not be surprising if background levels were high. In this case, if the IgA antibody in mammary secretion were serum-derived, the rate of its removal from the circulation must have equalled its rate of entry from GALT. Alternatively, small differences between groups in serum antibody levels could have been masked by the background effects and the relative insensitivity of the assay with respect to this isotype.

A further problem with suggesting a serum-derived origin for the IgA antibody, at least with regard to involution secretion, is the inconsistency this creates with the previous findings of lack of transport of radiolabelled IgA at this stage of lactation (Sheldrake *et al.*, 1984). This could be accounted for by differences between the two studies with respect to timing of involution, or by the fact that the antigen-specific IgA detected in this experiment in response to IP immunization may not reflect the behaviour of the population of radiolabelled IgA molecules which would have represented a wider range of antigenic specificities. In any case, it is considered that the results obtained here for IgA of a particular antigen specificity are likely to be more physiological than the behaviour of radiolabelled IgA described previously (Sheldrake *et al.*, 1984).

Despite previous reports that local immunization stimulates an IgA antibody response in mammary secretion of ruminants (Lascelles & McDowell, 1974; Lee & Lascelles, 1970; Chang, Winter & Norcross, 1981), IMam immunization alone failed to produce a significant number of IgA-AOCC in the gland (Tables 2 and 3). However, the sheep receiving IMam immunization alone were killed after only 7 days to provide the appropriate control for the IP/IMam group, whereas IP immunized groups were not killed until 14 days after this immunization. An IgA response may have been detected following IMam immunization alone if the sheep had been allowed a longer period to respond. It is also relevant that, in the experiments of the other investigators referred to above, either live or killed particulate antigens or complex soluble antigens were used which may have elicited a more vigorous local response, and the discrepancy may be explained on that basis.

As was reported previously for non-lactating sheep, significant numbers of IgA-specific AOCC were present in jejunal tissue in ewes receiving IMam immunization alone. In non-lactating sheep, these cells in the intestine were shown to be of GALT rather than mammary gland origin (Sheldrake *et al.*, 1985a). Those experiments showed that, after IMam immunization, antigen is transported to GALT via the circulation, either as free antigen or by antigen-presenting cells, and cells responding to this antigen leave GALT via the intestinal lymph duct, enter the circulation and return to the intestine as IgA-ACC. These findings reveal another dimension of the common mucosal immune system by which the gut–mammary link may operate in reverse.

The findings of the present study support those of Chang *et al.* (1981) that the presentation of antigen to the intestine results in an elevation of IgG1 and IgG2 antibody in both mammary secretions and serum, since a similar response was observed at all stages of lactation when antigen was presented IP only. However, the origin of this antibody has not been determined and may be GALT or, as Chang *et al.* (1981) suggested, spleen and peripheral lymphoid tissue. It might be expected that, since the bulk of the AOCC appearing in the immunized glands of the IP/IMam group were IgG1-specific, this would have generated an enhanced local IgG1 antibody response in these secretions. The data did not support this prediction, and the role of this large population of IgG1-specific AOCC in the mammary glands of IP-IMam immunized ewes is unclear. The amount of locally produced IgG1 antibody may have been too small to be of significance relative to the high levels of serum-derived IgG1-specific antibody in colostrum of IP immunized animals, consistent with the selective transfer of IgG1 from serum to mammary secretion during colostrum formation (Lascelles & McDowell, 1974). Nevertheless, in view of their potential contribution to local immune events in the mammary gland, the origin of the IgG1-specific cells should be investigated to determine whether they were systemically or GALT-derived.

The findings of this study indicate that, with respect to IgA precursor cell migration from GALT, the ovine mammary gland does not form part of the IgA-dominated common mucosal immune system as proposed by Bienenstock & Befus (1980), or as shown by Scicchitano *et al.* (1984) for the ovine respiratory tract. However, IP immunization does result in the appearance in mammary secretion of antigen-specific IgA antibody. Although the origin of this antibody is unclear from the data presented in this paper, it may be

derived from GALT tissues, reaching the mammary gland via the blood circulation where it is selectively transported into secretion. Thus, while priming of GALT does not contribute to the mammary gland immune response in terms of the relocation of IgA plasma cell precursors, there may be a contribution in terms of dimeric IgA molecules. The role of such a mechanism in protecting the suckling neonate, or the mammary gland itself, against infection needs to be evaluated.

ACKNOWLEDGMENTS

The authors acknowledge the skilled technical assistance of Mrs A. Tyler. This work was supported by grants from the Australian Meat Research Committee and the Australian Dairy Research Committee (ADRC). R. F. Sheldrake was supported by an ADRC postgraduate scholarship, and the NSW Department of Agriculture.

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