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The secondary immune response to *Staphylococcus aureus* vaccines in efferent popliteal lymph of sheep

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SUMMARY

The secondary immune response to live and killed *Staphylococcus aureus* vaccines was studied in efferent popliteal lymph of sheep. Animals were immunized with either live or killed *S. aureus* intracutaneously on the lateral hock, in an area draining into the popliteal lymph node. Six weeks later, an efferent popliteal lymphatic vessel in the vaccinated leg was cannulated, and 48 hr after surgery a second inoculation (identical to the primary) was placed in the skin adjacent to the primary vaccination lesion. A dramatic decrease in lymphocyte output ('cell shutdown') was observed in lymph collected from sheep given the secondary inoculation of live *S. aureus* during the first 8 hr after inoculation. However, only a moderate decrease in lymphocyte output occurred in lymph from animals receiving killed *S. aureus* or from control animals. The proportion of eosinophils in lymph collected from animals given live *S. aureus* increased to a peak (14% of total leucocytes in lymph) between 6 hr and 8 hr, and returned to prechallenge levels by 24 hr post-inoculation. The percentage of neutrophils in lymph peaked between 8 hr and 1 day after injection of live bacteria. This granulocyte response was not observed in animals given killed *S. aureus* or control animals. IgM-, IgG1- and IgG2- containing cells (-cc) in lymph were quantified by indirect immunofluorescence. Animals given live *S. aureus* produced lymph with greater numbers of Ig-cc of these isotypes than those given killed organisms. The ratio of IgG2-cc:IgG1-cc was significantly greater in lymph from animals given live *S. aureus* from Day 2 to Day 6 post-challenge. IgM and IgG1 anti-staphylococcal antibody levels increased in lymph collected from all vaccinated animals, but only sheep given live *S. aureus* showed any increase in levels of IgG2 antibody.

INTRODUCTION

Ruminants immunized with live *Staphylococcus aureus* vaccines are protected to a considerable degree from subsequent intramammary infection with homologous or heterologous strains of *S. aureus*. However, vaccination with killed *S. aureus*, with or without adjuvants, does not provide a comparable level of protection (Watson & Lee, 1978; Watson & Kennedy, 1981). Three mechanisms have been described which contribute to the greater protection from staphylococcal mastitis afforded by live compared to killed *S. aureus* vaccine in ruminants. First, animals immunized subcutaneously with live staphylococci can deploy neutrophils into mammary glands infected with *S. aureus* at a greater rate than can animals that have been similarly immunized with killed staphylococci (Colditz & Watson, 1982). Second, staphylococci grown *in vivo* have been shown to produce antigens that are not expressed when the bacteria are grown under standard *in vitro* conditions, and antibody specific for these '*in vivo* antigens' is important in immunity to staphylococcal infection (Watson, 1983). Finally, live staphylococcal vaccines have been shown to promote the synthesis of greater levels of IgG2 than IgG1 antibody in vaccinated animals, whereas a relatively greater IgG1 response is generated following inoculation of killed *S. aureus* (Watson, 1981; Watson, 1984). Ruminant neutrophils bear Fc receptors for IgG2 but not IgG1, and IgG2 anti-staphylococcal antibody is a very efficient opsonin for neutrophil-mediated phagocytosis of *S. aureus* (Watson, 1976).

Studies of immune responses *in vivo* have been greatly facilitated by techniques allowing collection and examination of lymph draining to and from regional lymph nodes (Hall & Morris, 1963). This approach has been exploited in our laboratory in a series of studies designed to elucidate mechanisms of immunity to *S. aureus* in ruminants (Kennedy, Watson & Bennell, 1981; Watson & Davies, 1985). Cells and antibody were examined in efferent lymph draining from popliteal lymph nodes of sheep during a primary immune response to either live or killed *S. aureus*. It was shown that lymph nodes stimulated by live staphylococci synthesized significantly higher levels of IgG2 than IgG1. In contrast, lymph draining from nodes of sheep

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given killed *S. aureus* contained relatively higher levels of IgG1 (Kennedy *et al.*, 1981).

It is well established that the anamnestic response measured in lymph draining from a primed node may be quite different from the response measured in lymph from a previously unstimulated node (Smith *et al.*, 1970). Therefore, the aim of the present study was to examine cellular and humoral parameters in lymph-draining popliteal lymph nodes of sheep given a secondary stimulation with either live or killed *S. aureus*, 6 weeks after directly priming the nodes with the same vaccine.

MATERIALS AND METHODS

Experimental animals

Sixteen adult cross-bred wethers and ewes were used in this study. They were kept indoors in cages and allowed free access to lucerne chaff and water. Feed was withheld during the 12 hr prior to surgery.

Preparation of bacteria

An attenuated non-haemolytic strain of *S. aureus* (WA79) was grown overnight in nutrient broth (Oxoid, Basingstoke, Hants, U.K.) on an orbital shaker at 37°. Killed bacteria were prepared by adding formalin (1%) to the broth culture and holding the mixture at 4°. Live or formalin-killed bacteria in broth cultures were washed twice with sterile phosphate-buffered saline (PBS) and adjusted to a final concentration of 1×10^9 bacteria per ml in sterile PBS by the spectrophotometric method of Hirsch & Strauss (1964).

Experimental method

The sheep were initially allocated at random to two treatment groups. Each animal was given an intracutaneous injection (1 ml) of either the live or killed *S. aureus* vaccine in an area of the lateral hock. Peripheral lymphatic vessels in this area drain directly into the popliteal lymph node (Heath & Brandon, 1983). Six weeks later, each animal underwent surgery to cannulate an efferent popliteal lymphatic vessel in the leg that had received the primary vaccine (Hall & Morris, 1962). Forty-eight hours later, five animals from the group given the live *S. aureus* vaccine were given a second dose of 1×10^9 live *S. aureus* (1 ml) into the skin adjacent to the primary injection site (Group 1). The remaining three animals from the group that received the primary live vaccine were given 1 ml sterile PBS in a similar manner to serve as controls (Group 2). In an identical regime, five animals from the group given a primary dose of killed *S. aureus* received a second injection of killed *S. aureus* (Group 3), and the three animals remaining were given 1 ml of sterile PBS to serve as controls (Group 4).

Samples of lymph were collected over a measured time-period (into siliconized glass tubes containing a few grains of powdered heparin) at intervals before and during the first 8 hr after the secondary inoculation, and then once daily for 7 days. Blood samples were taken into plain glass vacutainers whenever a lymph sample was collected. The blood was allowed to clot at 37°, then centrifuged and the serum decanted and frozen until needed. Lymph samples were centrifuged (100 *g*, 5 min) and the cell-free lymph was stored frozen. Cell pellets remaining were washed twice in Hanks' balanced salt solution containing 0·1% gelatin and resuspended in the same solution. Cytocentrifuge cell smears were prepared as described previously, then stored in a desiccator at 4° until required (Watson & Davies, 1985). Cell smears were either stained with Giemsa or subjected to indirect immunofluorescent staining to enumerate IgM-, IgG1- and IgG2-containing cells (Ig-cc) as described by Watson & Davies (1985). Levels of IgM, IgG1 and IgG2 anti-staphylococcal antibody were determined in lymph and serum using an ELISA (Watson & Davies, 1985). The concentration of antibody was derived from standard regression curves relating optical density measured in ELISA microtitre plate wells to antibody concentration (units per ml).

Statistical analysis

Differences between means of live and killed treatments and control groups were subjected to split-plot analyses of variance and Student's *t*-tests. Events during the first 8 hr after vaccination were analysed separately from those occurring during the subsequent 7 days of the response. Cell output and antibody concentration were subjected to $\log_{10}$ transformation and percentage data to arch sin $\sqrt{x}$ transformation before analysis.

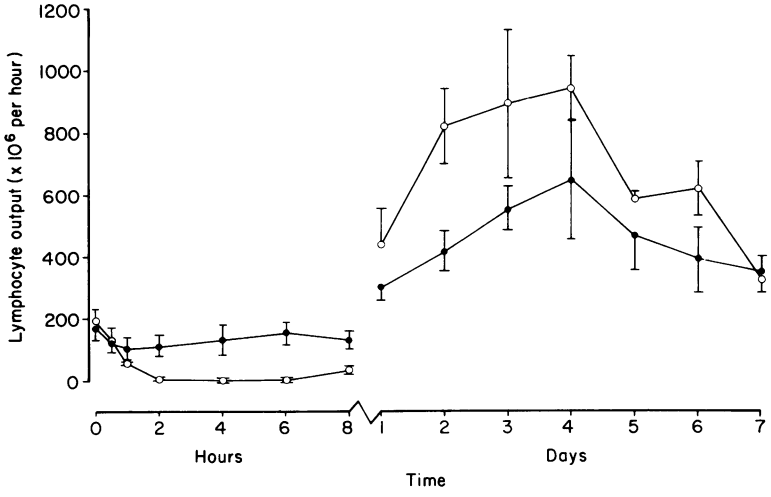

Figure 1. Lymphocyte output in lymph-draining sheep popliteal lymph nodes stimulated with secondary live (○) or killed (●) *S. aureus* vaccines. Each value is the mean ±SE of results from five animals.

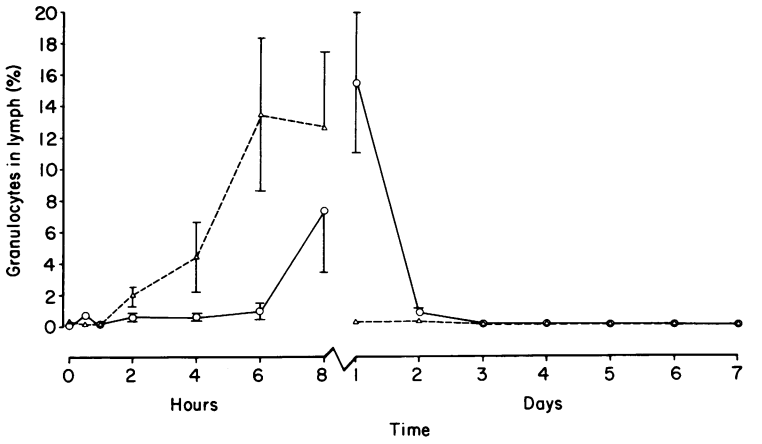

Figure 2. Granulocytes as a percentage of total leucocytes in lymph draining from sheep popliteal lymph nodes given a secondary live *S. aureus* vaccine. Lymph was collected at intervals and the percentage of eosinophils (△----△) and neutrophils (○——○) in each sample was recorded. Each value is the mean ±SE of results from five animals.

RESULTS

Animals given live *S. aureus* developed an acutely enlarged popliteal lymph node in the leg that received the bacteria, following both primary and secondary inoculations. The draining node was still palpably larger than the contralateral node 6 weeks after the primary injection (Groups 1 and 2). The secondary inoculation of live *S. aureus* in animals from Group 1 induced a transient temperature elevation of approximately 1°, which reverted to normal within 24 hr of receiving the injection. These two phenomena were not seen in animals given killed *S. aureus* (Groups 3 and 4). An abscess (approximately 20 mm diameter) developed following the secondary injection of live *S. aureus*. However, vaccination with killed *S. aureus* resulted in the formation of a granuloma that was smaller and more circumscribed than the abscess. No visible lesions developed following injection of sterile PBS in animals from either control group.

The output of lymphocytes in lymph following immunization with secondary live (Group 1) or killed (Group 3) *S. aureus* is shown in Fig. 1. The number of lymphocytes exported from lymph nodes stimulated with live *S. aureus* (Group 1) decreased rapidly following the injection of bacteria and reached approximately 1/50 of the prechallenge output between 2 hr and 6 hr post-inoculation. The lymphocyte output measured in efferent lymph collected from animals given killed *S. aureus* (Group 3), and animals from both control groups (Groups 2 and 4), decreased marginally during this period but were not significantly lower than the values recorded before inoculation. During this period of cell shutdown the mean lymph flow rate for sheep in Group 1 increased significantly above the preinoculation lymph flow rate at 6 ($P<0{\cdot}05$) and 8 ($P<0{\cdot}01$) hr after

Table 1. Output of Ig-containing cells (× 10^6 per hour) in lymph-draining sheep popliteal lymph nodes stimulated with secondary live or killed *S. aureus* vaccines

Time (days)	Vaccines							
	Live *S. aureus*				Killed *S. aureus*			
	IgM	IgG1	IgG2	G2/G1*	IgM	IgG1	IgG2	G2/G1
0	0·2±0·1†	0·3±0·1	0·4±0·3	ND‡	0·3±0·2	0·5±0·3	0·3±0·1	ND
1	2·9±1·8	3·9±2·5	4·9±3·1	1·52±0·24	0·1±0·1	0·4±0·2	0·2±0·1	0·75±0·25
2	15·1±5·3	36·2±9·7	40·4±14·6	1·10±0·11	0·4±0·2	0·8±0·3	0·8±0·2	1·00±0·00
3	41·4±7·9	217·3±33·4	244·6±41·3	1·02±0·12	11·5±4·6	17·6±6·1	23·5±10·0	1·16±0·24
4	45·7±7·9	209·9±45·7	188·4±31·0	0·94±0·06	18·5±7·1	64·6±23·1	53·0±21·8	0·81±0·11
5	31·1±10·2	102·8±34·3	86·4±12·7	1·11±0·08	6·5±5·8	23·7±12·9	14·2±7·5	0·69±0·06
6	24·3±16·1	59·6±8·9	73·4±12·8	1·21±0·07	3·5±3·1	10·7±6·0	8·8±5·6	0·72±0·11
7	2·9±1·4	14·1±3·8	14·2±4·8	0·96±0·24	1·8±1·4	7·8±2·8	7·0±2·3	1·02±0·02

* Ratio of IgG2-cc output: IgG1-cc output.
† Each value is the mean ± standard error for five sheep.
‡ ND, ratio not calculated because of insufficient output of Ig-cc.

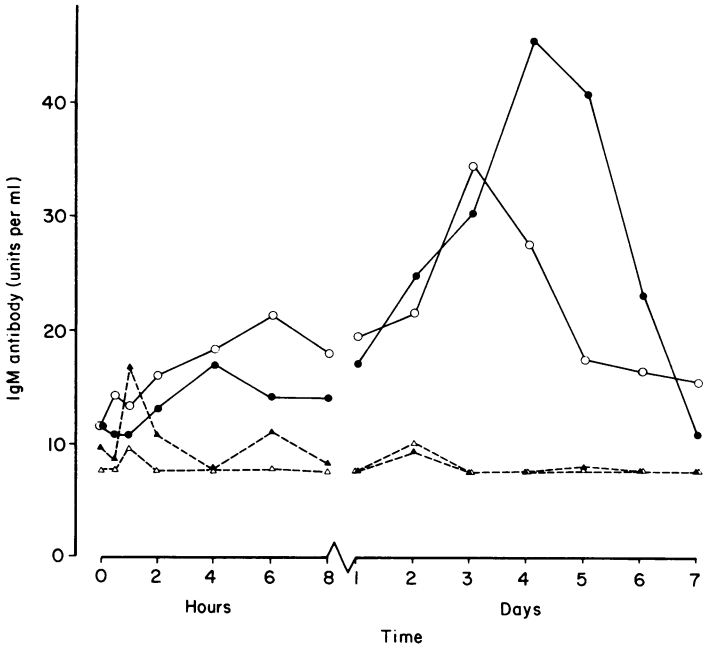

Figure 3. IgM anti-staphylococcal antibody concentration in lymph-draining sheep popliteal lymph nodes stimulated with secondary live (○——○) or killed (●——●) *S. aureus* vaccines. Each value is the mean of results from five animals. Animals given a primary dose of live (△----△) or killed (▲----▲) *S. aureus* vaccines received an injection of sterile PBS in a similar manner to serve as controls. Each point representing results from control animals is the mean from three sheep.

vaccination. No change in lymph flow rate above preinoculation values was recorded in animals given killed organisms or PBS. The lymphocyte output in lymph increased between 1 day and 4 days after the secondary inoculation with live or killed *S. aureus* (Groups 1 and 3). Subsequently, the output of lymphocytes decreased to almost preinoculation levels in lymph from animals in both treatment groups by 7 days after injection. No significant increase in lymphocyte output was observed in animals from either control group during this period.

The percentage of granulocytes in lymph-draining lymph

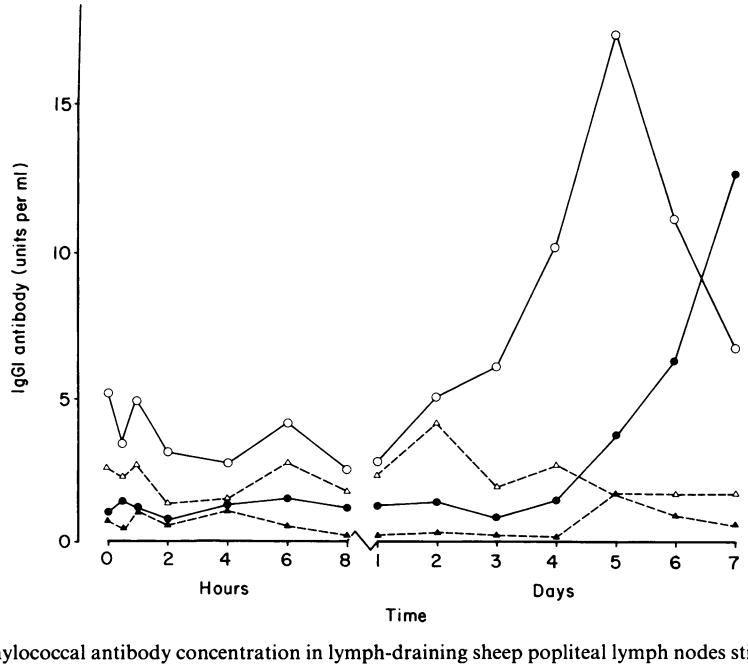

Figure 4. IgG1 anti-staphylococcal antibody concentration in lymph-draining sheep popliteal lymph nodes stimulated with secondary live (○——○) or killed (●——●) *S. aureus* vaccines. Each value is the mean of results from five animals. Animals given a primary dose of live (△----△) or killed (▲----▲) *S. aureus* vaccines received an injection of sterile PBS in a similar manner to serve as controls. Each point representing results from control animals is the mean from three sheep.

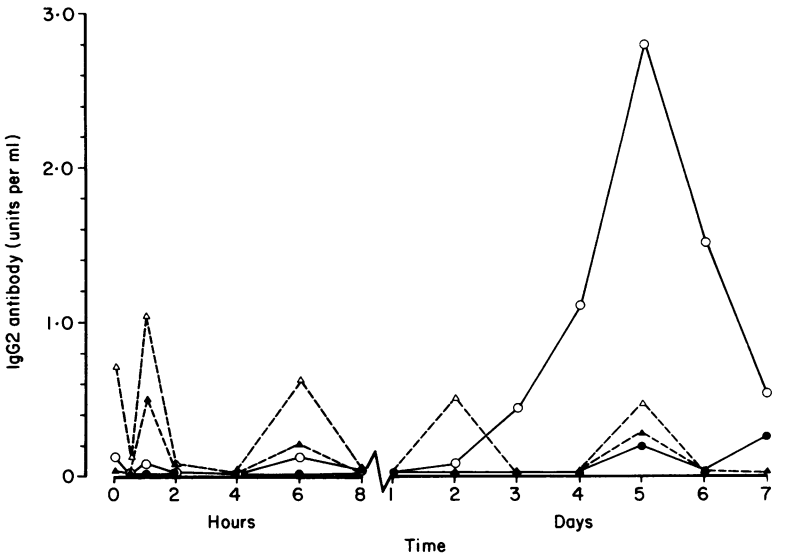

Figure 5. IgG2 anti-staphylococcal antibody concentration in lymph-draining sheep popliteal lymph nodes stimulated with secondary live (○——○) or killed (●——●) *S. aureus* vaccines. Each value is the mean of results from five animals. Animals given a primary dose of live (△----△) or killed (▲----▲) *S. aureus* vaccines received an injection of sterile PBS in a similar manner to serve as controls. Each point representing results from control animals is the mean from three sheep.

nodes challenged with a secondary dose of live *S. aureus* can be seen in Fig. 2. The percentage of eosinophils in lymph began to increase between 1 hr and 2 hr after injection, peaked at 6 hr, and was beginning to decrease by 8 hr post-inoculation. The eosinophil peak preceded an increase in the percentage of neutrophils in lymph which began between 6 hr and 8 hr after the organisms were injected. The percentage of neutrophils reached a maximum level in lymph between 8 hr and 1 day post-inoculation, and had returned to preinoculation values by 2 days after inoculation. These data correlate with increases in output of granulocytes in lymph following inoculation with live *S. aureus*. Preinoculation mean output were $0{\cdot}3 \times 10^6$ per hour (eosinophils) and $0{\cdot}04 \times 10^6$ per hour (neutrophils). At 8 hr post-inoculation the output of eosinophils had risen to $3{\cdot}5 \times 10^6$ per hour, and at 24 hr the neutrophil output had increased to 62×10^6 per hour. Little increase in the percentage of granulocytes in lymph were recorded from animals in the treatment group receiving killed *S. aureus* (Group 3) or either control group (Groups 2 and 4).

The output of Ig-cc measured in lymph collected from animals in treatment Groups 1 and 3 during the 7 days following inoculation is recorded in Table 1. Very few Ig-cc were observed in efferent lymph from animals in any group during the first 8 hr post-inoculation. Virtually no IgM-cc and only small numbers of IgG1- and IgG2-cc were seen in lymph from animals in both control groups (Groups 2 and 4) following the injection of sterile PBS. There were greater numbers of IgM-cc, IgG1-cc and IgG2-cc in lymph from animals given live *S. aureus* (Group 1) than in lymph from animals given killed *S. aureus* (Group 3), and the response to live organisms began at an earlier time after vaccination. There were greater numbers of IgG1-cc and IgG2-cc than IgM-cc in lymph from animals in both vaccinated groups (Groups 1 and 3). The Ig-cc response was most active from Day 2 to Day 6 post-inoculation which paralleled a concurrent peak of blast lymphocytes in lymph from animals in both treatment groups. The cumulative mean ratio of IgG2-cc:IgG1-cc in efferent lymph during this period was significantly greater in lymph from animals in Group 1 than the same ratio in lymph collected from animals in Group 3 ($P < 0{\cdot}05$), as determined by split-plot analysis of variance.

Anti-staphylococcal antibody levels were measured in lymph and serum collected at intervals after live or killed *S. aureus* or sterile PBS was given. No significant increases in antibody levels of any isotype were detected in serum samples taken following secondary inoculation with either live or killed bacteria. Anti-staphylococcal antibody concentrations in efferent lymph are recorded in Figs 3–5. Levels of IgM antibody in lymph were significantly greater from animals given live and killed *S. aureus* (Groups 1 and 3) than from animals given PBS (Groups 2 and 4). Lymph from animals given killed *S. aureus* had higher levels of IgM antibody than lymph from animals given the live bacteria, but this difference was significant only at Day 5 (Fig. 3). Levels of IgG1 antibody in lymph are shown in Fig. 4. The concentration of IgG1 anti-staphylococcal antibody in lymph from animals given live *S. aureus* (Group 1) began to increase from Day 1 post-inoculation to reach a peak 5 days after the vaccine was given. However, the levels measured in lymph from animals given killed *S. aureus* (Group 3) began to increase 4 days after vaccination and were still increasing by the end of the experiment, 3 days later. IgG2 antibody concentration in lymph is recorded in Fig. 5. There was a marked increase in the mean concentration of IgG2 antibody in lymph following secondary immunization with live *S. aureus*. The peak of the response occurred 5 days after inoculation, and levels had declined substantially by 7 days after vaccination. No significant increase in IgG2 antibody level was seen in lymph from animals in Group 3 (given killed *S. aureus*) or either control group (Groups 2 and 4).

DISCUSSION

The data from these experiments show that significant qualita-

tive and quantitative differences exist between the secondary immune responses to live and killed *S. aureus* vaccines. The effect of the primary vaccination was still evident in lymph 6 weeks after the nodes were stimulated with either vaccine. The leucocyte output at this time was approximately 10 times the value recorded in lymph issuing from previously unstimulated popliteal lymph nodes (Kennedy *et al.*, 1981). The secondary inoculation induced an immune response of considerably greater magnitude than the primary response to each vaccine measured previously in lymph, although this difference was most marked in animals given live *S. aureus* (Kennedy *et al.*, 1981).

A striking feature of the secondary response to *S. aureus* in efferent popliteal lymph was the marked decrease in lymphocyte output with a concomitant increase in lymph flow rate recorded during the first few hours following inoculation of bacteria. This phenomenon, termed 'cell shutdown' is thought to be mediated through complement fixation and consequent release and action of prostaglandin E_2 (PGE_2) within the node (McConnell, Hopkins & Lachman, 1980; Hopkins, McConnell & Pearson, 1981). Cell shutdown is observed more commonly during the secondary response to an antigen; the more marked cell shutdown observed during the secondary response to live or killed *S. aureus* in comparison with the primary response in a previously published report (Kennedy *et al.*, 1981) is consistent with results for other antigens (McConnell *et al.*, 1980). The difference in degree of shutdown observed between the secondary response to live and killed staphylococcal vaccines may be due to a greater inflammatory response and consequent release of larger amounts of PGE_2 as a result of local staphylococcal infection (Lundberg, Lebel & Gerdin, 1984). Also, bacterial replication in the site of infection may expose cells in the node to a greater quantity of antigen and so increase 'priming' of resident T cells in the node (McConnell & Hopkins, 1981).

A transient and relatively minor ($<2\%$ of total leucocytes) increase in proportions of neutrophils and eosinophils has been observed in efferent popliteal lymph following stimulation of nodes with a number of different antigens (Hall & Morris, 1962, 1963). Injection of *Haemonchus contortus* antigens, however, induced separate peaks of neutrophils and eosinophils in efferent popliteal lymph of similar magnitude to that seen in the present study (Hay, 1979). In contrast to our results, the *Haemonchus* antigens induced a neutrophil peak which preceded the eosinophil peak by more than 20 hr. A similar peak of neutrophils (without eosinophils) was observed following primary vaccination with live *S. aureus* (D. L. Watson, unpublished observations). These studies suggest that the kinetics of migration of each of these two granulocyte classes is controlled independently. Neutrophil chemotaxis induced by *S. aureus in vivo* appears to be mediated through the activation of complement and release of the chemotatic peptide C5a (Adlam & Easmon, 1983). However, the control of eosinophil chemotaxis has been shown to be directed by factors released by activated T cells (Tashiro *et al.*, 1985). It is possible that the lack of primed T cells in previously unexposed lymph nodes may explain the absence of eosinophils in lymph during the primary response to *S. aureus* vaccines, but does not provide an explanation for their absence during the secondary response to killed *S. aureus*. It has been suggested that eosinophils can exert a 'pro-inflammatory' effect by causing recruitment of neutrophils into affected tissues (Popper, 1984). Thus, the large neutrophil response to a secondary challenge with live *S. aureus* may be mediated in part by a prior influx of eosinophils.

During the peak of the secondary response to live *S. aureus*, almost 60% of the cells in efferent lymph contained cytoplasmic Ig, in contrast to approximately 10% recorded during the primary response (Kennedy *et al.*, 1981). However, as well as magnifying the response, there were qualitative changes in the antibody response to both vaccines. The number of IgM-cc in lymph draining from unprimed nodes exposed to either live or killed *S. aureus* was considerably greater than the numbers of IgG-cc (Kennedy *et al.* 1981). Predictably, during the secondary response to *S. aureus* this trend was reversed and much greater numbers of IgG1-cc and IgG2-cc were recorded in lymph than IgM-cc.

Experiments with single B-cell clones from mice have shown that the switch from IgM to a particular IgG subclass, or even other isotypes such as IgA, may be influenced by external factors (Rosenberg, 1982). It was important, therefore, to determine whether events responsible for such a major switch of emphasis from IgM to IgG during the secondary response to live and killed *S. aureus* favoured one or other of the two IgG subclasses in sheep. Secondary immunization with live *S. aureus* induced a significantly higher IgG2-cc: IgG1-cc ratio in lymph than did secondary immunization with killed *S. aureus*. Similar predication of the IgG subclasses in lymph has been shown during the primary response to live and killed *S. aureus* (Kennedy *et al.*, 1981), indicating that the critical conditions for preferential isotype expression are dictated by viability (or non-viability) of the organisms, rather than by prior immunization.

Both live and killed *S. aureus* vaccines stimulated a considerable IgG1 antibody response in lymph, although killed *S. aureus* provoked the response 4 days later than the live vaccine. However, the IgG2 anti-staphylococcal antibody concentration only increased in lymph draining nodes given live *S. aureus*. Serum levels of anti-staphylococcal antibody did not change following secondary immunization, a result that was expected with drainage of efferent lymph from the body (Hall *et al.*, 1967). Therefore, it is unlikely that any increase in antibody concentration in lymph resulted from transfer of serum antibody into interstitial fluid and thence into lymph. Thus, significant synthesis of IgG2 antibody occurred only in sheep that received the live bacteria; this antibody would have been synthesized in the vaccinal abscess and/or draining lymph node. However, as shown above, substantial numbers of Ig-cc of both IgG subclasses were recorded in lymph from animals given either live or killed *S. aureus*. This suggests that for anti-staphylococcal antibody the control of IgG heavy-chain expression is antigen-specific. These results concur with data from a recent study in which it was shown that the IgG subclass distribution observed in animals given live or killed *S. aureus* was not extended to the antibody response to a 'bystander antigen' combined with each of the vaccines (Kerlin & Watson, 1986). As a result, current work in our laboratory is concerned with isotype restriction of epitope-specific responses in sheep.

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