
Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

*Veterinary Immunology and Immunopathology*, 41 (1994) 275–294
0165-2427/94/$07.00 



# Humoral immune response to *Salmonella abortusovis* in sheep: in vitro induction of an antibody synthesis from either sensitized or unprimed lymph node cells

P. Berthon*, I. Gohin, I. Lantier, M. Olivier

*Institut National de la Recherche Agronomique, Centre de Tours, Equipe de Génétique et Immunité, Laboratoire de Pathologie Infectieuse et Immunologie, 37380 Nouzilly, France*

(Accepted 21 September 1993)

**Abstract**

In vitro culture conditions were determined to induce an anti-*Salmonella abortusovis* antibody synthesis from lymph node leucocytes of three immunized sheep and two unprimed lambs maintained in culture in the presence of heat-inactivated bacteria for 2 weeks. Humoral immune responses were assessed by enumerating specific antibody-secreting cells using ELISASPOT and by titrating immunoglobulins secreted into culture supernatants using ELISA techniques. Optimal secondary antibody response was observed from cultures performed with fetal calf serum (compared with horse serum) and with an antigen concentration of one to ten bacteria per cell. This kind of antigenic stimulation allowed induction of numerous antibody-secreting cells without adsorption of the secreted antibodies. Maximal numbers of antibody-secreting cells could reach a rate of 1% of the sheep leucocytes initially put into culture. Kinetic profiles of antibody production from boosted lymph node cells were characterized by an ascending phase from the sixth to the twelfth day of culture and then showed a plateau phase until Day 14. Most of the responses were composed of IgM and IgG1 antibodies, traces of IgG2 being detected at the end of experiments. From the twelfth day of antigenic stimulation, the IgM isotype was preferentially expressed with high antigen concentration (100 bacteria per cell), whereas the highest amounts of IgG1 were detected at lower concentration (one to ten bacteria per cell). While anti-*Salmonella* IgM appeared to be mainly specific for the lipopolysaccharide (LPS) cell wall fraction, some IgG1 recognized other bacterial antigens. Kinetic profiles and magnitudes of primary antibody responses induced in vitro from lamb lymph node cells did not differ from those defined in cultures of sheep boosted leucocytes. But these immune reactions were mainly made up of anti-LPS IgM. Few anti-*Salmonella* IgG1 were detected from the tenth day of culture. So these in vitro assays allowed induction of an antibody synthesis from either in vivo sensitized or unprimed sheep lymph node leucocytes. This methodology would permit achievement of more detailed studies on interactions between *Salmonella* and lymph node leucocytes, leading to a better understanding of the mechanisms controlling bacterial dissemination through the lymphoid tissue.

**Abbreviations**

APC, antigen-presenting cell; FCS, fetal calf serum; HS, horse serum; Ig, immunoglobulins; LPS,

*Corresponding author.
*SSDI* 0165-2427(93)05256-E

lipopolysaccharide; NLS, normal lamb serum; PBS, phosphate-buffered saline; SFC, spot-forming cells.

## Introduction

Facultative intracellular bacteria, such as *Salmonella*, *Brucella* or *Listeria*, are considered to be major pathogenic agents for all domestic animal species and could be transmitted to humans. The pathogenesis of these infections involves a step of bacterial dissemination from the inoculation site to the liver and spleen through the draining lymph nodes. Bacterial colonization of lymph node draining the inoculation area has been described after experimental infections by the conjunctival (Plommet and Plommet, 1975, 1976) and the subcutaneous routes (Pardon, 1977; Pardon and Marly, 1979; Lantier, 1987), but mechanisms restricting the bacterial dissemination are poorly understood.

Mechanisms of host defense involve both cellular and humoral immune reactions. Immune responses induced within lymph nodes seem to play a fundamental role in control of the infectious process. T-lymphocyte responses appear late, from nearly the tenth day following infection (Maskell et al., 1987), and are responsible for granuloma formation (Guilloteau et al., 1991). However, in cell-transfer experiments studying protective mechanisms, it has been shown that T-cells from lymph nodes were more efficient than spleen cells in the transfer of anti-*Brucella* immunity to mice (Plommet and Plommet, 1987). Antibody production from B-cells occurs earlier in infection and is usually considered as the first evidence of the bacterial contamination. With their agglutinin and opsonin properties, specific antibodies secreted within lymphoid tissue would be involved in control of the bacterial colonization of lymph nodes, as has been previously described (Pardon, 1977; Plommet and Plommet, 1989).

With a suitable size for the development of surgical procedures (Miyasaka and Trnka, 1985; Hopkins et al., 1985; Lascelles et al., 1988), sheep represent the best model for studying the role of lymph nodes in restricting bacterial dissemination from the inoculation site. The establishment of an experimental model of infection by *Salmonella abortusovis*, a serotype specific for sheep which induces abortion and sometimes mortality of newborn lambs (Pardon et al., 1990b), gives the opportunity to study the *Salmonella* pathogenesis pattern in its natural host. In mice, experimental inoculation of *S. abortusovis* results in an infection similar to the one developed after *Salmonella typhimurium* administration (Pardon and Marly, 1979; Lantier et al., 1981).

The intensity of bacterial dissemination through lymph nodes can vary according to the bacterial strain virulence (Fontaine et al., 1994) and according to the expression of the host genetic resistance (Plant and Glynn, 1976; Hormaeche, 1979a,b; O'Brien, 1986; Hormaeche and Maskell, 1989; Oswald et

al., 1992). Previous work has shown that after intravenous or subcutaneous inoculation of sheep with the vaccinal Rv6 strain of *S. abortusovis* (Lantier et al., 1983; Pardon et al., 1990a), blood levels of the specific humoral immune response determined by ELISA tests were closely related to the animal susceptibility to the disease, measured by the bacterial infection level in spleen, liver and lymph nodes. Greater bacterial numbers were detected from infected organs, and higher anti-*S. abortusovis* antibody titres were measured in blood (A.V. Gautier and F. Lantier, unpublished data, 1990).

To better understand the mechanisms controlling bacterial dissemination through lymph nodes and to study in detail interactions between *Salmonella* and host phagocytic cells (Buchmeier and Heffron, 1989, 1991; Finlay and Falkow, 1989) which play an antigen-presenting cell (APC) function towards lymph node lymphocytes, an in vitro assay allowing the induction of an antibody synthesis from both in vivo sensitized and unprimed sheep leucocytes had to be set up.

This report describes an in vitro assay performed with sheep lymph node cells, maintained in culture with *S. abortusovis* bacteria. The in vitro antibody synthesis induced after the antigenic stimulation was revealed, either by enumerating anti-*S. abortusovis* antibody-secreting cells using ELISASPOT assays, or by titration of antigen-specific antibodies secreted into culture supernatants using ELISA assays. The comparison between ELISASPOT and ELISA data allows study at the cellular level and leads to a more detailed approach of the ovine humoral immune response.

## Materials and methods

### *Experimental design*

The in vitro secondary antibody response has been assessed from three different culture experiments, carried out with in vivo primed lymph node cells from three sheep, respectively, of the Préalpes du Sud flock of the Laboratory of Infectious Pathology and Immunology. Those 10- to 19-month-old animals were immunized intravenously with $10^8$ bacteria of the live attenuated vaccinal Rv6 strain of *S. abortusovis* (Lantier et al., 1981; Pardon et al., 1990b) and then killed 1 month later. Unprimed lymph node cells from two 4-month-old Ile-de-France lambs were used to study the in vitro-induced primary humoral immune response. For each slaughtered sheep, cell cultures were performed from the prescapular, mediastinal, precrural, iliac and hepatic lymph nodes. Reported results represent mean values of the individual data and the standard errors (mean $\pm$ SEM).

*Bacteria and preparation of microbial suspensions*

Bacterial suspensions of the vaccinal *S. abortusovis* Rv6 strain (Lantier et al., 1981; Pardon et al., 1990b), either inoculated into sheep or a source of antigen for the in vitro lymph node cell cultures and the immunoenzymatic assays (ELISA and ELISASPOT tests), were prepared as previously described (Pardon and Marly, 1979; Lantier et al., 1981). Briefly, stored at −70°C in spleens of inoculated mice, bacteria were then grown on tryptic soy agar (Bio-Mérieux) slopes for 18 h at 37°C and harvested in phosphate buffered saline (saline, pH 6.85). These bacterial suspensions were standardized turbidimetrically. Purity of cultures and numbers of viable organisms were checked by plating serial dilutions.

Used as antigenic preparations in sheep leucocyte cultures, the bacterial suspensions were heat killed by treatment at 60°C for 2 h (Lantier et al., 1983) and then stored at −70°C. Before use, inactivated bacteria were washed twice in saline at 5000×*g*, 4°C for 20 min. Appropriate dilutions were performed in culture medium (see below).

For the coating step of ELISA and ELISASPOT microtiter plates, bacterial suspensions were first killed by heating at 60°C for 2 h, then incubated overnight at 4°C with 1% phenol in saline and washed twice at 2500×*g*, 4°C for 30 min in 0.5% phenol–saline. This stock suspension was standardized turbidimetrically, adjusted to the concentration of $3.8\times10^9$ bacteria $ml^{-1}$ by appropriate dilution in 0.5% phenol–saline and then stored at 4°C (adapted from Williams and Whittemore, 1973).

*Lymph node cell preparation*

Lymph node cell suspensions were prepared according to a technique previously set up for the isolation of sheep peripheral blood mononuclear cells. First, lymph nodes were washed in MEM (Gibco, 041-01380) supplemented with 2.5% heat-inactivated normal lamb serum (NLS), 200 U $ml^{-1}$ of penicillin (Sigma, P-3032) and 0.2 mg $ml^{-1}$ of streptomycin (Sigma, S-9137) (2.5% NLS–MEM).

Cells were released from organs by cutting the lymphoid tissues into small pieces with scissors in 2.5% NLS–MEM. Cellular suspensions were then filtered on gauze to remove cell debris and layered on Ficoll gradient (Histopaque 1077, Sigma, 1077-1). After a 1600×*g* centrifugation for 20 min at room temperature, lymph node leucocytes were collected at the Histopaque–MEM interface and washed three times with 2.5% NLS–MEM at 400×*g*, 4°C for 15 min. Cell viability was assessed by the trypan blue dye exclusion test and the cell suspensions were adjusted to a final concentration of $1.25\times10^7$ viable leucocytes per ml of culture medium. The culture medium used was RPMI-1640 (Gibco, 041-01870) supplemented with either 10% heat-inacti-

vated fetal calf serum (FCS, Gibco, 011-06290 M) or 10% heat-inactivated horse serum (HS, Gibco, 034-06050 H), 2 mM L-glutamine (Gibco, 043-05030 H), 1 mM sodium pyruvate (Gibco, 043-01360 H), $5\times10^{-5}$ M 2-mercaptoethanol (Serva, A 28625) and antibiotics (10% FCS–RPMI or 10% HS–RPMI).

*Lymph node cell cultures and in vitro antigenic boost*

Lymph node cell cultures, involving an in vitro antigenic stimulation, were carried out according to a technique previously set up (Berthon et al., 1990) and using different concentrations of either heat-inactivated *S. abortusovis* Rv6 or lipopolysaccharide (LPS) from *Salmonella abortusequi* (phenol extract, Sigma, L-5886) dissolved in culture medium. This LPS presents O-antigen specificities identical to LPS from *S. abortusovis* (Le Minor, 1984).

Antigenic boost in vitro was achieved by mixing, in each well of tissue culture 24-well plates (Linbro, Flow Laboratories, 76-033-05), 0.4 ml of lymph node cell suspension ($5\times10^6$ cells per well) with 0.1 ml of either heat-killed bacterial suspensions ($5\times10^6$, $5\times10^7$ and $5\times10^8$ bacteria per well, i.e. one, ten and 100 bacteria per lymph node cell) or LPS dilutions corresponding to final concentrations of 0.01, 0.1 and 1 $\mu$g ml$^{-1}$ (final volume of 0.5 ml per well). In both cases, cell cultures were performed using medium supplemented with 10% serum from fetal calf or adult horse. In control cultures, the addition of antigen was replaced with 0.1 ml of culture medium. Cultures were performed for 2 weeks and every 2 days supernatant samples were taken from each well (0.1 ml for the first sample and 0.4 ml for the others) and fresh culture medium (0.5 ml per well) was added. At the same time, lymph node cells from at least two wells were harvested and cell survival was assessed by enumerating viable leucocytes from samples using the trypan blue dye exclusion test. Results were expressed, according to the duration of culture, as percentages of the living lymph node cells initially put into culture.

*ELISASPOT test for enumerating antibody-secreting cells*

ELISASPOT tests were performed using microtiter plates with 96 flat-bottomed wells (Nunc, Immunoplates, 4-42404) coated with inactivated *S. abortusovis* bacteria. The enumeration of antibody-secreting cells by ELISASPOT test makes it necessary to perform an homogeneous and regular coating of antigen into microtiter plates; hence, the technique set up for coating bacteria on ELISASPOT plates was slightly different from the one used for ELISA plates (see below) and from the technique previously described with *Salmonella typhi* (Kantele, 1990).

Microtiter plates were first pre-treated with 0.1 ml per well of a 0.2% glutaraldehyde aqueous solution and incubated overnight at 4°C (Logtenberg et

al., 1985). After a wash with phosphate-buffered saline (PBS, pH 7.5), plates were filled with 0.1 ml per well of the stock bacterial suspension diluted 1:10 in saline ($3.8 \times 10^7$ bacteria per well) and incubated overnight at 4°C. Then, plates were twice washed carefully with PBS and the protein adhesiveness of the wells was blocked with 5% bovine skimmed milk in PBS for 15 min at room temperature, followed by a wash with PBS.

ELISASPOT tests were carried out with sheep lymph node cells primed in vivo and boosted in vitro. After every 2 days of culture, cell samples were prepared as described elsewhere (Berthon et al., 1990). Briefly, lymph node cells from several wells were harvested and washed in 5% FCS–MEM to remove free antibodies. Cell viability was assessed by the trypan blue dye exclusion test and several dilutions from each cell sample were prepared in 10% FCS–RPMI and put into microtiter plates. Negative controls were performed on plates coated with the B19 strain of *Brucella abortus*, which is a Gram-negative bacterium showing no antigenic cross-reactivity with *S. abortusovis* (Diaz and Bosseray, 1974), inactivated by heat and formol (gift from Dr. G. Dubray, INRA, Laboratory of Infectious Pathology and Immunology, Nouzilly, France). Cells were incubated at 37°C for 3 h and then removed. Antibodies secreted locally by B-lymphocytes were revealed as spots using an immunoenzymatic technique involving alkaline phosphatase conjugate (Sedgwick and Holt, 1983, 1986; Bianchi et al., 1990). In the present assay, a first overnight incubation at 4°C was performed using rabbit antibodies to sheep immunoglobulins (Ig) (Dako, Z 138), diluted 1:7500 in PBS containing 0.05% Tween 20 (Merck, 822 184) and 20% skimmed milk (Tween–milk–PBS). A second incubation was carried out at 37°C for 2 h with alkaline phosphatase-conjugated swine Ig to rabbit Ig (Dako, D 306) diluted 1:650 in Tween–milk–PBS. The enzymatic activity was revealed using as substrate 5-bromo 4-chloro 3-indolyl phosphate (Sigma, B-8503) in agarose (Sigma, A-6013) (Sedgwick and Holt, 1983, 1986), at the rate of 0.05 ml per well, incubated overnight at room temperature in the dark, enabling well-contrasted blue spots to be obtained. Alkaline phosphatase activity was stopped by adding 0.05 ml per well of 3 N sodium hydroxide solution and plates were stored at 4°C until reading. The numbers of spots per well were counted using an Olympus CK2 inverted microscope (40- and 100-fold magnification).

Since assays were performed on pools of several wells, they represented averages of several lymph node cell cultures. As shown by the results obtained from the different dilutions of samples, the numbers of spots were directly proportional to leucocyte concentration. Taking the cell culture recoveries into account, the results were expressed, according to the culture duration, as the decimal logarithm of the spot-forming cell (SFC) numbers per $10^6$ lymph node cells initially put into culture.

*ELISA tests for measuring secreted antibody*

Anti-*S. abortusovis* antibodies secreted into lymph node cell culture supernatants were detected according to different ELISA techniques. Amounts of the whole of specific antibodies were determined on microtiter plates coated with bacteria while Ig-isotype of in vitro produced antibodies were studied on both bacteria- and LPS-coated plates.

ELISA tests on bacteria were performed using 96-well microtiter plates (Greiner, Labortechnik, No. 655 161), filled with 0.05 ml per well ($1.9 \times 10^7$ bacteria per well) of a 1:10 dilution in saline of the stock suspension of inactivated *S. abortusovis*, also used for ELISASPOT tests. Plates were left to dry overnight at 37°C. After two washes with tap water and a wash with PBS, bacterial antigens were fixed for 20 min at −20°C with 0.2 ml per well of a cold 80% (v/v) acetone–water solution and then, washed as before. Non-specific binding was blocked by incubating plates with 0.1 ml per well of 5% bovine skimmed milk in PBS for 15 min at room temperature.

When the LPS fraction was used as immobilized antigen, 96-well microtiter plates (Nunc, Immunoplates, 4-42404) were coated with 0.05 ml per well of a 1 mg $ml^{-1}$ stock solution of LPS prepared in a 50% (v/v) PBS–glycerol mixture and diluted 1:100 in 0.05 M carbonate buffer (pH 9.6) (0.5 $\mu$g per well). After an overnight incubation at 4°C, plates were washed twice with tap water, once with PBS and then incubated with bovine milk as described previously.

In both cases, following two washes with PBS, culture supernatant samples with unknown antibodies were added (0.1 ml per well) after serial 1:3 dilutions in Tween–milk–PBS and incubated for 2 h at 37°C. Positive controls were performed with a specific anti-*S. abortusovis* serum from a hyperimmunized sheep used as reference sample in each test. Negative controls were carried out with 0.1 ml per well of fresh lymph node cell culture medium. Amounts of the whole of secreted anti-*Salmonella* antibodies were revealed using peroxidase-conjugated rabbit Ig to sheep IgG (H and L chains) (Nordic), 1:3000 diluted in Tween–milk–PBS and incubated for 90 min at 37°C. Ig-isotype was studied on bacteria- and LPS-coated plates using appropriate dilutions of mouse monoclonal antibodies to sheep IgG1, IgG2 (Beh, 1987) and sheep IgM (Beh, 1988) (ascitic fluids kindly provided by Dr. K.J. Beh, CSIRO, Division of Animal Health, McMaster Laboratory, Glebe, Australia). After a 90 min incubation at 37°C, monoclonal antibody binding was detected with peroxidase-conjugated goat Ig to mouse IgG (H and L chains) (Bio-Rad, No. 172-1011), 1:10 000 diluted in Tween–milk–PBS and incubated for 90 min at 37°C. Between each of the steps, plates were washed four times with tap water and three times with 0.05% Tween 20 in saline. The peroxidase activity was detected with 2,2′-azino-bis-(3-ethyl benzthiazoline-6-sulfonic acid) (Boehringer Mannheim)/$H_2O_2$ substrate solution and read

at 415 nm (Bernard et al., 1986). Optical densities were obtained according to the serial 1:3 dilutions of samples allowed to obtain ELISA logistic curves. These curves underwent conversion to logits to get linear functions enabling the determination of the sample dilution for the point of inflexion of the ELISA logistic curves.

Results were expressed as antibody titres. Antibody titres were equivalent to the logarithm base 3 ($\log_3$) of the sample dilution for which the point of inflexion of the corresponding ELISA logistic curve was reached.

## Results

### *Cell culture recovery*

With regard to the in vitro induction of a secondary antibody response from in vivo primed lymph node cells, boosted in vitro with either heat-inactivated *S. abortusovis* or LPS from *S. abortusequi*, cell recoveries decreased throughout the experiments (Table 1). From about 60% on Day 2 of culture, values fell to nearly 40% on Day 4 and to 35% on Day 6 until Day 10. Afterwards, lower survival rates (20%) could be observed from some samples, especially from those maintained with HS and stimulated with LPS. Despite antigenic boost in vitro, cell recovery from stimulated samples did not seem to significantly differ from control values. The greatest survival rates (45–50%) were obtained when cells were cultured in the presence of the highest concentration

Table 1
Characteristics of the antibody responses induced from in vivo sensitized lymph node cells, boosted in vitro with either heat-inactivated *S. abortusovis* (SAO) (one, ten and 100 bacteria per cell) or lipopolysaccharide (LPS) (0.01, 0.1 and 1 $\mu g\ ml^{-1}$). Cell cultures were carried out in the presence of fetal calf serum or horse serum. For each sample, reported data include the maximum of the antibody response (expressed as the $\log_{10}$ of the maximal number of spot-forming cells (SCF) per $10^6$ cells initially put into culture; mean ± SEM, $n=3$), the day of culture corresponding to this peak of the immune response and the cell culture recovery observed on this day (expressed as the percentage of living cells initially put into culture; mean ± SEM, $n=3$)

| Culture | Fetal calf serum | | | Horse serum | | |
|---|---|---|---|---|---|---|
| | Maximum of SCF ($\log_{10}$) | Day of maximal response | Cell recovery (%) | Maximum of SFC ($\log_{10}$) | Day of maximal response | Cell recovery (%) |
| Control | 0.44 ± 0.39 | 4 | 38.1 ± 0.6 | 0.18 ± 0.09 | 2 | 65.5 ± 5.5 |
| 1 SAO | 2.76 ± 0.36 | 10 | 27.2 ± 7.6 | 3.19 ± 0.05 | 10 | 29.5 ± 2.9 |
| 10 SAO | 3.04 ± 0.66 | 14 | 27.0 ± 12.1 | 3.77 ± 0.34 | 10 | 28.1 ± 6.4 |
| 100 SAO | 3.43 ± 0.66 | 14 | 48.8 ± 14.9 | 4.26 ± 0.11 | 14 | 44.0 ± 0.8 |
| LPS 0.01 | 1.04 ± 0.55 | 4 | 35.2 ± 1.1 | 0.47 ± 0.47 | 4 | 45.1 ± 4.7 |
| LSP 0.1 | 1.10 ± 0.64 | 4 | 28.8 ± 2.5 | 0.51 ± 0.48 | 4 | 45.5 ± 8.6 |
| LPS 1 | 0.92 ± 0.60 | 4 | 27.9 ± 6.3 | 0.60 ± 0.60 | 4 | 43.7 ± 5.0 |

Table 2
Cell culture recoveries observed from in vivo unprimed lymph node cells, stimulated in vitro with heat-inactivated *S. abortusovis* (SAO) (100 bacteria per cell) in the presence of fetal calf serum for 2 weeks. Results are expressed as percentages of the living cells initially put into culture (mean ± SEM, $n=2$)

| Day of culture | Cell culture recovery (%) | |
|---|---|---|
| | Control | 100 SAO |
| 2 | 56.3 ± 5.0 | 52.8 ± 1.5 |
| 4 | 40.5 ± 1.1 | 40.9 ± 1.7 |
| 6 | 25.3 ± 3.5 | 27.8 ± 10.0 |
| 8 | 22.8 ± 0.4 | 37.8 ± 7.7 |
| 10 | 25.1 ± 3.0 | 40.8 ± 8.9 |
| 12 | 22.0 ± 3.3 | 39.5 ± 9.6 |
| 14 | 22.5 ± 7.9 | 29.0 ± 9.1 |

of inactivated *S. abortusovis* (100 bacteria per cell), during the second week of culture. The stimulation in vitro with LPS (from 0.01 to 1 $\mu g\ ml^{-1}$) showed no mitogenic effect of this fraction on the cultured lymph node cells. Concentration of LPS higher than 1 $\mu g\ ml^{-1}$ was cytotoxic, whereas a concentration of 0.001 $\mu g\ ml^{-1}$ gave similar results to those reported (data not shown).

A similar range of cell recoveries was observed during the induction of the primary antibody response, from unprimed lymph node cell cultures in vitro stimulated with 100 *S. abortusovis* per leucocyte in the presence of FCS (Table 2).

### *Enumeration of antibody-secreting cells from boosted cell cultures*

From lymph node cells of immunized sheep, re-stimulated in vitro with either inactivated *Salmonella* (Figs. 1(a) and 1(b)) or LPS (Table 1), cells secreting specific antibody to *S. abortusovis* were enumerated at 2-day intervals using an ELISASPOT technique.

An humoral immune response mainly appeared when lymph node cells underwent an antigenic boost in vitro with whole bacteria, compared with the LPS stimulation (Table 1). Whatever the LPS concentration, this antigen led to an early and weak response, characterized by a peak on the fourth day of culture and a magnitude lower than ten SFC per $10^6$ leucocytes. No more SFC were detected by Day 8 (data not shown).

The profile and magnitude of the kinetics of the SFC numbers, induced after the boost with inactivated bacteria, were almost similar according to the antigen concentration (Figs. 1(a) and 1(b)). The lowest in vitro antigenic stimulation (one bacterium per cell) led to the earliest induction of antibody synthesis, detected as soon as the fourth day of culture. Maximal SFC num-

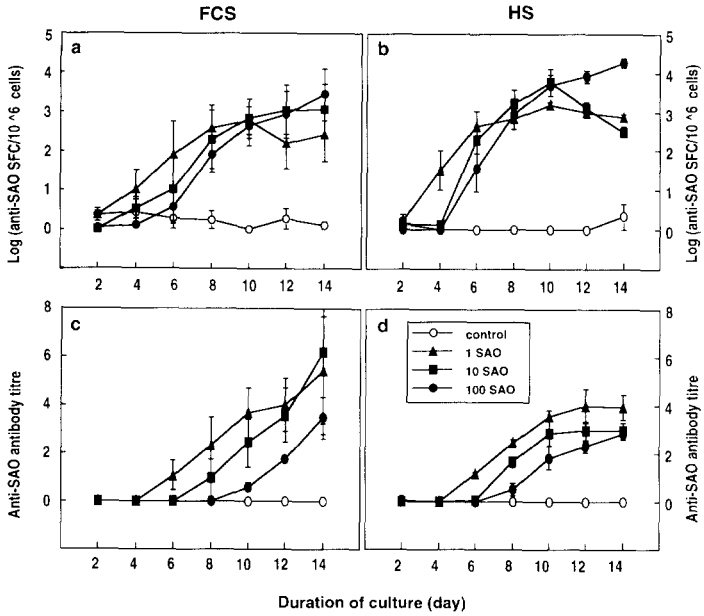


Fig. 1. Kinetics of the antibody synthesis from lymph node cells of three immunized sheep, boosted in vitro with different concentrations of heat-inactivated *Salmonella abortusovis* (SAO). Cultures were performed with either fetal calf serum (FCS: a,c) or horse serum (HS: b,d); a,b: enumeration of specific spot-forming cells (SFC) by ELISASPOT on fixed bacteria. Results were expressed as $\log_{10}$ of SFC numbers per $10^6$ cells initially put into culture (mean ± SEM, $n=3$). c,d: titration of antibodies secreted into culture supernatants by ELISA on whole bacteria. Antibody titres represented $\log_3$ of the sample dilution corresponding to the inflexion point of the ELISA logistic curve (mean ± SEM, $n=3$).

bers were observed from Day 10 and could represent nearly 1% of cells initially put into culture (or 3–4% of living cells tested from samples). No significant difference was observed in results, according to the kind of serum used as culture medium supplement (Table 1).

In both cases, a few or no SFC were detected from control samples, in which in vivo primed cells have been cultured without antigenic stimulation. Results obtained at the same time, using *B. abortus* B19 as coated antigen for the ELISASPOT assay, showed that all these SFC were secreting specific anti-*S. abortusovis* antibody (data not shown). Very few (less than four SFC per $10^6$ cells) or no anti-*B. abortus* SFC were detected from samples.

### *Assessment of the antibody secretion in vitro during the secondary immune response*

Parallel to the ELISASPOT technique which allowed the enumeration of specific antibody-secreting cells from lymph node cell cultures, ELISA assays

were carried out to determine amounts of anti-*S. abortusovis* antibodies secreted into culture supernatants (Figs. 1(c) and 1(d)).

Magnitudes of the antibody responses, measured by ELISA technique on the fourteenth day of antigenic stimulation, were higher from cell cultures performed with FCS than those observed from cultures with HS, whereas the kinetics of the SFC numbers did not differ with the kind of serum used (Figs. 1(a) and 1(b)).

Kinetic profiles induced in FCS increased throughout the culture duration while those characterizing responses in HS increased until Day 12 and then reached a plateau phase. After the LPS boost in vitro, no antibody was detected from samples maintained in HS (data not shown). A very weak immune response appeared from FCS cultures, with a peak on Day 6 (maximal antibody titre, obtained with the 0.1 $\mu$g ml$^{-1}$ LPS concentration, was equal to $0.48 \pm 0.11$), and no more antibody was revealed by Day 10 (data not shown). No antibody was detected from unstimulated lymph node cell culture supernatants.

Results on the amounts of secreted antibodies, when cells were re-stimulated with inactivated bacteria, showed an effect of the antigen concentration on the kinetics of the antibody response (Figs. 1(c) and (d)). The earliest immune reactions and the highest antibody titres were observed from samples stimulated with the lowest bacterial concentration. Maximal titres reached on Day 14 were, according to increasing antigen concentrations (one, ten and 100 bacteria per cell), $5.45 \pm 2.21$, $6.19 \pm 3.40$ and $3.47 \pm 0.87$ in FCS and $3.93 \pm 0.50$, $2.96 \pm 0.35$ and $2.81 \pm 0.19$ in HS, respectively.

Whatever the serum used, according to the increasing amounts of bacteria per cell, secreted antibody could be detected from Day 6, Day 8 and Day 10, respectively, while under identical conditions the SFC could be revealed from Day 4 in the presence of one bacterium per cell and from the sixth day of culture both in the presence of ten and 100 bacteria per cell. Hence, at low bacterial concentration (one and ten *S. abortusovis* per leucocyte), a 2 day lag was observed between the appearance of the SFC and the detection of antibodies secreted into culture supernatants. With a higher antigen concentration of 100 bacteria per cell, this interval increased and the lag reached 4 days. This suggests that large amount of bacteria could adsorb antibody and then induce a decrease of the antibody titres determined by ELISA. These results show that optimal in vitro culture conditions involved the use of FCS instead of HS and an antigen concentration of the order of one to ten bacteria per cell.

*Ig-isotype and antigen specificity of secreted antibody after the boost in vitro*

Ig-isotypes expressed by the anti-*S. abortusovis* antibodies secreted into supernatants of in vivo primed lymph node cell cultures were studied by an

ELISA technique performed on two kinds of fixed antigens: inactivated *S. abortusovis* whole bacteria and the polyosidic LPS fraction. Only data involving lymph node cell cultures carried out in the presence of FCS and boosted with heat-inactivated bacteria are presented in Fig. 2. Identical results have been obtained from samples maintained in HS (data not shown).

The humoral immune responses to *S. abortusovis* were mainly composed of anti-*S. abortusovis* IgM and IgG1 antibodies. No or very low levels of specific IgG2 were detected at the end of cell cultures. The earliest IgM and IgG1 antibody secretions were observed in vitro using the lowest antigenic stimulation. These anti-*S. abortusovis* antibody titres increased until Day 14, except the IgM responses induced with one bacterium per cell which decreased from Day 10. From the twelfth day of culture, the highest anti-*S. abortusovis*

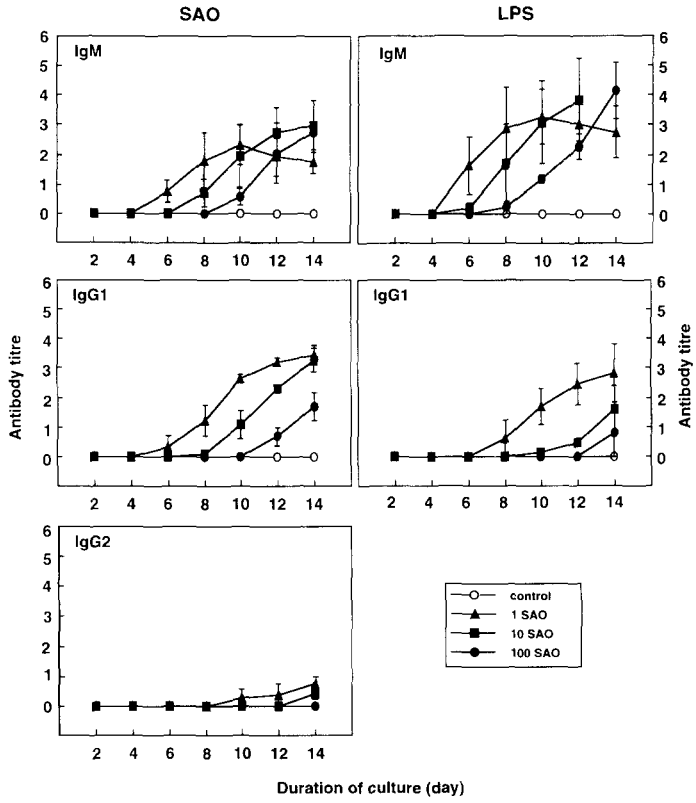


Fig. 2. Ig-isotype and specificity of the anti-*Salmonella abortusovis* antibodies secreted into supernatants of the cell cultures performed with fetal calf serum, during the secondary humoral immune response (see caption to Fig. 1). ELISA tests were carried out using whole bacteria (SAO) or lipopolysaccharide extract (LPS) from *Salmonella abortusequi* as immobilized antigens (mean ± SEM, $n = 3$).

IgM titres were obtained with ten and 100 bacteria per cell, whereas maximal IgG1 antibody synthesis was detected in the presence of one and ten bacteria per cell.

When humoral responses were studied on their specificity against LPS, their kinetic profiles were identical to those determined on inactivated whole bacteria. Titres of anti-LPS IgM were always higher than those measured on *S. abortusovis*. The anti-LPS IgG1 synthesis was detected later than the anti-*S. abortusovis* IgG1 production, with a 2 day lag, and showed a lower magnitude. The weak and late anti-*S. abortusovis* IgG2 response also contained some anti-LPS IgG2, detected on Day 14 of the culture, only from samples stimulated with one bacterium per cell (data not shown).

From lymph node cell cultures with FCS, a very small and early anti-*S. abortusovis* humoral response was induced with a LPS stimulation. These immune reactions were composed of IgM and IgG1 antibody (data not shown). Kinetics of IgM titres were identical to those described for the whole of anti-*S. abortusovis* antibodies, with a peak on Day 6 and no more antibody from Day 10. As previously, anti-LPS IgM titres were higher than the IgM titres

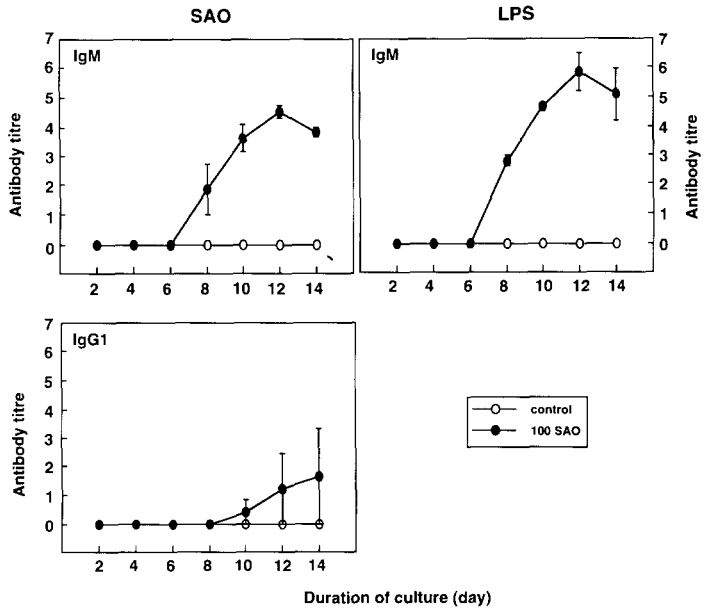


Fig. 3. ELISA data on Ig-isotype and specificity of anti-*Salmonella abortusovis* antibodies, secreted from lymph node cells of two unprimed lambs. ELISA were performed on whole bacteria (SAO) and on lipopolysaccharide extract (LPS). Leucocyte cultures were carried out in the presence of fetal calf serum and an antigen concentration of 100 bacteria per cell (mean ± SEM, $n=2$).

against whole *Salmonella*. Traces of IgG1 were detected only on Day 8 of the culture and only by ELISA performed on fixed whole bacteria.

*In vitro antibody synthesis during the primary immune response*

Figure 3 reports ELISA data obtained from in vivo unprimed lymph node cells of two lambs, which were maintained in vitro without or with an antigen concentration of 100 heat-inactivated bacteria per leucocyte for 2 weeks in the presence of FCS. Similar results were observed from cell cultures performed with HS (data not shown).

Results revealed that this kind of humoral response was mainly composed of IgM antibodies which appeared from the eighth day of culture. Defined either on whole bacteria or on LPS, the kinetic profiles of those IgM responses were characterized by a peak on Day 12. As previously observed during the secondary antibody response, anti-LPS IgM titres were always higher than titres of the anti-*S. abortusovis* IgM. Some anti-*S. abortusovis* IgG1 antibodies were detected on whole bacteria-coated plates during the second week of the culture experiments, from the tenth day. No IgG2 was revealed according to either kind of ELISA technique used and no antibody synthesis was shown from supernatants of leucocyte cultures performed without antigenic stimulation.

## Discussion

This study describes a new assay for an in vitro antibody synthesis, specific to bacterial antigens, from lymph node cells of unprimed lambs and sheep immunized by the intravenous route to get a general antigenic sensitization of the lymph nodes and killed 1 month later when no more bacteria were present within lymphoid tissues (Pardon et al., 1990a).

Culture conditions were derived from those previously established with pig leucocytes, stimulated in vitro with viral antigens (Berthon et al., 1990). From the sera available in the author's laboratory and checked by ELISA, only sera from fetal calf and adult horse were found devoid of antibody cross-reacting with bacteria (data not shown). Hence, FCS, considered to be Ig-free, and HS, with supposed low mitogenic properties, have been chosen to carry out sheep leucocyte cultures.

From cultures of either in vivo sensitized or unprimed sheep lymph node cells, according to the serum used as culture medium supplement, no great difference appeared concerning cell culture recoveries. Differences were rather induced with the kind of antigen stimulation performed in vitro. The highest cell recoveries were observed with the concentration of inactivated *Salmonella* of 100 bacteria per cell. Higher amounts of inactivated *S. abortusovis* per well have been tested (160 bacteria per cell), corresponding to the antigen

concentration of the stock suspension of *Salmonella*. These conditions led to similar results to those obtained with the former bacterial concentration (data not shown). Culture conditions defined in this report differ from those previously described for pig blood lymphocytes stimulated with bacterial antigens such as membrane components of *Haemophilus pleuropneumoniae* (Borthwick et al., 1988). In that case, the boost in vitro had to be carried out in the form of an antigenic pulse for 16 h to keep the cells clear of the cytotoxic effect of such antigens. Results on cell culture recoveries from sheep lymph node leucocytes stimulated with inactivated *S. abortusovis*, showed that these cells could be maintained in the presence of whole bacteria throughout the experiments. Some samples were cultured for 1 month (data not shown). Within the concentrations tested, inactivated *Salmonella* appeared to exert no cytotoxic effect over sheep leucocytes.

In vitro antigenic boost of in vivo primed leucocytes was also performed using LPS to study the effect of this component of the cell wall of Gram-negative bacteria on the immune response from sheep lymphocytes stimulated in vivo with living whole bacteria. The LPS used in these experiments, at higher amounts than 1 $\mu g\ ml^{-1}$, was cytotoxic for sheep leucocytes (data not shown). In the range of 0.001 to 1 $\mu g\ ml^{-1}$, no mitogenic effect was demonstrated throughout the culture duration. From in vivo primed lymph node cells, in vitro LPS boost induced a very weak and short immune antibody response, suggesting a lack of a polyclonal activation of the *Salmonella*-specific B-cells.

In vitro antigenic boost with inactivated whole bacteria of in vivo primed sheep lymph node cells led to the expression of a well-developed immune antibody response, detected either by ELISASPOT or by ELISA tests. This secondary humoral immune response was *S. abortusovis* specific and very low background was revealed from control cultures, whatever the serum used. Although identical numbers of SFC (measured by ELISASPOT) were induced from samples, antibody titres (determined by ELISA) were higher in the presence of FCS than those observed with HS. This would suggest that, according to the in vitro culture conditions, lymph node cells boosted in the presence of FCS were able to secrete, at the cellular level, more antibodies than the same cells maintained in HS. The antibody production in vitro was detected only if leucocytes, even after an in vivo immunization, were re-stimulated with bacteria. No spontaneous antibody secretion was shown from in vivo primed lymph node cells put into culture without antigen.

In the presence of an antigen concentration of one and ten bacteria per cell, SFC were first detected from the fourth and the sixth day of culture, respectively, while the corresponding antibody secretions into culture supernatants could be measured from Day 6 and Day 8. This 2 day lag between the enumeration of antibody-secreting cells and the titration of synthesized antibody has already been observed (Berthon et al., 1990). When leucocytes were re-

stimulated in vitro with higher bacterial concentrations (100 bacteria per cell), the SFC numbers slightly increased but the lag between ELISASPOT and ELISA results reached 4 days, suggesting that some antibodies secreted into culture supernatants were adsorbed by the antigen. So data from both techniques of antibody detection appeared to be less closely related. For this reason, an antigen concentration from one to ten bacteria per cell, allowing a good expression of SFC without great antibody adsorption, has been chosen for further experiments.

Kinetic profiles of the SFC numbers from in vivo primed cells, boosted in vitro, showed an ascending stage from Day 4 until about Day 10 and then reached a plateau phase to Day 14. Kinetic profiles of the SFC numbers previously determined from lymphocytes directly isolated from humans immunized with *S. typhi* vaccine (Kantele, 1990; Kantele and Mäkelä, 1991; Kantele et al., 1991) or from sheep vaccinated with a mutant strain of *S. typhimurium* (Lascelles et al., 1988), without an in vitro boost step, were characterized by a narrow peak on Days 5 or 7 after immunization. So, the antigenic re-stimulation step, performed in vitro with inactivated bacteria, seems to further a long-lasting humoral immune response. This might be dependent upon the nature of the antigen used and, especially on the presence of LPS into the cell wall of bacteria (Ruttkowski and Nixdorff, 1980).

These secondary antibody responses to *S. abortusovis*, from sheep lymph node cell cultures, were mainly composed of IgM and IgG1 antibodies. Similar Ig-isotypes were preferentially expressed in vivo, after experimental infection of sheep with *Salmonella* (Lascelles et al., 1988). In comparison with the IgG1 antibody secretion, the weak and late IgG2 response could be related to the differences in the respective biological activities of ovine IgG1 and IgG2 (Levieux, 1990).

During the second week of cell cultures, the highest IgM antibody titres were observed in the presence of a high antigen concentration (ten to 100 bacteria per cell), while the greatest IgG titres appeared with a low concentration (one to ten bacteria per cell). This would suggest that either Ig-isotype expression could require different culture conditions or that some Ig-isotypes (in this case, IgG) could be preferentially adsorbed on antigen. These hypotheses could be confirmed using ELISASPOT tests performed according to the Ig-isotypes.

If most of the anti-*S. abortusovis* IgM antibodies seemed to be LPS-specific, results concerning the IgG1 response showed that some anti-*S. abortusovis* IgG1 were specific for other bacterial antigens. These data are in agreement with those previously described from mice and humans (Brown and Hormaeche, 1989).

This methodology has also been applied to study in vitro-induced primary immune responses from lamb lymph node cell cultures. Experiments were carried out using high antigen concentration (100 bacteria per cell) because

those culture conditions led to slightly better cell culture recoveries and particularly allowed secretion of higher amounts of IgM antibody.

Kinetic profiles characterizing the antibody production from unprimed lymph node cells were similar to those defined from in vivo sensitized leucocytes. The main difference between the two kinds of humoral immune responses stood at the level of the Ig-isotype expression. From unprimed lymph node leucocytes, most secreted antibodies were IgM, while from boosted cells, synthesized antibodies involved both IgM and IgG1. The anti-*Salmonella* IgM appeared to be mainly composed of anti-LPS antibodies. The achievement of such primary antibody responses in vitro could be dependent upon the immunogenic properties of the Gram-negative bacteria used as antigen, but could also be related to the origin of cells put into culture. Thymus-dependent areas of lymph node contain interdigitating-dendritic cells considered to be the real effective APC, the only cells able to stimulate resting T-lymphocytes and to induce a primary immune response, in comparison with other kinds of APC such as macrophages or B-cells (Bujdoso et al., 1990).

This report describes for the first time the induction of an in vitro antibody synthesis specific for *Salmonella*, from either in vivo sensitized or unprimed lymph node cells. The assay, involving an in vitro antigenic stimulation of sheep leucocytes and an antibody detection using both ELISASPOT and ELISA tests, allows detailed studies on the ovine humoral immune response. Furthermore, characteristics of the immune reaction developed from in vivo primed leucocytes were identical to those observed in vivo. This suggests that these reported culture conditions might allow partial reproduction in vitro of some of the physiological mechanisms occurring in vivo. Such in vitro assays will give rise to more detailed studies on the interactions between *Salmonella* and lymph node APC and on the resulting immune responses, in relation to the expression of the host genetic resistance to the disease. This would lead to a better understanding of mechanisms controlling the bacterial dissemination through the lymphoid tissue.

## Acknowledgements

Our thanks are due to Dr. M. Pépin for performing the immunization of the adult sheep and to Drs. S. Bernard and L.T. Tô for their help in the computer treatment of ELISA data. We would like to acknowledge the gifts of monoclonal antibodies from Dr. K.J. Beh (CSIRO, Glebe) and of the *Brucella abortus* 19 bacterial antigen from Dr. G. Dubray (INRA, Nouzilly). We also wish to thank Dr. F. Lantier for his relevant comments and his interest in this manuscript.

## References

Beh, K.J., 1987. Production and characterization of monoclonal antibodies specific for sheep IgG subclasses IgG1 or IgG2. Vet. Immunol. Immunopathol., 14: 187–196.

Beh, K.J., 1988. Monoclonal antibodies against sheep immunoglobulin light chain, IgM and IgA. Vet. Immunol. Immunopathol., 18: 19–27.

Bernard, S., Lantier, I., Laude, H. and Aynaud, J.M., 1986. Detection of transmissible gastroenteritis coronavirus antigens by a sandwich ELISA technique. Am. J. Vet. Res., 47: 2441–2444.

Berthon, P., Bernard, S., Salmon, H. and Binns, R.M., 1990. Kinetics of the in vitro antibody response to transmissible gastroenteritis (TGE) virus from pig mesenteric lymph node cells, using the ELISASPOT and ELISA tests. J. Immunol. Methods, 131: 173–182.

Bianchi, A.T.J., Scholten, J.W., Jongenelen, I.M.C.A. and Koch, G., 1990. The use of monoclonal antibodies in an enzyme-immunospot assay to detect isotype-specific antibody-secreting cells in pigs and chickens. Vet. Immunol. Immunopathol., 24: 125–134.

Borthwick, N., Mackay, D. and Mackenzie, N., 1988. A rapid enzyme-linked assay for the enumeration of specific antibody forming-cells in blood. Poster abstract. Proceedings of the Joint Meeting of British and French Societies of Immunology, 7–9 April 1988, Cité des Sciences et de l'Industrie, La Villette, Paris. 205 pp. (unpublished).

Brown, A. and Hormaeche, C.E., 1989. The antibody response to salmonellae in mice and humans studied by immunoblots and ELISA. Microb. Pathog., 6: 445–454.

Buchmeier, N.A. and Heffron, F., 1989. Intracellular survival of wild-type *Salmonella typhimurium* and macrophage-sensitive mutants in diverse populations of macrophages. Infect. Immunity, 57: 1–7.

Buchmeier, N.A. and Heffron, F., 1991. Inhibition of macrophage phagosome–lysosome fusion by *Salmonella typhimurium*. Infect. Immunity, 59: 2232–2238.

Bujdoso, R., Harkiss, G., Hopkins, J. and McConnell, I., 1990. Afferent lymph dendritic cells: a model for antigen capture and presentation in vivo. Int. Rev. Immunol., 6: 177–186.

Diaz, R. and Bosseray, N., 1974. Estudio de las relaciones antigenicas entre *Yersinia enterocolitica* serotipo 9 y otras especies bacterianas Gram-negativas. Microbiol. Esp., 27: 1–14.

Finlay, B.B. and Falkow, S., 1989. *Salmonella* as an intracellular parasite. Mol. Microbiol., 3: 1833–1841.

Fontaine, J.J., Pépin, M., Pardon, P., Marly, J. and Parodi, A.L., 1994. Comparative histopathology in lambs infected with virulent and attenuated strains of *Salmonella abortusovis*. Vet. Microbiol., in press.

Guilloteau, L., Buzoni-Gatel, D., Blaise, F., Bernard, F. and Pépin, M., 1991. Phenotypic analysis of splenic lymphocytes and immunohistochemical study of hepatic granulomas after a murine infection with *Salmonella abortusovis*. Immunology, 74: 630–637.

Hopkins, J., McConnell, I., Bujdoso, R. and Munro, A.J., 1985. Studies of MHC class II products on sheep peripheral and afferent lymph cells. In: B. Morris and M. Miyasaka (Editors), Immunology of The Sheep. Roche, Basle, pp. 441–459.

Hormaeche, C.E., 1979a. Natural resistance to *Salmonella typhimurium* in different inbred mouse strains. Immunology, 37: 311–318.

Hormaeche, C.E., 1979b. Genetics of natural resistance to salmonellae in mice. Immunology, 37: 319–327.

Hormaeche, C.E. and Maskell, D.J., 1989. Influence of the *Ity* gene on *Salmonella* infections. Res. Immunol., 140: 791–793.

Kantele, A., 1990. Antibody-secreting cells in the evaluation of the immunogenicity of an oral vaccine. Vaccine, 8: 321–326.

Kantele, A. and Mäkelä, P.H., 1991. Different profiles of the human immune response to pri-

mary and secondary immunization with an oral *Salmonella typhi* Ty21a vaccine. Vaccine, 9: 423–427.

Kantele, A., Arvilommi, H., Kantele, J.M., Rintala, L. and Mäkelä, P.H., 1991. Comparison of the human immune response to live oral, killed oral or killed parenteral *Salmonella typhi* TY21A vaccines. Microb. Pathogen., 10: 117–126.

Lantier, F., 1987. Kinetics of experimental *Salmonella abortusovis* infection in ewes. Ann. Rech. Vet., 18: 393–396.

Lantier, F., Pardon, P. and Marly, J., 1981. Vaccinal properties of *Salmonella abortusovis* mutants for streptomycin: screening with a murine model. Infect. Immunity, 34: 492–497.

Lantier, F., Pardon, P. and Marly, J., 1983. Immunogenicity of a low-virulence vaccinal strain against *Salmonella abortus-ovis* infection in mice. Infect. Immunity, 40: 601–607.

Lascelles, A.K., Beh, K.J., Mukkur, T.K.S. and Willis, G., 1988. Immune response of sheep to oral and subcutaneous administration of live aromatic-dependent mutant of *Salmonella typhimurium* (SL 1479). Vet. Immunol. Immunopathol., 18: 259–267.

Le Minor, L., 1984. Genus III — *Salmonella* Lignières 1900, 389$^{AL}$. In: N.R. Krieg and J.G. Holt (Editors), Bergey's Manual of Systematic Bacteriology. 1. Williams & Wilkins, Baltimore, pp. 427–458.

Levieux, D., 1990. Immunoglobulines et transmission de l'immunité passive chez les ruminants. In: P.P. Pastoret, A. Govaerts and H. Bazin (Editors), Immunologie Animale. Flammarion Médecine-Sciences, Paris, pp. 596–599.

Logtenberg, T., Jonker, M., Kroon, A., Gmelig-Meyling, F.H.J. and Ballieux, R.E., 1985. Enumeration of (auto)antibody-producing cells in human using the spot-ELISA. Immunol. Lett., 9: 343–347.

Maskell, D.J., Hormaeche, C.E., Harrington, K.A., Joysey, H.S. and Lien, F.Y., 1987. The initial suppression of bacterial growth in a salmonella infection is mediated by a localized rather than a systemic response. Microb. Pathogen., 2: 295–305.

Miyasaka, M. and Trnka, Z., 1985. Sheep as an experimental model for immunology: immunological techniques in vitro and in vivo. In: I. Lefkovits and B. Pernis (Editors), Immunological Methods, III. Academic Press, Orlando, pp. 403–423.

O'Brien, A.D., 1986. Influence of host genes on resistance of inbred mice to lethal infection with *Salmonella typhimurium*. Curr. Top. Microbiol. Immunol., 124: 37–48.

Oswald, I.P., Lantier, F., Moutier, R., Bertrand, M.F. and Skamene, E., 1992. Intraperitoneal infection with *Salmonella abortusovis* is partially controlled by a gene closely linked with the *Ity* gene. Clin. Exp. Immunol., 87: 373–378.

Pardon, P., 1977. Resistance against a subcutaneous *Brucella* challenge of mice immunized with living or dead *Brucella* or by transfer of immune serum. Ann. Immunol. (Inst. Pasteur), 128C: 1025–1037.

Pardon, P. and Marly, J., 1979. Experimental *Salmonella abortus ovis* infection of normal or primo-infected CD1 mice. Ann. Microbiol. (Inst. Pasteur), 130B: 21–28.

Pardon, P., Marly, J., Lantier, F. and Sanchis, R., 1990a. Vaccinal properties of *Salmonella abortusovis* mutants for streptomycin: screening with an ovine model. Ann. Rech. Vet., 21: 57–67.

Pardon, P., Sanchis, R., Marly, J., Lantier, F., Guilloteau, L., Buzoni-Gatel, D., Oswald, I.P., Pépin, M., Kaeffer, B., Berthon, P. and Popoff, M.Y., 1990b. Experimental ovine salmonellosis (*Salmonella abortusovis*): pathogenesis and vaccination. Res. Microbiol., 141: 945–953.

Plant, J. and Glynn, A.A., 1976. Genetics of resistance to infection with *Salmonella typhimurium* in mice. J. Infect. Dis., 133: 72–78.

Plommet, M. and Plommet, A.M., 1975. Vaccination against bovine brucellosis with a low dose of strain 19 administered by the conjunctival route. I. Protection demonstrated in guinea pigs. Ann. Rech. Vet., 6: 345–356.

Plommet, M. and Plommet, A.M., 1976. Vaccination against bovine brucellosis with a low dose of strain 19 administered by the conjunctival route. II. Determination of the minimum dose leading to colonization of the regional lymph nodes of cattle. Ann. Rech. Vet., 7: 1–8.

Plommet, M. and Plommet, A.M., 1987. Anti-*Brucella* cell-mediated immunity in mice vaccinated with a cell-wall fraction. Ann. Rech. Vet., 18: 429–437.

Plommet, M. and Plommet, A.M., 1989. Immunity to *Brucella abortus* induced in mice by popliteal lymph node restricted strain 19 vaccination. Ann. Rech. Vet., 20: 73–82.

Ruttkowski, E. and Nixdorff, K., 1980. Qualitative and quantitative changes in the antibody-producing cell response to lipopolysaccharide induced after incorporation of the antigen into bacterial membrane phospholipid vesicles. J. Immunol., 124: 2548–2551.

Sedgwick, J.D. and Holt, P.G., 1983. A solid-phase immunoenzymatic technique for the enumeration of specific antibody-secreting cells. J. Immunol. Methods, 57: 301–309.

Sedgwick, J.D. and Holt, P.G., 1986. The ELISA-plaque assay for the detection and enumeration of antibody-secreting cells. J. Immunol. Methods, 87: 37–44.

Williams, J.E. and Whittemore, A.D., 1973. Avian *Salmonella*-stained microtest antigens produced on solid media. Appl. Microbiol., 26: 1–3.