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EXPERIMENTAL OVINE SALMONELLOSIS (*SALMONELLA* ABORTUSOVIS): PATHOGENESIS AND VACCINATION

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Summary.

Salmonella enterica subsp. *enterica* ser. Abortusovis, a sheep-adapted serotype, causes a contagious disease. Abortion is the major symptom and the main source of contamination. Research on this ovine disease may aid farmers, but may also contribute to comparative biological knowledge. Innate resistance partly controlled by the *Ity* locus, increased resistance to reinfection and humoral and T-cell-mediated immunity were observations gained with a murine model. In ewes, abortion regularly occurs following subcutaneous challenge carried out from the third month of gestation onwards. This ovine model was used to evaluate prevention methods for *Salmonella* Abortusovis infection. One subcutaneous injection of a live attenuated lyophilized vaccine containing a selected streptomycin-independent reverse mutant was shown to protect ewes against abortion and excretion of *Salmonella* Abortusovis. This vaccine could be administered simultaneously with other commercial live vaccines such as *Brucella melitensis* Rev.1 vaccine. In sheep, application of the vaccine to the conjunctiva (an easy, individual and hygienic route of mucosal vaccination) was followed by lymph node bacterial colonization and a serological response without local or general clinical reactions. The early events of natural infection remain to be explored, as do the mechanisms underlying the host specificity of *Salmonella* Abortusovis.

KEY-WORDS: *Salmonella* Abortusovis, Vaccine; Model, Sheep, Abortion, Pathogenesis.

Introduction.

Salmonella enterica subsp. *enterica* ser. Abortusovis, a sheep-adapted serotype, causes a contagious disease in sheep. Abortion is the major symptom and the main

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source of contamination. *S.* Abortusovis infection is one of the main causes of sheep abortion in France and has been isolated in many other countries, but never from humans. Bacteraemia leads to bacterial colonization of the foetoplacental unit, the principal site of *Salmonella* multiplication. Up to 60 % of pregnant ewes in a flock may abort (Jack, 1971). A massive peripartum vaginal excretion occurs with abortion, or sometimes with at-term lambing of a live lamb. Faecal excretion generally remains undetectable. Collective and repeated antibiotic treatment during an outbreak is expensive and sometimes disappointing. Prevention with antibiotics should be discouraged. In intensive farming, immunization is a cost-effective method of prevention against this disease (Pardon *et al.*, 1988). It was felt that a report of available information obtained on this particular salmonellosis would complement our knowledge in terms of comparative pathology and vaccinology.

Models of infection.

Methods for comparing vaccines and vaccinations are to be found in a natural host or in a relevant model (fig. 1). Kinetic studies after intravenous or subcutaneous challenge demonstrated the invasion and multiplication potentiality of *Salmonella* Abortusovis in normal CD1 mice (Pardon and Marly, 1979). Moreover, the spleens of OF1 mice can be colonized after oral administration of a high dose of virulent *Salmonella* Abortusovis (Lantier *et al.*, 1983).

In sheep, contamination by the oral (Jack, 1968), intragastric (Pardon *et al.*, 1983) or conjunctival (Jack, 1968) route was shown not to regularly reproduce infection leading to abortion, even with high challenge doses. The conjunctival route is one of the possible mucosal routes for natural contamination. It is used in other experimental ovine infections such as brucellosis (Fensterbank *et al.*, 1982). We reinvestigated conjunctival contamination with *Salmonella* Abortusovis in pregnant ewes.

Conjunctival contamination at mid-gestation with 1×10^{10} viable *Salmonella* Abortusovis strain 15/5 regularly induces a serological response; in 67 % (12/18) of pregnant ewes, this contamination leads to foetoplacental colonization and abortion or still-birth. This protocol does not enable us to consistently reproduce foetoplacental colonization (Sanchis *et al.*, submitted for publication).

A parenteral challenge does not stimulate early events in natural infection, which probably take place at the mucosal level. But abortion can be regularly reproduced by subcutaneous injection of *Salmonella* Abortusovis 15/5 from the third month of gestation (Pardon *et al.*, 1983; Sanchis and Pardon, 1984a). After bacteremia of short duration, a maximum of systemic colonization was observed about one week after inoculation (Lantier, 1987). This model was used to evaluate the means of diagnosis and prevention of *Salmonella* Abortusovis infection (Pardon *et al.*, 1980; Sanchis and Pardon, 1981).

Immunity.

Innate immunity against *Salmonella* Abortusovis has been documented in mice. The multiplication of *Salmonella* Abortusovis in spleen, liver and the peritoneal cavity of mice is controlled by the genetic background of the mouse, in a similar way to the control of resistance to *Salmonella* Typhimurium by the the locus *Ity* of mouse chromosome 1. In sheep, a large inter- and intra-breed variability was observed in rams inoculated subcutaneously. This variability is compatible with the hypothesis of a genetic control of sheep resistance to *Salmonella* Abortusovis infection. These observations might be of economic importance in the control of salmonellosis in domestic animals (Lantier *et al.*, 1988).

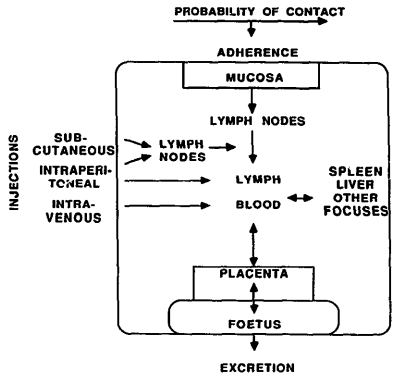

FIG. 1. — *Representation of major components of the conceptual model for* Salmonella *Abortusovis natural or experimental infection.*

A vaccination should reduce the probability of bacteraemic phases leading to placental colonization. (Adapted from Pardon and Marly, 1979; Lantier, 1987; Pardon *et al.*, 1988).

The potential use of non-specific immunity to protect animals against salmonellosis was explored by stimulation with a *Listeria monocytogenes* hypovirulent strain (Fensterbank, 1986) and challenge with *Salmonella* Typhimurium (Kaeffer *et al.*, 1989). Increased resistance is obtained from day 3 after intravenous *Listeria* injection to day 5 or 9 according to subcutaneous or intravenous challenge, respectively. Such acquired non-specific resistance to infection would not appear to completely remove the risks of invasion and multiplication of *Salmonella,* but can slow down infection and decrease pathologic manigestations without impeding the development of a specific immunity. In the best case, the stimulation of non-specific resistance seems to be limited to prevention of expected pathological risks over a relatively short period of time.

Vaccination is advisable in endemic areas. Three observations indicated existence of a serviceable immunity (Pardon *et al.*, 1988): 1) ewes infected with *Salmonella* Abortusovis usually abort once (Jack, 1971); 2) the cyclical evolution of abortive episodes inside a flock (Pardon *et al.*, 1979) or a sheep-rearing region (Sanchis, 1982) suggests the existence of group immunity; 3) primo-infected ewes exhibit an enhanced resistance to reinfection (Iliev, 1971).

Primo-infected mice manifest an increased capacity to withstand dissemination of the challenge bacteria from the inoculation site; these mice control the bacterial population in the draining lymph node and in the spleen (Pardon and Marly, 1979). The transfer to recipient mice of immune sera from primo-infected mice also reduces the level of splenic infection after a subcutaneous challenge (Lantier *et al.*, 1983). Adoptive transfer of splenic cells is under investigation using selective depletion of

T-cell subsets with monoclonal antibodies; some subsets of T cells seem to play a role in acquired immunity against *Salmonella* Abortusovis murine infection (unpublished results). More research is needed to set up an *in vitro* test of the immune status of the host or of the potential of an antigenic preparation.

Killed adjuvanted vaccines are easy to produce and have long been marketed. Usually two or three successive injections are recommended (Tadjebakche and Nadalian, 1980; Nicolas *et al.*, 1981b). Considering the low practicability of such vaccination, some authors were tempted to immunize non-pregnant ewe-lambs using a fully virulent strain (Nicolas *et al.*, 1981a). *Salmonella* Abortusovis being non-pathogenic for other animal species and human in natural conditions, and the infection in non-pregnant ewes being subclinical, the potential risk of an infection with a wild strain is low, but the maintenance or spreading of the infection in a flock or in a region cannot be excluded. Live attenuated vaccines may help control disease without constraints hardly compatible with sheep husbandry.

A live attenuated strain for parental vaccination.

1) *Selection of mutant strains.*

Several streptomycin-dependent mutants were designed to immunize human or animals against some infections due to enterobacteria (Anonymous, 1972; Pardon *et al.*, 1988). Our search for a live vaccine was undertaken in 1977 by analogy with the *Brucella melitensis* strain Rev.1 vaccine (Elberg, 1981; Pardon *et al.*, 1980). The vaccinal Rev.1 strain is a non-dependent reverse mutant selected from a streptomycin-dependent strain of *B. melitensis* (Elberg and Herzberg, 1953). This stable live vaccine provides strong immunity against brucellosis under farm conditions of many countries and seemed the best available *Brucella* valence for a polyvalent vaccine against sheep abortions.

Available genetic and physiologic knowledge of dependent and reverse mutations for streptomycin (Hancock, 1981) indicates that phenotypic variability observed between mutants of the same type corresponds to several possible sites of mutation. Reverse mutants originate from at least one mutation added to one of those leading to dependent strains from streptomycin-susceptible strains.

The technique of selection was adapted from that of Reitman and Iverson (1953). Clones were first screened according to bacteriological criteria: *in vitro* growth, morphology of colonies, serotyping. All mutants were of low *in vitro* growth capacity compared with the parental strain. Eight streptomycin-dependent and six reverse mutants were selected. Seven of the eight reverse mutants returned to the streptomycin-sensible phenotype (Lantier *et al.*, 1981). Screening of the vaccinal strain among these 14 mutant strains was done *in vivo* in mice and in sheep.

2) *Biologic screening.*

The subcutaneous route of vaccination was chosen with a view toward a polyvalent vaccine (Plommet *et al.*, 1987) including the *B. melitensis* strain Rev.1 vaccine, which is, at present, injected subcutaneously (Elberg, 1981). This route is of common use for vaccination of ruminants.

Virulence and immunogenicity studies in a murine model (Pardon and Marly, 1979) led to exclusion of 9 out of the 14 mutant strains (Lantier *et al.*, 1981). All mutant strains were hypovirulent. In view of such virulence attenuation, it was feared that residual virulence could be too low in view of the pursued objective: to render a vaccine efficient with one injection during a reproductive period, if not during the eco-

nomic life of a ewe. Mutant strains with longer *in vivo* persistence and with a higher level of biological reactions to injection were selected.

Further screening for virulence and immunogenicity using the murine model partly determined the choice of the reverse strain Rv6 for subsequent studies. This low-virulent and streptomycin-sensitive strain was stable *in vitro* and *in vivo*, immunogenic for two outbred strains of mice with different susceptibilities to *Salmonella* infection (Lantier *et al.*, 1983), and could be distinguished from wild strains (unpublished results). The plasmid found in wild *Salmonella* Abortusovis strains (Popoff *et al.*, 1984) is also found in Rv6 strain (unpublished results).

As no relationship was established between virulence and immunogenicity of mutant strains measured in the murine or ovine model, the choice of the vaccinal strain was made at the end of successive experiments in sheep, the natural host (Pardon *et al.*, 1983; Sanchis and Pardon, 1984a). The first experiments led to retain only two strains, one per category of mutants. Absence of bacteraemia detectable during the days following intravenous inoculation of the mutant strains was a direct indication of their low virulence, bacteraemia being a necessary pathogenic step towards abortion and excretion. A vaccination-challenge test in gravid ewes led to the choice of the strain Rv6 according to its immunogenicity and protective activity. In addition to the measure of the indirect effects of infection such as fever, local reaction and serological response, two criteria essential to test a vaccine against a contagious abortive disease were taken into account: the clinical issue of gestation and bacterial excretion. More analytical parameters measured before challenge could give information allowing replacement of *in vivo* challenge. The low persistence of virulent bacteria in tissues precludes a comparison based on the localization and intensity of colonization of samples at slaughter and the necropsy of ewes a few weeks after lambing; such parameters are used in experimental sheep brucellosis (Fensterbank *et al.*, 1982).

3) *Safety, efficacy, presentation and conservation.*

Virulence and immunogenicity tests done after serial cultures *in vitro* or serial passages in mice indicated stability of Rv6 bacteria. In mice orally infected by *Salmonella* Typhimurium, Vladoianu and Dubini (1975) concluded that there was stability in the reduction of virulence in dependent strains. Frequency of *in vitro* reversion from dependent strains of *Salmonella* Enteritidis or *Salmonella* Typhimurium serotypes is 10^{-9} to 10^{-10} (Vladoianu and Dubini, 1975); among these reverse mutants, about 1 % presents a reversion to virulence of the wild parental strain. Such a risk of reversion would be very small when a reverse strain is chosen directly as vaccinal strain.

Individual subcutaneous doses from 10^7 to 10^9 Rv6 induced non-significantly different types of protection, but intensity of local reactions was related to injected doses. A subcutaneous dose of 10^8 Rv6 was chosen for further experiments and for commercial vaccine: it was the highest protective dose inducing tolerable local reactions (unpublished results). Post-vaccinal serological titres measured by agglutination remained for at least 4 months above titers indicative of natural infection (Sanchis and Pardon, 1981). In terms of protection of ewes against a subcutaneous virulent challenge, one injection of Rv6 vaccination was favourably compared with two injections of commercial killed vaccines (Sanchis and Pardon, 1981; table I).

Immunogenicity was preserved after lyophilization. Resistance to heat and duration of conservation at 4°C were compatible with conditions of practical use. Viable Rv6 were not detected in organs remote from the injection region, and persisted no longer than one month in the prescapular lymph node draining the injection site (Pardon *et al.*, 1990a). Hypovirulence was also tested by injection in ewes at midgestation. A group of non-injected ewes was placed in contact with them. Fever,

TABLE I. — **One injection of live *Salmonella* Abortusovis strain Rv6 protects ewes at least as much as two injections of killed adjuvanted vaccine (adapted from Sanchis and Pardon, 1981).**

Groups (vaccine)	No. of ewes	Pregnant ewes	Before-term lambings	Infected lambings	Gestation length (mean, days)
No vaccine	10	8	8	8	121
Killed B (*)	10	10	5	5	128
Killed A (*)	10	9	4	4	129
Strain Rv6 (**)	10	7	0	0	146

All ewes were challenged subcutaneously in the right flank by 2×10^9 *Salmonella* Abortusovis strain 15/5.

(*) Two different commercial killed adjuvanted vaccines for subcutaneous injection, used as indicated by the manufacturers; first injection one month before fecundation, second injection 10 days later; injections in the left flank.

(**) Viable Rv6 (2×10^8) injected subcutaneously in the right flank 20 days before fecundation.

local reaction and serological response were observed only in injected animals. Since no vaginal or mammary bacterial excretion was observed, such vaccination created no environmental risks.

Such subcutaneous vaccinal injection was administered to thousands of ewes of different breeds, in several naturally contaminated rearing areas. Innocuity and efficacy were tested in field conditions (Sanchis and Pardon, 1984b; unpublished results). On the whole, the subcutaneous vaccination was judged safe, practical and effective.

Other presentations, uses and routes.

Salmonella Abortusovis, *B. melitensis* and *Chlamydia psittaci* var. *ovis* are the main causes of ovine abortions in ewes in France and in some parts of the Mediterranean. These three contagious bacterial infections may simultaneously affect flocks. The ability to simultaneously use two or three valences in a polyvalent vaccine could greatly reduce the cost of medical prophylaxis and the incidence of these bacterial infections. Polyvalent vaccines against ovine abortions are frequently made by mixing dead bacteria in adjuvant. Mixing live vaccines or simultaneous vaccination with live vaccines could also be considered, as shown by experiments in mice and in sheep (Plommet *et al.*, 1987; Souriau *et al.*, 1988; Pardon *et al.*, 1990b).

Cross-immunity between *Salmonella* serotypes was explored. Heterologous subcutaneous challenge in mice vaccinated subcutaneously with Rv6 strain indicated a better protection against *Salmonella* Typhimurium than against *Salmonella* Dublin infection (unpublished results). Therefore, parenteral vaccination with Rv6 strain could be used in sheep or in other species against abortion or other syndromes due to some other *Salmonella* serotypes.

A mucosal route of vaccination could have two advantages compared to subcutaneous injection: induction of local immunity and the absence of a needle. But oral challenge (Pardon *et al.*, 1983) or vaccination cannot be standardized easily in ruminants due to the complexity of the stomach. Conjunctival instillation of Rv6 bacteria was attempted based on previous results obtained with *B. melitensis* strain Rev.1 (Fensterbank *et al.*, 1985). A regular serological response of ewes was obtained after instillation of 10^{10} *Salmonella* Abortusovis strain Rv6. In cows, such a dose

and route of vaccination induced no serological response (unpublished results), but parenteral injection did so (Tadjebakche *et al.*, 1973).

Towards better vaccines and vaccination.

Economical constraints of vaccination against sheep disease not shared with humans and other animal species are sharp. Most of the preparation must be done before eventual takeover by an industrial team. But our research was favoured by the fact that *Salmonella* Abortusovis is a sheep-adapted serotype of *Salmonella*. Risks of transmission of the attenuated strain to humans or other animal species were very low, *i.e.* lower than the low risk of transmission of the wild strain. Doses for subcutaneous injection of the living attenuated *Salmonella* Abortusovis strain Rv6 are commercially available (Salmovis; Rhône-Mérieux, France). Other attenuated live vaccines against *Salmonella* Abortusovis infection were obtained by other teams (Pardon *et al.*, 1988; B. Schuster, personal communication). Exhaustive genetic studies on *Salmonella* Abortusovis have not been carried out. Techniques of genetic manipulation should be adapted to this particular serotype.

This vaccination represents progress compared with vaccination using existing killed vaccines. But many improvements should be considered. The duration of effective immunity should be further studied. New vaccinal preparations, diagnostic methods or conditions of use should allow better serological distinction between vaccinated and infected sheep. *Salmonella* Abortusovis infection can be further studied as a model for exploration of new means of control of *Salmonella* infections in ruminants.

Considering the tiny risk of transmission of *Salmonella* Abortusovis from sheep to other animal species and to humans, a live attenuated strain of *Salmonella* Abortusovis could be used as a vector for other immunizing antigens, resulting in another form of polyvalent vaccine. Parenteral injection of such recombinant vaccine could be protective in sheep and perhaps in other species. A mucosal route of administration of a live attenuated *Salmonella* Abortusovis strain could also be effective in sheep. In other species, the unknown mechanism underlying sheep specificity could hinder access of *Salmonella* Abortusovis to the immune system.

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