

Modulation of Immune Response in Lambs

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Summary

Experiments using different types of antigen-adjuvant preparations were conducted in outbred sheep to compare effects of adjuvants on immune responses. Trinitrophenyl-ovalbumin (TNP-ovalbumin) incorporated in a preparation with nonionic block copolymers elicited high antibody titers to both ovalbumin and TNP. Different humoral immune responses were observed when *Pasteurella haemolytica* lipopolysaccharide (LPS) was added to the preparations. Responses to ovalbumin and TNP were reduced when *Pasteurella haemolytica* LPS was added to copolymer L121. The antibody titers to ovalbumin or TNP were not affected by the addition of LPS to the preparation containing copolymer L180.5. Lymphocyte proliferation assays demonstrated high stimulation indices at day 17 to ovalbumin by lymphocytes from lambs receiving preparations containing copolymers without *Pasteurella haemolytica* LPS.

Introduction

Pasteurella haemolytica continues to be a major cause of pneumonia in neonatal and feeder lambs. Neonatal lambs are commonly affected even though the ewe has high levels of antibody against the organism. The problem is apparently related to a failure of transfer of anti-leukotoxin antibodies from the ewe to her lamb(s). The failure of transfer may be associated with the dominant isotype of antibodies present in the ewe, probably IgG2. If a procedure for vaccination of ewes that enhanced levels of leukotoxin antibodies of the IgG1 isotype could be developed, increased levels of antibody could be expected to be transferred to the lamb(s). This expectation is based on the selective transport of IgG1 antibodies into the colostrum of ruminant species.

The development of an effective vaccine requires not only an appropriate antigen, but usually a suitable adjuvant to enhance protective humoral and cell-mediated immune responses (Kensil, 1991). While various materials have been demonstrated to possess potent adjuvant activity, their use in humans and domestic animals has been limited due to significant side effects such as inflammation, granuloma formation, and

induction of arthritis. Freund's type adjuvants are a good example. They possess excellent adjuvant activity, but adverse effects are relatively common. In recent years extensive experimentation has been conducted on the adjuvant activity of a series of nonionic block copolymers (Takayama, 1991). These are surface active agents composed of a hydrophobic polymer of polyoxyethylene flanked by two hydrophobic polymers of polyoxyethylene. Some of these copolymers have been demonstrated to enhance humoral and cell-mediated responses to various antigens and to selectively induce production of different immunoglobulin isotypes in mice (Takayama, 1991).

The objective of this study was to determine the effect of two nonionic block copolymers on the immune response of sheep. The goal was to determine if these materials might be useful in the development of an efficacious vaccine for pasteurellosis.

Materials and Methods

Vaccine preparation

The trinitrophenyl (TNP) hapten was conjugated to ovalbumin, according to a published method by Garvey (1977). The hapten to protein ratio was determined spectrophotometrically using a molar extinction coefficient of 15,400 for TNP at 350 nm. The protein concentration was determined by the Pierce BCA protein assay procedure and the conjugated antigen contained 20 TNP hapten determinants per molecule of TNP-ovalbumin. The *Pasteurella* lipopolysaccharide used in the experiment was extracted by the phenol-water method from washed *P. haemolytica* bacteria. For the preparation of adjuvant-antigen mixtures, TNP-ovalbumin and *P. haemolytica* LPS were lyophilized. An oil-in-water emulsion was prepared by adding 500 µg. of TNP-ovalbumin, 1.0 mg. of purified *Pasteurella haemolytica* lipopolysaccharide if needed, 20 µl. of squalene, 50 µl. of the triblock nonionic copolymer; 930 µl. of a mixture of phosphate buffer solution and 0.2% v/v Tween 80 was added to account for the total final vaccine volume of one ml. The vaccines were used the same day they were made.

Animals and immunization

Twenty-two outbred lambs were used in this study. The animals were randomized into six groups using a statistical program (SAS). Each group was assigned to a particular treatment and pre-immunization bleedings were conducted. All lambs were given one dose of antigen-adjuvant preparation (1.0ml.) subcutaneously divided in each of two sites in the superior and lateral part of the neck, except for the controls. Each treatment group was composed of four lambs; a sixth group of two lambs served as unimmunized controls. Lambs in group 1 received TNP-ovalbumin plus polymer #1 (L121).

Lambs in group 2 received TNP-ovalbumin plus polymer # 1 together with lipopolysaccharide. Lambs in group 3 received TNP-ovalbumin plus polymer #2 (L180.5). Lambs in group 4 received TNP-ovalbumin plus polymer #2 together with lipopolysaccharide. Lambs in group 5 received TNP-ovalbumin in phosphate buffer solution and Tween 80. Lambs in group 6 were left used as unimmunized controls.

Lambs were bled at intervals after immunization for serum to be used in ELISA and for lymphocyte proliferation assays. The whole antibody titers to the antigens were determined with an ELISA-utilizing, peroxidase-labeled anti-sheep Ig and the positive/negative ratio method of analysis (Briggs et al., 1986). The lymphocyte proliferation assays were conducted using a standardized procedure. For determining antibody levels of different isotypes, the same ELISA procedure was used with the exception of using protein G and A horseradish peroxidase conjugate (BioRad, Hercules, CA) instead of anti-sheep immunoglobulin. Protein G binds to IgG1 and IgG2 antibody subclasses while Protein A binds only to molecules of the IgG2 subclass. Antibody titers to total IgGs were calculated using protein G and antibody titers to IgG2 were calculated using protein A.

Results

Sera collected at intervals over a period of approximately five weeks were tested with an ELISA to determine the antibody titer to the hapten (TNP) and to the carrier (ovalbumin). Lambs developed high antibody titers to both ovalbumin and TNP when the antigen was incorporated in preparations containing nonionic block copolymers L121 and L180.5 (Figures 1 and 2). The peak of the antibody response to both ovalbumin and TNP appeared to be at day 16. Antigen administered without the copolymers produced only very low concentrations of antibodies. In fact, there was not a significant difference between the control lambs and the lambs immunized with the antigen in saline. These findings are similar to the results obtained by Hunter and collaborators indicating that these nonionic copolymer surfactants have good adjuvant activity (Hunter, 1984).

Since the combination of some of the copolymers with LPS has been shown to be an effective adjuvant

system (Takayama, 1991), we evaluated the immune responses in lambs to ovalbumin and TNP using the copolymers together with *Pasteurella haemolytica* LPS. The addition of LPS did not influence the responses of lambs to ovalbumin or TNP in lambs given copolymer L180.5. Antibody responses to ovalbumin and TNP were minimal and much lower in lambs given copolymer L121 and LPS than in lambs administered only the copolymer. The anti-hapten antibody response in lambs that were immunized with copolymer L180.5 with or without *Pasteurella* lipopolysaccharide remained stable after 16 days. In contrast, the anti-ovalbumin response decreased after 16 days.

The character of antibodies present in sera on day 23 are shown in Figure 4. The predominant isotype of antibodies to ovalbumin or TNP elicited by immunization with ovalbumin-TNP were of the IgG1 subclass. Lambs given ovalbumin-TNP with copolymer L180.5 had greater levels of IgG2 anti-ovalbumin at day 23 than any other group. It is important to note that groups of lambs receiving copolymer L180.5 also had the highest total anti-ovalbumin antibody titers. The peak of the anti-ovalbumin IgG2 was shown to be around day 23. The anti-hapten IgG2 response was lower than the response to the carrier (data not shown).

The peripheral blood lymphocyte blastogenic response in the form of stimulation index was measured by the uptake of [³H] thymidine by cultured lymphocytes. The overall mean values with their respective standard errors for the different treatment groups are shown in Figure 3. The blastogenic response to ovalbumin was higher in lambs immunized with both preparations that contained the copolymers L121 and L180.5 without LPS. The highest stimulation indices to ovalbumin occurred at day 17 and there was no significant difference between groups of lambs administered preparations containing L121 and L180.5 without LPS. The cell-mediated response for the groups that were injected with copolymers alone was still present at day 23. This data agrees with earlier experiments conducted by Hunter et al. (1984) who reported that nonionic copolymers elicited cell-mediated responses. As expected, the stimulation indices to the hapten (TNP) were not significantly different than the control groups.

Figure 1. Antibody titers to ovalbumin in groups of lambs immunized with TNP-ovalbumin alone or incorporated in a nonionic block copolymer adjuvant with or without *Pasteurella haemolytica* lipopolysaccharide.

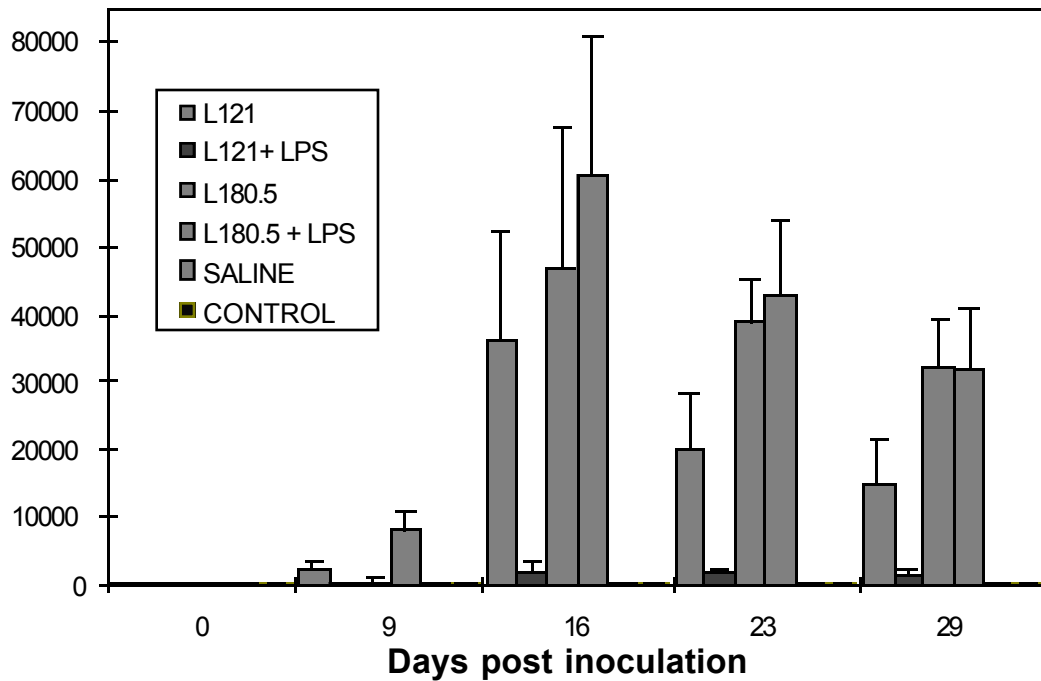


Figure 2. Antibody titers to TNP in groups of lambs immunized with TNP-ovalbumin alone or incorporated in a nonionic block copolymer adjuvant with or without *Pasteurella haemolytica* lipopolysaccharide.

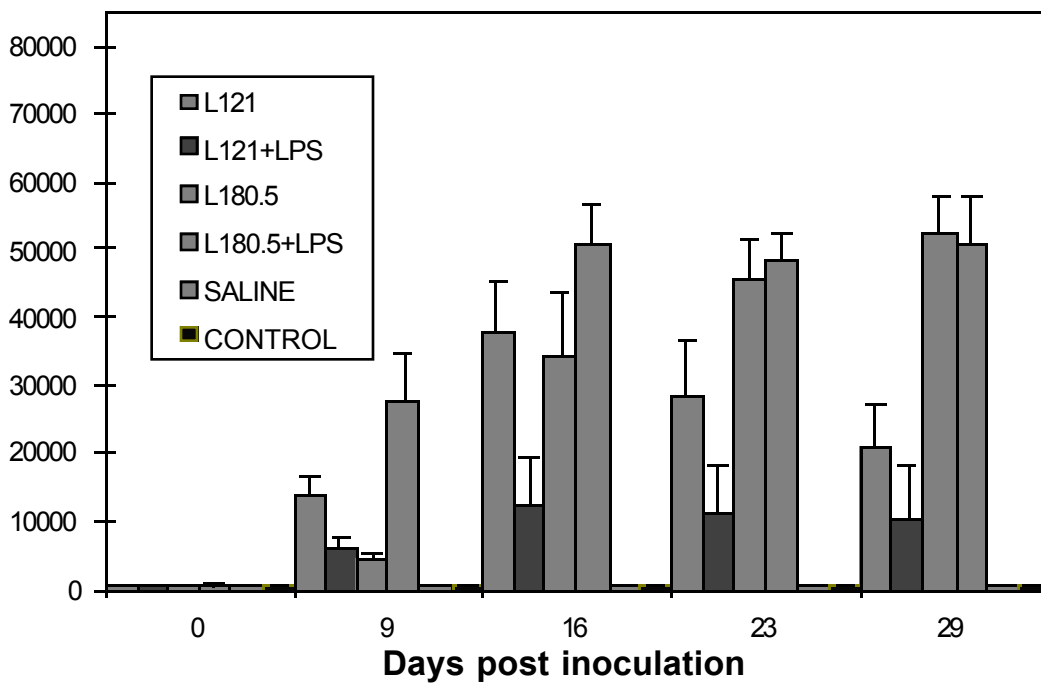


Figure 3. In vitro proliferation of blood lymphocytes from lambs immunized with TNP-ovalbumin and control lambs. Peripheral blood lymphocytes were cultured with 40ug./ml. ovalbumin as the antigen for 72 hours and then labeled with [³H] thymidine and harvested 18 hours later. Each bar represents the mean stimulation index (mean ± S.E.)

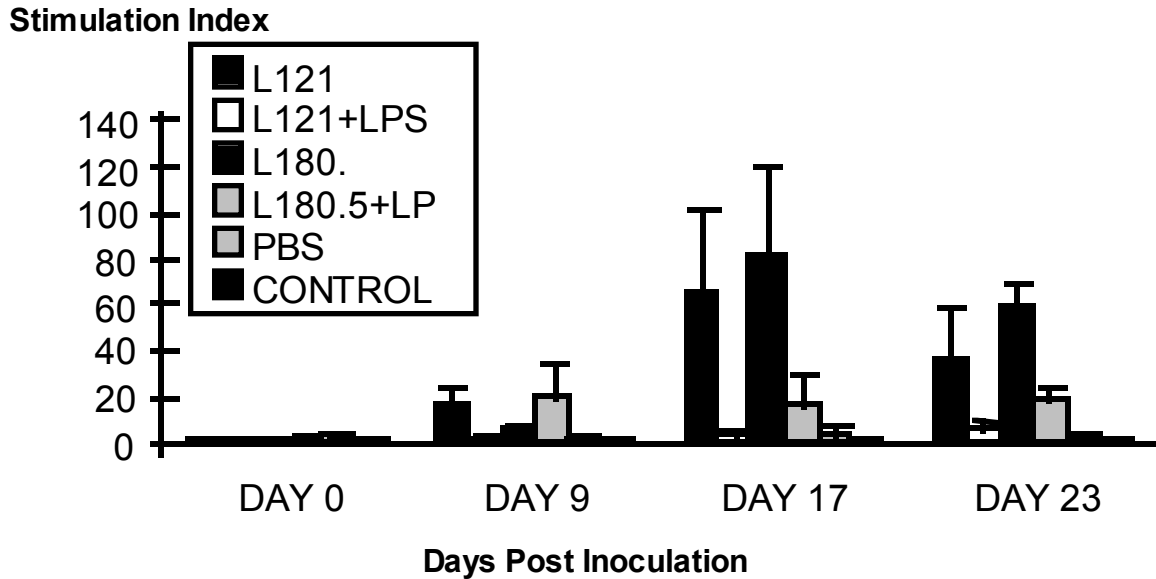
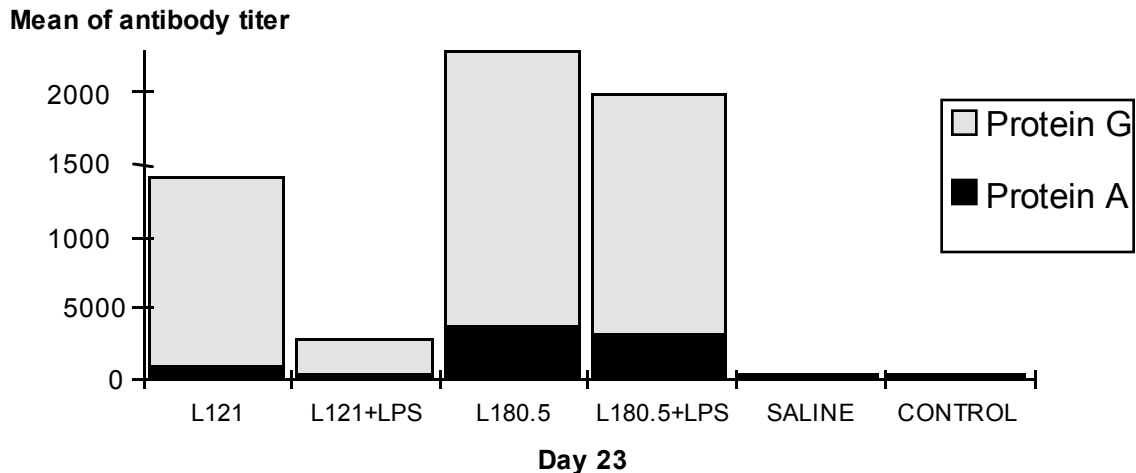


Figure 4. Ovalbumin antibody titers in serum of lambs on day 23 following immunization as determined by reactivity with protein A and G in ELISA. Protein G detects antibodies of both IgG1 and IgG2 isotypes while protein A detects only antibodies of the IgG2 isotype.



Discussion

Our objective for this experimentation was to determine if two nonionic block copolymers

alone or combined with LPS would enhance the immune response of lambs to a hapten-protein conjugated antigen. Both copolymers prepared

with the antigen as an oil-in-water emulsion markedly enhanced humoral and cell mediated responses to the protein and antibody responses to the hapten. The predominant isotype of antibodies produced belonged to the IgG1 isotype. However, the failure of LPS to further enhance the production of antibodies was disappointing since enhanced adjuvanticity had previously been reported (Takayama, et al 1991).

A further objective is to induce high levels of anti-leukotoxin antibodies of the IgG1 isotype to achieve transfer of these antibodies to the newborn lambs. The results of the experimentation indicate that the copolymers do have potential as adjuvants in a vaccine formulation. They markedly enhanced the response to a protein antigen and induced the appropriate type of antibodies. However, any efficacious *Pasteurella* vaccine prepared from the microorganism will probably contain LPS as a component. The failure of LPS to enhance the immune response in combination with L180.5 and apparent suppression when combined with L121 is a major concern. This certainly detracts from the potential for the use of these copolymers in *Pasteurella* vaccines that contain LPS. The application of one of these copolymers in a *Pasteurella* vaccine composed of relatively pure leukotoxin such as recombinant leukotoxin may be a possibility.

Most adult sheep in Iowa have some level of circulating antibodies to *Pasteurella haemolytica*. Attempts to boost the titer of anti-*Pasteurella* antibodies with a variety of vaccine preparations have not been very successful. There is a need to determine if these copolymer adjuvants or other adjuvant materials incorporated in the vaccine would overcome this problem. Experimentation reported by Reynolds et al (1990) indicated that aluminum hydroxide, diethylaminoethyl dextran, and complete Freund's adjuvants enhanced antibody responses of sheep to pure and conjugated protein antigens. They also reported that the IgG1 isotype was the predominant antibody produced. Whether or not these materials would be effective with *Pasteurella* antigens remains to be determined.

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