Vaccination of Calves with Leukotoxic Culture Supernatant from *Pasteurella haemolytica*

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ABSTRACT

In three experiments subcutaneous vaccination of calves with adjuvanted bacteria-free leukotoxic culture supernatant from log phase cultures of *Pasteurella haemolytica* A1 (toxin 1) was shown to induce some protection against intrabronchial challenge with live *P. haemolytica* A1. This toxin 1 vaccine was as effective as a whole cell bacterin in stimulating agglutinating antibody to *P. haemolytica*. Induction of leukotoxin neutralizing activity was variable; in some cases vaccination only primed the animal to produce an anamnestic response after challenge, whereas in other instances antitoxic activity increased in response to immunization. Two doses of vaccine were shown to be more effective than a single immunization. Vaccination with leukotoxic culture supernatant from the nonpathogenic *P. haemolytica* serotype 11 was as effective as vaccination with toxin 1 in stimulating antitoxic activity but was not protective. This implies that both serospecific agglutinating activity and an antitoxic response are needed for immunity.

RÉSUMÉ

À la faveur de trois expériences, la vaccination sous-cutanée de veaux avec le surnageant leucotoxique, c'est-à-dire la toxine #1 de cultures de *Pasteurella haemolytica* A1, en phase de croissance logarithmique, a révélé qu'elle pouvait procurer une certaine protection contre une infection de défi bronchique, avec cette bactérie. Le vaccin précité se révéla aussi efficace qu'une bactérine de cellules entières, pour stimuler la production d'anticorps agglutinants contre *P. haemolytica*. L'induction d'une activité neutralisante de la leucotoxine s'avéra variable: dans certains cas, la vaccination ne fit que préparer l'animal à produire une réaction anamnestique après l'infection de défi, tandis que dans d'autres l'activité antitoxique augmenta à la suite de l'immunisation. Deux doses de vaccin se révélèrent plus efficaces qu'une seule. La vaccination avec le surnageant leucotoxique de cultures du sérotype #11 non pathogène de *P. haemolytica* s'avéra aussi efficace que la vaccination avec la toxine #1, pour stimuler une activité antitoxique; elle n'exerça toutefois aucune influence protectrice. Ceci implique qu'une activité agglutinante sérospécifique et une réponse antitoxique sont toutes les deux nécessaires pour l'obtention d'une immunité.

INTRODUCTION

Pneumonia associated with *Pasteurella haemolytica* is a major cause of economic loss to the feedlot industry (1). Although bacterins incorporating *P. haemolytica* are commercially available and frequently used, their efficacy in preventing respiratory disease is disappointing. Evidence from field trials (2-4) and laboratory experiments (5,6) suggests an adverse effect of vaccination. Animals vaccinated with inactivated whole cell bacterins often show a higher incidence of pneumonia and more severe lung lesions at postmortem than do unvaccinated controls. This occurs despite the induction of serum antibody to *P. haemolytica* surface antigens, measured by bacterial agglutination or passive hemagglutination techniques (5,6). Paradoxically, the occurrence of an analagous antibody response as a result of natural or experimental infection with live bacteria can be correlated with protection (7-9).

During active growth, *P. haemolytica* is known to produce a soluble cytotoxin with specificity for ruminant leukocytes (10-12). This leukotoxin is believed to act as a virulence factor in the production of pneumonia by impairing pulmonary macrophage function and bacterial clearance or by the induction of damage to lung parenchyma through the release of proteolytic enzymes from lysed leukocytes. It has been shown that generation of an anticytotoxic immune response after natural or experimental infection with *P. haemolytica* can be related to protection against disease (13-16).

The failure of commercial bacterins to induce protection may result from the stimulation of an immune response to surface antigens of the bacteria which facilitates bacteria-macrophage contact and thereby enhances bacterial virulence in the absence of an adequate antitoxic response. On the other hand, infection with live bacteria may induce an immune response to leukotoxin as well as surface antigens and therefore be protective. Recently, live *P. haemolytica* vaccines have been shown to induce protection (15-18) and toxin neutralization in vaccinated calves (15). We decided to investigate

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the immune response induced by vaccination with bacteria-free leukotoxic culture supernatant and to compare that to the response generated by a commercial bacterin. In addition we examined the effect of vaccination with culture supernatant from a toxigenic but nonpathogenic serotype, *P. haemolytica* type 11 (eleven). *Pasteurella haemolytica* serotypes are distinguished on the basis of differences in surface antigens (19); however, all serotypes produce leukotoxin which can be cross-neutralized by type-specific antisera (20). We supposed that vaccination with serotype 11 might induce antitoxic activity which would be protective, without inducing surface specific antibody and the possible detrimental consequences.

MATERIALS AND METHODS

CALVES AND EXPERIMENTAL DESIGN

Holstein-Friesian bull calves, ranging in age from three to four months at the start of the experiments, were assigned to groups, vaccinated and challenged as outlined in Table I.

VACCINES

The commercial bacterin (Diamond Laboratories, Des Moines, Iowa) used in experiment I contained formalin-killed *P. multocida* and *P. haemolytica*. It was given in doses of 2 mL injected subcutaneously according to the manufacturer's instructions. Toxin 1 was lyophilized culture supernatant from a 1 h culture of *P. haemolytica* serotype 1, prepared for each experiment as described previously (11) and tested for cytotoxicity. This was resuspended to 10 mg/mL in phosphate buffered saline (PBS, 0.01 M, pH 7.4), filtered (45 μm), emulsified with an equal volume of incomplete Freund's adjuvant (IFA, Difco Laboratories, Detroit, Michigan) and administered by subcutaneous injection at two prescapular sites, 4 mL of vaccine per site. The vaccine designated toxin 11 (eleven) in experiment III was prepared and administered similarly, except that culture supernatant from *P. haemolytica* type 11 was used. All culture supernatant preparations were more than 90% toxic for BL-3 cells (21).

TABLE I. Protocol for Treatment of Calves with Commercial or Experimental *Pasteurella* sp. Vaccines

Experiment	Group	Vaccine[a]	Vaccination Day(s)	Challenge Day
I	1 (n = 8)	Toxin 1	0, 21	42
	2 (n = 8)	Bacterin	0, 21	
	3 (n = 8)	None		
II	4 (n = 8)	Toxin 1	0, 21	43
	5 (n = 8)	Toxin 1	21 only	
	6 (n = 8)	None		
III	7 (n = 3)	Toxin 1	0, 30	51
	8 (n = 3)	Toxin 11	0, 30	
	9 (n = 3)	None		

[a]Vaccines: Toxin 1 = cell-free culture supernatant from *P. haemolytica* type 1
Toxin 11 = cell-free culture supernatant from *P. haemolytica* type 11 (eleven)
Bacterin = formalinized *P. multocida*/*P. haemolytica* bacterin, (Diamond Laboratories, Des Moines, Iowa)

CHALLENGE

A 12 h brain heart infusion broth (BHIB) culture of *P. haemolytica* type 1 was centrifuged at 4000 *g* for 15 min and resuspended in sterile PBS to an optical density of 1.0 at 525 nm. The retrospective viable bacterial counts for each experiment were 2.3 x 10^9 colony forming units (CFU)/mL for experiment I, 3.6 x 10^9 CFU/mL for experiment II and 7.9 x 10^8 CFU/mL for experiment III. Calves were anesthetized by xylazine injection (Rompun, Haver-Lockhart, Mississauga, Ontario) and 25 mL of bacterial suspension, followed by 50 mL of sterile PBS, were delivered to the lung by means of an intratracheal catheter (6).

ASSESSMENT OF RESPONSE TO CHALLENGE

The respiratory rates and rectal temperatures of calves were monitored on a daily basis for three days prior to challenge and five days after. For each parameter, and for each calf, the mean plus two standard deviations of the prechallenge values served as the threshold value for evaluation of a change in that parameter after challenge. In experiments II and III calves were euthanized five days after challenge. The lungs were removed at necropsy and macroscopic lesions were graded according to the scoring system outlined in Table II.

ASSESSMENT OF IMMUNE RESPONSE

The systemic immune response to vaccination and challenge was assessed in serum collected prior to each vaccination, one week before challenge, at challenge, and five days after challenge. The immune response in the lung was determined in bronchoalveolar washing fluids (BAW) obtained by fiberoptic bronchoscopy and lavage (6) on the days outlined above, excluding the day of challenge. The BAW samples were dialyzed against distilled water, lyophilized and resuspended in PBS at a concentration of 30 mg/mL for titration.

The immune response to bacterial surface antigens was evaluated by an indirect (antiglobulin) microagglutination test using washed formalinized

TABLE II. Evaluation of Lung Lesions at Necropsy

Score: 0 — no visible lesions
1 — minimal focal changes in a single lobe
2 — consolidation, abscessation and/or adhesions in single or multiple foci, involving less than 25% of the lung
3 — as 2, involving 25-50% of the lung
4 — as 2, involving 50-75% of the lung
5 — as 2, involving 75% or more of the lung
+ 1 — additional, for pleural effusion or fibrinous adhesion to pleura or pericardium
Maximum score for each lung = 5

P. haemolytica type 1 as the antigen (8,9). In experiment III only, the indirect bacterial agglutination titer was also determined using *P. haemolytica* type 11 as antigen. Titers were expressed as the reciprocal $\log_2$ of the endpoint dilution.

Toxin neutralizing activity was determined in a microplate colorimetric assay as the ability of serial twofold dilutions of serum or BAW to neutralize the toxic effect of *P. haemolytica* type 1 culture supernatant for BL-3 cells, a bovine leukemia-derived B cell line (obtained from G. Theilen, Univ. of California, Davis, California) (21). Toxicity was determined by the inability of dead or lysed cells to incorporate the vital dye neutral red. Cell viability measured as the uptake of neutral red was determined by reading the optical density of each well at 540 nm using an automated spectrophotometer (Titertek Multiscan, Flow Laboratories, Mississauga, Ontario). The titer of each sample was expressed as the highest reciprocal $\log_2$ dilution which yielded at least 50% neutralization of toxicity.

The Student's t-test was used to compared differences between groups of calves.

RESULTS

Table III outlines the response to challenge with live *P. haemolytica* type 1 for each experiment. In experiments I and III all calves survived to the end of the observation period. In experiment II several calves died as a result of challenge: one in group 4 (toxin 2x), three in group 5 (toxin 1x), four in group 6 (controls). These were assigned the maximum clinical scores of five for each parameter during the remainder of the experiment. Lung lesions were evaluated as for other calves.

The immune response detected in sera and BAW is oulined in Figs. 1, 2 and 3. In experiment I toxin 1 (culture supernatant) was found to be as effective as the bacterin in stimulating agglutinating antibody to *P. haemolytica* type 1. Following immunization both groups of vaccinated calves had serum agglutinating titers significantly higher than unvaccinated controls (Fig. 1, days 21, 35, 42; Student's t-test $p < 0.001$). After challenge with live organisms the antibody titer in the control group also increased. Agglutinating titers in lung washing fluids (BAW) did not differ significantly among the groups, except on day 35 (one week prior to challenge) when both groups of vaccinated calves had higher levels than controls ($p < 0.05$, bacterin; $p < 0.001$, toxin 1). After challenge BAW agglutinating titers remained elevated in the toxin 1 group. Neither vaccine stimulated increased toxin neutralizing activity in serum or BAW prior to challenge. However, serum neutralizing activity was significantly higher in the toxin 1 vaccinated animals following challenge ($p < 0.01$).

In experiment II no significant differences were apparent in the agglutinating titer in either sera or BAW prior to challenge. After challenge the group which received two doses of toxin 1 vaccine had significantly higher serum titers than either of the other two groups ($p < 0.05$) and higher titers in BAW than unvaccinated controls ($p < 0.01$). These calves were also the most resistant to challenge. No significant differences among groups were found in the toxin neutralizing activity of sera or BAW, although the titers in vaccinated calves increased following immunization. Unfortunately, the control unvaccinated calves had relatively elevated serum neutralizing titers at the commencement of the trial. As with agglutinating activity, the highest serum neutralizing titers postchallenge were observed in the group which received two doses of toxin 1 vaccine.

In experiment III, the calves vaccinated with toxin 1 responded with increased serum agglutinating titers to *P. haemolytica* serotype 1, and at the time of challenge this group had the highest antitype 1 titers. However, titers in the toxin 11 and unvaccinated groups also increased over the course of the study, particu-

TABLE III. Response of Calves to Intrabronchial Challenge with *Pasteurella haemolytica* type 1

Experiment:	Clinical Score[a]				Lung Lesion Score[b]				Cumulative
	Temperature		Respiration		Left		Right		Score
Vaccine (n)	x + SD	Σ	x + SD	Σ	x + SD	Σ	x + SD	Σ	
Experiment I:									
Toxin 1 (8)	0.8 + 0.9	6	2.1 + 1.6	14	ND		ND		20
Bacterin (8)	1.6 + 2.1	15	2.8 + 1.9	22	ND		ND		37
Control (8)	2.3 + 2.0	15	2.3 + 1.5	18	ND		ND		33
Experiment II:									
Toxin 1, 2x(8)	2.9 + 1.8	23	3.0 + 2.0	24	2.4 + 2.2	19	0.8 + 1.8	6	72
Toxin 1, 1x(8)	4.0 + 1.2	32	4.1 + 1.1	33	3.3 + 2.1	26	1.4 + 1.5	11	102
Control (8)	3.5 + 1.7	28	3.9 + 1.4	31	3.5 + 1.9	28	1.6 + 1.8	13	100
Experiment III:									
Toxin 1 (3)	1.7 + 1.5	5	2.3 + 1.1	7	2.0 + 1.0	6	1.0 + 1.7	3	21
Toxin 11 (3)	2.7 + 2.5	8	4.7 + 0.6	14	1.3 + 2.3	4	2.0 + 2.6	6	32
Control (3)	3.0 + 2.0	9	5.0 + 0	15	1.7 + 2.9	5	3.3 + 1.5	10	39

[a]Clinical score: number of days postchallenge with change greater than prechallenge mean + 2 SD. Maximum score 5 for each calf. Calves dying prior to the end of the observation period were assigned the maximum score for the remainder of the experiment.

[b]Lung lesion score: scored on the basis of severity and distribution from 0 to 5 for each lung, outlined in Table II. ND = not determined.

x = mean, SD = standard deviation, Σ = sum of scores for all calves in the group.

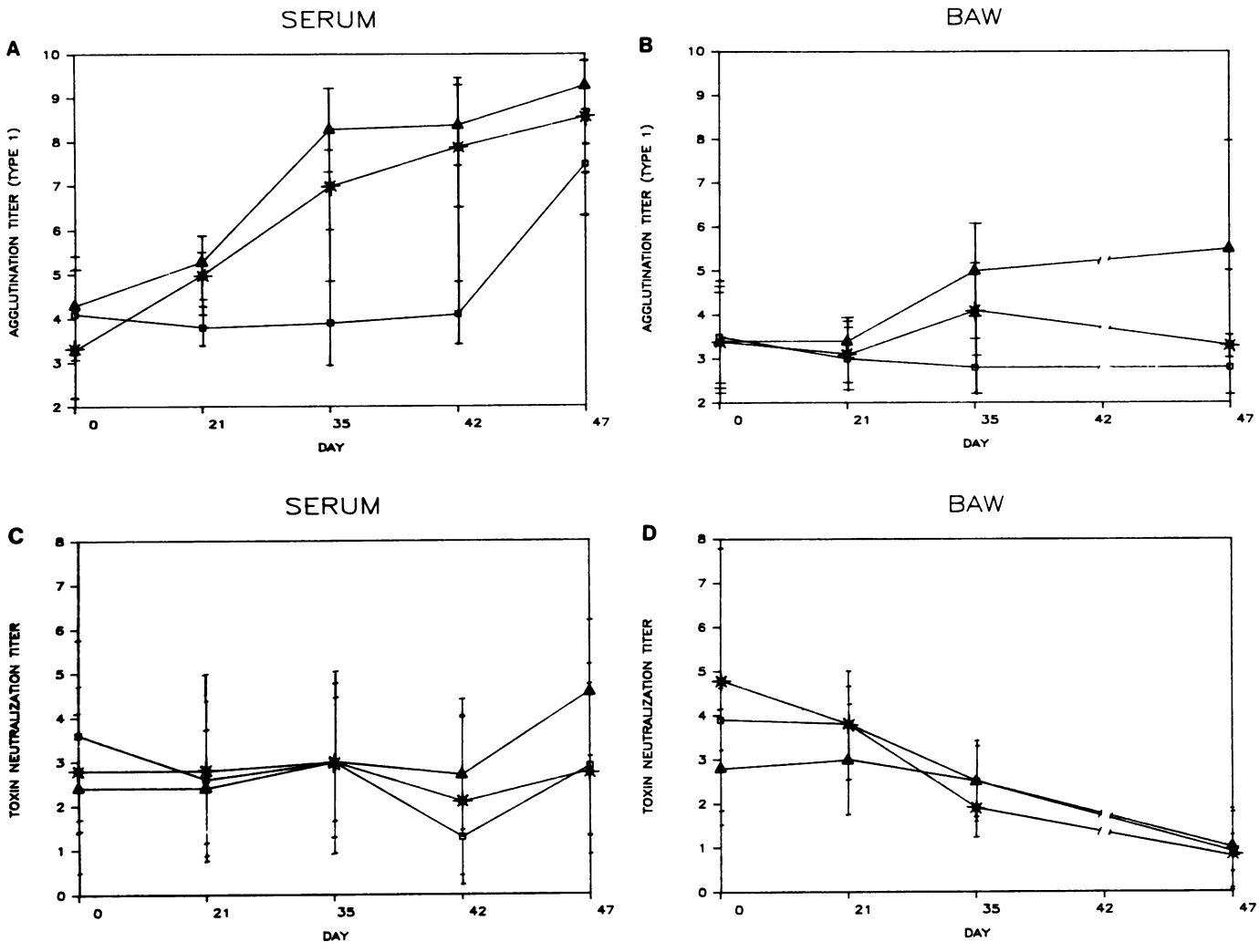

Fig. 1. Immune response of calves in experiment I. Indirect agglutination titer to *P. haemolytica* type 1 ($-\log_2$) in A. serum, B. lung washing fluid (BAW). Toxin neutralizing activity (50% endpoint, $-\log_2$) in C. serum, D. BAW. Points represent the mean value for eight calves, bars indicate standard deviation. Calves were vaccinated on days 0 and 21, challenged on day 42. □ controls, not vaccinated; ✱ bacterin vaccinated; ▲ vaccinated with toxin 1 (culture supernatant from *P. haemolytica* type 1). No BAW sample on day 42.

larly in the week prior to challenge and at no point were the differences in titers among the groups significant. Similarly type 1 agglutinating antibody in BAW increased in all groups of calves such that one week before challenge titers were essentially equal. Serum agglutinating titers to *P. haemolytica* type 11 changed minimally (one dilution) in calves receiving toxin 1 vaccine and in nonvaccinates, but increased in animals vaccinated with type 11 toxin to become significantly higher than controls on days 44 and 51 ($p < 0.01$). A slight increase was also seen in the agglutinating activity in BAW of type 11 vaccinates but the change was not remarkable. After challenge with live *P. haemolytica* type 1, agglutinating titers in all groups of calves were comparable against either type of *P. haemolytica*. Unfortunately toxin 1 calves had higher neutralizing activity in serum at initiation of the trial ($p < 0.05$ versus controls). Vaccination induced an increase in the neutralizing activity in both groups of vaccinated calves such that there was no significant difference in these groups by day 30 following the first vaccination or at challenge, and both groups had higher activity than nonvaccinates ($p < 0.05$ day 30; $p < 0.001$ day 51). Neutralizing activity in BAW did not differ significantly among groups although it was slightly higher in vaccinates.

DISCUSSION

In all experiments vaccination with toxin 1 provided some protection against experimental challenge with *P. haemolytica* A1. This vaccine induced serum agglutinating antibody to *P. haemolytica* type 1, comparable to that induced by a whole cell bacterin, indicating that the cell-free culture supernatant contains soluble surface antigens of the bacterium as well as leukotoxin. That these surface antigens have some serotype specific activity was shown by the finding that vaccination with toxin 1 was most effective in stimulating agglutinating antibody to type 1 and that only toxin 11 induced an increase in antitype 11 agglutinating activity in serum.

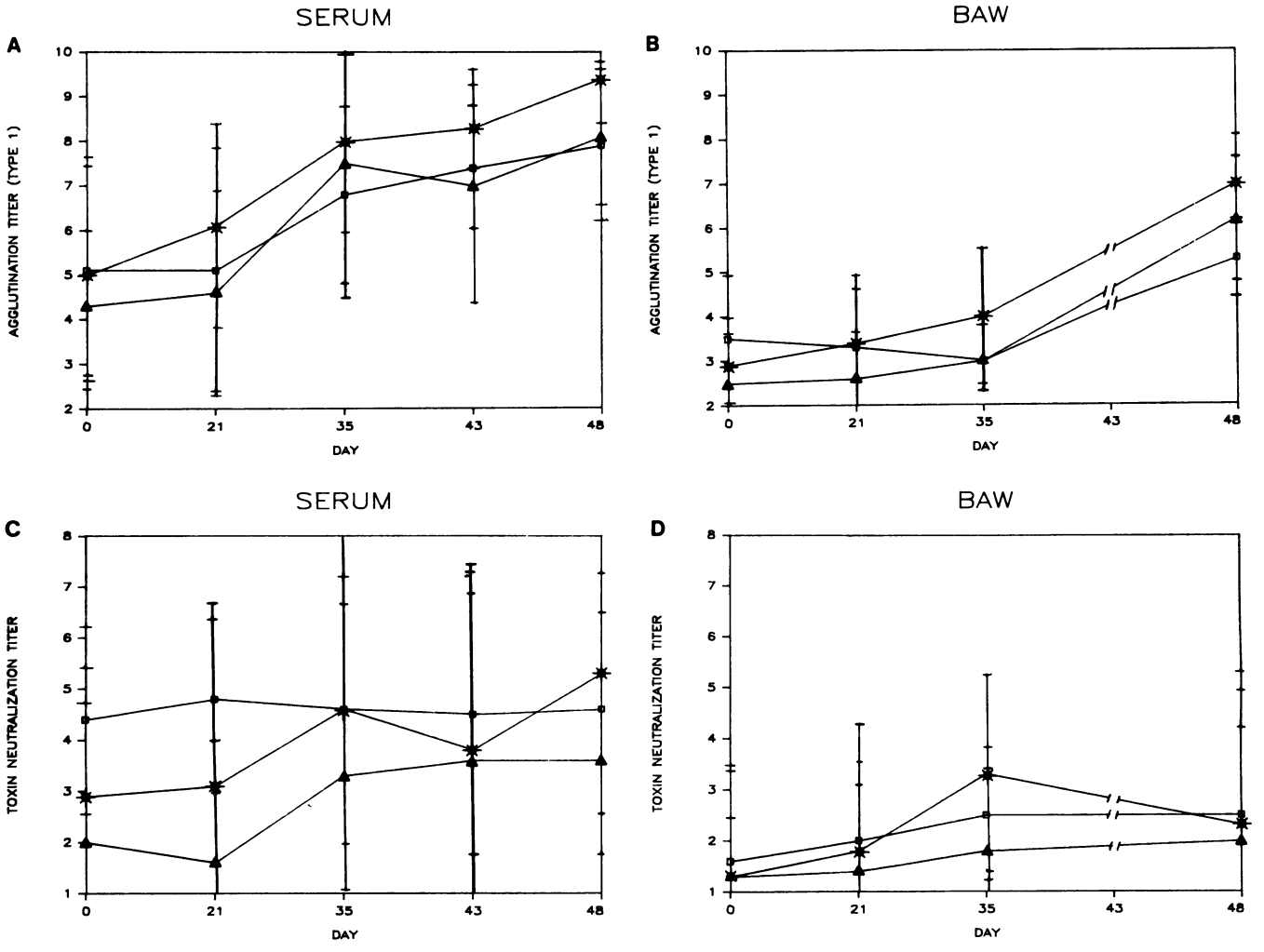

Fig. 2. Immune response of calves in experiment II. Indirect agglutination titer to *P. haemolytica* type 1 ($-\log_2$) in A. serum, B. lung washing fluid (BAW). Toxin neutralizing activity (50% endpoint, $-\log_2$) in C. serum, D. BAW. Points represent the mean value for eight calves, bars indicate standard deviation. Calves were challenged on day 43. □ controls, not vaccinated; ✱ vaccinated, toxin 1 (culture supernantant from *P. haemolytica* type 1) on days 0, 21; ▲ vaccinated, toxin 1 on day 21. No BAW sample on day 43.

During experiments II and III the serum agglutinating titer to *P. haemolytica* type 1 increased gradually in nonvaccinated controls. Since these were conventional calves housed in separate pens but within rooms which shared ventilation, this could reflect natural exposure to endogenous bacteria. Unfortunately, while this may mimic real life conditions it complicated interpretation of elevated agglutinating titers in vaccinated calves. Therefore, no attempt has been made to correlate the prechallenge agglutinating titer per se with resistance to development of pneumonia.

Induction of toxin neutralizing activity by vaccination with toxin 1 was variable. In the first experiment serum neutralizing activity developed anamnestically after challenge in toxin 1 vaccinated calves. In the second experiment immunization induced some increased activity prior to challenge, particularly in calves receiving two doses of vaccine. In experiment III both types of toxin vaccine (toxin 1 and toxin 11) were equally effective in stimulating antitoxic activity prior to challenge. In spite of this, however, vaccination with type 1 toxin was most protective, indicating that toxin neutralization by itself is insufficient and suggesting the concomitant need for serotype related antigens in immunity. This finding is in agreement with the work of other investigators who have shown that vaccination with capsular extracts of *P. haemolytica* type 1 may induce some protection against experimental aerosol (22) or intrathoracic challenge (23). We propose that effective immunity to pneumonic pasteurellosis requires a response to both leukotoxin and surface antigens of the bacterium.

In view of the reported adverse effects of vaccination with killed bacterins (2-6) and the production and storage problems associated with the use of live vaccines, we feel that the use of bacteria-free leukotoxic culture supernatant as a vaccine merits further investigation.

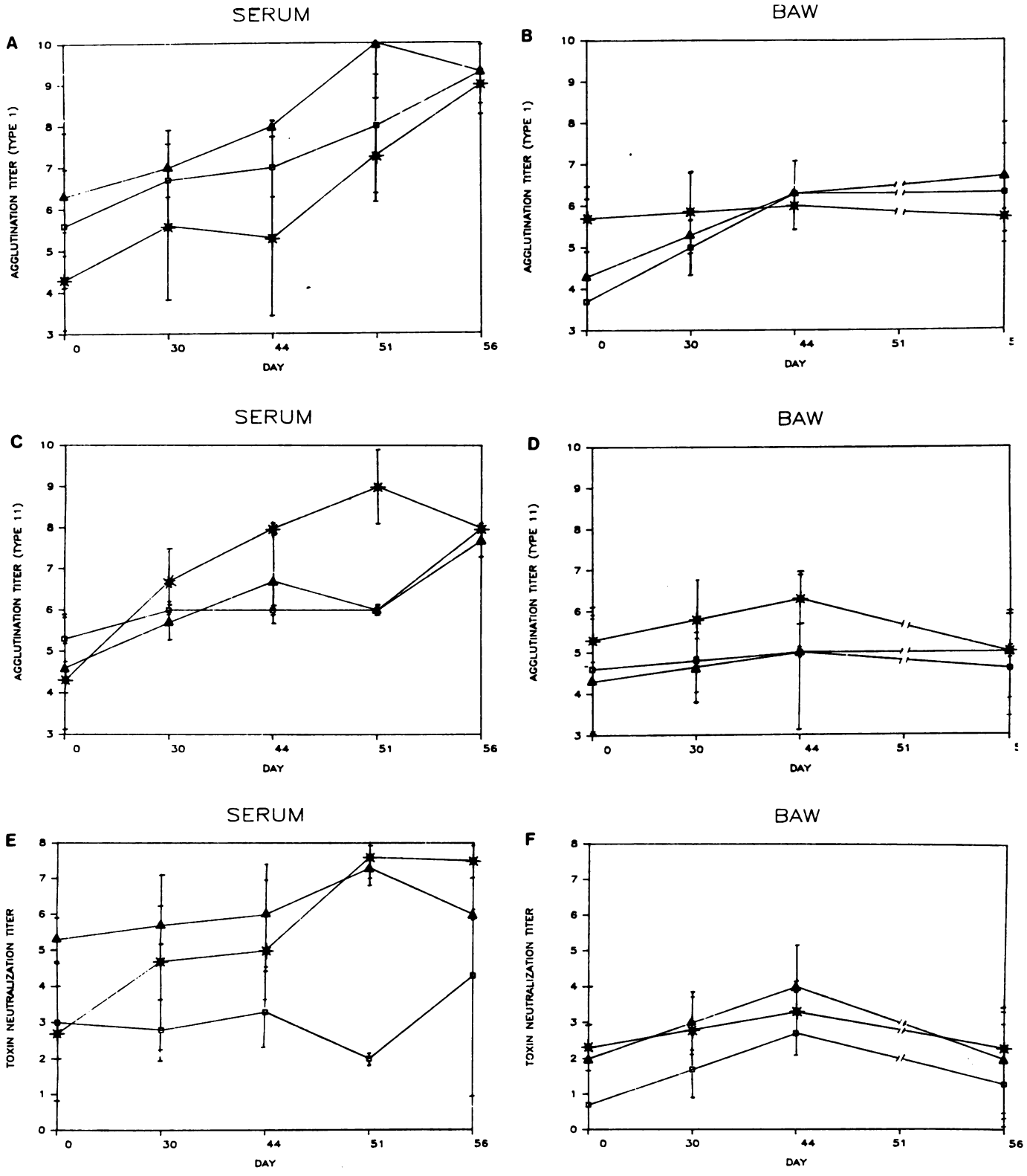

Fig. 3. Immune response of calves in experiment III. Indirect agglutination titer to *P. haemolytica* type 1 ($-\log_2$) in A. serum, B. lung washing fluid (BAW). Indirect agglutination titer to *P. haemolytica* type 11 ($-\log_2$) in C. serum, D. BAW. Toxin neutralizing activity (50% endpoint, $-\log_2$) in E. serum, F. BAW. Points represent the mean of three calves, bars indicate standard deviation. Calves were vaccinated on days 0 and 30, and challenged on day 51. □ controls, not vaccinated; ✱ vaccinated, toxin 1 (culture supernantant from *P. haemolytica* type 1); ▲ vaccinated, toxin 11 (culture supernate from *P. haemolytica* type 11). No BAW sample on day 51.

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