Intraperitoneal Immunization against Necrobacillosis in Experimental Animals

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ABSTRACT

Experiments employing recently developed mouse models indicated that intraperitoneal immunization with the cytoplasm (intracellular fraction) of Fusobacterium necrophorum protected the animals from a lethal challenge of the pathogen. The critical immunization schedule needed to achieve complete protection involved six weekly intraperitoneal doses of the intracellular antigen. Livers of immunized mice were cleared of infecting fusobacteria within 24 hours whereas those of nonimmunized mice harboured increasing numbers of the bacteria. Sera from both groups did not protect recipient mice from developing liver abscesses after challenge. Sheep immunized intraperitoneally with 20 mg of cytoplasmic protein given in three doses were protected against the development of abscesses induced by F. necrophorum.

RÉSUMÉ

Des expériences où on utilisait des souris, à titre de modèles récemment développés. révélèrent que l'immunisation intra-péritonéale de ces animaux avec le cytoplasme. c'est-à-dire la fraction intra-cellulaire, de Fusobacterium necrophorum les protégeait contre l'injection d'une dose léthale de cette bactérie. Le plan critique d'immunisation nécessaire pour obtenir une protection complète impliquait six injections intra-péritonéales hebdomadaires de l'antigène. Le foie des souris ainsi immunisées se débarrassa d'une dose infectante de la bactérie en l'espace de 24 heures; par contre, celui des souris témoins recela un nombre croissant de F. necrophorum. Le sérum de souris immunisées ou témoins n'empêcha pas le développement d'abcès hépatiques chez celles qui reçurent une dose infectante expérimentale. L'immunisation intra-péritonéale de moutons avec 20 mg de protéine cytoplasmique, répartis en trois doses, les protégea contre le développement d'abcès hépatiques attribuables à F. necrophorum.

INTRODUCTION

Necrobacillosis in livestock caused by *Fusobacterium necrophorum (Sphaerophorus necrophorus)* infection has been a constant veterinary and economic problem over the years. This organism causes several infections in feedlot cattle including liver abscesses (9) and plays a synergistic role in the development of footrot in cattle and sheep (3, 11). In spite of significant yearly economic losses caused by *F. necrophorum* infections, research efforts to prevent or reduce this problem have been few and sporadic. The classic studies of Jensen *et al* (10) have typified early difficulties in demonstrating protection in immunized animals against hepatic abscessation employing whole cell vaccines. A few years ago, however, we (7) found that an alum precipitated toxoid derived from the cytoplasmic fraction of ultrasonically disrupted *F. necrophorum* cells was partially effective in reducing the incidence of liver abscessation in feedlot cattle vaccinated subcutaneously with this toxoid. This preparation, however, induced formation of abscesses around the site of injection (2). This may have caused part of the deposited toxoid to be immobilized and also animals with large subcutaneous swellings were somewhat unattractive to prospective buyers. In order to obviate these problems other routes of inoculation

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based on a recently developed mouse model were investigated (1). The main thrust of this report, therefore, is to demonstrate through the use of experimental animals that intraperitoneal immunization of the cytoplasmic antigen could be an effective method in protecting animals against an overwhelming infection of *F. necrophorum*.

MATERIALS AND METHODS

Culture

F. necrophorum ADRI 19 (formerly LA19) was grown in prereduced Modified Casitone Broth (MC-3) for 16-18 h under oxygen-free CO_2 atmosphere at 37°C. MC-3 is composed of: casitone, 10 g; yeast extract, 3 g; beef extract, 3 g; K_2HPO_4, 5 g; $MgSO_4.7H_2O$, 0.2 g; cysteine, 0.5 g; resazurin (0.025%), 4 ml; dextrose, 10 g; maltose, 1 g; glycerine, 2 g; distilled water, 1 litre. Sugars were sterilized separately. The final pH of MC-3 was adjusted to 7.2. The culture was assessed for purity by gram staining and retrospective check on MC-3 agar + 5% sheep blood.

Antigen

Cytoplasm derived from the fractionation of *F. necrophorum* sonicated cells as described in a previous paper (5) was used throughout the study. The protein content of the antigen as determined by Biuret method was 2.8 mg/ml. The antigen was detoxified by incubating at 6-8°C with 0.3% formalin for at least six weeks prior to use.

Comparison of Different Routes of Immunization

White female inbred mice (CF-1 strain), weighing about 20 g, were divided into three groups of 20 mice and given the following treatments: *Group I* — intraperitoneal injection, ten weekly injections of 0.1 ml of the antigen, *Group II* — oral inoculation, ten weekly doses of 0.1 ml antigen and *Group III* — intranasal inoculation, ten weekly inoculations of 0.05 ml antigen. Saline treated controls were provided for each group. Five days after the last inoculation five mice from each group were bled to find out if circulating antibodies could be detected by immunodiffusion technique (5). Also at this time, these animals were injected with 0.02 ml of the antigen in the hind footpads and the reactions measured at various times up to 48 h according to the method of Tauber (12). The remaining 15 mice in each group were then challenged ip with a viable culture of *F. necrophorum* (about 2.5 x 10^8 viable cells). Mortality was observed for four weeks after challenge at which time the surviving animals were killed and necropsied.

To determine the critical stage of immunization when the animals become completely refractive to infection, groups of five mice were injected ip with one to nine weekly doses, then footpad tested and challenged as in the previous experiment.

Effect of Immunization on Fusobacterial Clearance in the Liver

Mice immunized past the critical stage, meaning completely protected against *F. necrophorum* infection, were challenged with about 2.5 x 10^8 organisms. Saline injected mice were similarly challenged. Four hours and 24 h later certain of the animals were killed by cervical dislocation and the livers aseptically removed and transferred into sterile plastic bags while being flushed with oxygen-free CO_2. The livers were then weighed and homogenized in a diluent salt solution (8) with a Colworth *Stomacher 400* (A. J. Seward). One ml of the homogenized liver suspension was removed from each bag for further dilution and subsequent plating in MC-3 agar + 5% sheep blood. *F. necrophorum* colonies on these plates were counted after three days anaerobic incubation at 37°C.

Effect of Serum on Abscess Development

Mice receiving nine weekly injections were bled and the serum was obtained and pooled as positive serum. Nonimmunized mice were similarly bled for negative serum. Both sera were tested for the presence of precipitating antibodies by immunodiffusion technique (5) Groups of five nor-

mal mice were injected intraperitoneally with the sera 48 h, 24 h or four h before challenge or together with a challenge dose of *F. necrophorum* (2.5 x 10^8 viable cells). Two weeks later the mice were killed and examined for abscesses.

Sheep Experiment

Twenty-three lambs, about six to eight months old, were maintained in temperature-air controlled cubicles and fed *ad libitum* with alfalfa pellets and a master dry and filling ration.[1] The animals were randomly divided into five groups and received intraperitoneally injected doses as indicated: *Group I* (four animals), 50 ml saline, negative controls; *Group II* (four animals), 50 ml saline, positive controls; *Group III* (five animals), one dose of 20 mg protein antigen in 50 ml saline; *Group IV* (five animals), an initial dose of 15 mg protein antigen in 50 ml saline and one booster dose of 5 mg protein antigen in 20 ml saline given ten days after the initial dose; *Group V* (five animals), an initial dose of 15 mg protein antigen in 50 ml saline + two booster doses of 2.5 mg protein antigen given at ten day intervals. Except for Group I (negative control), all groups were skin-tested with 0.2 ml of antigen 20 days after the last booster dose in Group V and then, four days later, challenged intraperitoneally with a viable culture of *F. necrophorum* (1.5 x 10^{10} cells) in 50 ml of MC-3 broth. The animals were slaughtered eight weeks after challenge and necropsied.

Sections of the livers and lesions produced were cultured for *F. necrophorum* as well as examined by fluorescent-antibody technique (6). Serum samples were obtained before the immunization schedule and prior to challenge for serological tests.

RESULTS

Comparison of Different Routes of Inoculation in Mice

The data shown in Table I give a clear indication that of the three routes only the intraperitoneal immunization protected the animals from a potent challenge of *F. necrophorum*. Subsequent necropsy further showed that livers of the ip immunized mice were free of any lesions suggestive of *F. necrophorum* infection. By contrast, all other groups inoculated with either the cytoplasmic antigen or saline succumbed to *F. necrophorum* infection. Postmortem examination of these groups revealed extensive abscessation of the liver and several other sites including the mesentery, reproductive organs, the diaphragm and at the site of inoculation. Only the serum from ip immunized mice formed a precipitation band with the *F. necrophorum* cytoplasm in the immunodiffusion assay.

Results of the footpad measurements (Table I) showed an initial increase in footpad size in all groups during the first eight h as a result of the injection of the antigen. The numbers represent the average differences in size between the right hind footpad receiving the antigen and the left hind footpad receiving saline. After 24 h, footpad size of both orally and intranasally vaccinated groups started to recede, whereas the intraperitoneally immunized group continued to maintain swollen footpads after 48 h. The differences, however, are less marked than those reported by Tauber (12).

At the termination of the experiment to determine the critical stage of immunization (five weeks postchallenge) only one out of 45 saline-treated mice survived (Table II). Even so this animal was gravely ill and was found by later postmortem examination to have multiple abscessation in the liver, mesentery, reproductive organs and around the site of inoculation. Of the cytoplasm-treated mice, only the group receiving one dose showed 100% mortality caused by the bacterial challenge. Survivors from the remaining cytoplasm-treated groups appeared healthy and had normal viscera free of gross lesions.

In this experiment complete protection was attained at about six weeks (Table II). Immunity appeared to be achieved gradually as indicated by a trend of decreasing mortality with increasing number of weekly injections of the cytoplasmic antigen. It is of interest that mice receiving no previous injection (nonimmunized control group) succumbed to the bacterial challenge earlier than the saline-treated mice. Within the first week postchallenge, 80% (4/5) of this group died and by the 2nd week 100% mortality was reached (Table I). On the

[1]Master Feeds, Prescott, Ontario.

TABLE I. Effect of Immunization Route on Footpad Response and Mortality in Mice

Route and Treatment	Footpad Response (mm)[a] 24 HPI[b]	48 HPI	Mortality (No. Dead/No. Challenged)
Intranasal:			
Cytoplasm	0.4 ± 0.3	0.3 ± 0.2	15/15
Saline	0.5 ± 0.2	0.2 ± 0.2	15/15
Oral:			
Cytoplasm	0.7 ± 0.3	0.4 ± 0.3	15/15
Saline	0.5 ± 0.3	0.3 ± 0.3	15/15
Intraperitoneal:			
Cytoplasm	1.0 ± 0.1	1.1 ± 0.1	1/15
Saline	0.6 ± 0.2	0.7 ± 0.3	15/15

[a]Mean difference between response from antigen (right pad) and saline (left pad)
[b]HPI: hours postinoculation

TABLE II. Effect of Weekly Injections on the Ability of Mice to Resist *F. necrophorum* Infection

No. of Weekly Injections	No. of Survivors Challenged with Bacterial Culture[a] Saline-injected 1 WPC[b]	2 WPC	3 WPC	5 WPC	Cytoplasm-injected 1 WPC	2 WPC	3 WPC	5 WPC
1	5	1	0	0	5	3	3	0
2	3	0	0	0	5	2	2	2
3	5	3	1	0	5	4	4	4
4	5	1	0	0	5	5	4	4
5	4	2	1	0	5	4	3	3
6	5	1	0	0	5	5	5	5
7	5	1	0	0	5	5	5	5
8	4	1	0	0	5	5	5	5
9	5	4	2	1	5	5	5	5
Nonimmunized control	1	0	0	0	—	—	—	—

[a]A 16-18 h culture, about 2.5 x 10^8 cells, inoculated ip to each of five mice per group
[b]WPC: week(s) postchallenge

other hand, only 9% of all saline-treated mice succumbed during the first week, then increasing to 69% and 94% by the second and third weeks postchallenge. Mortality among mice given cytoplasm for one to five weeks did not occur during the first week, while 60% and 75% of the 12 deaths occurred during the second and third weeks postchallenge.

Effect of Immunization on *F. necrophorum* Population in Mouse Livers

After challenge of immunized mice there were high numbers of *F. necrophorum* in the liver at four h followed by marked reduction at 24 hours (Table III). The pattern was similar whether the mice were challenged seven days or seven weeks after the last immunizing injection. Reduction in the liver population was about a thousandfold during the first four h and by the 24th hour the livers seemed to be cleared of *F. necrophorum* whereas the organism increased in livers of the nonimmunized groups.

Effect of Serum on Abscess Development

Passive transfer of sera from immunized mice did not protect recipient normal mice from necrobacillosis caused by *F. necrophorum* challenge (Table IV). Except for the group injected with positive serum 48 h before challenge which had less abscessed animals (3/5) compared to the negative serum group (5/5), no significant variation could be detected between groups receiving immune serum and normal serum. A similar experiment using whole blood instead of serum produced the same effect.

TABLE III. Immediate and Long-term Effects of Immunization on the Clearance of *F. necrophorum* in Mouse Liver

State of Immunization[a]	Viable Counts per gram of Liver			
	1 Week Postimmunization		7 Weeks Postimmunization	
	4 HPC[b]	24 HPC	4 HPC	24 HPC
Cytoplasm (6)	$\sim 7.0 \times 10^2$	0	—	0
Cytoplasm (7)	1.5×10^3	0	5.2×10^4	0
Cytoplasm (8)	$\sim 2.0 \times 10^2$	0	3.4×10^5	0
Cytoplasm (9)	$\sim 1.5 \times 10^2$	0	2.3×10^4	0
Nonimmunized	4.4×10^5	7.4×10^6	1.0×10^7	1.3×10^7

Challenge dose: one week postimmunization = 5.6×10^7 *F. necrophorum* cells/mouse
seven weeks postimmunization = 6.8×10^8 *F. necrophorum* cells/mouse
[a]Figures in brackets represent number of weekly injections
[b]HPC: hours postchallenge
$\sim$ = Approximate counts on lowest dilution

TABLE IV. Effect of Serum on Abscess Development in Mice

Treatment	No. Abscessed/ No. Challenged
48 h Prechallenge	
Negative serum	5/5
Positive serum	3/5
24 h Prechallenge	
Negative serum	5/5
Positive serum	5/5
4 h Prechallenge	
Negative serum	4/5
Positive serum	4/5
Mixed Serum + Culture	
Negative serum	5/5
Positive serum	4/5
Culture alone	4/5

Sheep Experiment

Table V summarizes the effectiveness of sheep vaccination against necrobacillosis. All skin tested animals in Groups IV and V developed an inner erythematous core surrounded by a circular, raised, thickened and hard ring of inflammatory tissues after 24 h. At 48 h this core became indurated to necrotic. Only two of three skin-tested animals in Group II showed a similar reaction but to a lesser degree. Animals skin-tested with saline did not develop any tissue inflammation. Although none of the animals developed liver abscesses, three of four saline control (Group II) animals developed abscesses in various areas after challenge (Table V). *F. necrophorum* was recovered from most of these abscesses. Abscessed sheep had one or more firm encapsulated nodules containing yellow-green viscous pus. They were 0.5 to 5.0 cm in diameter and occurred in the subcutaneous tissue at the injection site, muscles of the abdominal wall, pancreas, omentum and serosa of the liver and spleen (Table VI). None of the lambs receiving an initial dose plus two booster injections of the antigen had lesions which could be attributed to *F. necrophorum* challenge. There appeared to be a direct relationship between the number of vaccine injections and the number of abscess-free animals.

Of the livers cultured for *F. necrophorum* only those from sheep 43 and 46 harboured *F. necrophorum*. It should be noted that these two animals also had abscesses adjacent to the liver (Table VI).

DISCUSSION

Data presented here support our previous results (7) suggesting that a cell bound fraction of *F. necrophorum* as immunizing agent can protect against infection of this organism. The effectiveness of immunization may be related to the schedule and route employed. Our data suggest that a minimum of six weekly ip injections of the antigen can provide complete protection in mice against a lethal challenge of the organism. The level of immunity at this stage allows the liver to remove invading fusobacteria within 24 h. This immunity is retained even after seven weeks postimmunization. The actual mechanism by which this protection is achieved remains to be

clarified but experiments recently completed (unpublished) suggest that macrophages play a vital role in *F. necrophorum* infection. We have shown that abscesses in the mouse liver occur partly as a result of the ability of *F. necrophorum* to multiply in and subsequently destroy macrophages. Furthermore, immune serum alone does not appear to provide adequate protection against this organism although serum factors may be partly involved in enhancing phagocytosis. Presently, we are comparing macrophage activities in immunized and nonimmunized mice to help clarify this point. Our recent finding (unpublished) that there is significant lymphocyte stimulation in immunized mice over control animals suggests that cellular immunity may be a defense mechanism at least in mice and is therefore more significant than the slight increase in footpad swelling observed in this study. Recent data (1) showed

TABLE V. Vaccination of Sheep with Cytoplasmic Fraction of *F. necrophorum*

Group	Treatment	No. of Sheep	Total Dose (mg protein) of Vaccine	Challenged	Sheep with Abscesses No.	%
I	Saline control	4	0	−[a]	0	0
II	Saline control	4	0	+[b]	3	75
III	One dose of vaccine	5	20	+	3	60
IV	Initial dose + one booster	5	20	+	2	40
V	Initial dose + two boosters	5	20	+	0	0

[a]Not challenged
[b]Challenged with 1.5 x 10^{10} viable *F. necrophorum* cells in 20 ml saline

TABLE VI. Abscess Development in Sheep

Group	Treatment	Sheep No.	Abscess Formation	Abscess Location
I	Saline control, not challenged	11	−	
		41	−	
		44	−	
		45	−	
II	Saline control, challenged	32	+	draining abscess at injection area
		39	+	injection area (subcutaneous) and adjacent to the liver surface
		46	+	injection area (subcutaneous), omentum (multiple abscesses), among adhesions between omentum adjacent to rumen and abdominal wall, gastro-splenic ligament, and on ligament attached to the liver
		50	−	
III	One dose of vaccine	33	+	injection area (subcutaneous)
		38	+	muscle around injection site, between abomasum and left abdominal wall (multiple abscesses)
		42	−	
		43	+	injection area (subcutaneous)
		48	−	
IV	Initial dose and one booster	29	−	
		31	−	
		35	+	ventral surface of abomasum
		36	+	in adhesion between omentum and abdominal wall
		49	−	
V	Initial dose and two boosters	12	−	
		30	−	
		34	−	
		37	−	
		47	−	

that mice immunized with formalin-killed *F. necrophorum* cells for nine weeks and challenged two weeks after the last booster dose cleared up *F. necrophorum* infection within 24 h. However, there was no mention of possible mechanisms to explain the achieved resistance.

Results from the sheep experiment demonstrate that intraperitoneal immunization, while uncommon in large animal practice, may be of value in reducing necrobacillosis. Although hepatic abscesses did not develop in any of the challenged animals, the fact that multiple abscesses in other sites were found in nonprotected lambs indicates their susceptibility to *F. necrophorum* infection. It is widely recognized that liver abscesses can be reproduced consistently in large animals only by intraportal inoculation of the fusobacterium (10). Obviously, this is a direct, albeit more elaborate and artificial method of introducing the organism to the liver. Unlike previous workers (3, 10), however, we have succeeded in protecting lambs (Group V) against *F. necrophorum* infection. Whether the type of immunity developed in the mouse and sheep models is germane to that needed to control liver abscesses in cattle remains to be resolved.

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