

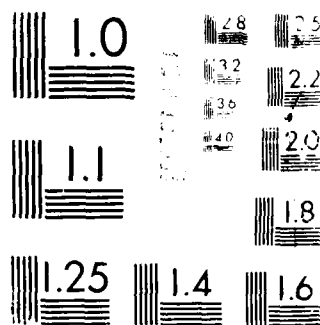
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DEVELOPMENT OF VACCINES TO PREVENT WOUND INFECTIONS DUE TO ANAEROBIC BACTERIA(U) BRIGHAM AND WOMEN'S HOSPITAL 1/1
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DEVELOPMENT OF VACCINES TO PREVENT WOUND
INFECTIOUS DUE TO ANAEROBIC BACTERIA

ANNUAL REPORT

Dennis L. Kasper, M.D.

October 11, 1985

U.S. ARMY MEDICAL RESEARCH DEVELOPMENT COMMAND
Fort Detrick
Frederick, Maryland 21701-5012

Contract DAMD-17-83-C-3239

Brigham and Women's Hospital
Boston, MA 02115

Approved for public release; distribution unlimited

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents.

SUMMARY

Progress on the two major objectives of this contract were made during this year. One lot of capsular polysaccharide of Bacteroides fragilis vaccine was tested for protective response. One human volunteer was immunized without adverse reactions occurring and the immunogenicity will be assessed. On the second specific aim, major progress was made in producing a specific T cell factor by cloning lymphocytes in vitro to produce the protective lymphokine. A small molecular weight antigen specific factor has been produced from hybridoma clones. This factor is of T cell origin and protects mice against experimental infection with Bacteroides fragilis.



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FOREWORD

In conducting the research described in this report, the investigator adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

For the protection of human subjects the investigator has adhered to policies of applicable Federal Law 45CFR46.

This annual report on DAMD 17-83-C-3239 represents only 8 months effort on this contract. Funding was delayed due to administrative decisions until February 1, 1985. The original proposal submitted in February, 1984, for an October, 1984 starting date was for 3 years. Despite the short time we have been working on the proposal, we have made significant progress. Although the original proposal contained three specific aims, only the first two have been pursued based on reviewers recommendations. We will report our progress on the two specific aims separately.

Specific Aim I: Phase I trial of capsular polysaccharide vaccine assessing safety and immunogenicity.

A single lot of Bacteroides fragilis capsular polysaccharide vaccine has been prepared from ATCC strain 23745. The organisms were cultured in a 12 liter fermentor in pre-reduced broth. Organisms were separated by centrifugation and the polysaccharide extracted with hot phenol-water. After removal of phenol and exhaustive dialysis of the water phase, the capsular polysaccharide was separated from the lipopolysaccharide by column chromatography in detergent containing buffer. The detergent was removed by alcohol solubilization and the precipitated polysaccharides treated with nucleases and protease to remove contaminants. After repeat chromatography to remove enzymes and digested materials, the final product was dialyzed exhaustively and lyophilized.¹ (See appendix A for detailed extraction procedure.) The vaccine eluted in the void volume of a Sepharose 4BCL column indicating that the size is large and probably aggregated. The vaccine lot (BF/-1R) was bottled at the Massachusetts Public Health Biology Laboratories. The safety and toxicity tests required by the Food and Drug Administration have been completed satisfactorily.

The bottled vaccine was shown to contain 1:9100 thimerosal which is used as a preservative. Sterility testing was performed and the lot was sterile; no growth was observed. Three rabbits were injected intravenously with 300 µg of polysaccharide (human dose expected to be 50 µg). The rabbits did not develop any fever to this injection over 4 hours observation. Two guinea pigs were injected with 500 µg of polysaccharide each, and normal growth and development was observed over a one week period. Two mice similarly were given 50 µg of polysaccharide with normal growth and development continuing.

The final bottled product was checked for chemical composition by comparison to the antigen lot prior to bottling. No significant chemical differences were noted. As a final test, the bottled material was checked for immunogenicity and protective capacity by immunization of C57/BL6 mice from Jackson Laboratories with a dose of 10 µg subcutaneously 3 times a week for three weeks. The immunization dose of 10 µg was delivered in 0.1 cc subcutaneously to each animal. A cohort group of littermate animals housed in the same room were used as a naive control. Eight animals were immunized with the capsular vaccine, and four animals were used as naive controls. Animals in both groups were challenged with 1 106 B. fragilis 23745 plus sterile cecal contents according to our previously published protocol.

¹Kasper DL, Weintraub A, Lindberg AA, Lonngren J. Capsular polysaccharides and lipopolysaccharides from two B. fragilis reference strains: Chemical and immunochemical characterization. J Bacteriol 1983; 153:991-7.

The animals were sacrificed for evaluation of abscess formation.^{2,3} of the eight animals which were immunized, only one animal exhibited an abscess which revealed polymorphonuclear cells on gram stain. All four naive recipients of B. fragilis demonstrated large abscesses within the abdominal cavity. These data are consistent with our previously published findings for the capsular polysaccharide.

The above information was transmitted to the FDA on April 29, 1985. After waiting for questions or required clarification without receiving a response, we were assured that we could proceed with a phase I trial.

The first volunteer was given 1 μ g of vaccine intradermally to assure absence in humans of an immediate hypersensitivity reaction. None were observed. Announcements have been made to recruit volunteers for receiving the vaccine according to the proposal we submitted. The trial will be completed before January 31, 1986, which is prior to the end of the first contract year, as proposed.

Specific Aim 2: In vitro production of specific T cell factor by cloning of lymphocytes in tissue culture and hybridoma technology.

As stated in the annual report of November 1, 1984, our early effort at producing active factor by a T cell clone showed promise. We have pursued this work further, fusing the cloned T cells with a thymoma parent line (BW), thereby immortalizing the cells. Nylon-wool column passed immune spleen cells were cultivated in vitro with Con A supernatant (IL-2), capsular polysaccharide and irradiated immune spleen cells to serve as stimulator cells. The growth factor dependent cell lines were cloned by limiting dilution. The putative clones then were recloned by the same methods. Cloned cells were mixed with BW 5147 thymoma cells at a ration of 5:1. After centrifugation, a solution of polyethylene glycol (PEG) and 15% dimethylsulfoxide (DMSO) in Dulbecco's Minimal Essential Medium (DMEM) without serum was added to the pellet. Cells were centrifuged and resuspended in DMEM-HAT medium with 20% fetal calf serum (FCS). Cells were aliquoted at 3×10^5 in wells of a microtiter plate and fed 10^5 syngeneic thymocytes to improve viability. Hybrids were fed HAT medium for one month to eliminate residual tumor parent cells and then switched to DMEM with 10% FCS. Fusion of a T cell line was ensured by sorting for Thy 1.2 cells on a fluorescence activated cell sorter and by selecting cells which tolerate HAT medium since BW thymoma cells are Thy 1.1 and sensitive to HAT medium. Supernatants from several fusions and from the BW parent line harvested without antibiotics in the medium were screened for ITF activity in the modified bactericidal assay. A supernatant referred to below as 2EB combined with C' killed B. fragilis, while the parent BW supernatant and other hybridoma supernatants did not produce killing. 2EB, BW and other supernatant, 2D3, then were tested for protective activity in mice. 2EB protected mice, while BW and 2D3 were not protective.

²Shapiro ME, Onderdonk AM, Kasper DL, Finberg RW. Cellular immunity to Bacteroides fragilis capsular polysaccharide. J Exp Med 1982; 154:1188-97.

³Zaleznik DZ, Finberg RW, Onderdonk AB, Kasper DL. A soluble suppressor T cell factor protects against experimental intraabdominal abscesses. J Clin Invest 1985; 75:1023-1027.

Table 1

Activity of Hybridoma Supernatants in Protection Against Abscesses

Lysate or supernatant transferred	# of mice with abscesses/total
Naive T cell factor	9/10 (10%)*
Immune T cell factor	2/10 (80%)
BW supernatant	12/13 (7.7%)
2D3 supernatant	10/14 (28.6%)
2E8 supernatant	2/15 (86.7%)

* () = % protection

To assess whether 2E8 was dialyzable, 3cc of 2E8 supernatant were placed in a dialysis membrane and dialyzed against 24cc of 5mM ammonium acetate pH 7.1 for 24 h at 4°C with two changes of the dialysate. 0.2cc of pooled dialysate was protective, while the dialysis bag contents lost protective capacity against experimental abscess formation.

Table 2

Activity of Dialyzed Hybridoma Supernatants in Protection Against Abscesses

Supernatant transferred	# of mice with abscesses/total
BW supernatant	4/4 (0%)*
2E8 supernatant	0/5 (100%)
2E8 supernatant dialysis bag contents	7/7 (0%)
2E8 supernatant dialysate	0/8 (100%)

* () = % protection

Specificity of the hybridoma-produced factor was assessed by incubation of 2E8 supernatant with sheep red blood cells coupled to B. fragilis CP, type III group B Streptococcus CP, or SRBC alone. Only incubation with SRBC linked to B. fragilis CP removed protective activity from the 2E8 supernatant.

Table 3

Antigen Specificity of T cell Hybridoma Supernatant

Supernatants	Treatment of supernatants	# of mice with abscesses/total
28E	absorbed with SRBC	0/8 (100%)*
2E8	absorbed with SRBC coupled to GBS polysaccharide	0/8 (100%)
2E8	absorbed with SRBC coupled to <u>B. fragilis</u> capsular polysaccharide	8/8 (0%)

* () = % protection

Therefore, we demonstrated that one of our hybridomas produced a small molecular weight, antigen-specific factor capable of protecting mice against intraabdominal abscesses caused by B. fragilis. The properties of this hybridoma supernatant were identical to the immune T cell factor (ITF) prepared by lysing splenic T cells from immunized mice. We, therefore, have accomplished our goal of producing active T cell factor in large quantities and can pursue further characterization.

GLOSSARY

BW	-	parent thymoma cell line used for producing hybridoma
IL-2	-	Interleukin 2
PEG	-	polyethylene glycol
DMSO	-	dimethylsulfoxide
DMEM	-	Dilbecco's Minimal Essential Medium
DMEM-HAT	-	above plus supplemental amino acids
FCS	-	fetal calf serum
SRBC	-	sheep erythrocytes
GBS	-	group B <u>Streptococcus</u>
CP	-	capsular polysaccharide
ITF	-	immune T cell factor

APPENDIX A

Description of Source and Preparation of Components for Lot B.f.-1R Capsular polysaccharide of Bacteroides fragilis prepared from strain 23745 (American Type Culture Collection). Other than the salt used for reconstitution and thimerosal, the polysaccharide is a pure bacterial product.

Methods, Facilities and Controls used for Manufacturing

Methods: (Materials list follows methods section)

1. Set up 12 l fermentor with pre-reduced Protease peptone-Yeast Extract media supplemented with 6% glucose and 10% fetal calf serum (FCS), and cysteine HCl.
2. Inoculate with 1500 ml broth culture of organisms, bubbling nitrogen with 3% hydrogen through the system.
3. Gram stain and subculture the inoculum aerobically and anaerobically to check for purity.
4. Incubate the 12 l for 18 h at 37°C with N₂ with 3% H₂ bubbling through continuously, maintaining pH 7.0 with 5 N NaOH using a pH titrator.
5. Collect organisms in Sharples centrifuge at 50,000 RPM to produce a 500 gm wet weight of organisms.
6. Suspend organisms in 1500 ml of sterile distilled water at 68°C.
7. Shake organisms vigorously with glass beads to suspend the organisms.
8. Mix an equal volume of 75% phenol at 68°C with the organism suspension and mix vigorously for 30 min at 68°C.
9. Mix overnight at 4°C.
10. Separate the phenol and water phases in 200 ml glass bottles at 8,000 rpm.
11. Remove the water phase on top which constitutes approximately one-half of the total volume into a vacuum flask using a vacuum aspirator and a Pasteur pipette.
12. Re-extract the residual phenol from the aqueous phase three times with equal volumes of anaesthetic grade ethyl ether.
13. Concentrate the aqueous phase and remove residual ether on a flash evaporator at 68°C.
14. Dialyze the aqueous phase for 48 h in deionized water at 4°C.
15. Concentrate the aqueous phase by partial lyophilization to 120 ml.
16. Dissolve dry ingredients of 3% sodium deoxycholate buffer in 120 ml of

sample and adjust pH to 9.

17. Chromatograph the material on a 10 x 90 cm column of S-400 (Pharmacia) in 3% deoxycholate buffer pH 9.
18. Antigen elutes just beyond void volume and is confirmed by Ouchterlony. Another peak elutes at 15,000 daltons and is the disaggregated LPS.
19. Pool Ouchterlony positive fractions and concentrate on Amicon TCF concentrator with a YM-10 membrane (Amicon).
20. Precipitate antigen from buffer with 80% ethyl alcohol at 4°C overnight. This removes the deoxycholate which is alcohol soluble.
21. Spin out precipitate at 12,000 x g for 20 min. Discard supernatant. Suspend precipitate in deionized water.
22. Dialyze for 48 h against distilled water with pH adjusted to 9.0 by adding a few drops of 0.1N NaOH. This is done to remove any trace amounts of remaining deoxycholate.
23. Dialyze for 48 h more against deionized water.
24. Lyophilize sample.
25. Suspend in 10 ml pH 7.3 0.05M Tris buffer at 37°C.
26. Add 1 mg DNase, 5 mg RNase with 3 drops of 0.1M MgCl₂ and 3 drops of 0.1 M CaCl₂. Incubate overnight at 37°C.
27. Repeat the above digestion procedure incubating for 3 h at 37°C.
28. Treat with 10 mg Pronase for 2 h at 37°C and again overnight.
29. Chromatograph on a 4.4 x 90 cm S-300 (Pharmacia) column monitoring with UV and RI monitors.
30. Identify antigen by Ouchterlony double diffusion using capsular antiserum against all fractions.
31. Pool the tubes with antigen and concentrate on PM-10 membrane (Amicon) to one-tenth of the original volume. These fractions are free of enzymes as identified by v/v absorption at 260 nm.
32. Precipitate antigen with 5 volumes of ethyl alcohol (95%) at 4°C.
33. Centrifuge at 12,000 g for 20 min.

34. Suspend pellet in deionized water and dialyze against deionized water pH 4 in 12,000 dalton exclusion size tubing for 2 days at 4°C. Dialyze against deionized water pH 7 for 2 days.
35. Lyophilize and determine dry weight (155.4 mg). Check for sterility anaerobically and aerobically.
36. Store in sterile containers until sent to Massachusetts State Biologics Laboratory for final bottling (86 mg).

Materials:

Proteose Peptone (Difco, Detroit, MI)
Yeast Extract (Difco, Detroit, MI)
Dextrose (Fisher Scientific)
Cystein-HCl (Fisher Scientific)
Fetal calf serum (Microbiological Associates, MA Bioproducts)
18 liter fermentor (LSL Biolafitte)
Nitrogen tank with 3% hydrogen
Sharples centrifuge (Penwalt Warminster, PA)
Ether, anesthesia (Baker Chemicals)
Water bath
Flash evaporator
Ethanol (Florida Distillers)
Glass beads
Sodium desoxycholate (Fisher Scientific)
Phenol (75% solution) (Fisher Scientific)
Glycine (For deoxycholate buffer) (Fisher Scientific)
EDTA (for deoxycholate buffer) (Fisher Scientific)
200 ml sterile glass centrifuge bottles
Vacuum flask 1000 ml
Tris buffer (Sigma Chemical Co.)
Deoxyribonuclease (Worthington)
Ribonuclease (Worthington)
Pronase (Calbiochem)

Labeling Information

B. fragilis capsular vaccine
IND No. 1615 Lot B.f.-1R
"Caution: New Drug - Limited by federal
law to investigational use."
Manufacture date 5-3-84
100 mcg/ml

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