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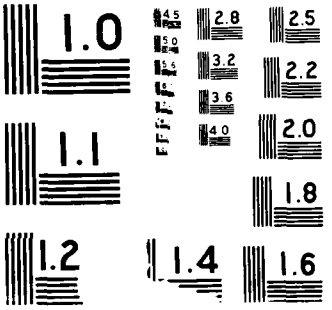
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Progress on the two major objectives of this contract has been made. The first objective was to bottle and provide safety testing on a Bacteroides fragilis capsular polysaccharide preparation. The antigen was prepared in our laboratory and bottled by the Massachusetts State Public Health Laboratory. The material passed all safety and pyrogenicity studies required by the Food and Drug Administration. The second objective was to study the lymphokine produced by immunization with the polysaccharide. We have shown that a lysate of splenic T cells from animals immunized with the capsular polysaccharide, transfers protection to recipients. The protective material appears to be a lymphokine which is a protein, of small molecular weight. This factor is antigen specific for the immunizing antigen.

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DEVELOPMENT OF VACCINES TO PREVENT WOUND INFECTIOUS DUE TO ANAEROBIC BACTERIA

ANNUAL REPORT

Dennis L. Kasper, M.D.

November 1, 1984

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

SUMMARY

Progress on the two major objectives of this contract has been made. The first objective was to bottle and provide safety testing on a Bacteroides fragilis capsular polysaccharide preparation. The antigen was prepared in our laboratory and bottled by the Massachusetts State Public Health Laboratory. The material passed all safety and pyrogenicity studies required by the Food and Drug Administration. The second objective was to study the lymphokine produced by immunization with the polysaccharide. We have shown that a lysate of splenic T cells from animals immunized with the capsular polysaccharide, transfers protection to recipients. The protective material appears to be a lymphokine which is a protein of small molecular weight. This factor is antigen specific for the immunizing antigen.

FOREWORD

In conducting the research described in this report, the investigator adhered to the "Guide for the 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.

The two major objectives of this year's work were:

1) Bottling and safety testing of a B. fragilis capsular polysaccharide (CP)

2) Study the T cell lymphokine produced by immunization with the CP

The third objective was not pursued at the suggestion of the review committee. This objective was to study synergy between B. fragilis and other bacteria.

1) In the past year we have extracted, purified, characterized, bottled and sterility and safety tested capsular polysaccharide vaccine from B. fragilis. This has all been done under IND BB-1615. We have been in regular communication with the FDA. The antigen was purified on sterile columns by methods described previously[1]. This involves growing organisms in a fermentor, extracting with phenol water and purification of the polysaccharide from the aqueous phase by chromatography. This antigen was a polysaccharide with the chemical characteristics described previously. It did, however, contain more glucose on a weight basis than some other lots. We believe this glucose represents a glycogen which is of bacterial origin[2]. The glycogen is not immunologically reactive. The antigen had the same specificity as prior lots of this material. Bottling and safety testing was done in collaboration with the Massachusetts State Public Health Laboratory. The bottled material passed all safety and pyrogenicity studies required by the FDA for polysaccharide vaccines, including rabbit pyrogenicity, mouse and guinea pig toxicity and limulus lysate. This material is awaiting use in the phase I trial listed on the continuation proposal.

2) Major accomplishments have been made in characterizing the lymphokine responsible for immunity to abscesses.

We have evidence that in addition to immune T cells, a cell-free factor (ITF) prepared from an immune splenic T cell population protects experimental animals from developing intraabdominal abscesses caused by B. fragilis. Preliminary purification of this factor reveals that the active material is low in molecular weight, can bind to B. fragilis CP, and induces antigen-specific immunity to abscesses. The material is heat-labile and loses efficacy after protease, but not nuclease, digestion.

Identification of a Protective Immune T Cell Factor (ITF):

Splenic cells from mice immunized with CP and naive mice were passed over nylon wool columns to eliminate B cells and macrophages, and varying numbers of T cells were lysed by sequential freezing and thawing. The cell-free lysates, immune T cell lysate (ITF) and nonimmune T cell lysate (NITF), were transferred i.c. to naive mice. Twenty-four h later, animals were challenged with B.

[1]Kasper 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.

[2]Kasper DL, Onderdonk AB, Reinap BG, Lindberg AA. Variations of Bacteroides fragilis with in vitro passage: Presence of an outer membrane associated glycan and loss of capsular antigen. J Infect Dis 1980; 142:750-6.

fragilis and sterile cecal contents and examined for abscesses 6 d later. Mice with one or more abscesses on exploration and examination of the abscess contents for PMN were recorded as positive. Animals listed as negative for abscesses contained no intraperitoneal collections. Results appear in Table I.

Table 1

Comparative Ability of Splenic T Cells and T Cell Lysates to Protect Mice Against Abscesses

Cells or factor transferred	# Cells or equivalents	# Mice with abscesses/Total	% protection
Nonimmune T cells	2.5×10^6	8/8	0%
Immune T cells	2.5×10^6	1/8	87.5%
Nonimmune T cell factor (NITF)	25×10^6*	10/10	0%
Immune T cell factor (ITF)	25×10^6*	1/8	87.5%
Immune T cell factor (ITF)	2.5×10^6*	1/8	87.5%
Immune T cell factor (ITF)	$.25 \times 10^6$*	3/8	62.5%

* Factor was prepared by freezing and thawing a known concentration of spleen cells, filtering and injecting a 0.2 cc volume of factor at the cell-equivalent dose listed.
$p<.001$ compared to nonimmune cells by the Fisher's exact test.
$p<.001$ compared to nonimmune T cell factor by the Fisher's exact test.
$p<.01$ compared to nonimmune T cell factor by the Fisher's exact test.

ITF at concentrations of $2.5 - 25 \times 10^6$ cell equivalents prevented the development of abscesses following challenge with viable B. fragilis to the same degree as 2.5×10^6 intact immune T cells. Neither 2.5×10^6 nonimmune T cells or 25×10^6 cell equivalents of NITF provided any protection. Even a dose of $.25 \times 10^6$ cell equivalents of ITF protected 5 of 8 animals from developing abscesses, a significant degree of protection ($p<.01$) compared to NITF. Crude factor prepared by lysing immune T cells, a process which left no cells intact by microscopic examination, was as active as intact cells in preventing abscess formation in mice. Protective ITF was prepared from mouse splenic T cells at least 46 d following completion of the immunization protocol.

Antigen-specificity of ITF: To evaluate the antigen-specificity of ITF activity, ITF was adsorbed with sheep red blood cells (SRBC) coupled with either B. fragilis CP or an unrelated capsular polysaccharide. CP of B. fragilis or type III group B Streptococcus (GBS) were bound to SRBC using chromium chloride. ITF was incubated with unbound SRBC or SRBC coupled to B. fragilis or GBS CP at a ratio of two and ten SRBC per cell equivalent of ITF. After incubation at 4°C for 30 min, SRBC were removed by centrifugation and 25 $\times 10^6$ cell equivalents of ITF in 0.2 cc transferred i.c. to naive mice. Mice were challenged as usual with B. fragilis 24 h later. Table II lists the

results.

Mice receiving unabsorbed ITF and ITF absorbed with SRBC alone or SRBC coupled to GBS CP were protected against abscesses caused by B. fragilis. Animals receiving ITF absorbed with SRBC coupled to B. fragilis CP developed abscesses as did control animals receiving NITF. Therefore, absorption of ITF with SRBC coupled to B. fragilis, but not to the unrelated polysaccharide, eliminated the protective effect of the factor. These results indicate that ITF derived from immune splenic T cells is capable of specific binding to the B. fragilis CP.

Table II

Antigen-Specificity of Protective Activity of T Cell Factor

Factor transferred	Prior adsorption of factor	# Mice with Abscesses/Total	% protection
Nonimmune T cell factor	---	9/10	10%
Immune T cell factor	---	0/10	100%
Immune T cell factor	Sheep RBC	2/20	90%
Immune T cell factor	B. fragilis capsular polysaccharide coupled to sheep RBC	18/20	10%
Immune T cell factor	Group B streptococcal capsular polysaccharide coupled to sheep RBC	0/20	100%

Factor prepared from 2.5×10^7 immune T cells was adsorbed with sheep RBC or capsular polysaccharides coupled by chromium chloride to sheep RBC for 10 min at $4^{o}C$. The ratio of sheep RBC to immune T cell-equivalents was 2:1 and 10:1 in groups of ten mice each. Data were pooled.
$p<.001$ compared to nonimmune T cell factor by Fisher's exact test.
$p=0.5$ compared to nonimmune T cell factor by Fisher's exact test.

Sizing and partial purification of ITF: To assess an approximate molecular size of the component which confers protection, ITF was dialyzed in a 12,000 MW exclusion dialysis membrane for 24 h at $4^{o}C$ against 5 mM Ammonium acetate pH 7.1. ITF, dialysis bag contents, and dialysate were tested in mice to determine protective capacity. Results appear in Table III.

Table III

Protective Capacity of Immune T Cell Factor (ITF) and Dialyzed Lysate

Factor transferred	# Mice with Abscesses/total	% protection
---	8/8	0%
Immune T cell factor	1/8	87.5%*
Dialysis bag contents	6/8	25%
Dialysate	0/8	100%

p<.001 comparing the two groups to dialysis bag contents by the Fisher's exact test.

Mice receiving non-dialyzed ITF were protected from developing abscesses caused by B. fragilis. Animals not receiving ITF all developed abscesses. The active component of the lysate appeared to be smaller than 12,000 MW since the dialysis bag contents lost protective capacity, while the dialysate was protective despite a thirty-fold dilution from the initial volumne of 1 ml.

The T cell lysate was purified partially by molecular sieve chromatography. ITF was loaded initially on an S-200 column and pooled fractions tested for protectivity activity in mice. In multiple runs protection was conferred by fractions near and at the bed volume of the column (data not shown). A protective peak which eluted at 26 ml from the S-200 column (where 12 ml represented void volume and 43 ml bed volume) was placed on a P2 Biogen column. Two ml fractions from the P2 Biogel column were pooled as marked on the elution profile recorded by RI monitoring. The P2 Biogel column has an exclusion size of 1800 MW for proteins. Doses of 0.2cc of these pooled fractions were transferred to mice which were challenged and examined for abscesses. Table IV presents the results.

Table IV

Testing P2 Biogel Column Fractions for Protection Against Abscesses in Mice

Group	P2 Biogel fractions transferred into mice	# Mice with Abscesses/Total
E	none	5/5
F	Fractions 27-30	3/5
G	Fractions 36-40	1/5
H	Fractions 55-72	1/5
I	Fractions 73-82	4/4

Mice given 0.2 ml of peaks G and H were protected from abscess formation, while bed volume peak I and voi volume peak F did not protect. All mice developed abscesses in the control group which did not receive any factor in transfer. These results are representative of eight experiments testing P2 column fractions in mice, and the elution profile was representative of other P2 column runs fractionating protective peaks from the S-200 column. On the P2

Biogel column protective activity resided in column fractions intermediate between the void volume and bed volume. In subsequent experiments pooled fractions from several column runs were utilized after testing for ability to protect mice against abscesses. Fractions capable of protecting >80% of mice in a test group were used.

Antigen-specificity of the protective action of column fractions: To assess whether the protective capacity of these fractions was specific for B. fragilis or induced more general immunity, a complex inoculum was given to mice. Anaerobes other than B. fragilis require a facultative aerobic organism to form abscesses in the animal model. Enterococcus was the aerobe employed, while B. distasonis served as the other anaerobe. The challenge inoculum was designed to provide two means of inducing abscesses, either B. fragilis alone or the combination of an anaerobe with an aerobic organism. Results of the experiment utilizing B. distasonis are listed in Table V.

Table V

Antigen Specificity of Protection Mediated by P2 Biogel Column Fractions

fractions transferred	challenge organism	# mice with abscesses/total	abscess cultures
Fractions 55-72^{o}	B. fragilis* B. distasonis Enterococcus	10/11	B. distasonis Enterococcus
---	B. fragilis B. distasonis Enterococcus	12/13	B. fragilis B. distasonis Enterococcus

Bacteroides fragilis ATCC 23745
Bacteroides distasonis ATCC 8503
Enterococcus 2988T (clinical isolate)
Mice received column fractions 55-72 (group H in Table IV) which previously had been found to protect >80% of mice.

Mice were not protected from abscess formation in either group in this experiment. Mice receiving active column fractions developed abscesses in 10 of 11 animals, but no B. fragilis was cultured from the abscess contents. Twelve of 13 mice in the control group not receiving factor developed abscesses, and all of these abscesses still contained the starting inoculum.

Another experiment similarly designed was performed substituting Fusobacterium varium for B. distasonis as the anaerobic organism. The conditions were identical otherwise, and the same active column fractions were transferred. Results of this experiment were similar. Twelve of 12 animals receiving column fractions developed abscesses compared to 12 of 13 animals not receiving fractions. Those mice receiving column fractions, however, did not

have B. fragilis cultured from the abscesses. Protection, then, was antigen-specific to B. fragilis alone without cross-protection even to another Bacteroides species.

Partial characterization of column-purified ITF: Column-purified ITF was evaluated for heat stability by incubating previously active P2 Biogel column fractions at different temperatures and injecting the fractions into mice to assess protective efficacy against abscesses. A control sample was incubated at 4°C since activity had been preserved at this temperature during the column chromatography processing. Table VI indicates results.

Table VI

Lability of Partially-Purified Immune T Cell Factor (ITF)

Fractions transferred	Treatment of fractions	# Mice with Abscesses/Total
--*	--	6/6
+°	4°C for 2 h	0/5
+	Room temperature for 2h	1/5§
+	37°C for 30 min	5/5
+	56°C for 30 min	5/5

* Mice received no fractions in transfer.
° Mice received fractions like group H in Table IV which previously had protected >80% of mice.
p<.01 compared to animals receiving no fractions by the Fisher's exact test
§ p>.05 compared to animals receiving no fractions by the Fisher's exact test

Animals receiving no factor in transfer developed abscesses while those receiving fractions incubated for 2 h at 4°C were all protected. Processing fractions for 2 h at room temperature preserved protective capacity, but heating to 37°C or 56°C for 30 min eliminated the protective effect. The extreme heat lability of column-purified ITF indicates that the active component was not antibody.

To determine whether the active constituent of ITF was a protein or nucleic acid, factor was subjected to enzyme digestion with either pronase or trypsin, or with the nucleases, DNase and RNase. Based on the previous experiment, enzyme digestions were performed at room temperature for 2 1/2 hours. After 2 h, trypsin soybean inhibitor was added to the trypsin tube to neutralize enzyme activity. To avoid manipulating ITF excessively but to remove the enzymes prior to injecting mice, ITF was dialyzed as before and 0.2 cc of dialysate was transferred to mice. The enzymes were retained in the dialysis bags. Control ITF was processed in an identical fashion without enzymes and also was dialyzed. Results appear in Table VII.

Table VII

Protective Efficacy of Enzyme Digested and Dialyzed Immune T Cell Factor (ITF)

Factor transferred	Enzyme Digestion	# mice with abscesses/total	% protection
--	--	10/10	0%
Immune T cell factor	--	1/15	93%
Immune T cell factor	Pronase	11/14	21.4%
Immune T cell factor	DNase/RNase	2/14+	85.7%
Immune T cell factor	Trypsin	8/8+	

Immune T cell factor was processed under identical conditions as enzyme-digested factor but without enzyme. Factor was incubated at room temperature for 2 1/2 h and then dialyzed against 5 mM ammonium acetate pH 7.1 at 4°C for 24 h with two changes of the dialysage. 30 cc of dialysate was pooled and 0.2 cc injected into mice.

* 1 cc of immune factor was incubated with 1 mg Pronase for 2 1/2 h at room temperature and dialyzed as above. Mice were injected with 0.2 cc dialysate.

o 1 cc of immune factor was incubated with 0.1 mg DNase and 0.5 mg RNase under the same conditions as for pronase digestion.

§ 1 cc of immune factor was incubated with 1 mg Trypsin for 2 h at room temperature. 1 mg Trypsin soybean inhibitor was added and the mixture incubated for 30 min at room temperature. Processing then was performed as for pronase digestion.

\+ $p<.001$ compared to immune T cell factor by the Fisher's exact test.

Mice not receiving factor all formed abscesses, while dialysate of ITF without enzyme treatment was protective. DNase/RNase treatment did not alter protection. Both pronase and trypsin digestion, however, eliminated the protective effect of ITF against abscesses caused by B. fragilis ($p<.001$ compared to ITF). These results suggest that ITF is a protein.

Ultimate utility of a small protein such as we are describing depends upon its availability and purity. Immune T cell factor extracted from spleen cells is time consuming to prepare, labile and difficult to obtain in workable quantities. We have overcome all these problems by cloning immune T cells in vitro, fusing them with a thymoma cell line. We have thus produced a hybridoma and after screening we have found one which specifically produces an active lymphokine.

Briefly, nylon wool passed T cells from an immune mouse were cloned in vitro in the presence of immune stimulator cells, IL-2 and capsular polysaccharide antigen. These cloned cells were fused with mouse thymoma line BW and grown in HAT media and screened for T cell marker thyl.2 antigen on a fluorescent activated cell sorter. After cloning by limiting dilution, several cell lines were established. In vitro supernatants from these cells were tested for protective activity in vivo by out mouse protection assay. A supernatant with comparable protective activity to ITF produced in vivo was found. Administration of supernatant from cell line E8 to naive mice produced over 90% protec-

tion compared to the parent BW supernatant which was not protective. Preliminary characterization of this supernatant is ongoing.

Needless to say, the vaccine implications of these data are potentially enormous. These cloning techniques are applicable to human cells and this work will be pursued in our renewal program.

Publications attributable to the work of this contract year.

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

GLOSSARY

FDA	-	Food and Drug Administration
ITF	-	immune T cell factor
NITF	-	nonimmune T cell factor
PMN	-	polymorphonuclear leukocyte
SRBC	-	sheep red blood cells
GBS	-	group B Streptococcus
MW	-	molecular weight
DNase	-	desoxyribonucleic acid degrading enzyme
RNase	-	ribonucleic acid degrading enzyme
IL-2	-	interleukin-2
HAT media	-	a maintenance media for cell cultures

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