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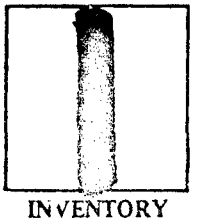

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6th Annual Report

Development of Vaccines to Prevent Wound Infections due to Anaerobic Bacteria

Formerly: Immunochemical Investigations of Cell Surface Antigens of Anaerobic Bacteria (DAMD 17-74C-4056)

ANNUAL REPORT

Dennis L. Kasper, M.D.

September 1979

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND

Fort Detrick, Frederick, Maryland 21701

Contract #DAMD-17-78C-8019
The Peter Bent Brigham Hospital
A Division of Affiliated Hospitals Center, Inc.
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Table of Contents

Section I

Summary of previous work done on this contract (1974-1979)

The purpose of this review is to summarize the most relavant data we have gathered in relation to Bacteroides fragilis infections. I have excluded many interesting issues from this review because they don't relate to the potential utility of the B. fragilis antigen as a vaccine. Such studies as we have done on B. melaninogenicus, B. thetaiotaomicron and rapid diagnostic methods are included as prior reports or elsewhere in this report.

Section I

Variations of *Bacteriodes fragilis* with *in vitro* passage:

A) The presence of an outer membrane associated glycan and loss of capsular antigen.

B) The effect of *in vitro* loss of capsular antigen on the results of tests involving neutrophil killing of *B. fragilis*.

A. Members of the species Bacteroides fragilis contain an immunologically identical capsular material which has been shown to be a virulence factor associated with abscess formation in an animal model of intraabdominal sepsis (1,2). This capsular antigen has been detected on fresh clinical isolates by both morphologic and immunochemical techniques (3,4). In the process of purifying this capsule, considerable lot to lot variation was noted in the carbohydrate composition. This study attempts to define this variation in capsular material. We have shown that after sequential in vitro passage of B. fragilis on blood agar plates, a colonial variant emerged from which an outer membrane associated glucan can be isolated. The glucan has a glycogen-like structure and its appearance on the outer membrane correlates with the colonial morphological transformation. There appears to be no concomitant alteration in outer membrane proteins or lipopolysaccharides but less capsular material is isolated and identified by electron microscopy after in vitro passage.

An original subculture of strain 23745, obtained from the ATCC was serially transferred on blood agar plates in gas-pak jars for 11 consecutive passages. Isolates from each passage were cultivated in 16 liter quantities and outer membranes extracted. The capsular antigen was purified from the membrane preparation by methods described previously (3,5-6).

It was found that capsular antigen purified from organisms grown from the first to the third passage of this strain had a low glucose content (Table 1) and a single peak eluted at 520 milliosmoles of buffer when chromatographed on DEAE Sephacel.

Table 1

Carbohydrate composition of the capsular antigen extract from high and low passages of B. fragilis strain 23745 prior to ion exchange chromatography

sugars	percent of carbohydrate low passage	high passage
incompletely identified early peaks*	56%	7%
galactose	30%	7%
glucose	8%	85%
glucosamine	3%	.1%

* relative retention times of TMS derivates compared to glucose < .81 but include fucose, mannose, and arabinose

On the fourth passage, the relative amount of glucose recovered began increasing, and by the sixth passage, 85% of the carbohydrate in a 1 mg sample was glucose. Concomitant with this qualitative change in carbohydrate composition, an increase in the total yield of carbohydrates from 1.7 mg to 1.5 mg per liter of broth was seen. However, in terms of relative serologic activity, the antigen isolated from low passage subcultures gave a capsule specific immunoprecipitins at 1 µg/mg in agar double diffusion, while the fraction with the high glucose content isolated from high passage subcultures reacted identically but at 10 µg/ml. No additional precipitin lines were seen in the high glucose preparation. These observations led us to believe that as the strain of B. fragilis was serially passed, we were isolating a non-serologically active constituent consisting primarily of glucose.

the non-serologically reactive glucose rich fraction was separable from serologically active capsular antigen by ion exchange chromatography on DEAE Sephacel (Fig. 1).

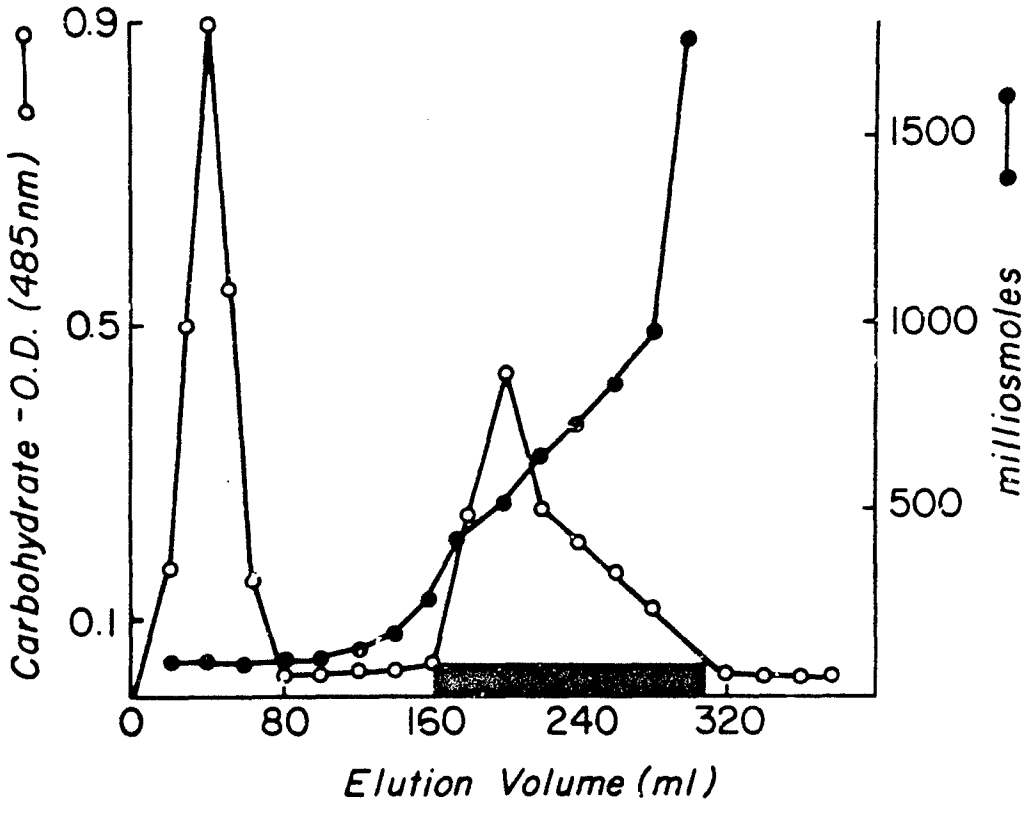

Figure 1. Column chromatography on DEAE-Sephacel (Pharmacia) of the capsular antigen extracted from B. fragilis strain 23745 which had been passed 10 times on blood agar plates. The glycogen-like material elutes at the void volume while the serologically active capsular antigen elutes at 520 milliosmoles of NaCl. 0-0-0 represents carbohydrate content, •-•-• milliosmoles of buffer, formed precipitins in agar gel diffusion with antiserum made to the capsular antigen (▬).

The glucose rich component was not retained by this column, while the serologically active fractions eluted at 520 milliosmoles of buffer. These latter fractions had a carbohydrate composition similar to that observed from the capsular polysaccharide of the earliest passages of strain 23745 (Table 1). The glucose rich fraction was subjected to methylation analysis (7): 2,3,4,6-tetra-, 2,3,6-tri-, and 2,3,-di-O-methyl-glucose were obtained in the approxiamtive ratio 2:4:1. These data suggest that the glucose rich fraction is a glucan with a glycogen-like structure.

Interestingly, cold 10% TCA (8) extraction of bacterial cells from early passages of strain 23745 and subsequent purification of the TCA soluble fraction of Sepharose 4B, also yielded a glycogen-like material. However, as indicated above, this material is not associated with the outer membrane in these early passage cells. This suggests that this material exsits inside the cell prior to *in vitro* passage and becomes associated with the outer membrane during subsequent passage.

Studies were done to determine whether this phenotypic change in the outer membrane was reversible. A later passage isolate (8th passage) was implanted into the rat peritoneum in the model of intraabdominal abscess formation (9). One week after implantation, the *B. fragilis* strain was reisolated from abscesses which were indistinguishable from abscesses formed using LCT alone. However, in contrast to the single colony type isolated from LCT implants, two colony types were noted on primary isolation of the abscess contents from SCT recipients (Fig. 2).

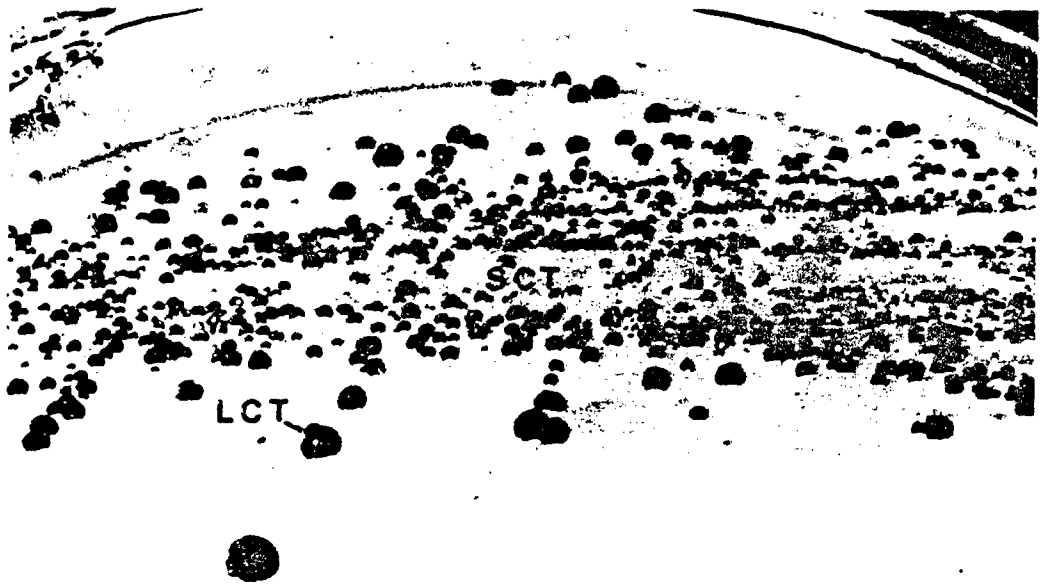

Figure 2. Two colonial morphotypes found in primary subculture of intra-abdominal abscess in a Wistar rat. The abscess had been induced by B. fragilis strain 23745 passed 10 times on blood agar plates. LCT - large colony type; SCT - small colony type.

These were a large smooth, rather mucoid appearing colony (LCT) and a small translucent appearing type (SCT). Several subcultures of both colony types were verified by biochemical reactions and GLC analysis of short chain fatty acids (10) as B. fragilis. After a second passage of the SCT organisms in animals, only LCT colony types were recovered.

Representative LCT and SCT colonies were each grown in 20 liter quantities, outer membranes extracted, and the capsular material purified. The capsule of the LCT's had a carbohydrate composition typical of the low glucose variety while the SCT's yielded primarily the glycogen-like material from their outer surface (although small amounts of the capsular antigen could be detected).

This phenotypic variation was limited to the addition of a glycogen-like material; other outer membrane associated antigens which were assayed did not vary. Thus, the carbohydrate composition of the LPS from both types were found to be similar (Table 2),

Table 2

Carbohydrate Content of the Lipopolysaccharide Isolated from the Two Colonial Variants of *Bacteroides fragilis*

	Large Colony Type (LCT)	Small Colony Type (SCT)
Galactose	57%*	58%
Glucose	15%	12%
Glucosamine	11%	14%
Fucose	6%	7%
Mannose	8%	9%

* Percent of total carbohydrates

containing roughly equimolar amounts of galactose, glucose, glucosamine, fucose and mannose. Furthermore, similar fatty acids were found in the LPS of both types.

Outer membrane protein band patterns from the two colony types were studied by SDS-PAGE (11) and the molecular weight of the individual peptides were found to be essentially identical (Fig. 3),

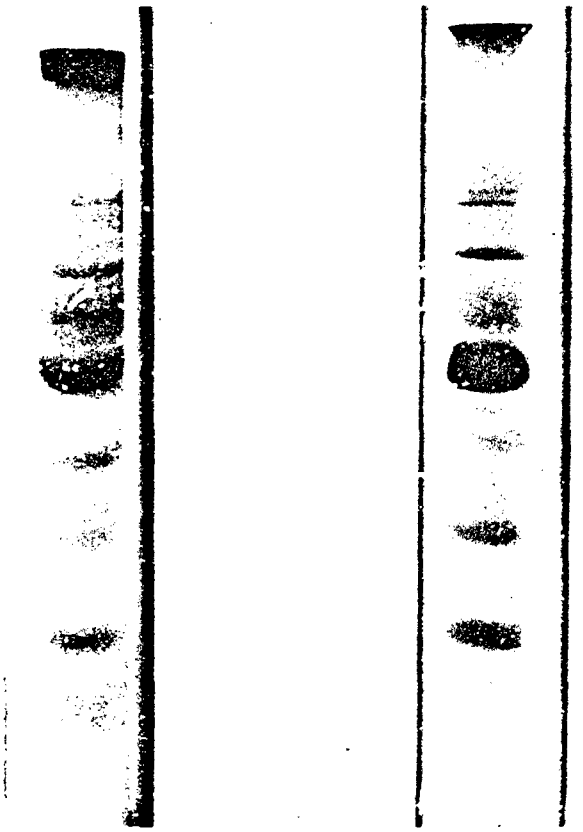

Figure 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the outer membrane proteins of B. fragilis strain 23745 of each colonial variety. Left - large colony type; right - small colony type.

although quantitatively some differences may exist.

These SCT strains were still fluorescence-positive in the IFA test using capsular antiserum (4). Therefore, the major outer membrane structural variation between the two colony types would appear to be that the SCT has relatively less capsular antigen and contain an abundance of the glycogen-like material.

The LCT's and SCT's were studied by electron microscopy (4,12) to morphologically define possible structural changes between the two colony types. In the LCT organisms a very dense layer was seen surrounding the cells when staining is done with ruthenium red (Fig. 4).

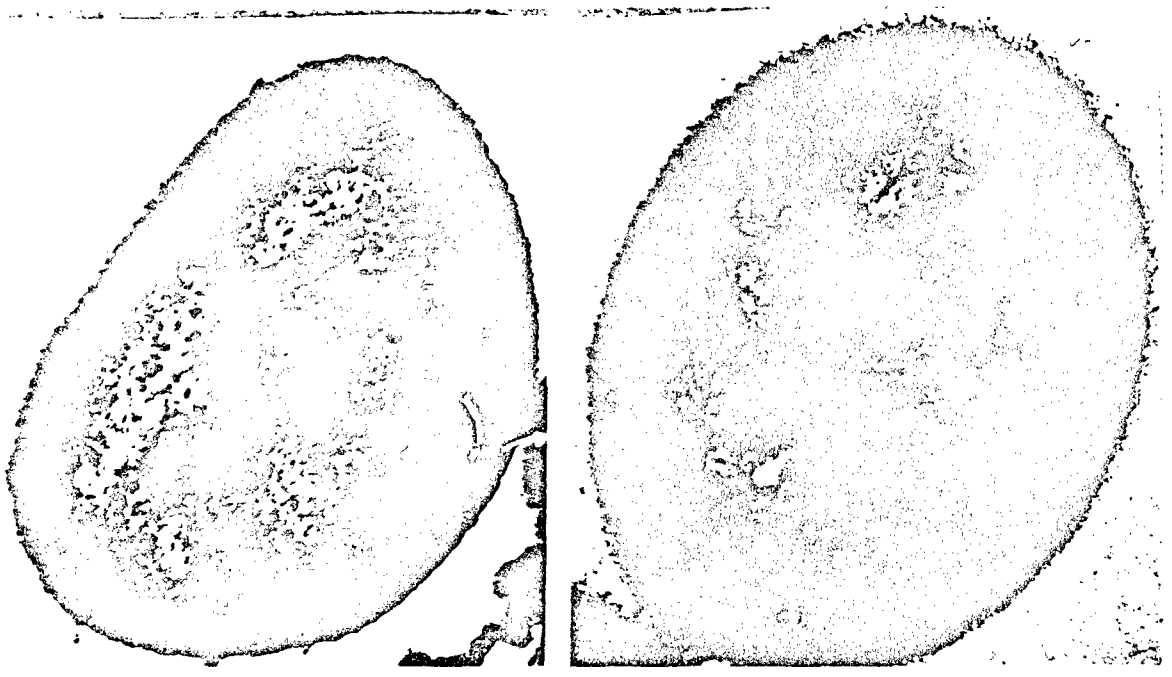

Figure 4. Electron micrograph of 2 colonial variations of B. fragilis strain 2375 stained with ruthenium red to demonstrate the surface polysaccharide. Left - large colony type with thickly stained capsule; right - small colony type.

A much less densely stained area is seen around cells of the SCT. Morphologically, these latter cells do not look significantly different than non-ruthenium red stained cells.

Both colony types were reacted with anti-capsular antibodies and stained with ferritin labelled anti-rabbit IgG. The specificity of staining with ferritin labelled anti-rabbit IgG was controlled for by reacting both colony types with normal rabbit serum.

More ferritin labelled antibodies were aggregated around the exterior of the LCT cells when compared to the SCT cells (Fig. 5).

Figure 5. Electron micrograph of 2 colonial variations of B. fragilis strain 23745 stained first with rabbit antibodies prepared to the capsular antigen and secondly with ferritin-labelled anti-rabbit IgG. Left - large colony type with large quantities of ferritin labelled antibodies aggregated on the surface; right - small colony type with only minimal amounts of ferritin on the surface.

Furthermore, only 10-15% of the SCT compared to >90% of LCT cells showed specific ferritin labelling. The normal controls of both colony types had fewer than 1% of cells with ferritin aggregation of the surface. These EM studies indicated that there is less capsular antigen being produced in the SCT cells or alternatively that access of the ferritin particles or ruthenium red to the capsule is being blocked by the nonreactive glycogen. The LCT cells consistantly have considerably more glycogen particles present in the cytoplasm than the cells of the SCT.

Significant morphologic changes have been described in several bacterial species associated with in vitro passage. For example, pneumococci are known to convert from smooth to rough colony types with a concomitant loss of capsular polysaccharide (13). Gonococci convert from the type T_1 to T_4 colony types during in vitro passage with a concomitant loss of pilation (14). For these two genera, a loss of virulence is associated with these variations in colonial morphology. We have described two colony types, LCT and SCT. The LCT appear to be isolated from clinical specimens or from infected experimental animals while the SCT appear after in vitro passage. Animal passage seems to select for the LCT. Phenotypic variations may depend on both genetic and environmental factors. It has been shown, for example, that some non mucoid Enterobacteriacae become mucoid if grown at low temperature or in media with an excess of carbon and insufficient amounts of nitrogen or phosphorus (15). Mucoid colonies such as are seen with the large colony types of B. fragilis may have an increased size because of the volume of capsular material present. This is an appealing explanation since it has been shown that there is a distinct loss in the quantity of recoverable capsular polysaccharide with in vitro passage and less capsule can be demonstrated on the surface of LCT cells by EM techniques. Although the total amount of extractable carbohydrate is greater in multiple passage isolates, the great majority is in the form of glycogen-like material.

Lindner et al (16) have shown that a glycogen with the same structure as that which we have identified during these studies can be found in cytoplasmic granules dispersed throughout B. fragilis cells. Synthesis of this glycogen appears to be dependent on the glucose concentration and pH of the medium, and the growth phase of the organisms. The data presented in this study suggests that a glycogen with structural characteristics similar to that described by Lindner et al. (16) is found associated with the outer membrane of the SCT cell, but not the LCT cell. The glycogen-like material can also be isolated from the LCT cells by a harsh extraction method with cold 10% TCA. Although it is possible that the glycogen found associated with the outer membrane from the SCT cells is merely contamination with intracellular glycogen granules, the fact that the LCT cells have more glycogen intracellularly and that the outer membrane of both colony types is extracted in identical manner, suggests that the glycogen in the SCT membrane fraction is not of intracellular origin. The genetic and/or environmental mechanism by which the glycogen-like material becomes associated with the outer membrane of strains passed in vitro is unknown at the present time. However, this appears to be a rather specific change in the outer membrane polysaccharides without any alteration in the LPS or the outer membrane proteins.

B. It is apparent from this study that it is exceedingly important not to use laboratory adapted strains for studies of B. fragilis structure or immunity to this organism. The use of cells with glycogen-like material associated with their surfaces could lead to misinformation not compatible with data obtained from fresh clinical isolates. This factor could result in a variety of interpretations regarding surface structure. In addition, the presence of this immunologically inactive glycogen-like material could well cause in vitro differences in assay systems used to measure the immunologic parameters of B. fragilis. This would be of particular concern if investigators continue to pass laboratory adapted strains in vitro and make comparisons to data generated using fresh clinical isolates. For example, conflicting in vitro data exists on the serum sensitivity of B. fragilis strains and the ability of these strains to be opsonized and killed by polymorphonuclear leukocytes (17-19). Studies by Bjornson, et al. (19) have indicated that there is no difference in the ability of B. fragilis and B. thetaiotamicron to activate the alternative pathway in vitro or to be opsonized by normal human sera. Yet there is a marked difference in the virulence of these two bacterial species (20) in vivo. However, using this in vitro assay we have shown that the SCT cells are significantly more sensitive to complement dependent opsonophagocytosis than the LCT cells.

The opsonophagocytic assay we have used in these studies is similar to that which we have used in our studies of group B streptococcus (22, 23). Reaction mixtures utilize pooled human serum as an antibody source, fresh frozen sera from an agammaglobulinemic donor as a complement source, and leukocytes obtained by dextran sedimentation. We have compared killing of the LCT and SCT both under aerobic and anaerobic conditions to determine the optimal parameters for this system (Figure 6).

AEROBIC AND ANAEROBIC NEUTROPHIL KILLING OF BACTEROIDES FRAGILIS

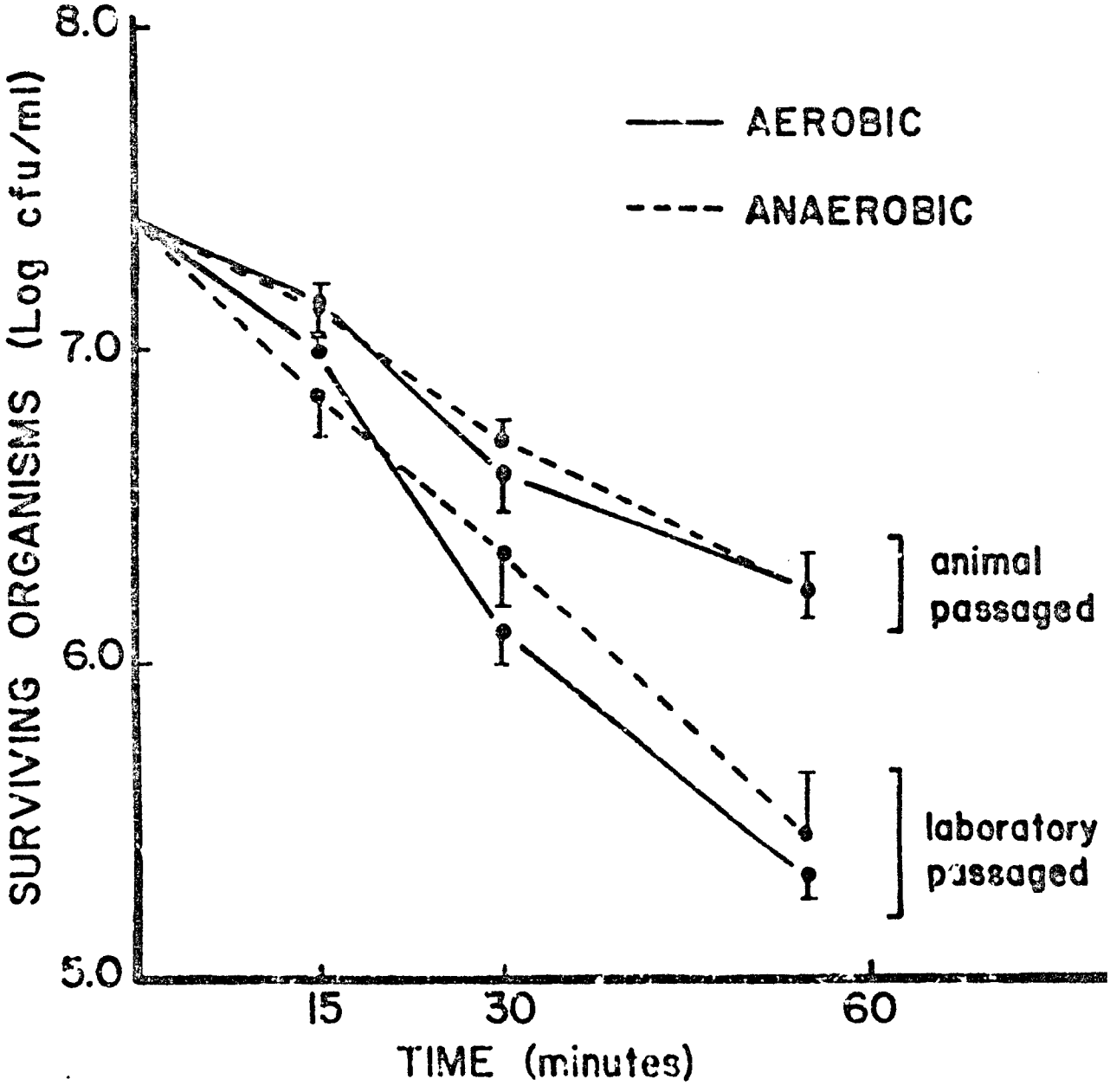

Figure C

The laboratory passed (SCT) strain is uniformly more readily opsonized and killed than the animal passed (LCT) strain, irrespective of the time of incubation. After 120 minutes incubation with leukocytes, the initial inoculum of 2.5×10^7 CFU/ml was reduced to 1.37×10^6 CFU/ml for the LCT and 1.89×10^5 CFU/ml for the SCT ($P<.01$). Controls with heat inactivated serum as the complement source showed growth to higher numbers of CFU than the initial inoculum.

A study was done to determine whether this serum resistance (of originally lab passed strains) induced by animal passage was a phenomenon unique to B. fragilis and whether Bjornson (18, 19) et al. data indicating similar susceptibility to opsonophagocytic killing of B. fragilis and B. thetaiotaomicron could be explained by their using lab passed (SCT) cells for study. A lab adapted clinical isolate of B. thetaiotaomicron was serially passed in vivo (10X) in the abscess model and the subsequent isolate compared to the original lab passed strain for suceptibility to opsonization and killing by neutrophils (Figure 7.)

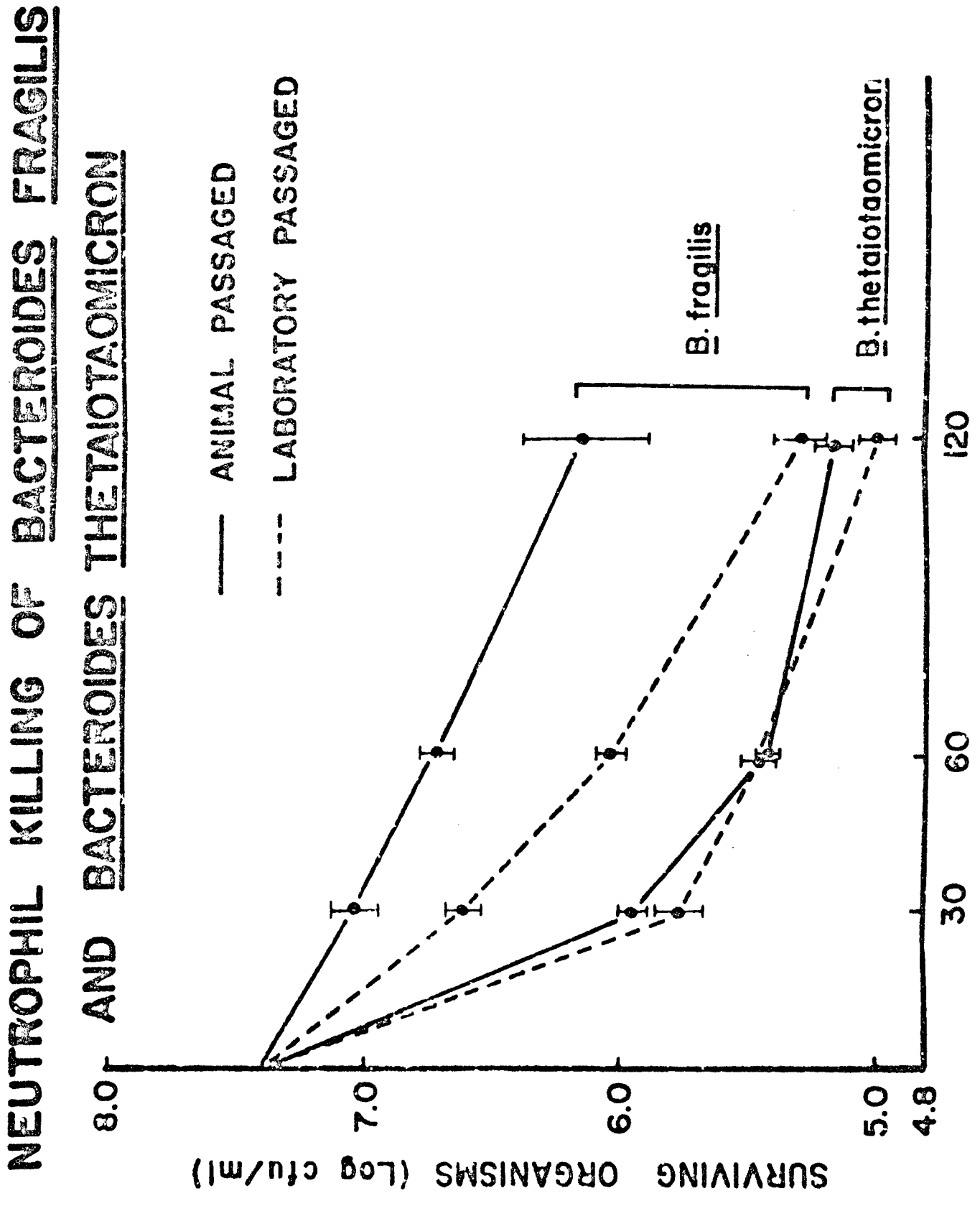
NEUTROPHIL KILLING OF BACTEROIDES FRAGILIS
AND BACTEROIDES THETAIOTAOMICRON
ANIMAL PASSAGED
LABORATORY PASSAGED
B. fragilis
B. thetaiotaomicron
SURVIVING ORGANISMS (Log cfu/ml)
8.0
7.0
6.0
5.0
4.8
30
60
120

Figure 7

The lab and animal passed B. thetaioataomicron strain remained as susceptible to killing as the SCT (lab passed) B. fragilis strain. The LCT B. fragilis was distinctly and significantly ($P<.01$) more resistant than the others. This data, combined with that presented above about structural changes with passage, suggests that in vivo passage of B. fragilis causes enhanced production of capsule and this likely accounts for the increased serum resistance. Furthermore, the data of Bjornsen et al (18, 19) most likely is due to their using a lab adapted B. fragilis isolate for comparative studies of killing to B. thetaioataomicron .

To confirm that this increased resistance of the B. fragilis animal passed (LCT) strain was likely due to increased capsular production, rabbit anticapsular antibody made to the purified antigen was added to the system (Figure 8).

INCREMENTAL KILLING DUE TO ANTICAPSULAR ANTIBODY

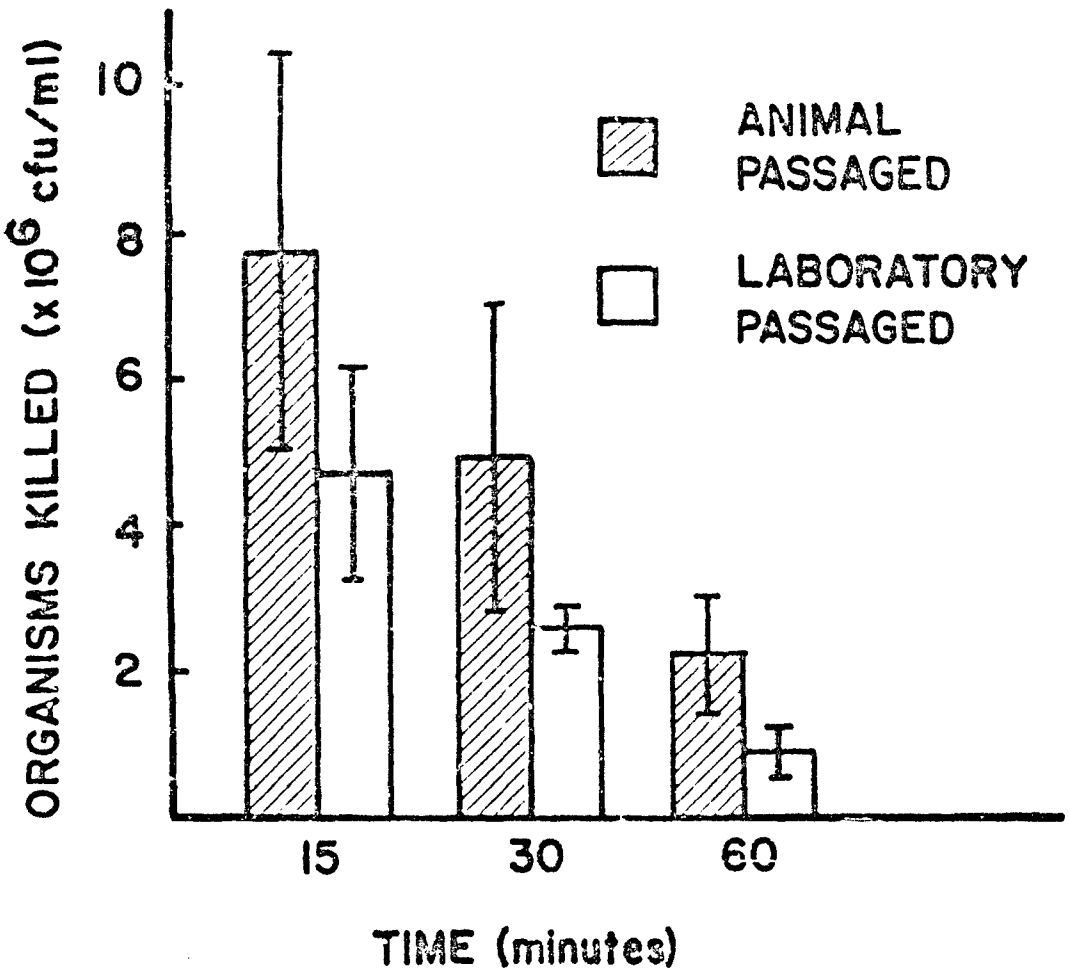

Figure 8

The number of additional animal-passed cells (LCT) killed was greater than the number of lab-passed cells (SCT). However, killing of both strains was significantly enhanced ($P<.05$). No effect was seen on killing of the B. thetaiotaomicron strain by this serum. These results suggest that with B. fragilis, the capsule contributes to its virulence by protecting the organism from neutrophil killing.

Section II

Mechanisms involved in immunity to experimental infection--role for both humoral antibody and cell mediated immunity.

The critical role played by Bacteroides fragilis in infection has been well documented (24,20). While B. fragilis is among the least common Bacteroides species in gut flora it constitutes 70-80% of Bacteroides species found in clinical isolates (20,25). The invasive potential of these bacteria clearly exceeds what would be expected by their random involvement in infectious processes.

B. fragilis possesses a unique polysaccharide capsule which has not been identified in other bacteria (1,3). The role of this capsule in the formation of intra-abdominal abscesses has been demonstrated in a rat model for the development of such abscesses (2). In this model it has been shown that B. fragilis species by themselves are capable of producing abscesses when they are the sole bacterial agents present in a fecal inoculum implanted in the abdominal cavity of rats. Other Bacteroides species require the presence of additional bacteria, such as enterococci or E. coli, before they can stimulate abscess formation (9). Furthermore a complex fecal inoculum lacking B. fragilis does not produce abscesses when implanted in the peritoneal cavity of rats but the same inoculum will cause abscess production if only the isolated capsule of B. fragilis is added to it (2). Intra-muscular immunization of rats with the capsular material from B. fragilis prevents the development of intra-abdominal abscesses after subsequent challenge with that organism (26). These studies examine the immunological basis for that protection.

The same immunization and challenge protocol was used in all experiments. Rats were immunized with three i.m. injections of 10 ug of a capsular polysaccharide preparation weekly for 3 weeks. A single booster injection was given in the 4th week and one week later the rats were either challenged or used as a source of immune spleen cells or immune serum. Bacterial challenge of both immune and non-immune rats consisted of surgical placement in the peritoneal cavity of a gelatin capsule containing sterile fecal contents, barium sulfate and 10^8 bacteria of the indicated species (29). The rats were sacrified 1 week after challenge and were examined in a blinded fashion for the presence or absence of abscesses, which were defined as loculated collections containing bacteria and polymorphonuclear leukocytes.

In this intra-abdominal abscess model, B. fragilis bacteremia occurs routinely during the first 48 hours after intra-peritoneal challenge. The ability of hyper-immune rabbit gamma globulin prepared by Cohn fractionation containing more than 50 ug/ml of B. fragilis anti-capsular antibody to protect against bacteria was studied in the first experiment. Just prior to peritoneal innoculation, groups of 3 and 4 rats received either non-immune or B. fragilis hyperimmune globulin. The rats were bled at intervals after innoculation, and the mean number of colony forming units of B. fragilis per ml. of blood was determined. At two, 8, 24, and 48 hours after challenge the level of bactermia was significantly reduced ($P<.01$) in the recipients of hyperimmune globulin (Table 3).

Table 3

Globulin protection against experimental B. Fragilis bacteremia

Hour after i.p. challenge	Mean $\log_{10}$ CFU/ml		
	Non-immune globulin	hyperimmune globuline	P
2	3.9 ± 1.9	2.9 ± 0.3	<.01
8	3.7 ± 0.1	2.0 ± 0.7	<.001
24	1.9 ± 0.5	0	<.02
48	1.3	0	<.02

In addition, by 24 hours bacteria had ceased in all of the rats receiving hyperimmune globulin, while bacteremia could still be documented in the recipients of non-immune globulin 48 hours after inoculation.

The efficacy of this same hyperimmune globulin in protection against abscess formation was evaluated in an experiment comparing the development of abscesses in rats receiving the hyperimmune globulin versus the appearance of abscesses in non-immune rats. The hyperimmune globulin was again administered just prior to intra-peritoneal challenge, no protection was afforded by the globulin, as all 10 rats in that group developed abscesses, as did all 10 rats in the non-immune group.

Table 4

Globulin protection against experimental intraabdominal abscess due to B. fragilis

Immunization status	Abscess incidence
1. None	10/10
2. hyperimmune globulin to B. fragilis capsule	10/10

From these it was concluded that antibody to the B. fragilis capsular polysaccharide offers some protection against bacteremia but does not prevent the development of intra-abdominal abscesses.

Since we knew that active immunization could protect rats against abscess development (26) we examined whether we could adoptively transfer this protection with spleen cells from immune rats. Forty million viable immune or non-immune cells from inbred Wistar Lewis rats were suspended in non-supplemented Hanks balanced salt solution and injected by the intra-cardiac route into non-immune syngeneic recipients. One the day after cell transfer the recipients of the cells were challenged intra-peritoneally with sterile fecal contents plus one of two bacterial inocula, either B. fragilis alone or a combined inoculum of Fusobacterium varium and enterococcus. Protection was afforded by passively transferred immune spleen cells.

Table 5

Spleen cell protection against experimental intraabdominal abscess due to B. fragilis

Group	Immunization status	Challenge inoculum	Abscess incidence
1	None	B. fragilis	5/5
		F. varium & entero	5/5
2	Active-capsule	B. fragilis	0/5
		F. varium & entero	9/10
3	Passive-immune Spleen cells	B. fragilis	2/9
		F. varium & entero	9/10
4	Passive-non-immune spleen cells	B. fragilis	9/10
		F. varium & entero	9/10

The first group of rats received no immunization, and it was clear that unprotected rats will develop abscesses when challenged with either of these bacterial regimens. Rats actively immunized with the capsular polysaccharide of B. fragilis were protected against challenge with that organism, as seen in group 2, in which none of B. fragilis immune rats developed B. fragilis abscesses, confirming our earlier data (26). However, nine of ten rats immunized with the capsule did develop abscesses when challenged with F. varium and enterococcus. This demonstrated the specificity of the protection achieved with the B. fragilis capsular polysaccharide. In group 3 the challenged rats received spleen cells from other rats that had been immunized. The same specific protection that was provided in group 2 by active immunization was again provided by adoptively transferred immune spleen cells, which protected all but 2 of 8 recipients from abscesses induced by B. fragilis, but effectively

provided no protection against F. varium and enterococcal challenge. Animals receiving only immune spleen cells showed no rise in anticapsular antibody as measured in the radioactive antigen binding assay (26). Therefore transfer of immune B cells was not inducing antibody as the mediator of this transferred protection.

To identify the type of cell responsible for the protection passively transferred with spleen cells, we actively immunized a group of congenitally athymic rats, which lack normal T cell function, and we also actively immunized their phenotypically normal littermate rats which are heterozygous for the athymic trait.

Table 6

Role of T cell immunity in rats challenged with B. fragilis

Thymus	Immunization	Abscess incidence	B. fragilis in abscess contents
+	None	5/5	+
+	Capsule	0/5	-
-	None	4/4	+
-	Capsule	9/9	+

100% of the phenotypically normal unimmunized rats developed abscesses while the phenotypically normal immunized rats developed no abscesses. Of greatest interest, however, was the observation that despite the presence of high antibody levels as measured in the RABA (27), congenitally athymic immunized rats were not protected by immunization with the capsular poly= saccharide preparation. A hundred per cent of both the immunized and the unimmunized athymic rats developed abscesses from which B. fragilis were cultured. These data clearly implicate the T lymphocyte as the mediator of protection against abscess formation caused by B. fragilis in this model.

From these studies it is apparent that both the cellular and humoral limbs of the immune system play important roles in protection against B. fragilis infection. As would be expected antibody continues to protect against bactermia with encapsulated pyogenic bacteria. But the distinctive pathological market of B. fragilis is abscess formation and these studies indicate that only T cell mediated immunity protects against that process in this experimental model.

A role for cell-mediated immunity in protection against encapsulated bacteria has not received great attention. Because previous studies have focused on

organisms such as the pneumococcus or meningococcus for which antibody plays a critical role in the susceptibility to the disease, it has been felt that humoral immunity is of primary importance with encapsulated organisms. Unlike these other organisms, B. fragilis presents with abscess formation as the primary disease process and it was focusing on that disease manifestation that directed us to understanding the nature of the immune response that protected against it.

Whether this observation is relevant to other abscess producing organisms remains to be determined. The ability of Staphlococcus aureus to induce a cell-mediated immune response has been well documented, and it is reasonable to speculate that such immunity would be activated in the face of tissue invasion by that organism as well. These studies with B. fragilis emphasize that efforts to understand the basis for protection against bacterial pathogens must be directed not only at the composition of the immunizing bacterial antigens but also at the characteristic pathology of the infection these bacteria induce.

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