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Development of Vaccines to Prevent Wound Infections due to Anaerobic Bacteria

ANNUAL REPORT

August 1982

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Summary of annual Report

Bacteroides fragilis is the major cause of anaerobic bacterial sepsis and abscess formation. We have found that immunization of rats with a purified capsular polysaccharide (CP) of a B. fragilis strain protects against experimental bacteremia and intraabdominal abscess formation. In this report we have adopted the animal model to the mouse because the mouse host allows better immunologic and genetic definition. These studies show that active immunization of the mouse with CP affords T-cell dependent protection. The T cell involved has been shown to belong to a subset of cells bearing Ly2+ phenotype on their surface and not to be H2 restricted, therefore conforming to the description of a suppressor cell.

Immunity to abscesses induced by CP is cross-protective when another B. fragilis strain is used as the challenge organism. However, immunity to bacteremia is specific to each of these strains and not corss-protective. This indicates distinct immunodeterminants are involved in stimulating immunity directed by B cells and T cells. Preliminary studies to define the mechanism of T-cell dependent immunity suggest that the cell required to kill B. fragilis in vitro is an immune T lymphocyte which acts in the presence of a heat-labile serum factor, presumably complement.

We have described an in vitro opsonophagocytic assay utilizing either human PMN or mononuclear cells for killing and found that human mononuclear cells require a significantly longer time to kill B. fragilis than PMN. Mouse intraperitoneal macrophages and PMN, in contrast were unable to act in a similar fashion.

Finally, we have studied mechanisms of synergy between enterococcus and Bacteroides distasonis in causing abscesses. Contrary to the theories of others, the facultative species is not contributing to an anaerobic microenvironment, but rather a cellular component is the synergistic effector. This component is sloughed by growing enterococci into the culture supernate and can easily be isolated.

I. Cellular Immunity in the Mouse Model

A. Background

During the past year, our studies have been designed primarily to define the mechanisms by which protection is afforded to animals after immunization with the capsular polysaccharide (CP) of B. fragilis. The decision to proceed along these lines, rather than to pursue vaccine trials, was based upon the recommendation of the site visit committee which reviewed our contract last fall.

As presented in last year's report, it had been shown that active immunization of rats with the capsular polysaccharide of strain 23745 of Bacteroides fragilis protected rats against abscess development following intraperitoneal challenge with this species (1). Passive transfer of hyperimmune globulin from immunized animals to non-immune recipients provided protection against B. fragilis bacteremia in challenged animals, but did not confer protection against abscess development. On the other hand, adoptive transfer of spleen cells from immunized animals to non-immunized recipients resulted in protection against abscesses following challenge with B. fragilis (2). These data suggested that a T-cell dependent immune response was involved in protection against abscess development after immunization with B. fragilis capsular antigen.

To determine the possible role of cell-mediated immunity prompted by the capsular antigen, inbred, congenitally athymic OLA/Rnu rats and their phenotypically normal littermates were actively immunized. Despite the development of high titers of anti-B. fragilis capsular antibody, 100% of actively immunized athymic rats developed abscesses, as did 100% of non-immunized athymic control rats. However, no phenotypically normal littermate control rats that were actively immunized developed abscesses, while 100% of phenotypically normal unimmunized rats developed abscesses. Additional studies showed that adoptive transfer of T cell-enriched spleen cell preparations from Wistar/Lewis rats immunized with the capsular polysaccharide to non-immune recipients also resulted in protection against B. fragilis induced abscesses (2).

We concluded that the protection afforded by immunization with B. fragilis capsule against intraabdominal abscesses caused by that organism was T cell-mediated and did not require the presence of serum antibody.

B. Adoption to Mouse Model

In last year's report, we demonstrated that it was feasible to use a murine model to study B. fragilis infection. During the current year, this model has been utilized to define better our understanding of immunity to this organism. This model had several features that made it preferable to other previously described model systems. The rat model of intraabdominal sepsis had the disadvantage of requiring surgical implantation of the inoculum, and both a gelatin capsule and $BaSO_4$ were required in order to obtain uniform abscess development (3). The murine model described by Joiner et al. involved subcutaneous implantation of the inoculum, resulting in subcutaneous abscesses at the site of inoculaion (4). The mouse model more closely paralleled disease in humans because no vehicle was required, and the result was intraperitoneal abscess formation. The use of sterile cecal contents provided a medium similar to that encountered in naturally occurring intraabdominal sepsis. Another advantage of the mouse model is that the immunogenetics of responsiveness and protection

can be studied in much greater depth than in the rat.

For the studies outlined below (5), 10^6 colony-forming units (CFU) of viable B. fragilis (ATCC 23745) were mixed 50:50 vol/vol with sterile cecal contents from meat-fed rats, and injected intraperitoneally into mice in a volume of 0.1 cc. In the specificity control, 5 x 10^7 CFU F. varium (TVDL3) and 5 x 10^7 CFU enterococcus (TVDL41) were mixed with sterile cecal contents and injected in a similar manner. The larger inoculum of these bacteria was required to produce abscesses. Mice were challenged 24 h after cell transfers.

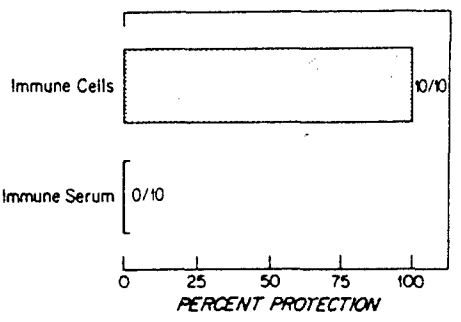
All animals were necropsied in a blinded fashion 6 d after bacterial challenge. Animals were considered to have an intraabdominal abscess if one or more loculated collections were identified containing a grossly purulent exudate that showed polymorphonuclear leukocytes and bacteria on gram stain. Each abscess was cultured to confirm the presence of the appropriate bacterial species and to ensure that contamination had not occurred. Statistical comparisons of the various groups were made by χ^2 analysis. Data are expressed as: percent protection = 100 x (1 - number with abscesses/number in group). Thus, percent protection refers to the fraction of mice that had no abscesses. All experiments were performed at least twice.

C. Effect of Immunization with CP on Abscess Formation in Mice.

C57BL/10 mice were immunized with 10 ug CP (ATCC 23745) without adjuvants three times a week for 3 wks, and weekly boosters were given beginning at week 5. Immune mice and normal controls were challenged with 10^6 CFU live B. fragilis and sterile cecal contents, and necropsied 7 d later. Abcesses were found only in 2 of 10 immunized mice as compared with 9 of 9 normal controls ($P<0.001$). This indicates that immunization with polysaccharide confers protection against abscess formation in the mouse as well as the rat.

D. Passive Transfer of Immune Cells.

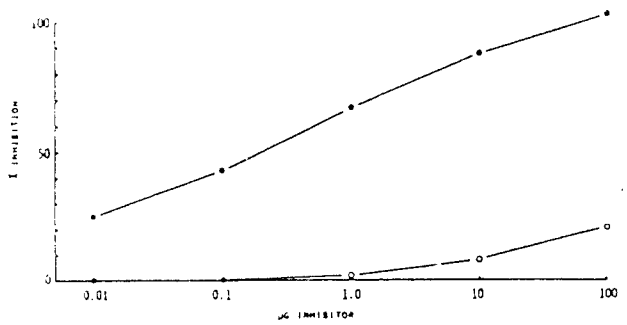
To determine the responsible agent for protection against abscess formation, serum or cells were transferred from immune animals into naive recipients. Mice received either 2.5 x 10^6 spleen cells or 0.2 ml of serum from CP-immunized mice by intracardiac injection. Immune cells were protective in 10 of 10 mice (Fig. 1), whereas immune serum was not (0 of 10 mice; $P<0.001$). As expected, anti-CP antibody titers were higher in the mice that received serum (data not shown).



E. Antigen Specificity of Immune Cells.

Mice received 2.5×10^6 spleen cells from immune donors or normal controls, and were challenged with either B. fragilis or a mixture of F. varium/enterococcus (both bacteria are required for abcess formation). Immune cells protected against B. fragilis abscesses ($P<0.01$) but not those caused by a combination of F. varium/enterococcus ($P>0.1$).

In last years annual report we described chemical analyses of two distinct capsules isolated from two different B. fragilis strains (ATCC 23745 and NCTC 9343). These are both complex carbohydrates, with one (23745) containing nine monosaccharides and the other (9343) containing six sugars. Immunologic data indicate that these two polysaccharides are distinct. Using antiserum prepared by immunization of different rabbits with each polysaccharide, an ELISA inhibition test was performed, the results of which are shown in this figure.



The ELISA reaction between 23745 CP and the antiserum prepared to 23745 CP was inhibited by prior incubation of the antiserum with homologous CP at several concentrations, but not inhibited by incubation with the 9343 CP. The converse experiment also confirmed the immunologic uniqueness of each CP. The serologic uniqueness of each CP led us to wonder why we had seen protection to intraabdominal abcesses inn rats immunized with 23745 CP despite challenge with several B. fragilis strains. Therefore, we conducted a cross protection experiment, immunizing groups of 10 animals with each polysaccharide and challenging with the homogolous and the heterologous strain:

	Organism Challenged (% protection from abscesses)	
Immunized with CP of Strain	23745	9343
23745	9/10	9/10
9343	9/10	9/10

Surprisingly, despite the lack of serologic relatedness of these two CP, they must share a common site for stimulation of T cell dependent immunity. This finding is certainly possible given the chemical complexity of these two CP

and the fact that a number of monosaccharides are common to both.

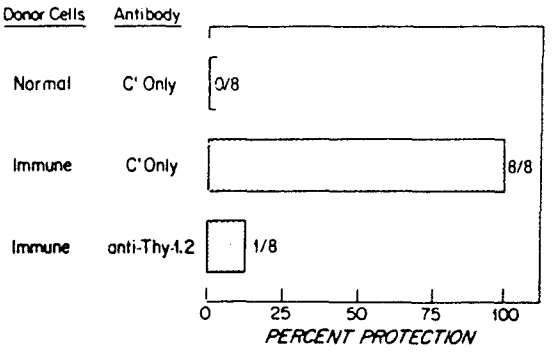
To define further these interesting results, we studied cross-protection of these two polysaccharides in the B. fragilis bacteremia model in rats which we have previously shown to be antibody-dependent (1). Following an intraperitoneal challenge of 5×10^7 B. fragilis, we have shown that 100% of non-immunized animals develop bacteremia. Prior immunization of these animals significantly decreases the degree of bacteremia (CFU/ml) and its duration. A cross protection experiment, similar to that described above for abscess formation, was conducted utilizing bacteremia as the endpoint. Each group contained five rats. Control groups of animals were challenged with each organism but not immunized.

	Organism challenge following immunization with 23745 CP		Organism challenge (animals not immunized)	
Time	23745	9343	23745	9343
2 h	2.73 ± .56*	4.14 ± .33	4.18 ± .25	3.84 ± .35
4 h	1.73 ± .56	4.19 ± .21	4.00 ± .06	4.08 ± .09
8 h	1.24 ± .52	3.65 ± .29	2.09 ± .01	3.55 ± .32
24 h	undetectable	undetectable	.68 ± .10	.20 ± .12
48 h	undetectable	undetectable	undetectable	undetectable

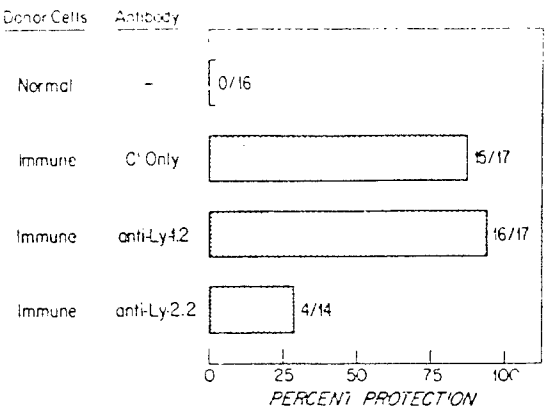
* Quantitative blood cultures reported as log CFU/ml

This experiment, in contrast to the abscess cross protection experiment, demonstrated serological specificity of protection against bactermia. These data, along with our earlier demonstration of the ability of immune globulin to protect against bacteremia, but not abscesses, further support our contention that distinct antigenic sites are responsible for inducing immunity based on B cells for bacteremia and T cells for abscesses. This information also emphasizes the necessity of pursuing an in vitro assay for protection dependent upon T cells in contrast to simple antibody determinations to examine protection against abscesses.

F. Surface Antigen Phenotyping of Immune Cells. Immune spleen cells were treated with anti-Thy-1.2 plus complement to deplete T cells prior to transfer. Anti-Thy-1.2 completely abolished the immune response ($P<0.001$), whereas complement alone had no further effect on the cells treated, identifying the immune cell as a T cell (5).

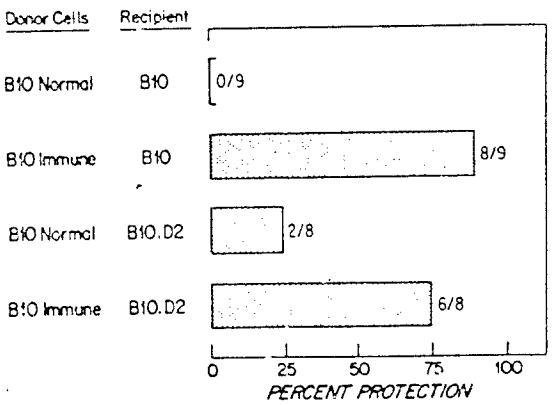


To further identify the cell, its Ly phenotype was also determined.



Pretreatment of immune spleen cells with anti-Ly-1.2 before transfer had no effect on the ability of the cell to confer immunity to abscesses. Anti-Ly-2.2 treatment abrogated the response ($P<0.001$). Thus, the immune cell is Ly-1^-2^+. The efficacy of the anti-Ly-1.2 plus complement treatment was confirmed by the elimination of an allogeneic proliferative response. Treatment with anti-Ly-2.2 did not affect the allogeneic proliferative response, but did prevent an allogeneic cytotoxic response (5). At the time of necropsy, animals from all groups had equivalent amounts of anti-CP antibody (data not shown).

G. H-2 Restriction of Immune Cells. 2.5×10^6 immune cells from C57BL/10J($H\text{-}2^b$) mice were transferred into syngeneic or allogeneic (B10.D2, $H\text{-}2^d$) mice.



Immune cells were significantly effective ($P<0.05$) in preventing abscess formation in allogeneic as well as in syngeneic mice. Similar results were obtained with transfers into B10.BR ($H\text{-}2^k$) mice. These results indicate that the protective action of the immune T cells is not H-2 restricted.

Bacterial polysaccharides have classically been thought of as T-independent

(TI) antigens. These antigens have been considered capable of generating antibody responses (primarily IgM in mice) in the absence of T cells and to be poor inducers of immunologic memory (6). In this sense the B. fragilis CP is a TI antigen, and antibody responses can be obtained using nude rats and nude mice. Studies of the type III pneumococcal polysaccharide by Baker et al. (7,8) and Braley-Mullen (9,10) have demonstrated that these putative TI responses can be amplified or suppessed by appropriately primed T cells. Recent work by Mond et al. (6) and Letvin et al. (11) has demonstrated a T cell requirement for the plaque-forming cell response to trinitrophenyl-Ficoll, another TI antigen. These authors suggest that helper T cells may be relevant to certain TI antigen responses in much the same way that T-dependent responses are controlled.

The T cell modulation of TI antigen responses reported by others have dealt with modification of B cell responses as measured by plaque-forming cell assays. Our model is entirely different. We have shown that T cells are capable of preventing abdominal abscesses. Cellular interactions relevant to abscess induction have not been adequately defined. At the cellular level, histological examination has revealed that abscesses are collections of PMN, macrophages, and bacteria with varying amounts of necrotic debris. Early in abscess formation (19 h), the PMN is the predominant cell type, whereas later on (>93 h) the macrophage dominates. Lymphocytes do not appear to account for >2-3% of the cells in the abscess at any time (5).

It is not possible at this time to assign the T cell(s) responsible for immunity to abscesses to any particular previously defined subset. Our data indicate that antigen-specific T cells are involved in the control of abscesses. The antigen-specific cell, however, is not H-2 restricted and is $Ly\text{-}1^{-}2^{+}$. Clearly, the cell is not a conventional cytotoxic or helper T cell. Both helper and cytotoxic cells act in an H-2-restricted manner. Cytotoxic cells are generally active against intracellular organisms, but not against soluble antigens or free bacteria. One might speculate that the immune T cell population is composed of suppressor cells.

A recent report suggests that T lymphocytes play a role in the defense against B. fragilis in humans (12). In a series of patients who underwent appendectomy while immunosupressed for renal transplantation, four patients developed B. fragilis bacteremia, and two had intraabdominal abscesses from which only B. fragilis was cultured. All patients who received anti-thymocyte globulin and had appendectomies developed B. fragilis bacteremia. Thus, T cells may be involved in immunity to B. fragilis in humans.

Although abscess formation is usually regarded as a host defense mechanism, it can also be regarded as a granulocyte failure that favors the persistence of bacteria. Thus, another possible interpretation of these data is that T cells prevent the bacteria from becoming established at all. This would explain the ability of the T cells to prevent abscess formation by implying that an effector T cell is active against bacteria.

II. In vitro Characterization of Mechanism of T-cell Dependent Protection.

In vitro studies of mechanisms of immune defense against B. fragilis strains have dealt only with serum factors, polymorphonuclear leukocytes (PMN) or a combination (13,14). These studies which have been performed by several groups have demonstrated a requirement for complement, antibody and PMN for killing of most Bacteroides strains, although killing of an occasional strain doesn't require the PMN. Therefore, most Bacteroides strains can be classified as serum rsistant. Notably lacking in all prior studies has been an investigation of mononuclear cells and their interactions with Bacteroides. This is of particular importance because one could easily view an abscess as a "poly failure", since the hallmark of abscess contents is numerous PMN and viable extracellular bacteria.

In prior polysaccharide vaccine studies, the measure of protection has always been antibody. As indicated above, we have shown that this is an inadequate measure of immunity to abscess formation. It became evident during discussions with site visit members last fall that without an in vitro assay for cellular immunity, vaccine efficacy could not be estimated. After approval by Dr. Gerald Sadoff, the project coordinator, we decided to delay vaccine studies until an in vitro assay could be established.

Our initial studies were designed to examine the conditions required for PMN killing of B. fragilis. Although some investigators have argued that anaerobic conditions are required for optimal killing, our own experience, as well as that of others, is that aerobic or anaerobic conditions do not affect killing (15). Therefore, tubes for killing experiments were held under aerobic conditions. Initial growth of organisms to log phase and plates for colony counts were incubated under anaerobic conditions. An overnight plate of strain 23745 (from stock culture frozen at -80°C) was swabbed and inoculated into 1 cc sterile saline. This suspension was transferred to a tube containing 10 cc brain heart infusion media supplemented with 1% yeast extract (BHI-S) prereduced with cysteine hydrochloride. Growth was monitored by optical density. The organism suspension was diluted 1:100 in Hank's Balanced Salt Solution supplemented with 1% gelatin and 2% BSA (GHBSS) to give a concentration of approximately 1 x 10^7 CFU/ml. Venous blood from a human volunteer was drawn and separated into a PMN-rich fraction by a dextran sedimentation protocol previously described (16). The final cell pellet was brought up in GHBSS to a concentration of 1 x 10^7 cells/ml (counts performed by trypan blue exclusion). Human serum was prepared for use as a complement source by well-established methods and frozen in small aliquots at -80°C thawed immediately prior to addition to the assay tubes. For some tests immune rabbit serum was added. The above ingredients were added in .1 cc volumes to polypropylene tubes and incubated at 37°C in a Fisher rotorack. Samples for colony counts were plated after initial dilution in distilled H_2O to lyse phagocytic cells. Quantitative colony counts were performed from blood agar plates incubated for 48 h in anaerobic gas pack jar at 37°C. Control tubes with constituents incubated with organisms separately or in combination were always prepared concomitantly.

A 1.5 to 2 log reduction in organism count was demonstrated after 2 and 4 h incubation using human PMN and fresh serum. The addition of rabbit antibody did not increase killing. Heat-inactivation of serum at 56°C for 30 min eliminated killing, and tubes with PMN plus organisms alone did not show a reduction in CFU. This killing by human PMN was then compared to killing by

human monocytes in this same system. For these experiments a mononuclear cell fraction (monos) was obtained from human venous blood by separation on a Ficoll-Hypaque gradient with subsequent processing of the remaining pellet by dextran sedimentation. After 2 to 4 h of incubation with monos, no killing was observed. The incubation was extended to 24 hr at which time a 1 to 1.5 log reduction occurred. Appropriate controls showed that this was specific complement dependent, monocyte-mediated killing. Similar experiments were then conducted using mouse peritoneal PMN obtained by lavage 24 h later or macrophages obtained 48-72 h after challenge with thioglycollate (17). In contrast to both PMN and mononuclear human cell fractions, neither type of mouse cell produced killing alone, with human serum, or with serum and specific rabbit antibody. This result raises several questions regarding the source and methods utilized to obtain cells for tests of in vitro killing. In addition, these findings raise the issue of how B. fragilis are eliminated intraperitoneally in immunized animals.

Since it has been established that immunity to abscess formation is T cell dependent, spleen cells from immunized and non-immunized mice were also utilized in the same type of opsonophagocytic assay. These cells, separated from other spleen cells on nylon wool columns as previously described (18), were tested alone, with human serum, and with various effector cells (mouse peritoneal PMN and MACS) to look for killing. Preliminary observations yielded most unexpected results which require further confirmation and explanation. The mixture of immune T cells with either effector cell type, with or without human serum, yielded no killing. However, the combination of immune T cells and complement demonstrated approximately 1 log reduction at 24 hrs with appropriate control tubes (such as non-immune T cells and complement) showing no killing. The explanation of this observation will require much further study (see proposal).

III. Studies of Mechanisms of Synergy in Intraabdominal Formation Between Bacteroides Species and Enterococcus.

Earlier studies in the rat model of intraabdominal sepsis demonstrated that although B. fragilis alone could induce intraabdominal abscesses, a combination with a facultative aerobe was required for other Bateroides species to cause abscesses. We have begun to study the mechanism by which this synergy occurs. A prominent theory (19) has been that the facultative bacteria produces a reduced microenvironment which promotes the ability of the Bacteroides species to cause abscesses. To investigate this question, groups of mice were challenged intraperitoneally with B. distasonis (ATCC 8503) and an enterococcus (2998T) strain isolated from a patient's urine. A dose response curve for each organism was determined. The optimal combination required for abscess formation was between 10^6 and 10^7 CFU/ml for each organism. Subsequently groups of animals were challenged with viable inocula of each species in combination with heat-killed (80 C for 60 min) organisms of the other species.

Group	Challenge Organism B. distasonis	Enterococcus	Number with Abscesses/Total
A	10^7	10^7	8/10
B	10^7	10^7 (heat-killed)	8/10
C	10^7 (heat-killed)	10^7	2/9
D	2 x 10^6 (heat-killed)	2 x 10^6 (heat-killed)	0/5

This experiment disproves the hypothesis suggested by others that the facultative species produces anaerobiasis. It suggests that a heat-stable organism constituent is donated by the enterococcus which is utilized by the Bacteroides to produce abscesses. This effect could either be on the bacterial cell or on the host.

In a subsequent experiment, enterococci were grown in a dialyzable complex media described by Carey et al. and Terleckyj et al (20,21). After removal of the cells, the culture supernate was dialyzed to remove media constituents and the residual bacterial components were lyophilized. This crude material released by the cells during growth was able to facilitate abscess formation in a dose-dependent fashion when combined with B. distasonis.

Group	Challenge Organisms B. distasonis	Enterococci	Crude Culture Supernate	Number with Abscess/Total
A	10^7	10^7	-	8/10
B	10^7	10^7 heat-killed	-	7/10
C	10^7	-	1000 ug	4/5
D	10^7	-	500 ug	4/10
E	10^7	-	100 ug	3/9

Therefore, a bacterial constituent produced by the enterococcus enables the B. distasonis strain to cause abscesses.

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