

CELLULAR IMMUNITY TO *BACTEROIDES FRAGILIS* CAPSULAR POLYSACCHARIDE*

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Bacteroides fragilis, an anaerobic bacterium that accounts for only a small fraction of the colonic microflora, is the most frequently isolated anaerobic species in many infectious processes (1), including intraabdominal sepsis (2). Strains of *B. fragilis* have a polysaccharide antigen on their surface that is not present on other *Bacteroides* species (3, 4). *B. fragilis* is unique among anaerobes in its ability to induce abscesses by itself, and in a rat model of intraabdominal sepsis, this ability has been shown to be dependent upon the presence of the polysaccharide capsule (5, 6). It has been shown that rats immunized with this capsular polysaccharide (CP)¹ are protected from intraabdominal abscess formation and bacteremia caused by this organism (7). In the rat, this protection can be passively transferred by nylon wool nonadherent spleen cells (8).

To better define the immune response to *B. fragilis*, we have developed a new experimental model for intraabdominal sepsis using the mouse. This model has several features that make it preferable to other previously described model systems (5, 9). The rat model of intraabdominal sepsis (5) has the disadvantage of requiring surgical implantation of the inoculum, and both a gelatin capsule and BaSO₄ are required in order to obtain uniform abscess development. The murine model described by Joiner et al. (9) involved subcutaneous implantation of the inoculum, resulting in subcutaneous abscesses at the site of inoculation. The model described below more closely parallels disease in humans because no vehicle is required, and the result is intraperitoneal abscess formation. The use of sterile cecal contents provides a medium similar to that encountered in naturally occurring intraabdominal sepsis.

In the studies detailed below, we have shown that mice immunized with CP are immune to the development of abscesses, and that this immunity is a function of T cells. The immune response is mediated by an antigen-specific, but non-H-2-restricted Ly-1⁻2⁺ cell. The application of this model to the study of immune responses to T-independent polysaccharides is discussed.

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¹ Abbreviations used in this paper: CFU, colony-forming unit; CP, capsular polysaccharide; PYG, peptone yeast glucose medium; TI, T independent.

Materials and Methods

Mice. C57BL/10J (H-2^b), B10.D2 (H-2^d), and B10.BR (H-2^k) mice were obtained from The Jackson Laboratory, Bar Harbor, ME, and maintained on Purina mouse chow and water ad libitum. Animals were housed in cages containing ≤ 10 animals each. Incoming mice were screened by viral serology for the presence of active murine viral infection, and were used at 8–16 wk of age.

Bacterial Strains. Cultures of *B. fragilis* (American Type Culture Collection ATCC 23745) were obtained from the stock culture collection of the Channing Laboratory, Boston, MA. Cultures of *Fusobacterium varium* (TVDL 3) and enterococcus (TVDL 41) were obtained from the stock culture collection of the Tufts University Veterinary Diagnostic Laboratory, Boston, MA. All cultures were grown in prerduced peptone yeast glucose medium (PYG; Scott Laboratories, Fiskeville, RI) within an anaerobic isolator (Labline Instruments, Melrose Park, IL) for 24–48 h. After incubation, cultures were divided into 5-ml aliquots, placed into gas-tight vials, and frozen at -70°C until used.

Immunization. The preparation of the *B. fragilis* capsular polysaccharide antigen used for those studies has been described previously (3). The antigen is free of contamination with protein or lipopolysaccharide. Amino acid analysis of the polysaccharide shows only trace quantities of all amino acids, except for alanine, which accounts for 3.8% of the antigen (D. L. Kasper, A. Weintraub, J. Lonngren, and A. A. Lindberg, manuscript in preparation).

C57BL/10J mice were immunized subcutaneously with 10 μg of CP in 0.1 ml PBS three times a week for 3 wk. The animals received a booster injection on week 5, and were available for challenge or adoptive transfer experiments on week 6.

Cell Transfers. Spleens were removed from immunized or naive mice and gently teased in L-15 medium (M. A. Bioproducts, Walkersville, MD) supplemented with 5% fetal calf serum. Spleen cells were counted using a Coulter FN counter (Coulter Electronics Inc., Hialeah, FL) and examined for viability by trypan blue exclusion. After washing, the cell density was adjusted to 12.5×10^6 cells/ml with medium and 2.5×10^6 spleen cells were transferred in a 0.2-ml vol by intracardiac injection.

Monoclonal Antibody. In some experiments, spleen cells were treated with monoclonal anti-Thy-1.2, anti-Ly-1.2, or anti-Ly-2.2 antibody (New England Nuclear, Boston, MA). The anti-Thy-1.2 was used at a 1:200 dilution, and the anti-Ly antibodies were used at 1:100. Cells were incubated in 0.5 ml of reagent per 50×10^6 cells for 45 min at 4°C . After incubation with antibody, cells were washed once and then incubated with rabbit Low-M-Tox complement (Cederlane Laboratory, Hornby, Ontario) diluted 1:6 with medium for 45 min at 37°C . After complement treatment, cells were washed once and the antibody-complement treatment was repeated a second time. The cells were then washed three times before transfer. Efficiency of antibody-complement treatment was confirmed by allogeneic proliferation and cytotoxic T lymphocyte assays. Immune cells were obtained from mice immunized for 8–12 wk.

Immune Serum Transfer. Immune mice were bled by retro-orbital puncture, and the sera were pooled. 0.2 ml of serum was transferred by intracardiac injection.

Serological Methods. Serum was obtained at the time of necropsy after bacterial challenge. Antibody to the *B. fragilis* CP was quantitated by the radioactive antigen binding assay described previously (10).

Preparation of Bacterial Challenge. Quantitative determinations of viable cell density were made by means of direct plate counts of each strain after freezing of the original culture preparations. Samples of each bacterial strain were thawed within an anaerobic chamber and

TABLE I
Protective Effect of Immunization with CP on B. fragilis Abscess Formation

Immunization status	Inoculum	Abscess incidence*
Nonimmunized control	10^6 CFU <i>B. fragilis</i> ‡	9/9
Immunized <i>B. fragilis</i> CP	10^6 CFU <i>B. fragilis</i>	2/10 ($P < 0.001$)

* Number with abscesses/number challenged.

‡ 50:50 with sterile cecal contents in 0.1-ml vol intraperitoneally.

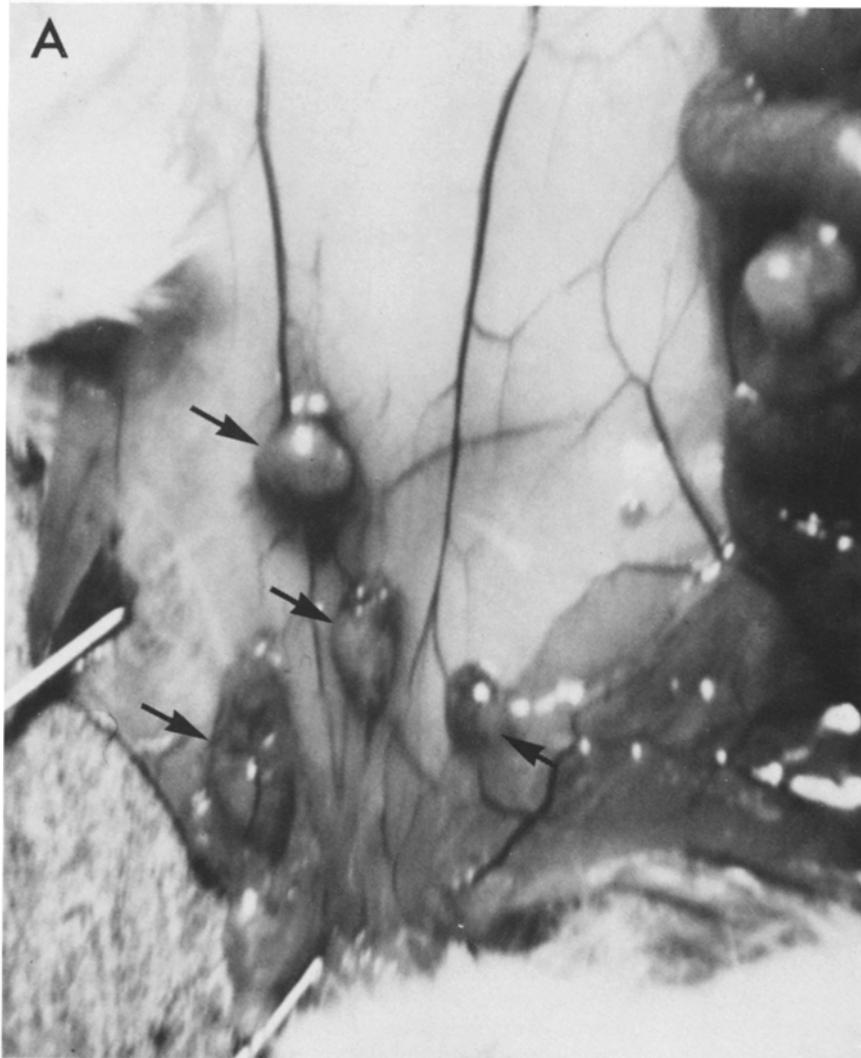


FIG. 1. Intra-abdominal abscesses in BALB/c mice. Mice were challenged with 10^6 CFU *B. fragilis* and necropsied 6 d later. Arrows identify abscesses adherent to the parietal peritoneum (A) and between intestinal loops (B).

serial 10-fold dilutions were made using sterile dilution salts (11). Duplicate 0.1-ml portions of each dilution were plated onto *Brucella*-base blood agar and allowed to dry. Plates were incubated for 48 h at 37°C within an anaerobic chamber and colonies were enumerated. Before implantation, bacterial cultures were diluted to the appropriate concentration with sterile PYG broth (12).

Bacterial Challenge. 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, 5×10^7 CFU *F. varium* (TVDL3) and 5×10^7 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.

Assessment of Results. All animals were necropsied in a blinded fashion 6 d after bacterial



FIG. 1B

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 (13). Data are expressed as: percent protection = $100 \times (1 - \text{number with abscesses}/\text{number in group})$. Thus, percent protection refers to the fraction of mice that had no abscesses. All experiments were performed at least twice.

Results

Effect of Immunization with CP on Abscess Formation in Mice. C57BL/10 mice were immunized with $10 \mu\text{g}$ CP without adjuvants three times a week for 3 wk, 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 (Table I). Fig. 1 demonstrates the typical appearance of the *B. fragilis*-induced abscesses in these mice. Abscesses were found in only 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.

Role of Cellular Immunity in Protection against Abscess. To determine what was responsible for protection against abscess formation, serum or cells were transferred from immune animals into naive recipients. Mice received either 2.5×10^6 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. 2), 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).

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 abscess formation). Immune

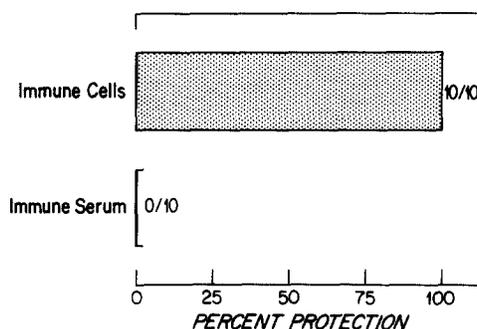


FIG. 2. Passive transfer of immunity to *B. fragilis* abscesses with serum or spleen cells from immune donors. 2.5×10^6 spleen cells or 0.2 ml of serum from immunized mice was transferred to C57BL/10 mice that were challenged 24 h later with 10^6 CFU *B. fragilis*. Necropsy was performed on day 7. The fractions indicate (number of mice without abscess)/(number of mice in group).

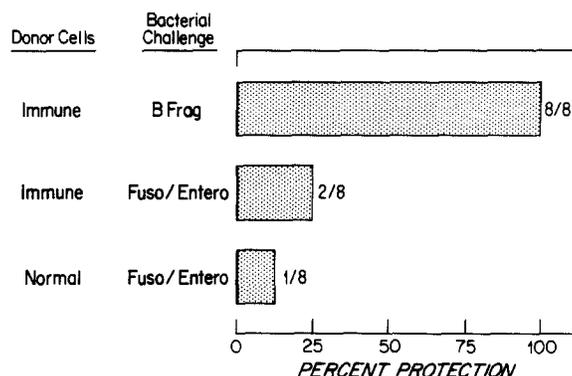


FIG. 3. Specificity of the passive protection against abscesses afforded by spleen cells from *B. fragilis* CP-immunized mice. Spleen cells were transferred as described (from either immunized or nonimmunized donors). Mice were challenged 24 h later with either 10^6 CFU *B. fragilis* or 5×10^7 CFU *F. varium* and 5×10^7 CFU enterococcus. The difference between the two fuso/entero groups is not significant ($P > 0.1$).

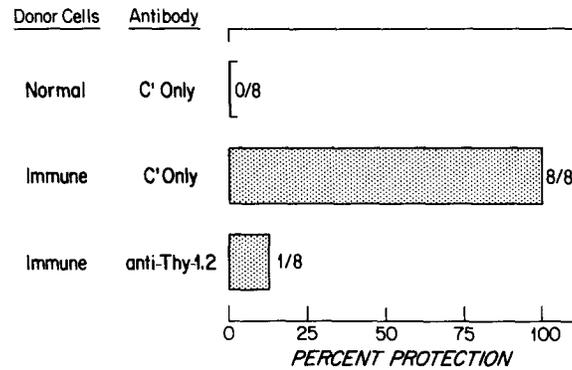


FIG. 4. Anti-Thy-1.2 plus complement treatment of immune spleen cells. Before transfer, immune cells were incubated with monoclonal anti-Thy-1.2 antibody and complement or complement alone as described.

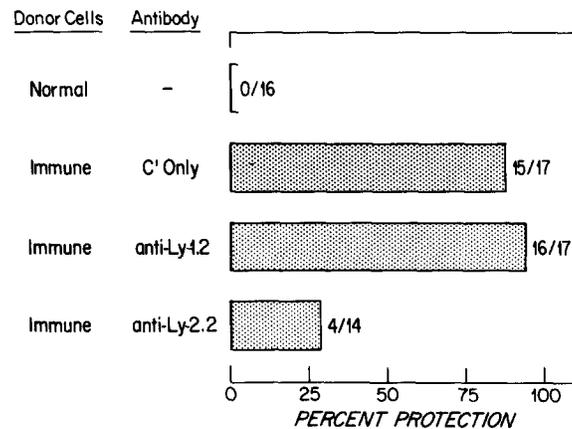


FIG. 5. Lyt phenotype of immune spleen cells. Before transfer, immune cells were treated with monoclonal Ly-1.2 plus complement, Ly-2.2 plus complement, or complement alone as described. 2.5×10^8 cells were then transferred into nonimmune recipient mice. Immune cells were from mice immunized for 8-12 wk.

cells protected against *B. fragilis* abscesses ($P < 0.01$) (Fig. 3), but not those caused by a combination of *F. varium*/enterococcus ($P > 0.1$).

Surface Antigen Phenotyping of Immune Cells. Immune spleen cells were treated with anti-Thy-1.2 plus complement before transfer to deplete T cells. Anti-Thy-1.2 completely abolished the immune response ($P < 0.001$), whereas complement alone had no effect on the cells treated (Fig. 4), identifying the immune cell as a T cell. To further identify the cell, its Ly phenotype was determined (Fig. 5). Pretreatment of immune spleen cells with anti-Ly-1.2 before transfer had no effect on the ability of the cells to prevent abscesses. Anti-Ly-2.2 treatment abrogated the response ($P < 0.01$). 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. At the time of necropsy,

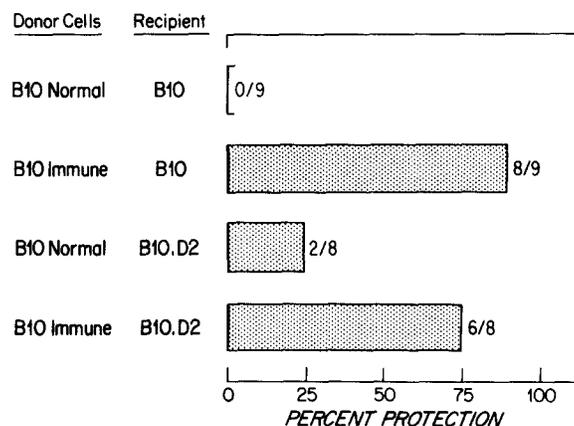


FIG. 6. Transfer of immune spleen cells into H-2 congenic mice. 2.5×10^6 immune or nonimmune C57BL/10 spleen cells were transferred into either syngeneic or B10.D2 congenic mice. Challenge with *B. fragilis* was as described.

animals from all groups had equivalent amounts of anti-CP antibody (data not shown).

H-2 Restriction of Immune Cells. 2.5×10^6 immune cells from C57BL/10J (H-2^b) mice were transferred into syngeneic or allogeneic (B10.D2, H-2^d) mice (Fig. 6). 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-2^k) mice (data not shown). Those results indicate that the protective action of the immune T cells is not H-2 restricted.

Discussion

The above experiments demonstrate the importance of cellular immunity (in vivo) to a polysaccharide antigen using a new murine model of intraabdominal sepsis. Previous studies have reported that rats immunized with the *B. fragilis* CP are protected against abscess development and bacteremia in an experimental model of intraabdominal sepsis (7). In further investigating this phenomenon, it was reported that protection against abscess formation could be passively transferred by immune spleen cells (8). Immune cells resided in the nylon wool nonadherent fraction of spleen cells, which suggested that this was a T cell-dependent phenomenon (8).

We have been able to show that mice immunized with the purified capsular polysaccharide from *B. fragilis* develop specific immunity as measured by protection against abscess formation (Table I). To examine this phenomenon in more detail, adoptive transfer experiments were used. The protection against abscesses is a cellular rather than humoral function. All animals tested develop specific anti-CP antibody during the period of bacterial challenge. Because the animals in the serum transfer group had higher antibody levels without protection against abscesses, we conclude that merely transferring helper T cells (with resultant higher antibody levels) cannot account for the protection observed. Moreover, the cell(s) involved in this immune response were identified as T cells belonging to the Ly-1⁻2⁺ subset. Thus, the responsible cell is unlikely to be a T helper cell, which would be expected to have an Ly-1⁺2⁻ phenotype.

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 (14). In this sense the *B. fragilis* CP is a TI antigen and antibody responses can be obtained using nude rats (8) and nude mice (data not shown). Studies of the type III pneumococcal polysaccharide by Baker et al. (15, 16) and Braley-Mullen (17, 18) have demonstrated that these putative TI responses can be amplified or suppressed by appropriately primed T cells. Recent work by Mond et al. (14) and Letvin et al. (19) 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 (14-19) 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. It is not known how the protective effect against *B. fragilis* abscesses is mediated, but antibody production does not appear to play a decisive role. Cellular interactions relevant to abscess induction have not been adequately defined. At the cellular level, histological examination has revealed that abscesses are collections of polymorphonuclear leukocytes, macrophages, and bacteria with varying amounts of necrotic debris. Early in abscess formation (19 h), the polymorphonuclear leukocyte is the predominant cell type, whereas later on (>93 h) the macrophage dominates (20). Lymphocytes do not appear to account for >2-3% of the cells in the abscess at any time (20). Whether lymphocytes play a role in the chemotaxis of neutrophils and macrophages to form abscesses has not been demonstrated.

What type of T cell is responsible for the immune response reported here? It is not possible at this time to assign the cell(s) 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-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. If this is so, what immune function is being suppressed? One possibility is that there is T cell control of the macrophage/neutrophil response that ultimately results in abscess formation. In this case, the transferred cells might be acting on abscess inducer T cells. Whether these putative inducer cells are stimulated by polysaccharide or protein antigens is another question that must be addressed. Recently Geha et al. (21) have described a suppressor cell defect in a human syndrome resulting in recurrent staphylococcal abscesses.

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. Such a T cell could function directly by binding or inactivating bacteria, or (more likely) by releasing a lymphokine that activates or attracts macrophages. It will require

further study to define the interactions occurring in the recipient animal to answer these questions.

A recent report suggests that T lymphocytes play a role in the defense against *B. fragilis* in humans. In a series of patients who underwent appendectomy while immunosuppressed for renal transplantation, four patients developed *B. fragilis* bacteremia, and two had intraabdominal abscesses from which only *B. fragilis* was cultured (22). 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.

Summary

The polysaccharide capsule of *Bacteroides fragilis* has been shown to be important in the virulence of the organism. The capsular polysaccharide (CP) of *B. fragilis* has been extensively purified. Using a murine model of intraabdominal abscess formation, we have been able to demonstrate cellular immunity to the capsular polysaccharide of *B. fragilis*. Immunization of C57BL/10J mice with the CP over 5 wk prevents abscess formation when the mice are challenged with *B. fragilis* intraperitoneally. This immunity can be transferred to naive mice with spleen cells from immune animals. The immune cells bear Thy-1.2 and Ly-2.2 antigens. The immune response has been shown to be antigen specific, but not H-2 restricted. The possibility that these immune cells are suppressor T cells is discussed. The experimental system presented provides a model for the examination of the cellular interactions responsible for abscess formation and the cellular response to bacterial pathogens.

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