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A Thesis Submitted to the Yale University School of Medicine in Partial Fulfillment of the Requirements for the Degree of Doctor of Medicine

by

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EFFECTS OF ADOPTIVE TRANSFER OF OSP A-SPECIFIC TH2 T CELLS ON THE EVOLUTION OF LYME BORRELIOSIS IN MICE. Paul H. Lee and Linda K. Bockenstedt. Section of Rheumatology, Department of Internal Medicine, Yale University, School of Medicine, New Haven, CT.

In many infectious diseases, the dominating effects of Th1 or Th2 T cell responses elicited by the pathogen in the host significantly alters the course of disease. In general, Th1 cells promote cell-mediated immunity whereas Th2 cells augment antibody production. Because specific antibody is important in prevention of *Borrelia burgdorferi* infection, it is surprising that only Th1-type T cells have been isolated from patients with chronic Lyme disease. We propose that the presence of Borrelia-specific Th2 cells may alter the course of Lyme borreliosis in mice. Dr. Bockenstedt's laboratory has previously defined a T cell epitope on the outer surface protein (Osp) A of *Borrelia burgdorferi*, strain N40, which elicits a Th2 response after immunization of C3H mice. In adoptive transfer experiments using T cells derived from mice immunized with the Osp A peptide or a control antigen (ovalbumin), we examined the effects of Osp A-specific Th2 cells at three critical stages of *Borrelia burgdorferi* infection: establishment of infection, disease evolution, and disease regression. The results suggest that while these cells appeared to have no effect during the initial phase of infection, Osp A-specific Th2 cells can attenuate the severity of arthritis during the disease regression phase. The accelerated disease regression occurred in the absence of specific antibody to Osp A. Possible mechanisms by which Th2 cells mediated their effects in early and late disease are explored.

Introduction

Lyme borreliosis is a tick-transmitted, multi-organ system disease caused by the spirochete *Borrelia burgdorferi*. Since the initial description in 1977 (1), Lyme borreliosis has become the most common vector-borne infection in the U.S and is a major public health concern (2). In most endemic areas of the Northeast, a significant percentage of the population have had clinical manifestations attributed to Lyme borreliosis. During the 1980's in Ipswich, Massachusetts, 35% of 190 residents living near a nature preserve had symptoms of Lyme borreliosis (3). In Fire Island, N.Y., 7.5% of the population had evidence of infection in a 5 year study (4). The major endemic areas of *B burgdorferi* in nature are the Northeast, Upper Midwest and West Coast states. This spread of disease as well as the concern over possible development of late, unremitting sequelae in patients who are not promptly treated has led to intense investigation into the early diagnosis and prevention of this disease. The investigation of the host immune response to *Borrelia burgdorferi* and spirochete evasion of host immunity has led to an experimental vaccine (5).

Clinical Manifestations in Humans.

The clinical manifestations of Lyme borreliosis can be described in three stages, similar to the staging system used in other spirochetal infections (e.g. syphilis) (6, 7).

Stage I (localized erythema migrans). Initially, the spirochetes are transmitted by the *Ixode* tick to the skin where 60-80% of Lyme borreliosis patients develop erythema migrans (EM) - often accompanied by fever and local lymphadenopathy. EM is the most specific manifestation of

Lyme borreliosis (1).

Stage II (disseminated infection). From days to weeks, the spirochetes disseminate via the bloodstream from the skin to all organs including heart, joints, and nervous system (8). During this stage, patients often have episodic manifestations of disease involving these organs but very little permanent or irreversible damage. Clinical signs include multiple annular skin lesions, migratory muscle pain, and brief attacks of oligoarticular arthritis. About 8% of patients have fluctuating atrioventricular blocks (9) and 20% of the patients develop meningoencephalitis, cranial neuritis, or peripheral radiculoneuropathy (10). Surprisingly, during this stage, spirochetes are few in number (11, 12) but elicit a vigorous immune response (6, 13). This observation has led to the assumption that it is the host immune response rather than the spirochete itself that leads to tissue damage and disease. Despite the intense immune reaction to infection, the spirochetes appear somehow to avoid elimination by the host. In fact, the immune response eventually becomes quiescent despite persistence of organism in tissues.

Stage III (chronic disease). After episodic manifestations of infection, some patients develop chronic, unremitting disease, particularly involving the joints or nervous system (14, 15). At this stage, irreversible damage may occur, resulting in permanent disability. Although the presence of spirochetes is thought to be driving the immune response even at this stage of infection, the propensity towards chronic disease may also be genetically linked to certain HLA haplotypes (15).

Host Defenses Against *Borrelia Burgdorferi*

Borrelia burgdorferi is a spirochete, similar to other *Borrelia* species in DNA homology and structure (16). It has an outer cell membrane containing many outer surface proteins (Osp) - important in host protective immunity (17, 18, 19). The abundant 31kDa Osp A and 34kDa Osp B comprise most of the outer membrane proteins in cultured North American organisms (20, 21). Beneath the outer layer, in the periplasmic space, is the 41kDa flagellin protein, necessary for motility (16).

In addition to Osps, other immunogenic proteins of the spirochete have been identified using infected human and animal sera. These include heat shock proteins, a 39KDa protein (p39) and 22kD proteins including Osp C (22, 23).

In patients with Lyme borreliosis, an IgM response develops first to flagellin, 3-6 weeks after infection, followed by IgG isotype switching (24). An early immune response has also been shown for 39kDa and 22kDa (Osp C) proteins. Chronic Lyme disease patients often develop a widening response to many other *Borrelia burgdorferi* proteins later in the course of the disease, including 60, 70, 83, 93 kDa proteins (22). Only a few patients, generally those with chronic disease, develop antibodies to Osps A and B (22). Furthermore, these Osp A and B antibodies arising in late disease are not capable of eradicating the spirochetes from infected patients. In contrast, sera (from patients with chronic disease) which contain Osp A and B antibodies have been shown to confer at least partial protection when used to immunize mice prior to challenge inoculation (25).

Mouse Model

Many animal models of Lyme borreliosis have been developed which can reproduce some of the pathology observed in human disease. The inbred weanling C3H/HeJ mouse is a particularly useful model for study of this disease because it has an intact, well-characterized immune system and reproducibly develops a syndrome resembling human Lyme disease within 2 weeks of *Borrelia burgdorferi* infection (26). N40 spirochetes inoculated into the skin of these mice disseminate via the blood to every organ, although disease is generally confined to heart and joints. Blood and spleen cultures are positive for *Borrelia* starting 3 days after intradermal inoculation and inflammation of the heart and joints appears by 7-10 days. The spirochetes are no longer detectable within the blood around 21 days after infection, a time coinciding with the onset of regression of carditis and arthritis. A subset of infected mice have recurrent spirochetemia and arthritis many months after infection, although arthritis tends to be milder and affects fewer joints (27). No immunocompetent animal model to date develops chronic unremitting disease similar to that found in patients, nor do mice develop neurologic disease.

The humoral immune response arising during experimental murine borreliosis parallels that seen in humans. Mice inoculated with less than 10^4 spirochetes seroconvert readily to flagellin, p39, and 22kDa proteins and only begin to develop Osp A and B antibodies after 6 months of infection (26).

The Role of Cellular Immune Response

Several theories have been introduced to explain this apparent lack of early response to Osps A and B in humans and mice (22): the Osp may be masked by host components which prevent an immune response until late in the illness (28), much like Schistosomula eggs, coated with host proteins, are not antigenic until they break open or die. Alternatively, Osps may undergo antigenic variation as in *Borrelia hermsii* (29) or be downregulated. A third possible explanation for the apparent lack of antibody response to Osps A and B is that the type of T cell response elicited by *B. burgderfori* early in infection favors cell-mediated over antibody -mediated effects. Similar to many diseases which have variable clinical presentations, specific T cell subtype (Th1 vs Th2) expression may retard the appearance of early borrelicidal antibodies to Osps A and B, thereby attenuating early, specific immune clearance of *Borrelia burgdorferi*. Thus, cell-mediated immunity may provide one explanation for the lack of an early antibody response to abundant protective proteins.

The cellular immune response, however, has been poorly characterized. Most of the investigations in humans of the cellular immune response have used peripheral blood mononuclear cells (PBMC) instead of specific or enriched T cells as representing cellular immune response. Also, whole spirochete or spirochetal lysates, which contain B cell mitogens, have often been used to stimulate this response. Therefore, proliferative response may be due to cell types other than specific T cells. With these limitations in mind, however, many patients have been shown to develop early and strong cellular immune reactivity to *Borrelia burgdorferi* (30, 31, 32). PBMC response increases above control 2-3 weeks postinfection with *Borrelia burgdorferi* (33). Patients with chronic

Lyme arthritis have a greater response to spirochetal antigens than those who resolve their disease. Treatment also appears to cause a concomitant decline in the response of PBMC, corresponding with clinical disease regression (31). A more specific investigation of the T cell response to recombinant Osp A has shown an elevated T cell response in early Lyme borreliosis, compared to healthy control patients (35, 36, 37). Characterization of Osp A-specific T cells has shown only Th1-type cells, suggesting that a preferential expression of a Th1 response in patients may prevent sufficient, potentially borrelicidal antibody response against the Osps.

The Effects of Th1 vs Th2 T-Cell Response in Other Diseases

Models of preferential expansion of a specific Th phenotype are found in many diseases, including leprosy, leishmania, and syphilis. The dominance of one Th response over another appears to cause vastly different disease progression and prognosis in patients and in animal models of these diseases. The understanding of the cell-mediated immune response in these diseases may help elucidate the lack of early antibody response to protective outer surface proteins in Lyme borreliosis.

In mice, there are two subsets of $CD4_+$ helper T cells (38). Th1 subset of T cells is important in induction of delayed type hypersensitivity (DTH), recruitment of macrophages, and host response to local and intracellular pathogens (39). These actions are in part mediated by the T cell secretion of a select repertoire of cytokines: interleukin 2 (IL-2), gamma-interferon (IFN-γ), and tumor necrosis factor beta (TNF-*B*) (38). The Th2 T cell response is important as a helper function for Ig synthesis. This subset of T cells is needed to produce a high and persistent antibody

production of IgM, IgG, IgA, and IgE and is needed for activation and prolonged survival of eosinophils and mast cells. Th2 T cells secrete interleukin 4 (IL-4), IL-5, IL-6, IL-10 (38, 40). A third subset of T cells called Th0 secretes IL-2, IFN-g, IL-4, and IL-5 and is believed to be the pluripotent precursor cell subset to both Th1 and Th2 T helper cells (41).

The murine immune system can switch from a predominant response of one subset of T cells to another or recruit Th0 cells into a Th1 or Th2 response (42). This switch or recruitment of T cells is based in part on the cytokine milieu predominating in the immune response. IFN-γ (produced by Th1 cells) promotes maturation of Th1 cells, activates macrophages, and stimulates natural killer (NK) cells, while inhibiting Th2 cell proliferation and IL-4 production (43). IL-4 (produced by Th2 cells) potentiates proliferation and cytokine synthesis of Th2 cells. In fact, low levels of IFN-g and high levels of IL-4 can cause a Th2 cell predominant response (44). High levels of IFN-γ can promote differentiation of precursor cells into Th1 cells in vitro (45) and in murine models of Leishmania (46).

In humans, healthy donor CD4+ clones have mostly an unrestricted cytokine profile, resembling the murine Th0 cells. T cell subsets with different cytokine secreting profiles are also found in human disease states (47). Human T cell clones specific for *Borrelia burgdorferi* are able to secrete IL-2 and IFN-γ but not IL-4 or IL-5. In fact, as in mice, the Th1 response appears to be directed at viruses and mostly intracellular bacteria while the Th2 cell response is directed towards allergens and parasitic infections. These responses correlate with cytokine profiles and action of Th cells.

The subset of T cells activated in some diseases have a significant

effect on the type of resulting pathology, clinical presentation, and often mortality in patients with the disease and in murine models. One of the most dramatic differences in disease states (due to differential T cell response) occurs in Hansen's Disease (48), caused by infection with *mycobacterium leprae*. Tuberculoid leprosy is a form of this disease which is slowly progressing with large numbers of lymphocytes, epithelioid and giant cell granulomas and very few acid-fast bacilli. In contrast, the lepromatous form of leprosy is a rapidly progressing disease with significant parasitization of macrophages and Schwann cells and few T cells and plasma cells in tissue. The difference in these forms of the same disease appears to be the host immune response. The milder tuberculoid form has a Th1-like response with IL-2 and IFN-γ expressed by the T cells while the severe lepromatous form has a Th2 like response, expressing IL-4 and IL-5. (49)

A similar effect is observed in murine models of *Leishmania major* infection. In BALB/c mice, the predominant response to infection is a Th2-like cytokine pattern with high levels of IL-4 and IL-5 but little IFN-γ (46). These mice have poor delayed-type hypersensitivity even with high antibody titers and go on to develop localized lesions, parasite replication, visceral disease and, finally, succumb to the disease. On the other hand, C57/Bl/6 mice develop a Th1-like response to *Leishmania* infection with high levels of IFN-γ and little IL-4 or IL-5 (50). This Th1-like response is associated with strong DTH, low antibody titers, and disease regression.

T cell subset response and disease progression, in fact, can be controlled by providing specific cytokines. Mouse strains which normally have Th1 response to infection with *Leishmania* revert to a strong Th2 response with disease progression when given a single dose of anti-IFN-γ

(51). Giving a single dose of IFN-γ to BALB/c mice early in infection, causes a Th1 like response rather than a Th2 in these mice (52).

Extracellular parasites, trypanosoma, and schistosoma also display a Th1 and Th2 subset-dependent disease progression. Th2 response depresses Th1 response and leads to chronic infection in both of these diseases(53). Also, *Treponema pallidum*, a spirochete and causative organism of syphilis, induces variable disease progression based on the level of delayed-type hypersensitivity (DTH) (53). Syphilis is particularly interesting as a comparative disease to Lyme borreliosis because of the similarity in the latency and chronic course of disease and because of evidence of T cell subset dependent response to a spirochetal and nonintracellular organism. It is postulated that those patients who develop tertiary syphilis have a weak DTH or Th1 response while those who undergo disease regression without long-term infection have a strong DTH reaction (54). In a rabbit model of syphilis, early Th1-like response were down regulated 9-14 days after infection while the Th2 response remained elevated up to 90 days after infection.(53).

Although in leishmania, leprosy, and other diseases including syphilis the Th1 response to the pathogen appears to lead to disease regression or to a slowly progressing form of the disease, the opposite may be true in Lyme borreliosis. Antibody is known to be the most important factor in disease prevention, and only a Th1 T cell response (not Th2) has been found in diseased patients - even those that are chronically infected with B burgderfori. This finding is striking since a Th2-type response is needed to mount a sufficient and persistent IgG1 and IgG3 antibody response.

Hypothesis

We propose that Osp-specific Th2 T cells may alter the course of Lyme borreliosis in mice. This knowledge may be valuable in explaining the apparent lack of early antibody response to abundant Osps in patients with Lyme borreliosis. These patients all appear to express Th1 phenotype after infection with Borrelia spirochetes. The lack of a Th2 response to the *Borrelia* spirochetes may thwart the host immune system's attempt to mount a sufficient and specific antibody response to the outer surface proteins.

Th2 cells can secrete cytokines that result in a dominance of Th2 over Th1 cell maturation. Dr. Bockenstedt has recently found a synthetic peptide spanning amino acid 179-193 of Osp A can elicit an Osp A-specific Th2 response in mice. This study examines the effects of adoptively transferred Osp A peptide primed Th2 cells on the onset, progression and regression of Lyme borreliosis in mice.

Experimental Design: Overview

Th2-type T cells were primed in 6-8 week old C3H/HeJ mice by immunization with Osp A peptide 179-193 in complete Freund's adjuvant (CFA). Ten days later, immunized T cells were isolated from regional lymph nodes and spleen and adoptively transferred into mice at 3 stages of infection: 1) immediately prior to inoculation with *Borrelia burgdorferi*, 2) after infection was established during disease evolution, and 3) during disease regression. We then sacrificed the mice to assess the pathology in the hearts and joints, to examine for spirochete by cultures of various organs, and to assess Osp A antibody response to the spirochete in a Th2 phenotype by immunoblots..

Materials and Methods

Mice

Weanling and 6-8 weeks old female C3H/HeJ mice were purchased from Jackson Laboratory (Bar Harbor, ME) and housed in filter-top cages. Food and water were provided *ad libitum*. The mice were killed with carbon dioxide gas.

Borrelia burgdorferi

A cloned N40 strain of *B. burgdorferi* with proven infectivity and pathogenicity in C3H/HeJ mice was used. The spirochetes were grown in modified Barbour-Stoenner-Kelly (BSK II) medium (55) at 33° C. Spirochetes were inoculated after the third in vitro passage at log-phase of growth and after dilution to 10^4 Borrelia/ml with BSK II medium.

Culture Media

Click's media supplemented with 1% penicillin, 1% gentamicin, 1% L-glutamine, $5x10^{-5}$ M 2ME (Sigma), and 5% FCS was used for cell cultures.

Derivation and characterization of T cells

The synthetic Osp A peptide 179-193 and ovalbumin were provided by Dr. Linda Bockenstedt. Ovalbumin-specific T cells were derived and characterized in the same way as the Osp A specific T cells. Osp A peptide-specific T cells were generated from C3H mice immunized with 50ug of peptide emulsified in CFA. Six to eight days after injection of peptide to peritoneum, foot pad, and base of tail of each mouse, the spleen and regional draining lymph nodes were aseptically collected. Lymph nodes

and spleens of one or several mice were combined and made into a single cell suspension. After erythrocytes were lysed, the remaining cells were incubated overnight (5% CO_2, 37° C) with 10ug/ml of specific peptide. Expanded T cells were enriched over a nylon wool column and used for adoptive transfer.

Prior to nylon wool enrichment, $5x10^5$ peptide-primed cells were placed into wells of 96-well microtiter plate to characterize the cells. To each set of duplicate wells, recombinant Osp A (rOsp A) at different concentrations (100ug/ml, 10ug/ml, 1ug/ml), ovalbumin (100ug/ml), PPD (10ug/10ul), and media only were added. The cells were incubated for 4 days (5% CO_2, 37° C), then pulsed with 1uCi [^{3}H]-thymidine/well overnight and harvested. [^{3}H]-thymidine incorporation was assessed using a Beckman Beta-plate scintillation counter.

Prior to pulsing with thymidine, 50ul of supernatant from each well was transferred to another 96-well microtiter plate to assess cytokine secretion of the cell. The IL-4 dependent CT.4S cell line ($5x10^3$ cells/well) was added to each supernatant and incubated (5% CO_2, 37° C) for 24hrs. These cells were then pulsed with [^{3}H]-thymidine (Amershan) and incubated overnight and harvested. The thymidine incorporation was assessed using a Beckman Beta-plate scintillation counter.

Immunoblot

Borrelia burgdorferi N40 lysate and recombinant Osp A and C antigens prepared by Dr. Linda Bockenstedt were electrophoresed on 12% SDS-PAGE polyacrylamide minigel. The proteins were transferred to nitrocellulose sheets and stained with Ponceau Red (0.1% Ponceau S in 1% acetic acid) to confirm transfer. After destaining with dd H_2O, the

nitrocellulose sheets were blocked for 1hr in 5% nonfat milk in PBS. Antigen strips were incubated with test sera diluted 1:100 at room temperature for 1hr. The strips were washed three times in PBS with 0.05% Tween 20 (PBST), then incubated for 1 hr. with alkaline phosphatase-labeled goat anti-mouse immunoglobulin diluted 1:5000 in PBST. After washing three times with PBST, the strips were developed with BCIP system with nitroblue-tetrazolium (One Component system, Kirkegaard and Perry Laboratories).

Experimental plan

C3H mice were divided into three groups each containing 10 mice: 5 receiving Osp A-specific T cells and 5 receiving ovalbumin-specific T cells. All mice were inoculated subcutaneously with 10^3 N40 *Borrelia burgdorferi* in 0.1ml BSK II medium at set time intervals from the T cell transfers. Group I mice were inoculated with *B. burgdorferi* and injected with T cells on Day 1 and sacrificed 14 days later. Group II mice were inoculated with *B burgdorferi* on Day 1, received adoptive transfer of T cells 3 and 10 days later, and were sacrificed 14 days after spirochete inoculation. Group III mice were inoculated on Day 1, received adoptive transfer of T cells 14 and 21 days later, and were sacrificed 30 days after inoculation with spirochetes. The adoptive transfer of T cells into mice were done by two methods: subcutaneous and retro-orbital injections. Some groups of mice received subcutaneous injection of $1x10^6$ to $7x10^6$ T cells in 0.1ml PBS on the back (on a site opposite to the *B. burgdorferi* inoculation). Mice receiving retro-orbital injections were anesthetized with methoxyflurane. The number of Osp A-specific and ovalbumin-specific T cells used in the T cell transfers were the same in each group of

mice and ranged from 1-7 x 10^6 T cells depending on the experiment.

Histology and tissue cultures

All cultures and histopathology were done by Dr. Stephen Barthold's laboratory. Blood, ear punch (1.5 mm diameter), and urinary bladder specimens were aseptically collected from each mouse for culture. Two drops of blood were placed directly into 8-ml glass screw top tubes containing 7ml of modified BSK II medium without antibiotics. Duplicate ear punches were also collected and cultured with or without Ciprofloxacin (which does not kill the spirochete). Bladder was cultured similarly. The spleen was aseptically collected and homogenized in 4x volume of BSK II medium. Then, 0.5ml of homogenate was placed in a glass screw top tube containing 7 ml BSK medium and cultured. All cultures were incubated for 2 weeks after which the presence of viable spirochetes was confirmed by darkfield microscopy.

The heart and tibiotarsal and knee joints were also collected and examined for histopathology. These tissues were immersion-fixed in 10% neutral buffered formalin, pH 7.2. Decalcifying solution (Baxter Health Corp., McGaw Park, IL) was used to demineralize bone in joints. Tissues were embedded in paraffin, sectioned by standard technique, and stained with hematoxylin and eosin then examined for inflammation and pathology. Examiners were blinded to experimental group, and the severity of inflammation were recorded as (-) for no inflammation to (+++) for severe inflammation.

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Results

Because only Th1 phenotype cells are found in *B burgdorferi* infection, we adoptively transferred Osp A specific and control Th2 cells into mice to determine the effects of these cells on T cell dependent antibody production and the onset, progression, or regression of Lyme borreliosis in mice. Group I mice received T cells on the day of spirochete inoculation, Group II mice received T cells after infection was established and during the evolution of arthritis and carditis (3 and 10 days after infection), and Group III mice received T cells during disease regression (14 and 21 days after infection). Identical numbers of ovalbumin-primed T cells Osp A-primed T cells were used in all transfers. Evidence of change in disease onset, progression, or regression were assessed by cultures of blood, bladder, spleen, and ear and histologic examination of the heart and knee and tibiotarsal joints.

T cells used in adoptive transfer experiments were analyzed for antigen specificity and cytokine secretion. The transferred cells all had much greater proliferation with the presence of specific peptide compared with nonspecific peptide or no peptide, particularly in T cells incubated in 100ug/ml (Figures 1-6). Furthermore, all the Osp A-primed T cells had a dose-dependent proliferation to their specific antigen. The ovalbumin-primed T cells did not appear to have a dose-dependent response to specific peptide concentrations. These cells mainly had strong specific proliferative response to 100ug/ml concentration of recombinant ovalbumin. All cells had Th2 phenotype with production of IL-4. The IL-4 production of cells was quantified using the proliferation of the IL-4 dependent murine T cell line CT.4S. Therefore, the cells transferred into

experimental mice were specific for the Osp A or ovalbumin and had either an effector Th0 or Th2 phenotype.

Fig. 1: Group I - Assay for IL-4 production by transferred T cells.

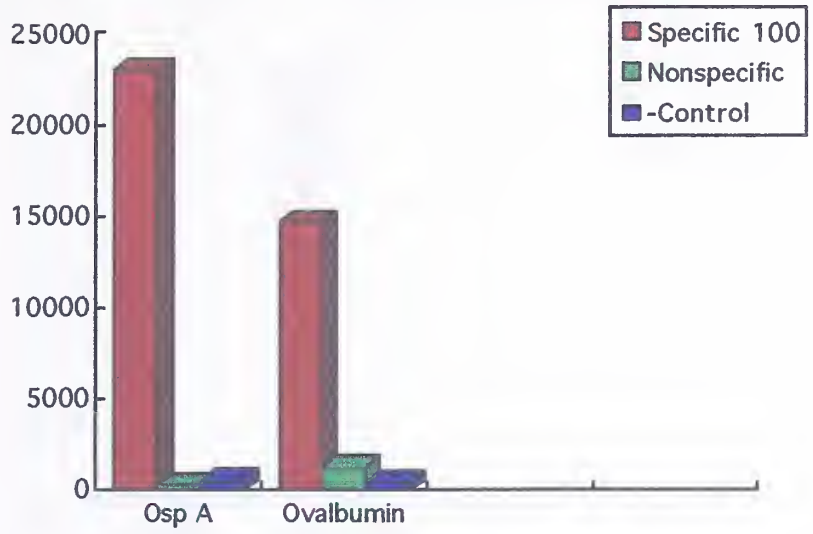


Fig. 1-6: Osp A or Ovalbumin-primed T cells used for adoptive transfer were incubated in specific peptide at concentrations of 100, 10, 1ug/ml ("Specific 100", "Specific 10," "Specific 1"). In the Osp A-primed T cell assay, ovalbumin at 100ug/ml was used for "nonspecific" response. Osp A at 100ug/ml was used for "nonspecific" response in the ovalbumin-primed T cell assays. The response of T cells in PPD was used to assess the success of immunization ("+ control") and the response of T cells in media alone was used as "- control." The supernatant from the proliferation assays were analyzed for IL-4 by thymidine incorporation of IL-4 dependent murine T cell line. The results are duplicated and the mean of the dose dependent IL-4 production of T cells are given above as counts per minute (CPM).

Fig. 2: Group II, First transfer- Assay for IL-4 production by transferred T cells.

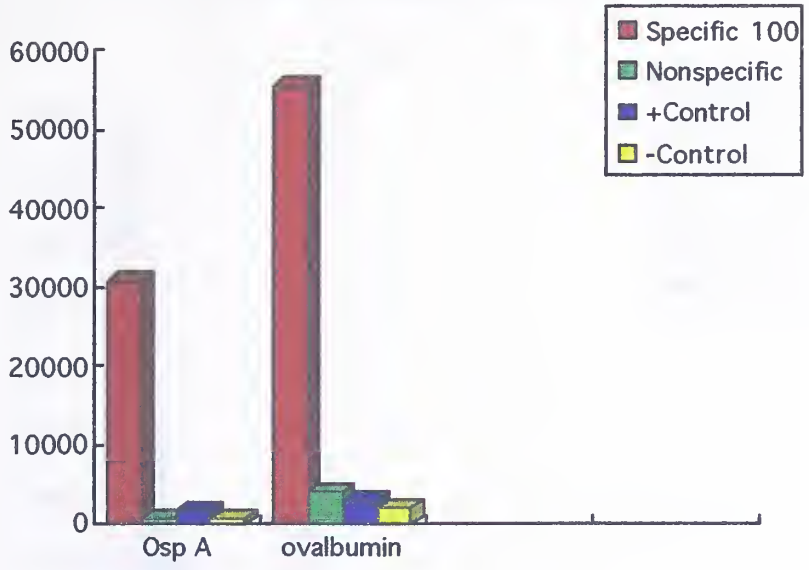


Fig. 3: Group II, Second transfer - Assay for IL-4 production by transferred T cells.

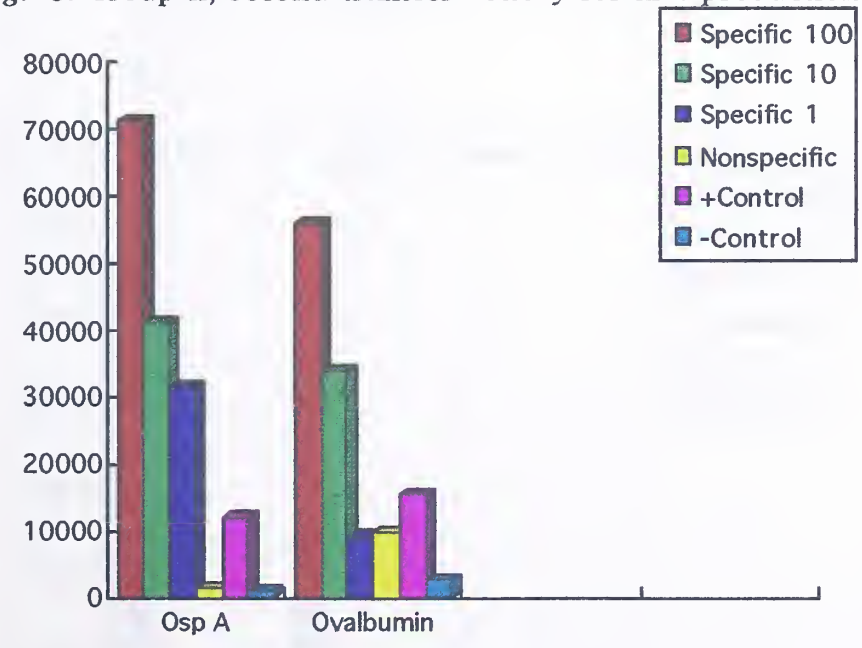


Fig. 4: Group II (Repeat), Second transfer - Assay for IL-4 production by transferred T cells.

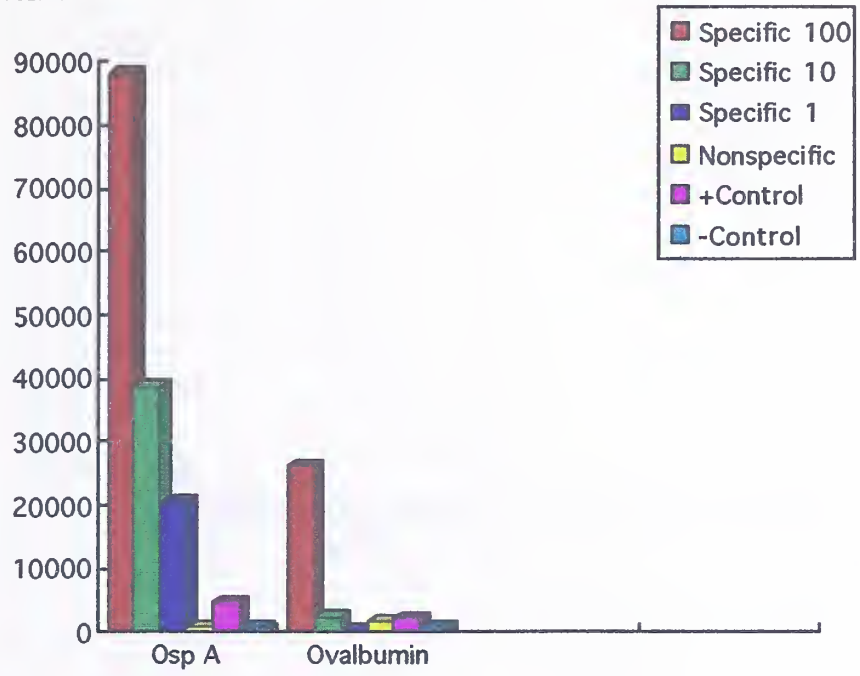


Fig. 5 Group III, First transfer - Assay for IL-4 production by transferred T cells.

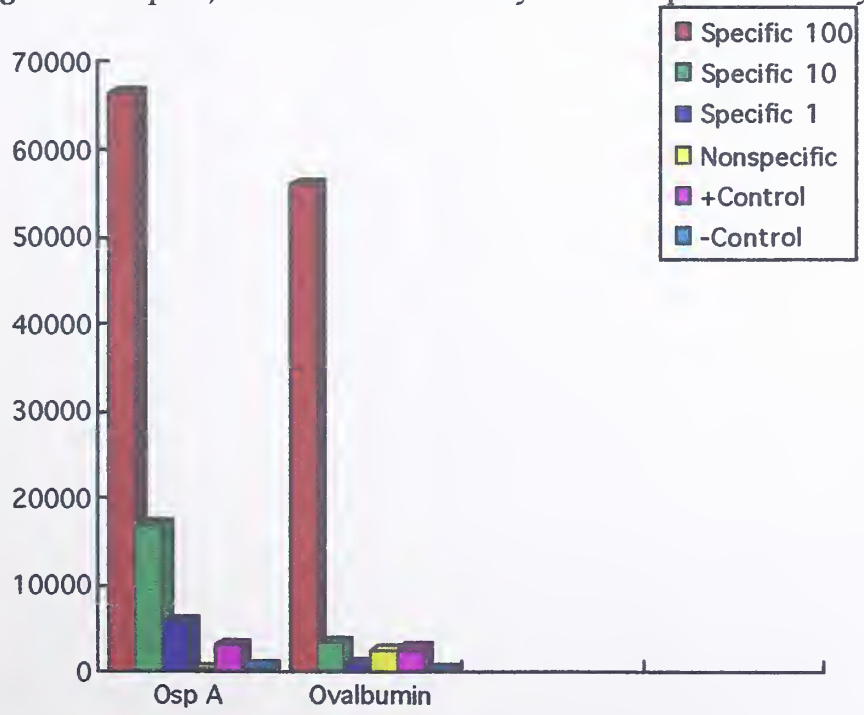
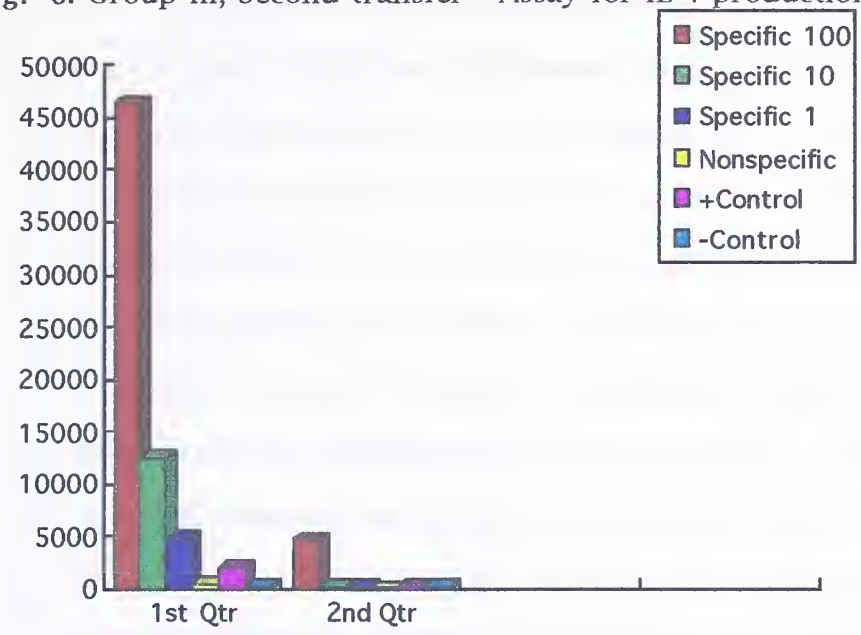


Fig. 6: Group III, Second transfer - Assay for IL-4 production by transferred T cells.



In the first Group I experiment, the blood and organ cultures from mice showed significant differences in isolation of spirochetes between mice receiving Osp A T cells and control T cells (Table 1). This group of mice were injected subcutaneously on the back with purified T cells from immunized mice. The five mice that received ovalbumin-primed T cells all had spirochete infected organs. However, only 2 of the 5 mice injected with Osp A-primed T cells had any infected organ. The number of mice with carditis also paralleled the culture results. The severity index for arthritis, however, was similar in both sets of mice. One mouse in the Osp A set had completely negative culture or histology results. Two of the five mice in the Osp A set clearly had arthritic disease but no spirochetes in the other organs and no carditis. Based on these limited number of mice, it appears that the spread of spirochetes to organs and inflammation of the heart was attenuated in mice receiving Osp A peptide primed T cells.

The Group I study was repeated using Osp A peptide and ovalbumin-primed T cell clones, injected retro-orbitally into mice. In this repeat study, all mice of both Osp A and control sets had multi-organ presence of spirochetes by culture. The Th2 T cells did not appear to attenuate or affect the spread of spirochetes in the repeat experiment. The histology of the repeat study showed that mice receiving Osp A-primed cells appeared to have slightly more severe joint disease (severity index, Osp A vs ovalbumin mice: 2.6 vs 1.8, $P < 0.025$) but the same degree of carditis (Table 2).

In Group II mice, purified T cells from immunized mice were transferred (by subcutaneous back injection) after spirochete dissemination and before regression of disease. The cell transfer was repeated 1 week later to ensure continued presence of Th2 cells. Again,

the study was repeated in similar experimental conditions, using greater number of purified T cells transferred retro-orbitally. One mouse in the repeat study died during the injection of T cells and was excluded from the analysis. No significant difference in the severity of arthritis (Index, ovalbumin vs Osp A: 1.9 vs 2.6 ($P > 0.10$) and 1.7 vs. 1.5 ($P > .25$)) and carditis and the presence of spirochetes in organs could be appreciated between the Osp A and ovalbumin-primed mice in both the experiments of Group II (Table 3 and 4).

The third group of mice were injected retro-orbitally with purified T cells from immunized mice during the third and fourth week of infection - at the time of disease regression. In these mice, there was a significant difference in the severity of disease with appreciable attenuation of arthritis (severity index, ovalbumin vs. Osp A: 3.0 vs. 1.8 ($P=0.005$)) and carditis in the mice receiving Osp A primed T cells (Table 5). Of the five mice with Osp A primed T cells, only three had carditis, while the other two had only trace or mild disease. In the mice which received ovalbumin-primed T cells, all the mice had significant carditis.

Table 1

The effects of Osp A and ovalbumin-primed T cells on the presence of spirochetes and degree of arthritis and carditis in infected mice.

Group I		Cultures	Arthritis	Carditis
Oval mice	1	Pos	0	Pos
	2	Pos	0	Mild Pos
	3	Pos	2	Pos
	4	Pos	1	Pos
	5	Pos	1	Pos
Average of		5/5	0.8	5/5
oval mice			(SD 0.8)	
Osp A	1	Neg	0	Neg
	2	Pos	3	Pos
	3	Neg	1	Neg
	4	Neg	2	Neg
	5	Pos	2	Pos
Average of		2/5	1.6	2/5
Osp A mice			(SD 1.0)	

Individual mice were considered positive (Pos.) if spirochetes could be grown from any of the cultured organs (blood, spleen, ear, or bladder). The disease severity index for arthritis was calculated by adding the severity (designated from - to +++) in the most inflammed tibiotarsal joint. The index average and standard deviation is given for each set of five mice.

Table 2

Group I	Repeat	Cultures	Arthritis	Carditis
Oval	1	Pos	2	Pos
	2	Pos	1	Pos
	3	Pos	2	Pos
	4	Pos	2	Pos
	5	Pos	2	Pos
Average of oval mice		5/5	1.8 (SD 0.5)	5/5
Osp A	1	Pos	2	Pos
	2	Pos	2	Pos
	3	Pos	3	Pos
	4	Pos	3	Pos
	5	Pos	3	Pos
Average of Osp A mice		5/5	2.6 (SD 0.6)	5/5

Table 3

Group II		Cultures	Arthritis	Carditis
Oval	1	Neg	0	Neg
	2	Pos	2.5	Pos
	3	Pos	3	Pos
	4	Pos	2	Pos
	5	Pos	2	Pos
Average of oval mice		4/5	1.9 (SD 1)	4/5
Osp A	1	Pos	3	Pos
	2	Pos	2	Pos
	3	Pos	3	Pos
	4	Pos	2	Pos
	5	Pos	3	Pos
Average of Osp A mice		5/5	2.6 (SD 0.5)	5/5

Table 4

Group II Repeat		Cultures	Arthritis	Carditis
Oval	1	Pos	2	Pos
	2	Pos	2	Pos
	3	Pos	0.5	Pos
	4	Pos	2	Pos
	5	Pos	2	Pos
Average of oval mice		5/5	1.7 (SD 0.7)	5/5
Osp A	1	Pos	2	Pos
	2	Pos	2	Pos
	3	Pos	1	Pos
	4	Pos	1	Pos
Average of Osp A mice		4/4	1.5 (SD 0.6)	4/4

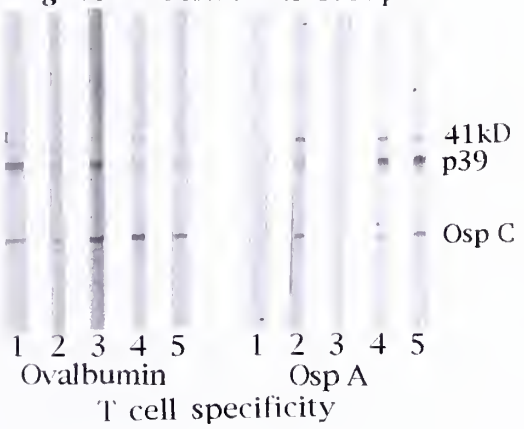
Table 5

Group III		Cultures	Arthritis	Carditis
Oval	1	Pos	3	Pos
	2	Pos	3	Pos
	3	Pos	3	Pos
	4	Pos	3	Pos
	5	Pos	3	Pos
Average of oval mice		5/5	3 (SD 0)	5/5
Osp A	1	Pos	2	Pos
	2	Pos	1	Mild Pos
	3	Neg	1	Trace
	4	Pos	2	Pos
	5	Pos	3	Pos
Average of Osp A mice		4/5	1.8 (SD 0.8)	3.5/5

Sera obtained at the time of sacrifice were tested by immunoblots of recombinant Osp A and C (not shown) and N40 spirochete lysate for antibodies to B burgdorferi. None of the mice appear to have IgG antibody response to Osp A during the first few weeks of infection. However, almost all the mice developed IgG antibodies to 22kDa Osp C, P39 and 41kDa flagellin (Figures 7-11). Group II ovalbumin mice also developed antibodies to a protein in the 41-43kDa range (Figure 9). Only one of the Group II Osp A mice developed this antibody. Most of the Group III mice had widening antibody response to low molecular weight proteins and to a protein in the 41-43kDa range (Figure 11). All the mice which had no culture or histopathologic evidence of infection also had no antibody response detected against *B. burgdorferi* antigens. One mouse in the Osp A Group I with very mild arthritis also had no antibodies specific for Borrelia. The transfer of Osp A-specific T cells did not appear to alter significantly the antibody response when compared to the antibody profile in mice receiving ovalbumin-specific T cells.

Figures 7-11. IgG Immunoblot of *B. burgdorferi* N40 lysates using mice sera diluted at 1/100. The number in the lane corresponds to the mouse numbered in Tables 1-5.

Figure 7: Sera from Group I



T cell specificity

Figure 8: Group I repeat

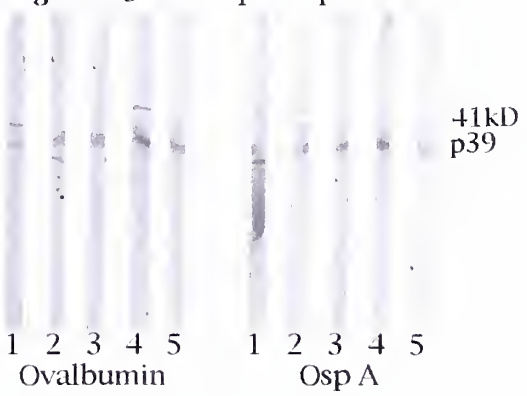


Figure 9: Group II

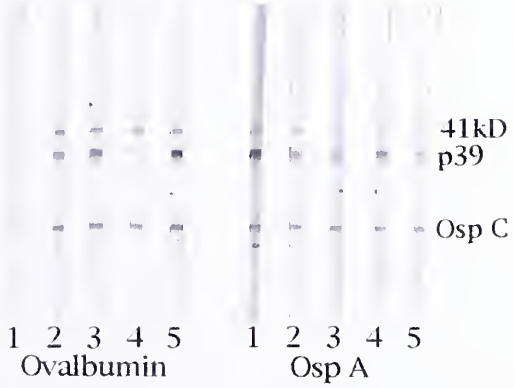


Figure 10: Group II repeat

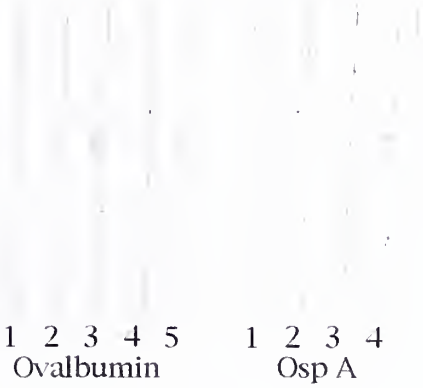
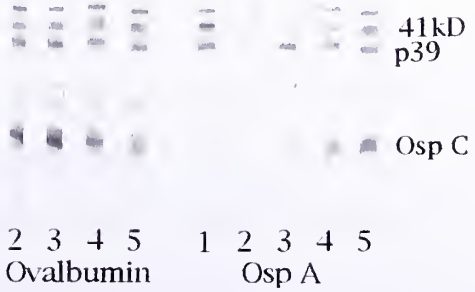


Figure 11: Group III



Discussion

The T cell immune response to Borrelia burgdorferi infection in mice has been the focus of recent efforts into understanding the adaptation of this spirochete to the mammalian host. The finding of enhanced production of gamma-interferon (a cytokine produced by Th1 cells) (36,37) and the presence of activated macrophages in B. burgdorferi infected mice supports a bias in the differentiated T helper cell repertoire towardscell-mediated immune responses. More direct evidence comes from characterization of T cell lines isolated from the synovium of patients with Lyme arthritis, in which only those T cells that secrete a cytokine profile produced by Th1 cells (and not Th2 cells) are found (37).

Osp A antibodies can protect naive mice from challenge infection. Yet, these antibodies do not arise until quite late, if at all, during experimental and natural infection, despite the dominance of Osp A on spirochetes examined in culture and in ticks (27). We hypothesized that the lack of early Osp A antibody production in infected mice was, in part, due to the type of Th cell response elicited during infection. Recently, Dr. Bockenstedt's laboratory has produced T cell hybridomas from splenocytes of C3H mice sacrificed at day 14 of infection. Some of these T cell hybrids proliferate in response to Osp A and secrete both IL-2 and gamma-interferon, consistent with a Th1 phenotype. Therefore, immunologic exposure to Osp A occurs early in infection even though specific antibodies cannot be detected, and such exposure elicits a Th1-type T cell response. Although Th1 cells can promote IgG1 and IgG3 antibodies, this Th1 response does not appear to lead to early seroconversion to Osp A. Dr. Bockenstedt has also identified a T cell epitope on Osp A which induces

differentiation of T cells from C3H mice into Th2-type cells after immunization with the peptide in CFA. Mice primed with the Osp A T cell epitope, then infected with low-dose spirochetes (10^3) can seroconvert to Osp A within the first 2 weeks of infection. The purpose of this study was to determine if primed Th2 T cells transferred at different stages of early infection in C3H mice could affect the antibody response to Osp A and/or course of disease.

The preliminary experiment in which mice received ovalbumin (Ova) or Osp A primed Th2 cells at the time of infection was suggestive that Osp A specific T cells may have an effect on the early evolution of disease. Whereas 5/5 mice receiving Ova-specific T cells were infected and had disease, 4/5 mice in the Osp A group were infected and only 3/5 mice had significant disease. A second experiment failed to confirm this finding and even suggested that Osp A T cells may increase the degree of arthritis (several index of 2.6 vs 1.8 ($P < 0.025$)). The difference between the two experiments was that in the second, Th2 cell lines had been established in culture and likely consisted of a completely differentiated Th2 population. The first experiment (and all other experiments in this study) were performed using purified T cells from mice immunized with either Osp A or ovalbumin. These T cells secreted IL-4 in response to the specific antigen, but likely contained both undifferentiated Th0 as well as differentiated Th2-type cells. It has been suggested that Osp A specific T cells can actually enhance arthritis in humans, based on the finding that Lyme disease patients with chronic arthritis have a greater prevalence of Osp A specific T cells (56).

Studies of T helper cell differentiation suggest that naive T helper cells develop into effector Th0 cells when primed with antigen (42). It is

from this unrestricted Th0 cell population (which secrete IL-2, gamma-interferon, and IL-4) that Th1 and Th2 cells arise upon restimulation with antigen in the presence of cytokines (57). In murine leishmaniasis, IFN-gamma given prior to infection causes CD4+ T cells to differentiate into Th1 phenotype cells, while IL-4 promoted a Th2 phenotype (45, 46). The effect of the cytokine environment is critical to maintenance of Th cell phenotype. It has been demonstrated that the Th0 and Th1 phenotypes are relatively unstable, with Th1 cells incubated in the presence of IL-4 converting to a Th2 phenotype. In contrast, the Th2 phenotype does not appear to readily convert to a Th1, no matter what the cytokine environment (58). In our study, the assays for cytokine secretion of T cells detected the presence of IL-4 which can be produced by both Th2 and Th0 cells. Therefore, the cells used in the transfer may have had significant numbers of undifferentiated Th0 effector cells. The effects of the dominant Th1 response in early infection likely produced a cytokine environment rich in IL-2 and gamma-interferon. When the T cells were then transferred into this environment, the Th0 cells could differentiate into a Th1 phenotype. Adoptively transferring a population of cells which contain Osp A specific Th0 cells may therefore enhance the arthritis if they differentiate, because of the cytokine environment existing in early infection, into Th1-type cells. The effect appears to be antigen specific as we did not see similar exacerbation of arthritis in mice receiving Ova-specific T cells. We did not attempt in these studies to reisolate T cell lines from mice to determine the relative prevalence of Th2 vs. Th1-type Osp A specific T cells, which could lend support to the above theory.

Although transfer of IL-4 producing Osp A-specific T cells did not have any beneficial effect on the host during the first 2 weeks of infection,

they did appear to have significant effect on the resolution phase of disease. In the Group III mice, those receiving Osp A-specific T cells had substantially less severe arthritis than those receiving Ova-specific T cells (severity index 1.8 vs 3.0 (P=0.005)). This is consistent with the notion that cytokines secreted by Th2 cells oppose the action of gamma-interferon on macrophages and suppress the production of gamma-interferon by Th1 cells, thereby accelerating the resolution of inflammation ("disease") (59).

The differentiation of Th0 and Th1 cells into Th2 cells is due, in part, to the antigen dose. In leishmania infection, when the antigen level is decreased by the effects of the Th1 response in the first phase of infection, Th1 or Th0 cells may convert to Th2 cells which serve to downregulate the inflammatory response (leading to healing of leishmania lesions) (58). If we draw parallels between the Leishmania system and ours, the initial phase of the T cell reaction in our mice appears to be a Th1 phenotype, which serves to control the spirochete infection by nonspecific immune mechanisms including macrophage activation. However, as the spirochetal load decreases, disease regression occurs, in part, because the naturally arising Th0 cells may convert now more readily to a Th2 phenotype and secrete cytokines which will promote terminal differentiation of Th1 cells into a Th2 phenotype. Thus, when the Osp A-specific Th0/Th2 cells are transferred at this phase of infection, the Th0 cells could conceivably differentiate into Th2, and the combined effects of lower spirochetal load and cytokines elaborated by Th2 cells would be to accelerate the resolution phase of disease.

The effects of the Osp A-specific T cells at all stages of infection appear to be independent of antibody, as none of the mice had detectable

serum levels of Osp A antibodies. In addition, minimal differences could be seen in the degree of heart inflammation, suggesting that the role of Osp A in disease in this organ may be different than disease in the joints. This is particularly interesting because carditis in CD4+ "knock-out" mice, which have no CD4+ T cells, is essentially unchanged from that seen in the immunologically intact littermate (60). If Osp A-specific T cells have a role in disease (either exacerbation or attenuation), then it is most readily demonstrated in arthritis. In humans, the finding of higher titer Osp A antibodies and greater numbers of peripheral T cells reacting with Osp A in patients with chronic, treatment-resistant Lyme arthritis has led to the speculation that persistent disease may be due to molecular mimicry between Osp A and joint tissue, leading to an autoreactive phenomenon (56). An alternative view is that these patients, because of their genetic background, may effectively lower their spirochetal loads by the Th1 response and/or not readily convert to a Th2 phenotype, resulting in a persistent inflammatory response.

In summary, although the numbers of mice used were small, the experimental data suggest that IL-4 producing Osp A-primed T cells transferred into B. burgdorferi infected mice do not appreciably affect the course of Lyme borreliosis early in disease, but accelerate the resolution of disease once it is established. The attenuation is possibly due to T cell production of cytokines that downregulate the inflammatory response at the time when the antigen load has reached a critical lower threshold which favors the development of Th2 cells in the host. Our results do not implicate a critical role for Osp A-specific antibodies in this process. Future experiments will be designed to map the cytokine environment during the first four weeks of infection and to determine whether

terminally differentiated Osp A-specific Th2 cells can help regulate arthritis, even in the early stages of disease evolution.

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