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Spring/Summer 2002 Volume 9

ORIGINAL ARTICLES

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In Vivo Immunotherapy of Treponemal Infection by Depletion of CD4 T Cells

Hsi Liu, DVM, PhD[*†]; *Bret M. Steiner, PhD*[†]; *and Ronald F. Schell, PhD*[*‡]

ABSTRACT

Syphilis and yaws infections in the hamster model have been shown to mimic human infections. We have shown previously that in hamsters, both the CD4 and CD8 subsets of T cells can transfer immune protection against infection with *Treponema pallidum* subsp. *pertenue* (*T pertenue*). In this study, temporal changes over a 10-week period were followed in *T pertenue*-infected and noninfected control hamsters using monoclonal antibodies (mAb) that identified hamster lymphocyte subsets. A reduction in the percentage of T cells was observed between the 4th and 6th week postinfection; this development was accompanied by an increase in the proportion of B cells in the infected nodes. The proportion of $CD8^+$ cells did not change.

These findings suggest a decrease in the ratio of CD4/CD8 cells (T helper/T cytotoxic ratio). The total number of lymphocytes increased in the infected lymph nodes until 8 weeks postinfection and then decreased to near normal.

Next, we studied the role of $CD4^+$ T cells in immunity against *T pertenue* infection. We depleted $CD4^+$ T cells from *T pertenue*-immune hamsters and then challenged them with *T pertenue*. This group showed decreased protective immunity. In contrast, normal hamsters that were depleted of $CD4^+$ T cells and then challenged with *T pertenue* had a reduced number of treponemes recovered from the lymph node. The depletion of $CD4^+$ T cells was verified by our observations that the proliferative response to KLH was completely inhibited in hamsters that received mAb GK 1.5, whereas hamsters that received a control antibody showed no loss of responsiveness to KLH. We concluded that T cells are important host factors in determining the outcome of infection with *T pertenue*.

Key words: yaws, syphilis, treponemes, spirochetes, CD4, anti-CD4, mAb, immunity, hamster

INTRODUCTION

Yaws, caused by *Treponema pallidum* subsp. *pertenue* (*T pertenue*), is a contagious disease that primarily affects rural populations in warm humid areas. *T pertenue* is morphologically and antigenically indistinguishable from the organism that causes syphilis, *T pallidum* subsp. *pallidum*.[1] These organisms are collectively named treponemes. Immunity to treponemal infection develops during the course of the disease. Cross-protection has been observed[2]; however, complete protection is usually lacking and reinfection is possible.

Inbred hamsters have been used as a model system to study the immune response to treponemal infections.[2-7] When infected, inbred hamsters produce chronic skin lesions that mimic human disease. Also, the regional lymph nodes of infected hamsters increase in weight and contain large numbers of treponemes.[2,7] In prior studies using this animal model, humoral-mediated protective response has been demonstrated. Passively transferred yaws immune serum confers complete protection against challenge when the serum is administered either before or at the time of challenge.[3] Some protection is conferred when serum is administered several days after challenge; the serum recipients, compared to the control group, showed reductions in the size of lesions, the weight of the lymph nodes, and the number of treponemes found in the

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lymph nodes. However, when immune serum is administered more than one week after challenge, no protective effect can be detected.[3] Interestingly, while the hamsters evidently develop an antibody-mediated protective immune response to treponemal infection, this response is incapable of eliminating treponemes from the infected hamsters that serve as serum donors.

The role of cell-mediated immune response to *T pertenue* infection is less clear. Immune-T cells have been shown to confer protection against challenge with *T pertenue* on recipient hamsters.[4] Immune T cell subsets, $CD4^+$ and $CD8^+$ cells, can also confer partial protection against subsequent challenge.[5] Several lines of evidence suggest that the mechanism of protection by T cells is mediated by direct treponemicidal activity of immune T cells[5,8]: (1) antitreponemal antibody is not detected in recipients of immune T cells; (2) hamsters infused in the popliteal lymph node region with immune T cells do not demonstrate significant accumulation of macrophages in the lymph node[5]; and (3) a lymphotoxin-like lymphokine, secreted by immune T cells, is reported to be responsible for the direct killing of treponemes.[8] Collectively, these findings suggest that the T cell component of the cell-mediated immune response plays an important role in protection against infection with *T pertenue*.

The in vivo administration of anti-CD4 antibody has been shown to selectively deplete $CD4^+$ T lymphocytes.[9-15] Moreover, the depletion of $CD4^+$ T cells has been shown to result in multiple effects including inhibition of delayed-type hypersensitivity reactions,[9,11] reduced production of cytotoxic T cells,[9] reversal of autoimmune diseases,[12-14] prevention of infection with *Plasmodium berghei* in mice,[16] and a therapeutic effect on *Leishmania major* infection in susceptible mice.[15]

Here we have demonstrated that: (1) the numbers of B cells, T cells and T cell subpopulations underwent temporal changes in treponemal infected animals; (2) immune hamsters depleted of $CD4^+$ T cells by mAb treatment were still resistant to challenge with *T pertenue*; however, the protection was weaker in comparison to the immune hamsters treated with control antibody; and (3) in contrast, normal hamsters treated with mAb GK 1.5 and then challenged with *T pertenue* yielded significantly reduced numbers of treponemes. These results suggest that $CD4^+$ T cells have an important role in the pathogenesis of treponemal infection.

MATERIALS AND METHODS

Animals

Male and female inbred LSH/Ss LAK hamsters, 6 to 9 weeks old, were obtained from Charles River Breeding Laboratories, Inc. (Wilmington, MA). The hamsters weighed 80 to 100 g, and were housed 3 or 4 per cage at an ambient temperature of 21°C. Female inbred nude mice (nu/nu), to be used as a source of ascitic fluid, were obtained from Sprague-Dawley (Madison, WI). They were housed in sterile cages in a vertical laminar flow hood. Sterile food and water were available ad libitum.

Organism

Treponema pallidum subsp. *pertenue* (Haiti B) was originally isolated from a lesion on the lower abdomen of an 11-year-old boy with typical generalized frambesiform yaws.[17] The strain has been maintained by passage in hamsters.[7] The inguinal lymph nodes of the hamsters were removed aseptically 5 to 6 weeks after the hamsters had been injected intradermally in the inguinal region with $1x10^6$ viable treponemes. The nodes were teased apart in RPMI 1640 medium (Gibco, Long Island, NY) containing 5% fetal bovine serum (Hyclone Lab. Inc., Logan, UT) and gently forced through sterile 60-mesh stainless-steel wire mesh filters. After centrifugation at 270 x g for 3 minutes to remove cellular debris, the number of treponemes in the supernatant was determined by darkfield microscopy.[18] The suspension of treponemes was dispensed into vials at a concentration of 5×10^6/mL and stored at −70°C until used.

Antibody Reagents

The following mAbs were used in this study. Hybridoma cell line 14-4-4s, producing monoclonal murine anti-Ia antibody (mAb 144-4s, hamster pan B cell marker) was provided by Dr. Colleen Hayes (University of Wisconsin, Madison). This mAb recognizes the Ia.7 specificity present on the $I\text{-}E^k$ in the mouse[19] and was used to identify hamster B cells.[4,5,20] The hybridoma cell line was grown in RPMI 1640 medium containing 10% fetal bovine serum. Five days after cultivation the supernatant was centrifuged at 270 x g, collected, dispensed in 5 mL aliquots, and stored at −20°C. The aliquots were then thawed on the day of experiment and used at a final dilution of 1:10. MAbs 20 (hamster pan T cell marker) and 38 (hamster cytotoxic T cell marker) ascitic fluid were provided by Dr. Joan Stein-Streilein (University of Miami, FL).[20] The ascitic fluid was maintained at −70°C, thawed, and used at 1:50 dilution. MAb anti-L3T4 (hamster CD4 cell marker), derived from hybridoma GK 1.5 (ATCC), was grown as ascites in pristane (Sigma, St. Louis, MO) primed inbred nude mice. The antibody concentration of the ascitic fluid was calculated from the total protein concentration determined by absorbance at 280 nm. Fluorescein (FITC) conjugated and unconjugated goat antihamster immunoglobulin and FITC conjugated

goat antirabbit immunoglobulin, purchased from Organon Teknika-Cappel Corp. (West Chester, PA), were used at a final dilution of 1:20. MAbs 14-4-4s, 20, GK 1.5, and 38 were labeled with biotin.[4] Briefly, 2 mg of antibody solution were incubated with 0.25 mg of biotin-HO-succinimide (Sigma, St. Louis, MO) in 100 μL of dimethyl sulfoxide (Sigma, St. Louis, MO) and stirred for 2 hours. Each antibody was then dialyzed overnight against Dulbecco's phosphate buffered saline (DPBS, Sigma, St. Louis, MO) with several changes of buffer. The resulting solution was sterilized by filtration (pore size 0.45 μm; Corning Glass Works, Corning, NY) and stored at −70°C until use. Avidin-FITC and Streptavidin phycoerythrin conjugates were purchased from Becton-Dickinson (Mountain View, CA).

Flow Cytometric Analysis

Two million lymph node cells were incubated with a 1:10 dilution of FITC conjugated goat Ab to hamster immunoglobulin (antiheavy and light chain) for 30 minutes, washed twice with DPBS, fixed with 2% formaldehyde, and stored in the dark at 4°C until analyzed. Alternatively, 2 x 10^6 cells were incubated with biotinylated mAbs 14-4-4s, GK 1.5 or 38 for 30 minutes at 4°C, then washed twice with DPBS, and incubated with avidin-FITC (Becton Dickinson Immunocytometry Systems, Mountain View, CA) for 30 minutes at 4°C. Surface immunofluorescence of 10,000 cells was determined with a Coulter Epics C cell sorter (Coulter Immunology, Hialeah, FL). The percentage of macrophages was determined by forward and 90° angle light scatter analysis of each lymph node sample.[5]

Experimental Design

Hamsters received 600 μg of GK 1.5 crude ascitic fluid intramuscularly 1 day before infection and at days 3 and 14 postinfection (**Figure 1**). This procedure is known to deplete $CD4^+$ T cells in many animal species. Control hamsters received the same concentration of an unrelated myeloma protein, MOPC-195 (Litton Bionetics, Inc., Kensington, MD). Hamsters were then injected in the hind paw with 5x10^5 *T pertenue*. The animals were sacrificed 26 days after infection to determine the number of cells in the lymph node and the proliferative response to KLH.

Preparation of Lymph Node Cells

Single cell suspensions were prepared from lymph nodes by teasing the nodes apart with forceps and gently pressing them through a stainless steel 60-mesh screen into RPMI 1640 medium containing 5% fetal bovine serum (FBS). The cells were then washed twice with RPMI 1640 medium and resuspended in RPMI 1640 medium containing 10% FBS to a concentration of 2x10^7 cells/mL.

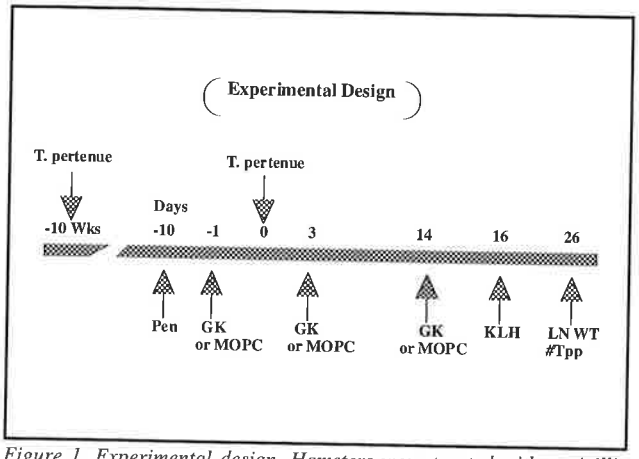

Figure 1. Experimental design. Hamsters were treated with penicillin (Pen) 10 days before infection and injected with either mAb GK 1.5 (GK) or MOPC 195 (MOPC) 1 day before infection. T pertenue *(TPP), MAb GK 1.5 or MOPC 195 was injected at the indicated time.*

Assessment of Protection

The number of treponemes in the lymph node was adopted as the criterion for quantitative assay of protection against treponemal infection.[2] The number of treponemes per lymph node was determined according to a modification of the procedure described by Miller.[18] In brief, duplicate slides of each homogenized lymph node were prepared and 100 fields per slide were examined for treponemes by darkfield microscopy. Data are presented as the mean number of treponemes per lymph node.

Immunization and Assay of KLH Proliferative Response

Hamsters were immunized subcutaneously at two sites in the inguinal region with 100 μg of keyhole limpet hemocyanin (KLH, Calbiochem, La Jolla, CA) in 0.5 mL of RPMI 1640 medium. Ten days later, lymph node cells were assayed for proliferative responses to KLH.

Culture Conditions

Suspensions of lymph node cells were prepared and diluted to 2x10^6 cells/mL in RPMI 1640 medium containing 10% heat inactivated FBS supplemented with 100 units of penicillin and 100 μg of streptomycin (Hazelton, Denver, PA) per mL. For evaluation of the in vitro KLH proliferative response, suspensions containing 2x10^5 lymph node cells in 0.1 mL medium were cultured with 30 μg/mL of KLH in flat-bottomed 96-well microtiter plates (Costar, Cambridge, MA) at a final volume of 0.2 mL per well. Cultures were incubated in triplicate at 37°C

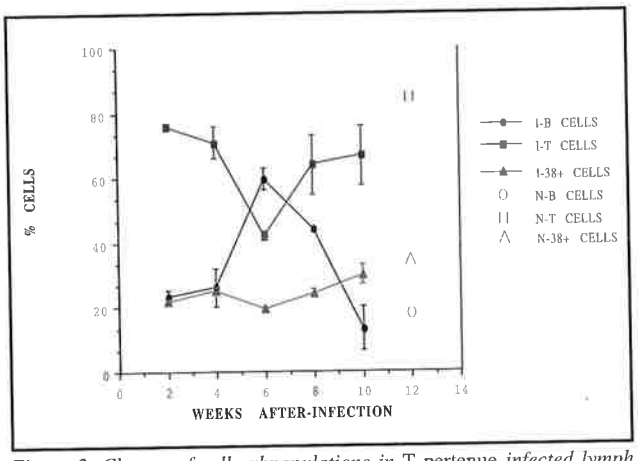

Figure 2. Change of cell subpopulations in T pertenue *infected lymph nodes. mAb 20 was used to identify T lymphocytes; mAb 14-4-4s and goat anti-hamster Ig were used to identify B cells; mAb 38 was used to identify hamster T cell subsets (cytotoxic/suppressor T cells, Tc). Lymph node cells were obtained from animals infected with* T pertenue *at 2, 4, 6, 8, and 10 weeks postinfection. Single cell suspensions were prepared and cells were stained with the antibody reagents. I-B=B cells from infected lymph node were recognized by goat anti-hamster Ig and mAb 14-4-4s. I-T=T cells from infected lymph nodes were recognized by mAb 20. I-38+ =cytotoxic T cells were recognized by mAb 38. N-B=B cells were from normal animals; N-T=T cells were from normal animals; N-38=cytotoxic T cells were from normal animals. Normal values were obtained from age-matched control hamsters.*

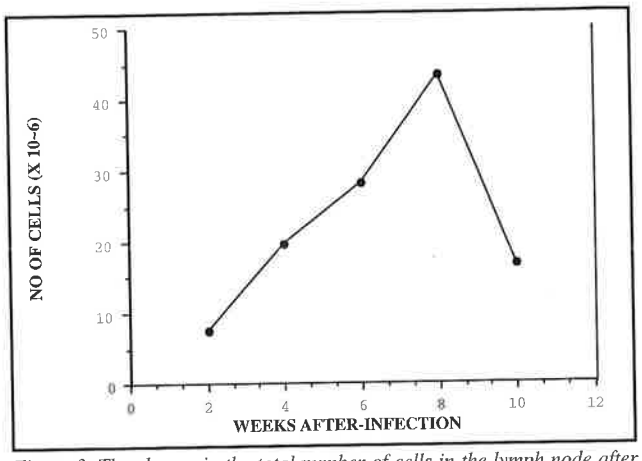

Figure 3. The change in the total number of cells in the lymph node after infection with T pertenue.

for 6 days in a humid atmosphere of 5% CO_2 in air. Eight hours before harvesting, each culture was pulsed with 50 μCi/mL of ^{3}H-thymidine (specificity 6.7 μCi/mole, New England Nuclear, Boston, MA). Cultures were harvested with a multiple-cell-harvester (Whittaker, M.A. Bioproducts, Inc., Walkersville, MD) onto fiberglass filter paper. The filter-paper disks were placed in Biosafe II liquid scintillation fluid (Research Products International Corp., Mount Prospect, IL) and counted in a Beckman scintillation counter. Results are expressed as counts per minute (CPM) ± standard error.

Statistical Analysis

Data were analyzed by the analysis of variance (ANOVA) procedure. When a significant F ratio indicated reliable mean differences, Student's t test was used to examine pairs of means. The alpha level was set at 0.05 before the experiments were started.

RESULTS

Kinetics of Cell Subpopulations in *T pertenue* Infected Lymph Nodes

Using monoclonal antibody reagents 14-4-4s, 20, 38, and GK 1.5, the kinetic changes in cell subpopulations were studied in lymph nodes of infected hamsters at biweekly intervals for 10 weeks (**Figure 2**). Changes in subpopulations were also determined using age matched noninfected hamsters. The percentage of macrophages remained between 2% to 5% in both infected and noninfected hamsters throughout our study. The percentages of B cells, T cells, and T cells subpopulations were similar in noninfected hamsters and hamsters infected with *T pertenue* for 4 weeks or less. At the 6th week after infection, a sharp reduction in the percentage of total T cells (from 80% to 40%) was detected. Concurrently, the proportion of B cells increased from 20% to 60%. Additionally, the total number of cells in the *T pertenue*-infected lymph node continued to increase until week 8 after infection (**Figure 3**) and then declined. Although the proportion of T cells decreased (**Figure 2**), the actual number of T cells in the infected lymph nodes increased. The proportion of cytotoxic T cells (mAb 38+) did not change. Taken together, there was a decrease in the 38−/38+ cell ratio (or T_H/T_C ratio). This change in T_H/T_C ratio prompted us to study the consequence of depletion of CD4 T cells.

Effects of CD4$^+$ T Lymphocyte Depletion on Resistance Against *T pertenue* Challenge in Immune Hamsters

Following the experimental design illustrated in **Figure 1**, hamsters were immunized 10 weeks earlier by injecting with $5x10^5$ *T pertenue*, and the immunizing infection was cured by treatment with penicillin 10 days before reinfection. The immune hamsters were treated with mAb GK 1.5 or MOPC 195 according to the schedule in **Figure 1**. Immune hamsters that received mAb GK 1.5 exhibited a significant increase ($P< 0.05$) in the number of treponemes ($17\pm15x10^3$) in their lymph nodes (**Table 1**). In contrast, immune hamsters that received

Table 1. Effects of in vivo depletion of CD4+ T cells in T pertenue *immune and normal hamsters.**

Ab Injected‡	Lymph Node† No. of Treponemes x10³
Immune Hamsters	
MAb GK 1.5	17±15§
MOPC 195	<4
Normal Hamsters	
MAb GK 1.5	5±5§
MOPC 195	2457±670

*Hamsters were sacrificed 26 days after infection.
†Values are mean±SE.
‡Three hamsters per group.
§P<0.05 determined by ANOVA followed by Student's t test.

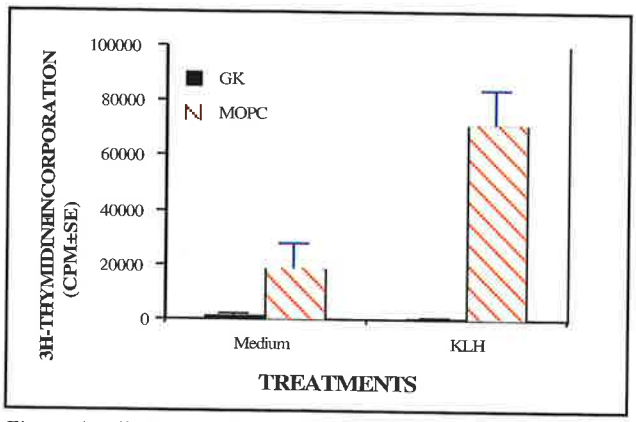

Figure 4. Effects of depletion of CD4+ T cells on the proliferative responses of hamster lymph node cells to KLH.

control antibody MOPC 195 yielded a minimum number of treponemes (<4000; ie, 1 of 3 hamsters had 3.6 x10³ treponemes/LN; the others had no detectable treponemes) in their lymph nodes. These results suggest that treatment with mAb GK 1.5 abrogates protective immunity to *T pertenue* in immunized hamsters.

Next, we tested whether depletion of CD4 T cells can affect the responsiveness to *T pertenue* infection in a normal hamster. Previously uninfected hamsters were depleted of CD4+ T cells 1 day before they were infected with *T pertenue* (**Figure 1**). The lymph nodes of the hamsters depleted of CD4+ cells yielded significantly fewer treponemes ($P<0.05$) in comparison with the lymph nodes of hamsters that received MOPC 195 (**Table 1**).

Effects of CD4+ T Lymphocyte Depletion on Antigenic Stimulation in Treponemal Immune Hamsters

Finally, we tested the degree of CD4 T cell depletion by examining the responsiveness of lymph node cells to antigenic stimulation in vitro. Treponemal immune hamsters were depleted of CD4+ T cells and immunized with KLH, according to the experimental design illustrated in **Figure 3**. For the control group of hamsters that received control antibody MOPC 195 and were immunized with KLH, the lymph node cells responded to stimulation with KLH in an in vitro proliferation assay. In contrast, the proliferative response to KLH was significantly depressed in cells of hamsters that received mAb GK 1.5 (**Figure 4**).

The number of cells in the lymph nodes of animals receiving mAb GK 1.5 (**Table 2**) was also significantly reduced ($P<0.05$). These results suggest that the depletion of CD4+ T cells abolished the normal proliferative response to antigenic stimulation.

DISCUSSION

The mechanism of resistance against infection with treponemes is poorly understood. In this report, we examined the course of infection with *T pertenue* in the popliteal lymph nodes of inbred LSH hamsters. We used the number of treponemal organisms in the lymph node to determine the level of protection.[4,5] In the course of a non-interrupted infection, the numbers of *T pertenue* reached a peak at week 5 of infection, then gradually declined. The proportion of B cells also increased and peaked at 5 to 6 weeks postinfection, while the percentage of macrophages (2%-5%) did not change during the course of infection. The kinetic change of cell subpopulations after infection revealed a decrease in the T_H/T_C ratio in infected lymph nodes 6 weeks after infection. We next defined the role of CD4+ T cells in relation to the pathogenesis of *T pertenue* in immune as well as in normal hamsters. The depletion of CD4+ T cells appeared to abrogate protective immunity in immune hamsters. By contrast, in vivo depletion of lymph node CD4+ T cells in normal hamsters also reduced the number of treponemes in the lymph node.

*Table 2. Effects of depletion of CD4+ T cells on the number of cells in the lymph nodes.**

Treatments‡	Lymph Node† Immune x10⁵	Lymph Node† Normal x10⁵
MAb GK 1.5	8.5±1.3	15.7±3.2§
MOPC 195	25.0±5.0	198.0±67.8

*Hamsters were sacrificed 26 days after infection.
†Values in column 2 and 3 are mean±SE.
‡Three hamsters per group.
§P<0.05 determined by ANOVA followed by Student's t test.

Previously, Bagasra et al[21] reported a large increase in the percentage of null cells (36%) in the lymph nodes of infected hamsters. However, at the time of that study, reliable monoclonal reagents that recognized hamster lymphocyte subpopulations were unavailable. In this study, we used a set of previously defined mAbs 20, 14-4-4s, GK 1.5, and 38 to identify T cells, B cells, T helper cells (T_H), and cytotoxic T cells (T_C), respectively. These monoclonal reagents are able to identify most (95%-98%) of the peripheral lymphocytes in the hamster.[4,5,19,20]

Our results indicate that lymph nodes of infected hamsters increased in weight for several weeks and showed a significant increase in the total number of cells. While absolute numbers of both B and T cells increased (**Figures 2 and 3**), the increase in total cell numbers was mostly in the B cell fraction. This was accompanied by a reduction in the proportion of T cells at 4 to 6 weeks postinfection. An influx of lymphocytes into the lymph nodes and/or local proliferation of B lymphocytes in the follicles might have caused these changes; also, T cells appeared not to proliferate or accumulate in the lymph node at the same rate as B cells. The lower percentage of T cells reflected a relative decrease in the helper T cells (38-/GK1.5+ cells). This decrease resulted in a lower ratio of T helper to T cytotoxic cells (CD4/CD8) that was most evident 6 weeks postinfection. One explanation for the reduced CD4/CD8 ratio could be that recruitment of $CD4^+$ T cells into the regional lymph node is reduced or slowed. Alternatively, the ratio could be caused by preferential recruitment, proliferation, and activation of $CD8^+$ T cells in the regional lymph nodes. This latter hypothesis may be substantiated by findings that primary and secondary syphilitic lesions contain mRNA of activated $CD8^+$ T cells.[22,23] The biological significance of our observations warrants further studies to explain the cause of this decreased helper/cytotoxic T cell ratio and how it affects the pathogenesis of yaws.

Prior investigators have suggested that a period of immunosuppression occurs during the early course of treponemal infection.[24-28] This hypothesis is based on findings of a reduced proliferative response to mitogen by peripheral blood mononuclear cells in syphilitic subjects. Other investigators, however, have reported that lymph node or spleen cells from syphilitic rabbits responded normally to mitogen even when peripheral blood mononuclear cells of the same animals responded poorly.[29-30] The low responsiveness to mitogens might be caused by reduced peripheral blood T lymphocytes in the early stage of syphilitic infection.[24,31,32] Our observation of a reduced helper/cytotoxic T cell ratio is consistent with these reports. Furthermore, examination of the clinical pictures of patients infected with *T pallidum* does not lead to a conclusion of obvious immunosuppression. Patients infected with *T pallidum* exhibit little or no increased susceptibility to the bacterial or viral infections associated with immunocompromised individuals. In addition, there is no evidence of increased opportunistic infections in hamsters infected with *T pertenue*.

The administration of mAb GK 1.5 eliminates $CD4^+$ T cells from mice. This is evident from the reduced antibody- and cell-mediated immune responses.[9-11] In the present study, the effect of depletion of $CD4^+$ cells was demonstrated by the complete absence of a KLH-proliferative response in hamsters immunized with KLH. The background proliferative response was higher than normal >4000 CPM). The cells that proliferated in response to stimulation with *T pertenue*—B cells, T cells, or both—or remaining KLH antigen in the lymph nodes may have caused this high background response. The key observation, though, is the complete suppression of the response to KLH in anti-CD4 antibody treated hamsters, which suggests a complete depletion of CD4 cells.

Our experiments demonstrate that $CD4^+$ T cells play a key role in the pathogenesis of infection with *T pertenue*. Several hypotheses may explain the complex interactions of $CD4^+$ cells in normal and immune hamsters. (1) In the early stage of infection, $CD4^+$ T cells may be important for the growth of *T pertenue*, since treponemes are often attached to in vitro co-cultured cells,[33] and can be cultivated in vitro only in the presence of eukaryotic cells.[34] However, this theory is weakened by findings that treponemes can still multiply in hamsters whose lymph nodes have been destroyed by radiation.[4,5] (2) Subpopulations of $CD4^+$ T cells, T_H1 and T_H2 cells, may be stimulated at different levels by treponemes. It is known that certain pathogens stimulate predominantly T_H2 cells,[35] which are preferentially involved in the production of IgG1 antibody through the secretion of IL-4.[36] It has been shown that hamster IgG1 has less treponemicidal activity than IgG2, because it does not bind well to complements and prevents phagocytosis. In contrast, IgG2 does fix complements and promotes phagocytosis.[37,38] Thus, the preferential activation of T_H2 cells in the early stage of treponemal infection may stimulate predominantly the production of IgG1 antibody. The IgG1 antibody protects the treponemes from phagocytosis by macrophages and from the treponemicidal activity of IgG2 antibody. Elimination of $CD4^+$ T cells also eliminates the T_H2 cells, thus preventing the production of blocking antibody (IgGl). One study that has observed predominantly T_H1 type cytokine profile in primary and secondary syphilitic lesions would argue against this hypothesis.[23] (3) Another plausible hypothesis is that depletion of CD4 T cells also reduces recruitment of

macrophages in the lymph node. In this case, treponemes were not trapped in the regional lymph nodes and move on to the next or other lymphoid tissue in the lymphoid systems. Further studies are needed.

We hypothesize that in the later immune stages of infection, both T_H1 and T_H2 cells are activated when lymphocytes encounter treponemes. T_H1 cells may compete with T_H2 cells in stimulating the production of IgG2 antibody, which fixes complements and promotes phagocytosis.[37,38] This theory is supported by the results of Azadegan et al,[3] which demonstrated that the IgG2 fraction in the immune serum is treponemicidal, whereas IgG1 is not. Alternatively, CD4+ T cells activated at the later stages of the infection may exert a direct treponemicidal effect.[5,8] In this theory, $CD4^+$ T cells are needed in the early stages of treponemal infection for immune recruitment; depletion of $CD4^+$ T cells (**Table 2**) eliminates the ability of regional lymph nodes to mount an effective response to detain the treponemes, which then move on to other parts of the host animal. At a later stage of the infection, both $CD4^+$ and $CD8^+$ T cells become important in host defense against *T pertenue*. This is evident from previous adoptive cell transfer experiments that demonstrated that both T cell subsets, helper, and cytotoxic T cells, confer protection,[4,5] perhaps by a direct treponemicidal activity, such as secretion of lymphotoxin (**Table 1**)[4,8] The elimination of one subset, ie, the $CD4^+$ T cells, abrogates part of the protective immunity. We may conclude from these data that $CD4^+$ T cells play a crucial role in the pathogenesis of treponemal infection.

The protective immune responses against treponemal infection are complicated. We still do not have a complete picture of the host-parasite interactions, but the present study, together with some key observations by others, suggests the following outline. Macrophages and polymorphonuclear neutrophils phagocytize treponemes and may be vital for the early defense of the infection.[39-41] The activation of macrophages, however, does not mount a complete protection against infection.[6,42] It appears that professional phagocytes ingest and remove dead treponemes, but they are ineffective in disposing of live motile treponemes.[39,40,43] It is also known that immune serum confers complete protection against challenge with *T pertenue* when transferred into recipients before challenge.[3] It is the IgG2b fraction that has the treponemicidal activity. Immune T lymphocytes and T cell subsets (CD4 and CD8), when passively transferred to recipient hamsters, mediate protective immunity against challenge. Although immune lymphocytes and immune serum can adaptively transfer protection, it takes donor hamsters 10 weeks to develop this protective immune response. It is possible that immune maturation is required for the host immune system to generate a protective immune response. The immune response that produces the immune sera and lymphocytes in donor hamsters cannot, however, clear the treponemes from the lesions and lymph nodes of donors.[3] This incomplete protective response in donor animals may be partially explained by recent findings that there are few outer membrane proteins on the surface of treponemes.[44,45] Current efforts to develop an effective vaccine against treponemal diseases will likely require further studies on molecular structure of treponemal proteins and on host-treponeme interactions.

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Borrelia burgdorferi Persists in the Gastrointestinal Tract of Children and Adolescents with Lyme Disease

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ABSTRACT

This study documents the persistence of *B burgdorferi* DNA in the gastrointestinal tract of pediatric patients who have already been treated with antibiotics for Lyme disease. Ten consecutive patients between the ages of 9 and 13 years presented with an erythema migrans (EM) rash, a positive western blot for Lyme disease, chronic abdominal pain, heartburn, or bright red blood in the stool. Endoscopy assessed the gastrointestinal (GI) mucosa for inflammation and biopsies were examined for *B burgdorferi* using a Dieterle stain and with polymerase chain reaction (PCR) to the outer surface protein A (Osp A) of *B burgdorferi.* As controls, 10 consecutive patients with chronic abdominal pain were also tested by GI biopsies and PCR. *B burgdorferi* persisted in the GI tract in all 10 patients with Lyme disease as shown by Dieterle stain of biopsies and with PCR. None of the control subjects' biopsies were PCR positive for *B burgdorferi*. Chronic gastritis, chronic duodenitis, and chronic colitis were found in Lyme disease patients and associated with the detection of *B burgdorferi* DNA in the GI tract despite prior antibiotic treatments. We have concluded that the DNA of *B burgdorferi* persisted in patients with Lyme disease even after antibiotic treatment.

Key words: Lyme disease, abdominal pain, heartburn, blood in the stool, *B burgdorferi,* gastritis, duodenitis, colitis, polymerase chain reaction

INTRODUCTION

Two previous studies have described the presence of *B burgdorferi* in the stomach, intestines, and colon of children.[1,2] To address the possibility of the persistence of *B burgdorferi* in the gastrointestinal (GI) tract, a prospective study was made of 10 consecutive patients who had a physician-documented erythema migrans (EM) rash followed by symptoms of Lyme disease that persisted for a year before diagnosis and antibiotic treatment was instituted. They had chronic GI symptoms that persisted during this same period and after completing antibiotic therapy. The purpose of this study is to address the possibility of persistence of *B burgdorferi* DNA after antibiotic treatment in patients with Lyme disease.

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MATERIAL AND METHODS

All of the patients included in our study had an EM rash with no prior history of GI complaints. They were referred to the Pediatric Gastroenterology and Nutrition Service of Jersey Shore Medical Center for evaluation of chronic abdominal pain, heartburn, or bright red blood in the stool that persisted for at least one year after the onset of the EM rash. In all cases, antibiotic therapy for the treatment of Lyme disease was instituted one year after the EM rash and initial symptoms of Lyme disease. Ten consecutive patients satisfying the above clinical criteria[3] were evaluated prospectively from January 1993 through July 2000. There were 5 boys and 5 girls evaluated (mean age 14±3.6 years, range 9-12). Each case included a history, physical examination, complete blood cell count, liver function tests, esophagoduodenoscopy (EGD), and/or colonoscopy. One year after the EM rash, a Lyme IgG western blot confirmed a *B burgdorferi* infection by using the commercially available Marblot strip test system (MarDx Diagnostics, Carlsbad, CA). A positive western blot contained the presence of 5 or more of the following Borrelia bands: 18, 23, 28, 31, 34, 39, 41, 45, 58, 66, 93kDa. The interpretation of the *B burgdorferi*

western blot satisfied the surveillance case definition of *B burgdorferi* infection of the Centers for Disease Control and Prevention. Ultrasonography of the abdomen was performed when the history suggested a diagnosis of biliary tract disease, gallstones, or pancreatitis. Stool samples were examined for occult blood, *Salmonella*, *Shigella*, *Yersinia*, *Campylobacter*, ova and parasites, and *Clostridium difficile* toxins A and B. GI biopsies assessed the mucosa by microscopy for *Helicobacter pylori* (on EGD) and for the presence of *B burgdorferi* by Dieterle stain.

Biopsy specimens were taken from areas of the GI tract that looked inflamed during EGD or colonoscopy. The biopsies were repeated on the dates shown in the **Table** because of the persistence of GI complaints despite the antibiotic treatment of Lyme disease. The biopsies were randomly assigned to five histopathologists who were blinded to the diagnosis of the specimens they received. Biopses were reported as acutely inflamed when polymorphonuclear cells were present in the mucosa and chronically inflamed if 6 or more plasma cells and lymphocytes were present in the gastric mucosa without polymorphonuclear cells. Chronic duodenitis or chronic colitis was diagnosed when greater than 6 intraepithelial lymphocytes per 100 surface absorptive cells were present in tissue biopsies in conjunction with a distortion in glandular architecture.

A polymerase chain reaction (PCR) to detect the DNA of *B burgdorferi* outer surface protein A (Osp A) was performed on all biopsies by Medical Diagnostic Laboratories, New Jersey.

DNA Extraction

As a target for DNA amplification, the gene coding for the Osp A of *B burgdorferi* was analyzed. The tissue was dissolved in 470 μL of tris-edetic acid (EDTA) buffer (10 mM tris-hydrochloride [pH, 8.0] and 1 mM EDTA), 25 μL of 10% sodium dodecyl sulfate, and 12 μL of freshly prepared deoxyonuclease-free proteinase K (10 mg/mL). The mixture was incubated at 55°C for 2 hours; DNA was extracted with phenolchloroform extraction and ethanol precipitation. The purified DNA was dissolved in pyrogen-free, double distilled water and quantified using a Genesys-5 spectrophotometer (Spectronic Instruments, Rochester, NY). The purified quantitated DNA was used as a template for *B burgdorferi* PCR analysis.

Primers

The PCR primers for the identification of *B burgdorferi*, as well as the sensitivity and specificity of the *B burgdorferi* primers are well described. The primers were synthesized by Research Genetics (Huntsville, AL) and purified by high-performance liquid chromatography.

Polymerase Chain Reaction

The PCR mixtures (50 μL) contained extracted DNA (5 μL, 2 μg/μL), P24E, and P12B primers (50 nM), 10 mM trishydrochloride (pH 8.3), 50 mM potassium chloride, 3 mM magnesium chloride, 0.001% (wt/vol) gelatin, the nucleotides dATP, dCTP, dGTP, and dTTP (each at concentrations of 200 mmol/L), and 2.5 U of Taq DNA polymerase (Perkin-Elmer, Foster City, CA). The PCR was carried out in 0.2 mL tubes. The thermocycler was a Perkin-Elmer Gene AMP PCR system 2400. The PCR program ran for 3 minutes at 94°C, followed by 40-one minute cycles at 94°C, 1 minute at 56°C, and 1.5 minutes at 72°C. The program finished with an additional 10-minute extension step at 72°C. A 30 μL sample of the final reaction product was run on 1% agarose gel containing 0.5 μg of ethidium bromide per mL, and the gel was photographed under ultra violet (UV) light.

Histone PCR

Alliquots (5 μL) of the newly extracted DNA were mixed in a 50 μL PCR reaction mixture containing 10X PCR buffer (Perkin-Elmer), 3 mM magnesium chloride, 200 mM dNTP, 2.5 μL of Taq DNA polymerase (5 U/μL), and 1 μL (8 pmol) of 5' and 3' histone amplifier primer set. The histone primers are complementary to the DNA of a constitutively expressed human histone gene H3.3 as described. The amplification process was subjected to 30 cycles of PCR (each cycle at 94°C for 45 seconds, 60°C for 45 seconds, and 72°C for 90 seconds) in a 2400 Perkin-Elmer DNA thermocycler. The histone primers served as internal controls for the sample's DNA integrity, presence of inhibitors, and intersample equivalency of total amount of DNA analyzed.

Precautions Against Contamination

The extraction of DNA and PCR were performed under sterile conditions and in separate rooms. All positive samples were confirmed by re-extraction from the original sample, followed by amplification in triplicate. DNA-positive status was defined as samples that were positive initially and in at least one of the replicates after re-extraction. Pyrogen-free water was used in the isolation of DNA from the biopsy specimens. The Eppendorif microcentrifuge tubes and the PCR tubes were sterilized in an autoclave and UV irradiated. New Finn pipettes were used solely with the filter tips for PCR. Disposable plastic trays were used to prepare PCRs in a UV irradiated PCR biohood. GI biopsy samples from 10 patients with chronic abdominal pain who had no history of tick-borne disease or antibiotic use in the year prior to endscopy were used in the PCR assays as negative controls. The laboratory performing the PCR analysis was blinded to the diagnosis of all specimens they received.

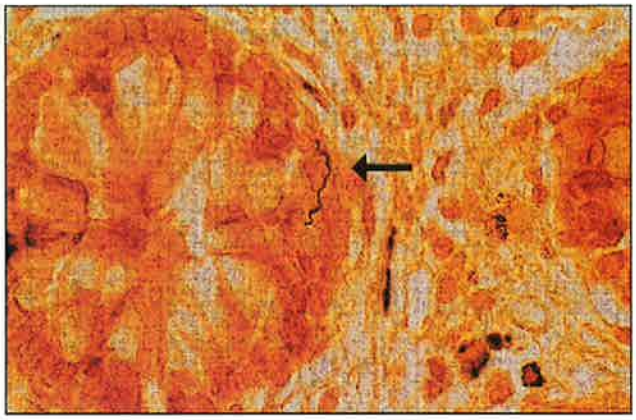

Figure 1. Colon biopsy specimen from a patient with Lyme disease. Abdominal pain and blood in the stool were symptoms. Arrow shows typical helical-shaped spirochete characteristic of Borrelia burgdorferi *(Dieterle stain-magnification x522).*

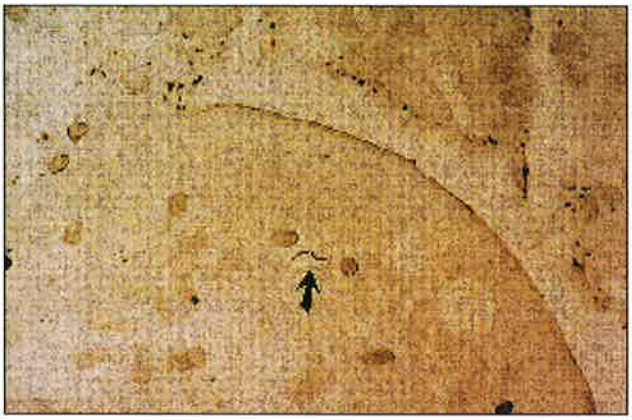

Figure 2. A spirochete (arrow) consistent with the microscopic appearance of Borrelia burgdorferi *is seen in the basal layer of the columnar epithelium from a duodenal biopsy specimen of a patient with Lyme disease and chronic abdominal pain (magnification x790).*

RESULTS

Lyme disease patients presented with chronic abdominal pain and heartburn (n=4, 40%), or chronic abdominal pain and bright red blood in the stool (n=6, 60%). All 10 patients had evidence of inflammation at a GI biopsy site with the microscopic detection of *B burgdorferi* by Dieterle stain that was also confirmed by the detection of *B burgdorferi* DNA by PCR despite 2 prior months of antibiotic therapy (**Figures 1 and 2**). Two of the control patients (#4, #7) had antral gastritis and *H pylori* on biopsy but no evidence of *B burgdorferi* on microscopy or PCR in biopsy specimens from the GI tract (**Table**).

In patients 1 and 10, *B burgdorferi* DNA was detected in the colon on 2 separate colonoscopies performed at least 4 years apart after having received 2 months of intravenous antibiotics for the treatment of Lyme disease. The biopsies of all the Lyme disease patients revealed no evidence of granulomas or terminal ileitis. The IgG western blot was positive in all the patients and negative in all of the control patients. *Salmonella, Shigella, Yersinia, Campylobacter,* and *Clostridium difficile* toxins A and B were not detected in any of the stool samples from patients or controls.

Ultrasonography of the abdomen did not reveal any gallstones, biliary tract disease, or evidence of pancreatitis. The lab performing the PCR had a false positive rate of 1 in 500 by analyzing 25,000 specimens from January 1998 through January 2002.

DISCUSSION

The persistance of Lyme disease as seen by light microscopy and confirmed by tissue PCR in patients with chronic GI symptoms has not been described before. Oksi[3] described the use of PCR in clinical relapse of disseminated Lyme borreliosis to guide the use of additional antibiotic therapy. According to Oksi, the treatment of Lyme borreliosis with appropriate antibiotics for even more than 3 months may not always eradicate the spirochete. The biopsy and PCR results described here suggest the persistence of *B burdorferi* in the stomach, duodenum, and colon at the sites of inflammation despite antibiotic therapy.

Several other authors have suggested that *B burdorferi* may persist in humans and animals for months or years, despite strong humoral or immune responses.[4-7] One possible explanation for this persistence is the failure of the host to produce borreliacidal Osp A antibodies. Another possible explanation is the invasion of poorly vascularized connective tissues by spirochetes or even an intracellular location of spirochetes.[8-11]

Other etiologic infections that are also transmitted by a tick bite should be considered in the differential diagnosis of heartburn, abdominal pain, and blood in the stool in Lyme disease patients with persistent symptoms. Coinfection with either Babesia, Ehrlichia, and Bartonella have recently been reported.[12-14] In areas where both babesiosis and Lyme disease have been reported, the possibility of concomitant babesial infection should be considered. In one such study[14] of 240 patients diagnosed with Lyme disease, 26 (11%) were coinfected with babesiosis. These coinfected patients experienced fatigue, headache, sweats, chills, anorexia, nausea, and splenomegaly more frequently ($P< 0.05$) than those with Lyme disease alone. This pathogen may survive Lyme antimicrobial therapy and be one of the agents responsible for causing persistent symptoms in Lyme disease patients.

Table. The biopsy results of patients with chronic persistent abdominal pain and Lyme disease versus controls.

Patient No	Sex	Age	Diagnosis Date	Chief Complaint	Antibiotics (months)*	Date of Biopsy	Gastric Biopsy	Gastric PCR	Duodenal Biopsy	Duodenal PCR	Colon Biopsy	Colon PCR
Controls												
1	F	17	April 1999	Abdominal pain	0	April 1999	(—)**	(—)	(—)	(—)		
2	F	14	April 1999	Abdominal pain	0	April 1999	(—)	(—)	(—)	(—)		
3	F	11	March 1993	Abdominal pain	0	March 1993	(—)	(—)	(—)	(—)		
4	M	17	January 1996	Abdominal pain	0	January 1996	*H pylori*	(—)	(—)	(—)	(—)	(—)
5	F	15	February 1998	Abdominal pain	0	February 1998	(—)	(—)	(—)	(—)	(—)	(—)
6	F	8	February 1995	Abdominal pain	0	February 1995	(—)	(—)	(—)	(—)		
7	F	15	April 1999	Abdominal pain	0	April 1999	*H pylori*	(—)	(—)	(—)	(—)	(—)
8	F	17	April 1999	Abdominal pain	0	April 1999	(—)	(—)	(—)	(—)	(—)	(—)
9	F	16	January 1993	Abdominal pain	0	January 1993	(—)	(—)	(—)	(—)		
10	M	13	March 1999	Abdominal pain	0	March 1999	(—)	(—)	(—)	(—)	(—)	(—)
Lyme Disease Patients												
1	M	9	January1993	Abdominal pain, blood in stool	4	March 1993					Bb,‡ colitis	DNA
			December 1998	Abdominal pain, blood in stool	4	February 1999	Gastritis	DNA†	Duodenitis	(—)	Colitis	DNA
2	M	13	January 1999	Abdominal pain, blood in stool	2	March 1999	(—)	(—)	(—)	(—)	Bb, colitis	DNA
			February 2000	Abdominal pain, blood in stool	2	April 2000	(—)	(—)	(—)	(—)	Colitis	DNA
3	F	12	December 1994	Abdominal pain, heartburn	4	February 1995	Gastritis	(—)	Bb, duodenitis	DNA		
			January 1999	Abdominal pain, heartburn	5	March 1999	Gastritis	(—)	Duodenitis	DNA		
4	F	7	March 1997	Abdominal pain, heartburn	2	May 1997	Bb, gastritis	DNA	(—)	(—)		
			March 1999	Abdominal pain, heartburn	2	May 1999	Gastritis	DNA	(—)	(—)		
5	F	11	December 1992	Abdominal pain, heartburn	2	February 1993	Gastritis	(—)	Bb, duodenitis	DNA		
			April 1993	Abdominal pain, heartburn	2	June 1993	(—)	(—)	Duodenitis	DNA		
6	M	9	March 1993	Abdominal pain, blood in stool	2	May 1993	(—)	(—)	(—)	(—)	Bb, colitis	DNA
			May 1994	Abdominal pain, blood in stool	2	July 1994	(—)	(—)	(—)	(—)	Colitis	DNA
7	F	10	February 1995	Abdominal pain, heartburn	5	April 1995	Bb, gastritis	DNA	(—)	(—)		
			January 1999	Abdominal pain, heartburn	4	March 1999	Gastritis	DNA	(—)	(—)		
8	M	12	January 1999	Abdominal pain, blood in stool	2	March 1999		(—)			Bb, colitis	DNA
			January 2000	Abdominal pain, blood in stool	2	March 2000					Colitis	DNA
9	M	11	June 1993	Abdominal pain, blood in stool	4	August 1993	Gastritis	DNA	Bb, duodenitis	DNA	Bb, colitis	DNA
			June 1994	Abdominal pain, blood in stool	4	August 1994	Gastritis	DNA	Duodenitis	DNA	Colitis	DNA
10	F	10	April 1994	Abdominal pain, blood in stool	2	June 1994	Bb, gastritis	DNA	Duodenitis	DNA	Bb, colitis	DNA
			December 1998	Abdominal pain, blood in stool	4	February 1999	Gastritis	DNA	Duodenitis	DNA	Colitis	DNA

**Number of months of antibiotic treatment for Lyme disease prior to endoscopy in the last year.*

*** (—) denotes no histological pathology detected on biopsy and no detection of B burgdorferi DNA by polymerase chain reaction (PCR).*

†DNA denotes the detection of B burgdorferi *DNA (the outer surface protein A) by PCR of biopsy specimens. DNA denotes the detection of B burgdorferi DNA.*

‡Denotes the microscopic appearance of B burgdorferi on biopsy specimens with the use of a Dieterle stain.

Unfilled spaces denotes areas of the gastrointestinal tract that were not biopsied.

CONCLUSION

Inflammation found in the stomach, duodenum, and colon of patients with Lyme disease who had persistent heartburn, abdominal pain, or blood in the stool may be attributed to multiple etiologies, one of which is the presence and or persistence of Lyme DNA. Babesia, Ehrlichia, and Bartonella as etiologies of persistent GI symptoms unresponsive to Lyme antimicrobial therapy deserves further investigation.

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Lyme Vaccination Safety

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ABSTRACT

The purpose of this analysis was to examine serious arthritic, immunologic, neurologic, and gastrointestinal adverse reactions following recombinant Lyme vaccination in the adult population of the United States. A certified copy of the Vaccine Adverse Events Reporting System (VAERS) database was obtained and analyzed from December 1998 through October 2000 using Microsoft Access. We analyzed the following serious adverse reactions: arthritis, neuropathy, convulsions, thrombocytopenia, lymphadenopathy, flu syndrome, alopecia, gastrointestinal disease, and paralysis. A χ^2 statistical 2x2 contingency table was used to determine whether the noted elevated incidence rates of serious adverse reactions in Lyme vaccine recipients achieved statistical significance over those reported following Tetanus-diphtheria (Td) and rubella vaccinations received by adults. We observed adverse reactions fairly evenly divided between men and women between the ages of 35 to 62 years old within several weeks of Lyme vaccination. Because of the molecular design of the recombinant form of Lyme vaccine, it was assumed that this vaccine would be well-tolerated and result in few serious adverse reactions. This prediction was not borne out by our analysis of the VAERS database. Rather, our analyses showed a statistical increase in adverse reactions over those reported following Td or rubella vaccination in adults. Our results indicate a less reactogenic Lyme disease vaccine is needed. The withdrawal of Lyme vaccine in early 2002 seems well justified based upon the results of this study and Lyme vaccine probably should not be used until processes have been developed to produce a safer vaccine.

Key words: adverse reaction, arthritis, gastrointestinal disease, Lyme vaccine, neuropathy, VAERS

INTRODUCTION

Lyme disease is a bacterial infectious disease of considerable importance. In the United States, Lyme disease is caused by the spirochete *Borrelia burgdorferi* sensu lato; in Europe *B afzelii* and *B garinii* have also been identified as causes of Lyme disease.[1] Ticks of the Ixodes ricinus complex—*I Scapularis* and *I Ricinus* in Europe—transmit spirochetes after feeding for 36 to 72 hours.[1-3] The most important reservoir for *B burgdorfei* is the white-footed mouse of North America, where both adult and nymph forms of the tick can transmit infection.[4]

The most commonly reported vector-borne infection in the United States is Lyme disease. Lyme disease is most prevalent in the northeast, north central, and western costal regions of the United States, although cases of it have been reported in all 50 states. Children from 5 to 14 years old and adults between 30 and 55 years old are most often affected by the disease. In the United States from 1982 to 1996, a 30-fold increase in the number of cases of Lyme disease has been observed.[5]

In Europe and the United States, case definitions for Lyme disease have been developed.[6-7] The initial symptoms of the disease are characterized by erythema migrans and flu-like illness, followed by neurologic, cardiac, or musculoskeletal manifestations following weeks or months after exposure.[4]

The early attempts to develop a vaccine against Lyme disease involved making whole-cell preparations of *B burgdorferi*.[8-9] The observed reactogenicity/toxicity of the whole-cell preparations caused the pursuit of a recombinant vaccine.[10] The outer surface protein A (OspA) of *B burgdorferi* was selected and proved to be immunogenic

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in animal models.[11-13] OspA expression occurs primarily in the tick and is upregulated after passage from the vertebrate host to the tick.[14] Additionally, *Borrelia* OspA is primarily found in the United States, although this is not true in Europe.[15]

The OspA-based Lyme vaccine confers protection in a unique way. Bactericidal antibodies generated against OspA eliminate *B burgdorferi* from this vector during feeding, preventing infectious spirochetes from entering the host.[16-17] To be effective, a seroprotective level of antibody must be obtained before exposure to infected ticks, and a bactericidal antibody can be achieved through vaccination or infection.[18-20]

The Food and Drug Administration (FDA) of the United States in 1998 licensed an adjuvanted vaccine (LYMErix™, SmithKline Beecham Pharmaceuticals) containing 30 µgrams of lipidated recombinant OspA for the prevention of Lyme disease and asymptomatic infection in persons 15 to 70 years old.

In a multicenter double-blind trial of 10,936 participants, this vaccine was administered in a 0, 1, and 12 month schedule, demonstrating efficacy after 2 to 3 doses. Antibody titers 1 month after dose 2 were determined to be 1,227 IU/mL and 1 month after dose 3 were determined to be 6,006 IU/mL. These levels of antibody were achieved in 90% and 95% of recipients, respectively. Efficacy in preventing symptomatic Lyme disease was 76% after 3 doses of vaccine and 49% after 2 doses. Efficacy for preventing asymptomatic disease was 100% after the third dose and 83% after the first 2 doses. LYMErix vaccine was described as "well-tolerated" in the trials.[21] Additionally, two studies were published, one by Steere et al and another large clinical trial by Sigal et al, that were unable to substantiate inflammatory arthritis as a complication of Lyme vaccination.[21-22]

The purpose of this analysis was to examine serious arthritic, neurologic, immunologic, and gastrointestinal adverse reactions reported to the Vaccine Adverse Events Reporting System (VAERS) database following recombinant Lyme vaccination in the adult population of the United States. The VAERS database is an epidemiological database maintained by the Centers for Disease Control and Prevention (CDC) since 1990. All adverse reactions following vaccination are to be reported to this database as mandated by US law. The protocol for reporting all serious reactions to VAERS requires written and telephonic confirmation by the CDC. The CDC also follows up all serious reactions 1 year after they occur to determine whether or not the patients had fully recovered from their reaction. The VAERS Working Group of the CDC analyzes and publishes epidemiological studies based upon the VAERS database. A recent study by the VAERS Working Group of the CDC has stated that VAERS is simple for reporters to use, flexible by design, and that its data are available in a timely fashion.[23] We and other authors have found that the massive size of the VAERS database makes it a unique and useful tool to analyze adverse reactions to vaccines. Our recent studies have shown an association between hepatitis B vaccination and arthritic, immunologic, and gastrointestinal symptoms based upon our analysis of the VAERS database.[24-31] We have also reported on the incidence of adverse reactions following childhood vaccines, adverse reactions following vaccination in the state of Texas, arthritic symptoms following rubella vaccination, and adverse reactions following anthrax vaccination in light of biological warfare scenarios based upon analysis of the VAERS database.[32-39]

It was our aim in this study that by examining the VAERS database we would gain a broad perspective of the effects of Lyme vaccination in the United States population based upon many millions of doses of vaccine that is virtually unattainable by any other methods of analysis.

MATERIAL AND METHODS

To further examine adverse reactions reported following Lyme vaccination, we made a retrospective examination of the information reported to the VAERS database from December 1998 through October 2000 using Microsoft Access. Our analysis was limited to only adverse reactions reported among those residing in the US, within 60 days of immunization, in the adult population for which the FDA has approved the Lyme vaccine. The adverse reactions reported to the VAERS database examined in this study were: arthritis, neuropathy, convulsions, thrombocytopenia, lymphadenopathy, flu syndrome, alopecia, gastrointestinal disease, and paralysis. The chronic adverse reactions examined, wherein the patients had not recovered from their adverse reaction based upon a 1-year follow up, were: arthritis, neuropathy, lymphadenopathy, flu syndrome, gastrointestinal disease, and paralysis. We also examined the number of reaction reports, emergency room (ER) visits, life-threatening reactions, hospitalizations, disabilities, and deaths reported to the VAERS database following vaccination. These categories for adverse reactions were based upon descriptions of adverse reactions by those reporting them and by defined reporting fields contained in the VAERS database. The incidence rates calculated in this study were based upon the estimates of the Biological Surveillance Summaries that we obtained from the CDC for the number of doses administered during the period examined. The use of these numbers to calculate the incidence of adverse reactions reported to the VAERS database has

Table 1. General adverse reactions reported following Lyme vaccination in comparison to adult tetanus-diphtheria (Td) vaccination.

Type of Vaccine	Incidence per Million Vaccines of Reaction Reports	Incidence per Million Vaccines of Emergency Room Visits	Incidence per Million Vaccines of Life-threatening Reactions	Incidence per Million Vaccines of Hospitalizations	Incidence per Million Vaccines of Disabilities	Incidence per Million Vaccines of Deaths
Lyme	474	154	7.8	17	18	1.4
Td vaccine	56	23	0.46	2.0	0.28	0.06
Relative risk	8.5	6.7	17	8.5	64	23
Attributable risk	7.5	5.7	16	7.5	63	22
Percent association	89	87	94	89	98	96
Statistical significance	$P< 0.0001$	$P< 0.0001$	$P< 0.0001$	$P< 0.0001$	$P< 0.0001$	Not significant

been validated in several of our recent publications.[27-39] The CDC estimates indicated that 1,400,000 Lyme vaccinations were administered during this study period. Additionally, as a control, we analyzed the adverse reactions in adults residing in the US to the adult tetanus-diphtheria (Td) vaccine within 60 days of receiving immunization as reported to VAERS from 1991 through 1999, so as to maximize the background reporting rates of adverse reactions reported to the VAERS database. The CDC estimates indicated that 129,293,354 Td vaccinations were administered from 1991 through 1999 to adults. The incidence rates of adult adverse reactions in Td vaccine recipients provided a background rate to compare against the incidence rates of adverse reactions in Lyme vaccine recipients.

We believe an unbiased search of the incidence rate of a specific adverse reaction to one vaccine would be expected to be similar to the incidence rate following another vaccine administered to a similar aged population, because whatever the inherent limitations in the accuracy of reported adverse reactions to the VAERS database, they should be expected to equally effect the reports of both vaccines under study. Similarly, the number of doses of a type of vaccine administered based on the Biological Surveillance Summaries of the CDC should be unbiased because whatever the inherent limitations of the Biological Surveillance Summaries, they should apply equally to each vaccine under study. In performing our statistical analyses, the assumption of equal reactogencity between vaccines forms the basis of our null hypothesis. In performing our statistical analysis, we used a 2x2 χ^2 contingency table where we assume that the total number of an adverse reaction following our control vaccine and the number of doses administered based upon our Biological Surveillance Summaries for the time period examined are the expected values and the total number of an adverse reaction following our vaccine under study and the number of doses administered based upon our Biological Surveillance Summaries for the time period examined are the observed values. The use of vaccine control groups to determine if there was a statistical relationship between a vaccine and specific types of reactions using χ^2 statistical analysis has been validated in several of our recent publications.[27-39] We used the statistical package contained in Corel's Quattro Pro and accepted a P value of 0.05 as statistically significant.

We have also used the incidence rate of an adverse reaction following our vaccine under study in comparison to the incidence rate of an adverse reaction following our control vaccine group to determine the relative risk, attributable risk, and the percent association of the adverse reaction following our vaccine under study. The relative risk value is obtained by dividing the incidence rate of the adverse reaction following our vaccine under study by the incidence rate of the adverse reaction following our vaccine control group. The attributable risk value is obtained by subtracting 1 from the relative risk following the vaccine under study. The percent association value is calculated by dividing the relative risk value by the relative risk value plus 1 and multiplying this computed value by 100.

RESULTS

Table 1 summarizes the incidence per million vaccinations of total reaction reports, ER visits, life-threatening reactions, hospitalizations, disabilities, and deaths reported to the VAERS database following Lyme and adult Td vaccinations among those residing in the United States. This table shows that there was a statistical increase in the incidence of total reaction reports, ER visits, life-threatening reactions, hospitalizations, and disabilities reported to the VAERS database following Lyme vaccination in comparison to our adult vaccine control group. **Table 2** summarized the number of male and female reaction reports,

Table 2. A summary of serious adverse reactions to Lyme vaccination.

Type of Reaction	Number of Female Reports	Number of Male Reports	Mean Age (Years)	Mean Onset (Days)	Incidence per per Million Vaccinations
Arthritis	24	14	51.0±13.0	10.4±15.1	27
Neuropathy	5	2	57.8±6.8	2.1±2.4	5.0
Convulsions	1	3	39.7±13.0	0.75±0.83	2.9
Thrombocytopenia	1	2	56.2±1.7	3.7±5.2	2.1
Lymphadenopathy	5	5	48.0±13.7	8.7±9.1	7.1
Flu syndrome	47	42	47.4±14.0	5.0±9.8	64
Alopecia	2	0	55.5±1.5	1.5±0.5	1.4
Gastrointestinal disease	4	1	48.6±14.1	18.8±20.0	3.6
Paralysis	3	1	45.7±19.8	4.2±5.1	2.9

mean age in years, mean onset in days, and the incidence per million vaccinations of adverse reactions reported following Lyme vaccination among those residing in the United States. This table illlustrates that most of the adverse reactions analyzed were reported to the VAERS database following Lyme vaccination occurred in patients about 50 years of age within several weeks of immunization. The data further shows that the number of adverse reactions reported following Lyme vaccination were fairly evenly divided between men and women. **Table 3** compares the reactivity of adult Td vaccine and Lyme vaccine administration among those residing in the United States. This table details the statistical increase in both acute and chronic adverse reactions reported to the VAERS database following Lyme vaccine in comparison to our adult Td vaccine control group.

We also compared the incidence of arthritic reactions reported following Lyme vaccination with those reported following adult rubella vaccination to the VAERS database. We have previously reported on arthritic conditions reported to the VAERS database following adult rubella vaccination.[31] The incidence of arthritis reported to the VAERS database following adult rubella vaccination from 1991 through 1999 was 8.0/million rubella vaccinations within 60 days of immunization among those residing in the US. The Institute of Medicine (IOM) of the US

Table 3. Serious adverse reactions following Lyme vaccination in comparison to adult tetanus-diphtheria (Td) vaccination.

Type of Adverse Reaction	Incidence per Million Td Vaccines	Incidence per Million Lyme Vaccinations	Relative Risk	Attributable Risk	Percent Association	Statistical Significance
Arthritis	0.22	27	123	122	99	$P< 0.0001$
Chronic arthritis	0.054	16	296	295	99	$P< 0.0001$
Neuropathy	0.36	5.0	14	13	93	$P< 0.0001$
Chronic neuropathy	0.12	2.1	18	17	95	$P< 0.0001$
Convulsions	1.2	2.9	2.4	1.4	70	Not Significant
Thrombocytopenia	0.070	2.1	30	29	97	$P< 0.0001$
Lymphadenopathy	2.2	7.1	3.2	2.2	76	$P< 0.0005$
Chronic lymphadenopathy	0.18	2.1	12	11	92	$P< 0.0001$
Flu syndrome	1.0	64	64	63	98	$P< 0.0001$
Chronic flu syndrome	0.11	24	218	217	99	$P< 0.0001$
Alopecia	0.39	1.4	36	35	97	Not Significant
Gastrointestinal disease	0.039	3.6	92	91	99	$P< 0.0001$
Chronic gastrointestinal disease	0.023	2.1	91	90	99	$P< 0.0001$
Paralysis	0.12	2.9	24	23	96	$P< 0.0001$
Chronic paralysis	0.054	1.4	30	29	97	Not Significant

National Academy of Sciences reported in 1991 that the evidence indicated a causal relationship between the currently used rubella vaccine and acute and chronic arthritis.[40] In comparing the incidence rate per million vaccinations of arthritic reactions reported to VAERS following Lyme vaccination to adult rubella vaccination, a statistical increase in the incidence of arthritis was observed ($P < 0.0001$, relative risk =3.4, attributable risk =2.4, percent association =77). We also compared the incidence of chronic arthritis following Lyme vaccine in comparison to adult rubella vaccine (3.3/million rubella vaccinations). We found a statistical increase in the incidence of chronic arthritis adverse reactions following Lyme vaccine in comparison to adult rubella vaccination ($P < 0.0001$, relative risk =4.8, attributable risk =3.8, percent association =83).

DISCUSSION

The prediction, both from the vaccine design and early clinical trials on Lyme vaccine, that this type of vaccine would be well-tolerated and result in few adverse reactions is not borne out by our analysis of the VAERS database. Our analysis showed a statistical increase in numerous adverse reactions reported to occur in adults after Lyme vaccination when compared with our adult Td vaccine control group. The adverse reactions we observed following Lyme vaccine were also of particular interest because they appear to reflect the serious effects of the natural disease. This suggests that OspA may indeed play a significant role in the development of serious sequella following natural Lyme disease infection.

The overall rate of developing a serious arthritic, neurologic, or gastrointestinal adverse reaction following Lyme vaccine, based upon the reactions we examined, was 1 in 8,621 doses of Lyme vaccine. However, since serious adverse reactions following Lyme vaccine were unexpected, the adverse reactions reported to VAERS undoubtably are under reported. Doctors need to become aware of these reactions and, should they occur following Lyme vaccinations, they should be reported to VAERS if the vaccine is reintroduced.

A model system has been developed by Croke et al to observe adverse reactions following Lyme vaccination. Their work provided direct evidence that OspA can induce arthritis in Hamster animal model systems.[41] They showed hamsters vaccinated with 30, 60, or 120 micrograms of recombinant OspA and challenged with *B burgdorferi* developed swelling of the hind paws that was detected in 100%, 100%, and 50%, respectively. The authors stated that their findings suggested recombinant OspA vaccines should be modified to eliminate epitopes of OspA responsible for the induction of arthritis, especially considering the FDA has approved recombinant OspA vaccine for use in humans.

The mechanism with which Lyme vaccine potentially causes the adverse reactions observed in this study involves the histocompatibility type HLA-DR4. This histocompatibility group has demonstrated an increased frequency of migratory arthritis following natural infection with *B burgdorferi*.[42] Additionally, an epitope of OspA, when bound to transgenic mouse HLA-DRB1*0401, is cross reactive with human leukocyte function-associated antigen-1, prompting speculation that so-called "treatment-resistant" Lyme arthritis is an autoimmune phenomena. Evidence for the role of a cross reactive T-cell response in the pathogenesis of Lyme arthritis remains theoretical.[43-44] We believe this type of molecular mimicry response may also play a significant role in the development of the other serious adverse reactions we observed following Lyme vaccine. The combination of the adjuvant with a potentially cross-reactive Osp A antigen may be why the reactions we observed following Lyme vaccine are so serious and occur in fairly close temporal association with vaccination.

We have recently published an article detailing how to design vaccines accompanied by fewer serious adverse reactions.[39] We suggested that vaccines should be single antigen, highly purified and checked to determine if any of the epitopes they contain are cross reactive with human lymphocytes. They should come in single dose sealed vials so that preservatives are not necessary, and should contain enough antigenic material so that adjuvants are not needed.

A recent study by Lathrop et al concluded that, following the first 19 months of Lyme vaccination and based upon analysis of the VAERS database, there were no unexpected or unusual patterns of reported adverse events following Lyme vaccination, other than hypersensitivity reactions, compared with adverse events observed in clinical trials.[45] It is interesting to note that the early clinical trials reported that Lyme vaccine was "generally well-tolerated" and that apparently these clinical trials, as reported by Lathrop et al, observed similar rates of reactivity that we observed in this study to make their decision that Lyme vaccine was "generally well-tolerated." Our data does not support the observation that Lyme vaccine was "generally well-tolerated."

CONCLUSION

Our study showed that there was a statistical increase in the incidence rate of serious arthritic, immunologic, gastrointestinal, and neurologic adverse reactions reported to the VAERS database following Lyme vaccine in comparison to our adult Td vaccine control group. Even more

remarkable, our analysis showed Lyme vaccination to have a statistical increase in the incidence rate of both acute and chronic arthritis reactions in comparison to adult rubella vaccination that has been determined by the IOM to have a causal relationship with acute and chronic arthritis. The observed adverse reactions in this study were unexpected based upon the Lyme vaccine design as a single antigen, genetically engineered, purified vaccine. Our results indicate that additional study of Lyme disease is necessary to allow for the production of a safer vaccine. The withdrawal of Lyme vaccine in early 2002 seems justified based upon the results of this study and Lyme vaccine probably should not be used until processes have been developed to produce a safer vaccine.

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Cat-Scratch Disease—A New Tick-Borne Disease?

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ABSTRACT

A broad outline of the different Bartonella species is presented. The epidemiology, pathology, and clinical presentation of Bartonella henselae is covered. Co-infection rates of 327 patients tested for tick-borne illnesses revealed that more patients were positive for *Bartonella henselae* (92/327) than for *B burgdorferi* (64/327); 22 patients were positive for both. This correlates with the infection rates found in ticks analyzed in 2001. Of those patients with positive Bartonella polymerase chain reaction, 83% had positive antibodies. A patient with both Bartonella and Lyme disease is presented. This patient developed a sarcoid-like presentation as a complication of her infection.

Key words: Bartonella, tick-borne infections, sarcoid

INTRODUCTION

Cat-scratch disease, caused by the bacterial organism Bartonella, has been recognized since 1950. It was always thought to be transmitted by a cat scratch, a puncture wound, a previous break in the skin or, rarely, the bite of a cat or other animal. It was postulated in several articles, though, that it can be transmitted by an insect vector. It was in the 1990s that the organism responsible for this disease was identified as *Bartonella henselae.*

A recent article published by Eskow and Mordecai[1] demonstrates the coexistence of both Lyme disease and Bartonella in several cases. This has raised the possibility that Bartonella is yet another infection that can be transmitted by deer ticks.

Other species of Bartonella are responsible for several other vector-borne infectious diseases, namely:

- Carrion's disease: *Bartonella baccilliformis* transmitted by sand flies.
- Trench fever: *Bartonella quintana* transmitted by human lice.
- Several new species identified in animals, mites, and ticks.

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Epidemiology

The epidemiology of cat-scratch disease is interesting because 72% of cases in the United States were seen between June and December. This seasonality would not be explained if the disease were transmitted only by cat scratches alone.

Over the past 1 to 2 years, several studies have been published wherein the relationship between *B henselae* and tick-borne illnesses has been studied. In July 1999, a study of deer ticks conducted in the Netherlands found polymerase chain reaction (PCR) evidence of Bartonella in 70% of ticks tested compared with a 45% positivity for *Ehrlichia* and a 13% positivity for *Borrelia.*[2] A subsequent study performed in the United States on deer ticks in California demonstrated an infection rate of 19%.[3] This organism has been isolated from a wide range of mammals, many of which also carry *Borrelia, Ehrlichia,* and *Babesia.* A study in the midwestern United States revealed that the white footed mouse, a common reservoir of Lyme disease, was 46% positive for *B Burgdorferi* by culture, 12% positive for *Babesia* by PCR, 5 to 10% positive for Bartonella by culture.[4] In an ongoing study in New Jersey, we currently estimate that 20% of deer ticks are positive by PCR (unpublished).[5]

Prevalence statistics of Bartonella in cats specifically are: California, 85.7%; North Carolina, 89.5%; and Hawaii, 42.3%.[6] Although transmission of this disease by

Table 1. Analysis of 327 patients tested by polymerase chain reaction (PCR) for Bartonella species.

Total Patients	327
Positive Bartonella PCR	92
Positive Lyme and Bartonella PCR	22
Positive Lyme PCR	64

Table 2. Polymerase chain reaction (PCR) sensitivity.

	+PCR	−PCR
+AB	48 (83%)	62 (76%)
−AB	10 (17%)	19 (24%)

ticks has not been conclusively established, we know that it is transmitted in the feline population by fleas.[7]

MATERIALS AND METHODS

Data on the clinical presentation of cat-scratch disease consists of one large review of cases published by Carithers in 1985[8] and numerous case reports found in ophthalmology, neurology, infectious disease, and rheumatology literature. The initial rash in the classic description of cat-scratch disease is described as a papule that progresses through a vesicular, crusty stage (resembling the lesions seen in chickenpox) over a period of several days. Within 1 to 2 weeks, regional lymphadenopathy appears. In 75% of cases, the illness is mild and self-limiting. Biopsies of lymph nodes reveal pathology often indistinguishable from sarcoidosis. There also are reports of biopsies strongly suggestive of lymphoma. Atypical or unusual manifestations reported in the literature include:

- Oculoglandular syndrome: preauricular adenopathy and conjunctivitis associated rarely with suppurative lymph nodes.
- Encephalopathy occurring 1 to 6 weeks after the initial infection.[8-11] Ten percent overall had some residual neurologic deficits. The cause has been postulated to be both infectious[12] and vasculitis, as seen in several reports demonstrating beading of the cerebral arteries.[13]
- Myelitis including 1 case of transverse myelitis.[14]
- Pradiculitis.
- Sarcoid-like presentation.
- Osteolytic lesions.
- Erythema nodosum (notably associated with sarcoidosis).
- Ophthalmologic manifestations: peripapillary angioma, branch retinal artery occlusion with vision loss,[15] Bartonella neuroretinitis.[16]
- Arthritis.[17]
- Chronic demyelinating polyneuropathy.[18]

Analysis of 327 Patients Tested for Tick-borne Infections

Table 1 demonstrates that of the 327 patients tested for tick-borne infections, 92 tested positive by PCR for Bartonella species. This number exceeds those who tested positive for *B burgdorferi* (64). Additionally, 22 patients tested positive for both infections. These data are similar to studies done in ticks in 2001, wherein a higher percentage of ticks tested positive for Bartonella, than for *B burgdorferi* (unpublished).

Table 2 illustrates the sensitivity and specificity of antibody production in patients with a positive PCR. Of those patients with a positive PCR for Bartonella, 83% had positive antibodies. Of those with negative PCRs, however, 76% also had positive antibodies. The presence of antibodies cannot, therefore, be used as the sole criterion for establishing a clinical diagnosis. While the sensitivity is 83%, the specificity is only 24%. Of 3 patients who underwent a spinal tap, 3/3 had a positive PCR for Bartonella. One of these 3 also had a positive PCR for *B burgdorferi*.

CASE REPORT

A 30-year-old female was diagnosed by a dermatologist as having erythema migrans rash. She was placed on oral doxycycline. Within a week, the rash became vesicular and crusty and antiviral medication was added. Within 2 weeks, the patient had progressive loss of balance, bilateral symmetrical leg weakness, loss of bladder control, headaches, and neck stiffness. A MRI revealed enhancing lesions in the thoracic and cervical spinal cord. The brain was normal. Both blood and cerebral spinal fluid were positive for Bartonella PCR. Subsequent serologies for *Bartonella henselae* were strongly positive. The patient has had a protracted course with an initial response to 3 months of intravenous antibiotics. This was followed by a relapse in which she developed total paralysis of both legs, traverse myelitis, and hilar lymphadenopathy. Hilar node biopsy was consistent with sarcoidosis. Protracted treatment with a combination of steroids and intermittent antibiotics has resulted in substantial improvement. The patient does have residual weakness and is currently experiencing some relapsing symptoms associated with another positive Bartonella PCR (which had become negative after treatment).

Other Bartonella Infections

The most comprehensive review of Carrion's disease was recently reported by Maguina et al[19] based upon a review of 145 patients from Peru. The organism responsi-

ble for this illness is *Bartonella bacilliformis*. In this disease, cases are separated by clinical criteria into two groups: hematic phase (47%) and eruptive (verruga) phase (53%).

The hematic phase is usually associated with fever, lymphadenopathy, and hepatomegaly (with abnormal liver enzymes). Of these patients, 17% had ophthalmological findings including flame-shaped hemorrhages, cotton wool exudates, and papilledema. Twenty-six percent had central nervous system symptoms and/or findings. In 30%, cardiomegaly was present on a chest roentgenograph. Intraerythrocytic organisms were present in 100% of patients, visible on a blood smear stained with Giemsa. Only 71% grew positive cultures and the average time to yield a positive culture was 18 days. Most patients were anemic with an elevated bilirubin. The death rate in this group was 9%.

The eruptive phase is generally benign and usually associated with skin lesions, most commonly miliary lesions on the lower extremities.

CONCLUSION

Bartonella is an emerging infectious disease that is carried by deer ticks, isolated in animal reservoirs known to also be reservoirs for Lyme disease, and is being found in association with *B Burgdorferi* in patients. It is being isolated from a substantial number of deer ticks in New Jersey, and has been tested in several patients this year in whom both the infecting tick and the patient had positive PCRs.

It would be advisable to consider this infection in the same light as the other more recently discovered infections transmitted by the bite of deer ticks, even in the absence of conclusive proof that it is transmitted by tick bites. Further studies should be performed in laboratory animals to gain better knowledge of this issue.

The relevance of testing for this infection would lie in the different treatment approaches. Some of the first line choices of antibiotics for the treatment of Lyme disease would not be the first choice in Bartonella.

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Dedicated to science and art in spirochetal and tick-borne diseases

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