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IFNγ production in peripheral blood of early Lyme disease patients to hLFAα_L (aa326-345)

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Abstract

Background: It has been proposed that outer surface protein A (OspA) of *Borrelia burgdorferi* sensu stricto contains a T helper 1 (Th1) cell epitope that could play a role in an autoimmune response to hLFA1.

Methods: We used two peptides, hLFAα_L (aa326-345) and *Borrelia burgdorferi* OspAB31 (aa164-183), as stimulating antigens to measure Th1 proinflammatory IFNγ cytokine production in peripheral blood of Lyme disease patients presenting with EM without history of arthritis, as well as in peripheral blood of healthy individuals.

Results: IFNγ responses to hLFA1 peptide were observed in 11 of 19 Lyme disease patients and in 3 of 15 healthy controls. In contrast, only 2 of 19 of the Lyme disease patients and none of the controls responded to the homologous OspAB31 peptide.

Conclusions: IFNγ was produced in response to stimulation with peptide hLFAα_L (aa326-345) in peripheral blood of 58% of patients with early Lyme disease without signs of arthritis, as well as in peripheral blood of 20% of healthy individuals, but not in response to stimulation with the homologous OspAB31 (aa164-183) peptide ($p < 0.05$). Our results suggest that reactivity to the hLFA1 peptide in peripheral blood may be the result of T cell degeneracy.

Background

Lyme disease is a multisystem disease caused by the spirochete *Borrelia burgdorferi* [1,2]. Although most patients respond to two to four weeks of antibiotic therapy, a few individuals continue to have clinical manifestations after treatment. These patients do not respond to additional courses of antibiotics raising the possibility that a post infectious immunologic process is responsible for these persistent clinical manifestations.

T helper 1 responses are associated with antibiotic refractory Lyme disease [3,4]. Outer surface protein A (OspA) from *B. burgdorferi* sensu stricto contains a helper T cell epitope with a similar sequence to a segment in human leukocyte function-associated antigen-1 (hLFA1). Gross et al. found that individuals with treatment resistant Lyme arthritis, but not other forms of arthritis, generated Th1-IFNγ responses to OspA, hLFA1 and their highly related peptide epitopes in synovial fluid. These authors pro-

posed that cross-reactivity between an OspA epitope and hLFA1, allows OspA primed T cells to remain activated by continued stimulation through cells expressing hLFA1, even after elimination of the spirochetes by antibiotic therapy, initiating an autoimmune response to hLFA1 in the joint [5,6]. Thus, the ongoing clinical manifestations in refractory Lyme disease may not depend on persistent infection, but to the immune response to a cross reactive epitope in the etiological agent [7].

Since an autoimmune cross reactivity between *B. burgdorferi* OspA, human LFA1 and antibiotic resistant Lyme arthritis has been suggested [6] and it is known that only a subpopulation of Lyme arthritis patients are resistant to treatment we wanted to further dissect the involvement of OspA as well a possible reactivity to the hLFA1 peptide in a population of early Lyme disease patients using healthy individuals as controls. We used borrelia whole cell sonicate and the two peptides first identified by Gross et al. [6], hLFAα_L (aa326-345) and OspAB31 (aa164-183) to measure antigen-specific T cell reactivity in peripheral blood of two groups, early Lyme disease patients without history of arthritis and healthy individuals. Th1-IFNγ production in response to the peptide hLFAα_L (aa326-345) was more frequent than Th1 reactivity to the OspAB31 (aa164-183) peptide in peripheral blood of Lyme disease patients presenting with erythema migrans as well as in a subpopulation of healthy individuals.

Methods

Patients and controls

Samples were obtained from nineteen adult patients from an endemic area enrolled in ongoing Lyme disease studies at the Lyme Disease Center at the State University New York at Stony Brook, NY and at New York Medical College, Valhalla, NY. Sixteen of these patients presented with early localized infection typified by the presence of well defined erythema migrans (EM). They had no other signs of disseminated infection. None of these patients developed Lyme arthritis. Seven patients were evaluated within 1 month of EM presentation (acute) and nine different patients at 3 months post EM presentation (convalescent). Also, three patients presenting with Lyme disease symptoms (EM or arthralgia, mialgia) that had been previously vaccinated with recombinant OspA were tested. All patients were treated with suitable antibiotics at the time of diagnosis. We did not test antibiotic resistant Lyme arthritis patients because we didn't have any in that category during the course of the study. We don't see those patients often in either Long Island or Westchester County, highly endemic areas for Lyme disease. Twenty-one healthy adults from the same endemic area, with no prior history of Lyme disease and no prior serologic screening, were recruited as controls. Whole blood samples were drawn from each enrolled patient into a heparinized tube. The blood was kept at room temperature and used within 24 h after it was obtained.

Antigens

Two synthetic, twenty amino acid peptides, *B. burgdorferi* OspAB31 (aa164-183), GYVLEGTLTAEKTTLVVKEG and hLFAα_L (aa326-345), acetyl-ELQKKIYVIEGTSKQDLTSF, were custom contracted through the Small Scale Peptide Synthesis laboratory at the HHMI Biopolymer / Keck Foundation Biotechnology Resource Laboratory (Yale University School of Medicine, CT). Suitably protected peptide chains were constructed in an automated, stepwise solid-phase protocol using FMOC N-protection for N-alpha protection at each cycle. The peptides were all synthesized on substituted benzhydrylamine-resins to yield amidated C-termini. The amide group is a better approximation of peptide bonds than are free carboxyl termini.

Since T cell receptors recognize structures rather than amino acid sequences and both peptides of 20 residues are about 70% different we did not use an unrelated peptide as control. Borrelia whole cell sonicate derived from *B. afzelii*, strain Pko, was developed by CSL Biosciences (Melbourne, Australia) in collaboration with CDC (Fort Collins, Colorado, USA), as a stimulating antigen for the T cell Lyme assay. This antigen was found to have a higher stimulation rate in blood from Lyme disease patients than that of *B. burgdorferi* sonicate used in [8]. This antigen was kindly provided by Dr. Roland Martin (CSL Biosciences) and was used as an internal control to check the performance of the assay.

T-Cell assay

We used the QuantiFERON®-CMI test (CSL Biosciences, Melbourne, Australia). This assay allows detection of cytokine production, which may occur in the absence of proliferation. In persons exposed to *B. burgdorferi*, an early, vigorous, and sustained specific T-cell response develops that precedes a measurable antibody response [9]. The production of IFNγ 16–24 hours after antigen stimulation is dependent on the presence of T-cells capable of rapid response kinetics (antigen-primed effector and/or antigen-primed memory population). These T-cell populations are short lived and are strictly dependent on the presence of antigen [10]. This assay is based on the release of gamma-interferon (IFNγ) from sensitized lymphocytes during a 16 to 24 hour incubation with antigens specific for the pathogenic agent and subsequently quantification of IFNγ levels by a single step ELISA. The ratio of IFNγ response to antigen versus the response to negative control is quantified to determine if IFNγ production is due to prior exposure. The assay is simple to perform and does not require the isolation of lymphocytes [8]. Incorporation of the positive control mitogen in the test allows for the detec-

Table 1: IFNγ Specific Human Response (HR>10%) in LD Patients with EM and Healthy Individuals

	N. of Patients	Borrelia Whole Cell Sonicate N.Pos (%)
LD, EM, < 1 mo.	7	5 (71%)
LD, EM, 3 mo.	9	4 (44%)
LD, OspA Vaccinated	3	3 (100%)
LD total	**19**	**12 (63%)**
Healthy	21	6 (29%)

LD, Lyme disease; EM, erythema migrans; N.Pos., number of positives

tion of immunocompromised individuals (eg. due to AIDS) and thereby removes the risk of detection of false negatives.

The test was carried out as instructed by the manufacturer. A healthy individual is expected to have 0.8–1.2 × 10^6 Th cells/ml of whole blood. First, heparinized whole blood was aliquoted at 1 ml/per well (about 10^6 T helper cells/ml) in a 24 well cell culture cluster (Costar) and stimulated with 120 μl of the following antigens and controls: sonicated borrelia or WCS at 2.5 μg/ml, peptide hLFAα_L (aa326-345) at 2.5 μg/ml, peptide OspAB31 (aa164-183) at 10 μg/ml, mitogen (PHA) and negative (PBS) controls.

After overnight incubation (16–20 h) at 37°C in a humid chamber, the plasma was removed and the amount of IFNγ was determined by a rapid single step ELISA provided with the test kit, as per protocol. The single step ELISA was run against a standard curve of human IFNγ standards. The OD results from patient blood stimulated with *B. burgdorferi* antigen (corrected for the saline control) were converted into International Units per ml (IU/ml) from a standard curve of the human IFNγ standards. The IU/ml IFNγ values were used to calculate the % specific response to the antigen for each sample. The % specific response is the ratio of IFNγ response to test antigen (Ag) minus negative control (N) versus the response to mitogen (M) minus negative control (N), times 100. We determined the cutoff at 10% specific response.

Statistical analysis

The comparison between the response to OspAB31 (aa164-183) and hLFAα_L (aa326-345) was done using the McNemar's exact test for correlated proportions [11] because the same population of patients was tested in a paired-sample design. P values of < 0.05 were considered statistically significant.

Results

Samples were obtained from nineteen (19) adult patients and twenty-one (21) healthy adult individuals from endemic areas. The production of IFNγ in peripheral blood stimulated with borrelia whole cell sonicate antigen (WCS), hLFAα_L peptide (aa326-345) or OspAB31 (aa164-183) peptide was evaluated. Sixteen of the nineteen patients presented with early localized infection typified by the presence of well defined erythema migrans (EM). They had no other signs of disseminated infection and did not develop Lyme arthritis. Seven patients were evaluated within 1 month of developing EM (acute) and nine other patients were evaluated 3 months post EM presentation (convalescent). The additional three patients tested were OspA vaccine failures who presented with acute Lyme disease. These samples were not screened for anti-OspA antibodies. The 21 healthy individuals samples were used as controls.

The optimal concentration of antigen used in the assay varies for each specific antigen. The concentration of the WCS (2.5 μg/ml or 2.5 μg/~10^6 Th cells), was previously determined at CSL Biosciences by dose response curve as the optimal concentration of antigen to be used in this T cell assay [8]. We used the peptide OspAB31 (aa164-183) at 10 μg/ml (or 10 μg/~10^6 Th cells). This was the same concentration of OspA peptide used in Gross et al. for 3 × 10^5 Th cells [6]. The hLFA1 peptide was used at 2.5 μg/ml (or 2.5 μg/~10^6 Th cells), the same concentration determined for the WCS. All antigens at the proposed concentrations were tested in blood from known healthy individuals to determine the background level stimulation.

To confirm the performance of the Th1-IFNγ assay, we evaluated the results obtained with the borrelia whole cell sonicate antigen (WCS) in both Lyme disease and healthy individuals (Table 1). Of the 19 Lyme disease patients studied, 5 acute, 4 convalescent and 3 infected OspA vaccinated patients (63%, 12/19) produced IFNγ when stimulated with WCS. Of the 21 healthy individuals tested, 6 produced IFNγ when stimulated with WCS (29%). The sensitivity of the assay obtained for the Lyme disease pa-

Table 2: IFNγ Specific Human Response (HR>10%) in LD Patients with EM and Healthy Individuals that were WCS Negative

	Lyme Disease patients with EM N.Pos/total (%)				Healthy, WCS neg N.Pos/total (%)
	< 1 month	At 3 months	OspA vacc.	LD total	Healthy total
hLFAaL(aa326-345)	4/7 (57%)	5/9 (56%)	2/3 (67%)	**11/19 (58%)**	**3/15 (20%)**
OspAB31(aa164-183)	1/7 (14%)	1/9 (11%)	0/3 (0%)	2/19 (11%)	0/15 (0%)

WCS, whole cell sonicate; LD, Lyme disease; EM, erythema migrans; hLFA, human leucocyte associated function antigen; OspA vacc., OspA vaccinated; N.Pos., number of positives

tients was equivalent to data obtained independently with the a borrelia whole cell sonicate stimulating antigen [8].

When we compared the Th1 reactivity between the two peptides in the Lyme disease patients (Table 2) we found that 58% (11/19) produced IFNγ when stimulated with the hLFAα_L (aa326-345) and 11% (2/19) produced IFNγ when stimulated with the OspAB31 (aa164-183) peptide ($p < 0.05$, McNemar's exact test for correlated proportions). There was no correlation between the IFNγ response weather the patient was studied at less than one month or three months post presentation ($p > 0.05$). Twenty percent (3/15) of the healthy individuals that did not react to borrelia WCS produced IFNγ when stimulated with the hLFAα_L (aa326-345) and none (0/15) produced IFNγ when stimulated with the OspAB31 (aa164-183) peptide.

Observing the three vaccinated patients that came down with Lyme disease, we verified they were all responsive (100%) to the Borrelia WCS, 2/3 were positive for hLFA peptide (67%) and none (0%) was positive for the OspA peptide. This last result was surprising since these patients had been previously vaccinated with OspA. However, this could be explained by the obvious vaccine failure which is supported by the fact that all three patients reacted with borrelia whole cell sonicate.

Discussion

Our results show that the Th1 proinflamatory cytokine IFNγ is produced in peripheral blood of patients with early Lyme disease without any signs of arthritis when stimulated with the peptide hLFAα_L(aa326-345) but not when stimulated with the homologous OspAB31 (aa164-183) peptide. This observation was also verified for healthy individuals.

It has been postulated that hLFA1 autoreactive T cells play a major role in the pathogenesis of chronic Lyme arthritis [6]. However, the hLFAα_L (aa326-345) peptide was not recognized by any of the OspA (aa164-175)-specific T cell hybridomas generated from a *B. burgdorferi* OspA immunized DR4 transgenic mouse, illustrating that individual T-cell receptor (TCR) possess different patterns of cross-reactivity [12]. In addition, analysis at the clonal level demonstrated that hLFAα_L (aa326-345) behaves as a partial agonist for OspA-reactive T cells, not as a full agonist [13].

Antigen specificity of a single T-cell clone can be degenerate and yet the clone can preferentially recognize different peptides derived from the same organism. This demonstrates that flexibility in T cell recognition does not preclude specificity. If recognition were not degenerate, the immune system would be incompetent since the limited number of T-cells in an organism would allow recognition of small fraction of a mixture of randomized peptides [14]. There is ample evidence suggesting that cross-reactivity leading to T cell activation is a very frequent event [15–17,14,18,12]. In fact, the presence of cross-reactive T cells is normal and required for the maintenance of memory T cell responses. T cell reactivity between a microbial peptide and a self-peptide alone is not sufficient to induce autoimmune disease [12].

TCR recognition of peptide-major histocompatibility complex antigens can elicit a diverse array of effector activities. Itoh et al., analyzed TCR engagement and the production of multiple cytokines in a clonal Th1 CD4+ cell population. They verified that low concentrations of TCR ligand elicit only IFNγ production. Their findings provide strong support for a model of T cell activation in which different effector functions have a hierarchical arrangement of elicitation thresholds, such that the ratio of elicited effector molecules changes with antigen concentration [19]. Rogers et al., used a model of differentiation that is dependent on the initial dose and affinity of peptide presented to a naïve CD4 cell. They used this system to confirm that LFA 1 interaction can suppress differentiation of cells secreting Th2 cytokines and that this interaction is only seen over a range of peptide doses [20].

We detected a Th1 response to hLFAα_L (aa326-345) in peripheral blood of patients with Lyme disease without any signs of arthritis and in a subpopulation of healthy individuals. We observed that the reactivity to hLFA1 was significant and was dissociated from the response to OspA. The hLFA1 peptide was used at the same concentration determined for the WCS and four times less the concentration used for the OspA peptide. We anticipated that differences in data obtained for a concentration of 25 μg/ml for the hLFA antigen compared to 10 ug/ml of the OspA antigen as in Gross et al. [6] could cause skepticism. For that reason, and to minimize the amount of variables in the study, we chose a semi-empirical route.

Taking into account all previous findings, the greater response to the hLFA peptide as compared to the OspA may be explained by the different doses of hLFA peptide and OspA used. An additional, and in our opinion better explanation, is that reactivity to the hLFA1 peptide in peripheral blood is in large part the result of T cell degeneracy. This could explain the number of healthy individuals who reacted to the hLFA1 peptide. An alternative to this model could be that some individuals may have been infected with a previous pathogen that triggers activation of T cells reactive to the hLFA1 peptide. In any case, this does not rule out the possibility that primed hLFAα_L reactive Th1 cells homing to a specific milieu such as the joint could potentially be involved in the pathogenic mechanisms associated with chronic Lyme arthritis. Additional studies are required to define the role of T cell reactivity in the immunopathogenesis of antibiotic refractory Lyme disease.

Conclusions

We detected a significant Th1 response to the peptide hLFAα_L(aa326-345) in peripheral blood of patients with early Lyme disease without any signs of arthritis and in a subpopulation of healthy individuals. Our results suggest that reactivity to hLFA1 peptide in peripheral blood may be the result of T cell degeneracy.

Competing interests

None declared.

Authors' contributions

MGS carried out the T cell assays, performed the statistical analysis and drafted the manuscript. GPW participated in the design and coordination of the study. MGS and RJD conceived of the study, and participated in its design and coordination.

All authors read and approved the final manuscript.

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References

1. Benach JL, Bosler EM, Hanrahan JP, Coleman JL, Habicht GS, Bast TF, Cameron DJ, Ziegler JL, Barbour AG, Burgdorfer W, Edelman R, Kaslow RA: **Spirochetes isolated from the blood of two patients with Lyme disease.** *N Engl J Med* 1983, **308(13):**740-2
2. Nadelman RB, Wormser GP: **Lyme borreliosis.** *Lancet* 1998, **352(9127):**557-65
3. Dong Z, Edelstein MD, Glickstein LJ: **CD8+ T cells are activated during the early Th1 and Th2 immune responses in a murine Lyme disease model.** *Infect Immun* 1997, **65(12):**5334-7
4. Kang I, Barthold SW, Persing DH, Bockenstedt LK: **T-helper-cell cytokines in the early evolution of murine Lyme arthritis.** *Infect Immun* 1997, **65(8):**3107-11
5. Gross DM, Steere AC, Huber BT: **T helper 1 response is dominant and localized to the synovial fluid in patients with Lyme arthritis.** *J Immunol* 1998, **160(2):**1022-8
6. Gross DM, Forsthuber T, Tary-Lehmann M, Etling C, Ito K, Nagy ZA, Field JA, Steere AC, Huber BT: **Identification of LFA-1 as a candidate autoantigen in treatment-resistant Lyme arthritis.** *Science* 1998, **281(5377):**703-6
7. Sigal LH: **Immunologic mechanisms in Lyme neuroborreliosis: the potential role of autoimmunity and molecular mimicry.** *Semin Neurol* 1997, **17(1):**63-8
8. Sikand VK, Rothel JS, Martin RM: **Diagnosis of Lyme borreliosis by a whole-blood gamma interferon assay for cell-mediated immune responses.** *Clin Diagn Lab Immunol* 1999, **6(3):**445
9. Dattwyler RJ, Volkman DJ, Halperin JJ, Luft BJ, Thomas J, Golightly MG: **Specific immune responses in Lyme borreliosis. Characterization of T cell and B cell responses to *Borrelia burgdorferi*.** *Ann N Y Acad Sci* 1988, **539:**93-102
10. Bell EB, Sparshott SM, Bunce C: **CD4+ T-cell memory, CD45R subsets and the persistence of antigen – a unifying concept.** *Immunol Today* 1998, **19(2):**60-4
11. Rosner B: **Fundamentals of Biostatistics.** *Boston, Massachusetts: PWS-Kent Publishing Company* 1990
12. Maier B, Molinger M, Cope AP, Fugger L, Schneider-Mergener J, Sonderstrup G, Kamradt T, Kramer A: **Multiple cross-reactive self-ligands for *Borrelia burgdorferi*-specific HLA-DR4-restricted T cells.** *Eur J Immunol* 2000, **30(2):**448-57
13. Trollmo C, Meyer AL, Steere AC, Hafler DA, Huber BT: **Molecular mimicry in Lyme arthritis demonstrated at the single cell level: LFA-1alpha(L) is a partial agonist for outer surface protein A – reactive T cells.** *J Immunol* 2001, **166(8):**5286-91
14. Hemmer B, Gran B, Zhao Y, Marques A, Pascal J, Tzou A, Kondo T, Cortese I, Bielekova B, Straus SE, McFarland HF, Houghten R, Simon R, Pinilla C, Martin R: **Identification of candidate T-cell epitopes and molecular mimics in chronic Lyme disease.** *Nat Med* 1999, **5(12):**1375-82
15. Tabira T: **A view for understanding the pathogenesis of multiple sclerosis.** *Rinsho Shinkeigaku* 1995, **35(12):**1493-5
16. Hemmer B, Vergelli M, Pinilla C, Houghten R, Martin R: **Probing degeneracy in T-cell recognition using peptide combinatorial libraries.** *Immunol Today* 1998, **19(4):**163-8
17. Ufret-Vincenty RL, Quigley L, Tresser N, Pak SH, Gado A, Hausmann S, Wucherpfennig KW, Brocke S: ***In vivo* survival of viral antigen-specific T cells that induce experimental autoimmune encephalomyelitis.** *J Exp Med* 1998, **188(9):**1725-38
18. Grogan JL, Kramer A, Nogai A, Dong L, Ohde M, Schneider-Mergener J, Kamradt T: **Cross-reactivity of myelin basic protein-specific T cells with multiple microbial peptides: experimental autoimmune encephalomyelitis induction in TCR transgenic mice.** *J Immunol* 1999, **163(7):**3764-70
19. Itoh Y, Germain RN: **Single cell analysis reveals regulated hierarchical T cell antigen receptor signaling thresholds and intraclonal heterogeneity for individual cytokine responses of CD4+ T cells.** *J Exp Med* 1997, **186(5):**757-66
20. Rogers PR, Croft M: **CD28, Ox-40, LFA-1, and CD4 modulation of Th1/Th2 differentiation is directly dependent on the dose of antigen.** *J Immunol* 2000, **164(6):**2955-63

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