Modulation of Natural Killer Cell Activity by *Borrelia burgdorferi*[a]

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INTRODUCTION

Natural killer (NK) cells are a heterogenous subpopulation of peripheral blood lymphocytes (PBL) that have the ability to kill various tumors and tumor cell lines in the absence of prior sensitization. These cells have generated considerable interest and have been implicated in natural resistance to tumors,[1-5] resistance to viral infection,[5-7] transplantation rejection,[5,8] and immunoregulation of both T and B cells.[9-11] Recently, there has been evidence that NK cells or large granular lymphocytes (LGLs) are also involved in host resistance against bacterial infections. This has been demonstrated by the bactericidal activity of NK-like cells against certain bacteria such as *Shigella* and *Salmonella*.[12-14] In addition to these interactions, there are several reports that demonstrate bacterial activation and/or recruitment of NK and NK-like cells presumably through the production or action of interferon, interleukin-2, and/or lipopolysaccharide (LPS).[12,16-18] Under certain conditions this augmentation may be inhibited by LPS,[17] which attests to the complex nature of the microorganism-host interactions. Furthermore, the NK cell killing of infected host cells[15] or local inflammation secondary to the killing of extracellular microorganisms may be responsible for the tissue destruction seen in various bacterial infections.

The humoral and cellular responses in Lyme borreliosis have recently been under active investigation. While the humoral antibody response to *Borrelia burgdorferi* is well characterized, the cellular immune response is not well defined. However, it is known that a strong specific T cell response to *B. burgdorferi* occurs early in the course of the disease. This response may even precede the development of a measurable antibody response in some cases.[19] Once established, this response is long lasting.

In this report, the cellular immune response to *B. burgdorferi* is further investigated. Specifically, the modulation of NK cells and NK cell activity by *B. burgdorferi* both *in vivo* and *in vitro* is characterized and discussed.

MATERIALS AND METHODS

Cell Preparations

Normal human peripheral mononuclear cells (PBMs) were obtained from healthy volunteers or from patients with Lyme disease by separation on Ficoll-Hypaque density gradients as previously described.[20]

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Immunofluorescent Assays and Cell Sorting

For immunofluorescent phenotyping a microlabeling procedure was developed due to the constraints on cell numbers. This procedure is basically the same as the standard published labeling techniques,[21] with the exception that only 5–10 × 10^4 cells were labeled with appropriately reduced amounts of antibody. In addition, the labeling was done in conical tubes rather than the round bottom tubes standardly used. The monoclonal antibodies used were CD3 (Leu-4, T cells), CD4 (T4, helper-inducer cells), CD8 (T8, cytotoxic-suppressor cells), Leu-7 (NK cells), CD16 (Leu-11, cytotoxic NK cells), and NKH-1 (also NK cells). Leu series antibodies were from Becton-Dickinson, Mountainview, CA. T4, T8, and NKH-1 were from Coulter, Hialeah, FL.

Cytotoxic Assay

^{51}Cr Release Assay. Cytotoxic activity was determined in a chromium release microcytotoxicity assay as described in detail elsewhere.[23] Briefly, chromium-labeled target cells were added at 5 × 10^3 per well in V-bottom 96-well tissue culture plates. Effector cells were then added at various effector to target cell ratios in a total volume of 0.2 ml of RPMI 10% Ab. Each ratio was performed in triplicate. Total release was determined by substitution of 100 μl of 1% Triton X-100 for effectors. The plates were then centrifuged. After incubation at 37°C for 1–2 hours, they were centrifuged again and 100 μl collected from each well and counted in a gamma counter. The percent cytotoxicity was calculated according to the following formula:

$$\text{percent cytotoxicity} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{total release} - \text{spontaneous release}} \times 100.$$

Statistical Analyses. Results are expressed as the mean ± SE % cytotoxicity from triplicate wells. Significance was established using single classification analysis of variance for two groups (equivalent to the Student's *t*-test for the difference between two means).

Targets. The suspension tumor cell line used as targets was the K562 human erythromyeloid cell line. The cells were maintained in RPMI 10% FCS. The cells were mycoplasma free as determined periodically by the Hoescht fluorescent staining method.[23]

IL-2 Lymphocyte Cultures. IL-2 cultures were set up as previously described[24] with modifications. Briefly, 20 BRMP U/ml of TCGF (interleukin-2, Cellular Products, Buffalo, NY) was incubated with the responding cells (usually plastic depleted and nylon fiber column passed) at a concentration of 1 × 10^6/ml in RPMI 10% for 3 days or more depending on the experiment.

RESULTS

NK Activity and Phenotypic Analysis in Lyme Borreliosis

PBL from patients with various stages and treatment of Lyme borreliosis were examined for NK functional activity against K562 tumor targets. These results were compared to normal controls drawn and processed on the same day. FIGURE 1

demonstrates that there was a marked and highly significant inhibition ($p < 0.0005$) of *in vivo* endogeneous NK activity in two patient populations: (1) patients in the early stages of Lyme disease (ECM stage) and before treatment, and (2) patients exhibiting chronic active stages of the disease. There was no significant inhibition of NK activity in three other patient populations: (1) patients in the early stages of Lyme disease (ECM stage) after treatment; (2) chronic inactive patients; and (3) seronegative chronic Lyme disease patients before treatment. The seronegative patients were diagnosed with Lyme disease based on the clinical symptoms and a positive mitogenic responsiveness of PBL to *B. burgdorferi*.

The phenotypic analysis of PBL from patients with early Lyme disease and chronic Lyme disease demonstrates that there were no significant changes in the absolute numbers of T cells (CD3) and T cell subsets (CD4 and CD8, FIG. 2). However, FIGURE 3 demonstrates that there was a significant ($p < 0.0001$) increase in the absolute

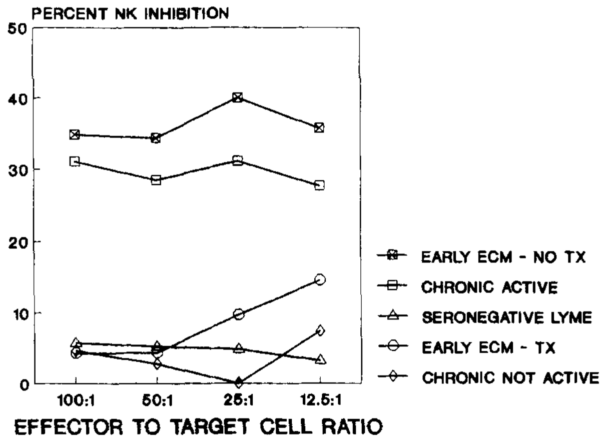

FIGURE 1. NK inhibition by *B. burgdorferi in vivo.* Patients exhibiting the following stages and treatment of Lyme borreliosis were tested by NK activity against K562 tumor targets and compared to normal healthy controls: (1) early ECM, no treatment ($n = 7$, $p < 0.0005$); (2) chronic active Lyme ($n = 11$, $p < 0.0005$); (3) seronegative Lyme ($n = 5$, not significant); (4) early ECM after treatment ($n = 5$, not significant); (5) chronic lyme, not active ($n = 4$, not significant).

number of circulating $CD16^+$ NK cells in the Lyme disease patients over the controls. It is interesting to note that while these patients exhibit an approximately fourfold *increase* in absolute numbers of CD16 NK cells, the NK activity is *decreased* by 30–40% without being normalized for the increase in absolute numbers. There was no significant change in the absolute number of Leu-7 and NKH-1 positive cells. In addition to the absolute numbers, these differences and similarities were also reflected in the percentage of fluorescent positive cells (data not shown).

Effect of B. burgdorferi *Culture on Normal PBL*

PBL from normal healthy donors were cultured for 7 days with and without *B. burgdorferi* in BSKII media or in RPMI-1640 with 10% FCS. On days 3, 5, and 7 the NK activity was examined. FIGURE 4 demonstrates that when lymphocytes are

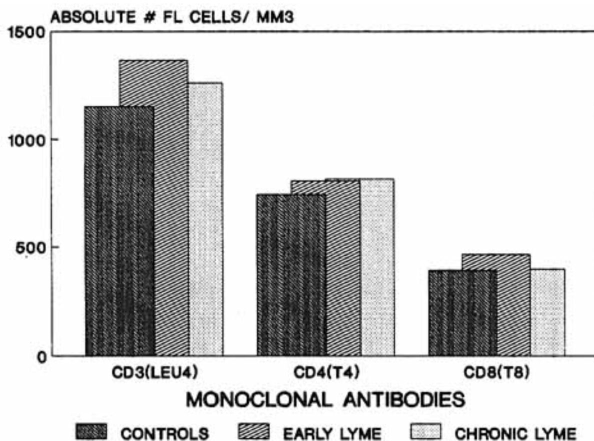

FIGURE 2. Lymphocyte phenotypic analysis in Lyme disease patients. PBL were examined in early Lyme, chronic Lyme, and normal healthy controls for expression of T cell antigens (CD3, CD4, and CD8). No significant differences were detected when compared to controls.

cultured in the presence of growing *B. burgdorferi* (in BSKII media) there is a marked inhibition ($p < .0005$) of NK activity on days 3, 5, and 7 when compared to lymphocytes cultured in BSKII media in the absence of spirochetes. This effect is not due to a selective depletion of or toxicity to endogeneous NK since viability studies and monoclonal antibody studies demonstrate no significant changes after culture with the organism (FIG. 5). The inhibition is directly attributable to the organism or its

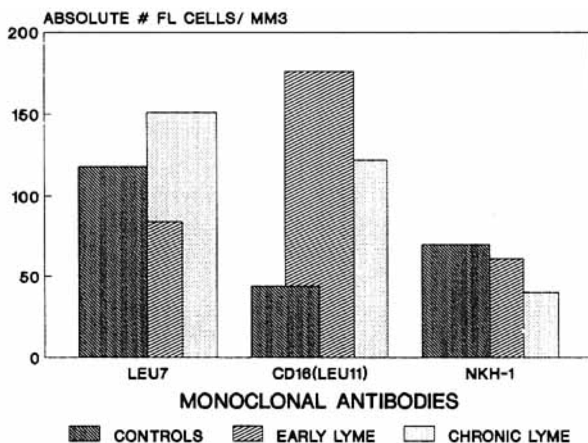

FIGURE 3. Lymphocyte phenotypic analysis in Lyme patients. PBL were examined in early Lyme, chronic Lyme, and normal healthy controls for expression of NK cell antigens (Leu-7, CD16, NKH-1). Early Lyme and chronic Lyme increased over control for CD16 ($p < 0.0001$ for both).

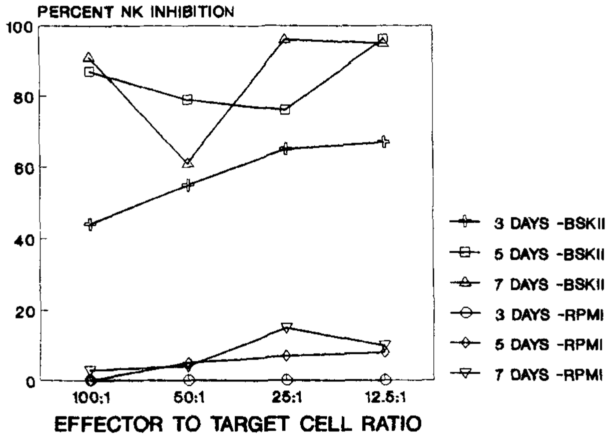

FIGURE 4. *B. burgdorferi* inhibition of NK *in vitro.* PBL from normal healthy donors were cultured for up to 7 days with spirochetes. On days 3, 5, and 7 NK activity was assessed and compared to those PBL cultured in the same media without spirochetes. In all cultures viability was >95%; $p < 0.0005$ for spirochete-PBL cultures in BSKII media.

products, since supernatants from the organism cultures also inhibit endogeneous NK without prior exposure (data not shown).

PBL cultured in the presence of *B. burgdorferi* in RPMI-1640 media exhibited no inhibition when compared to lymphocytes cultured in RPMI-1640 in the absence of spirochetes. Whether this lack of inhibition is related to inhibited spirochetal growth or metabolism is not known and is currently under investigation.

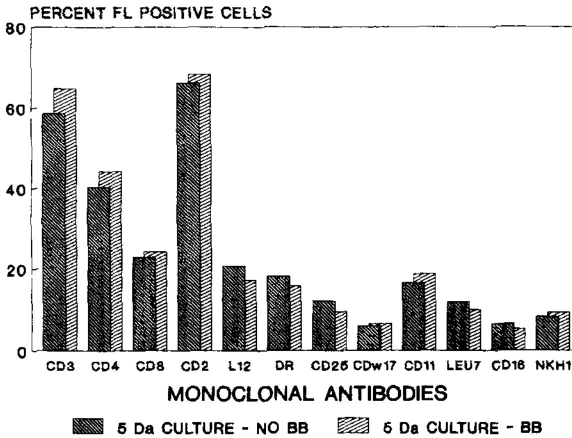

FIGURE 5. Lymphocyte phenotypic analysis of normal PBL after culture with *B. burgdorferi* in BSKII media. Cultures were grown for 5 days, then the PBL recovered and subjected to phenotypic analysis with monoclonal antibodies.

Effect of Interleukin-2 on PBL Cultured with B. burgdorferi

PBL from normal healthy donors were cultured for 5 days with and without spirochetes (in BSKII media). The PBLs were then washed and resuspended in RPMI-1640 10% FCS or RPMI-1640 10% FCS with 20 BRMP units/ml TCGF (IL-2) for an additional 3 days. The cytotoxic activity was then determined against K562 targets. Viability was greater than 98% (by trypan blue exclusion). FIGURE 6 demonstrates a representative experiment. PBL incubated with spirochetes for 5 days were unable to spontaneously recover NK activity when switched to RPMI-1640 in the absence of proliferating spirochetes (the majority of spirochetes are removed by the slow-speed centrifugations during the washes). However, PBL cultured with spirochetes for 5 days, then incubated with interleukin-2, recovered the cytotoxic activity to levels indistinguishable from IL-2-exposed PBL that were not cultured with *B.*

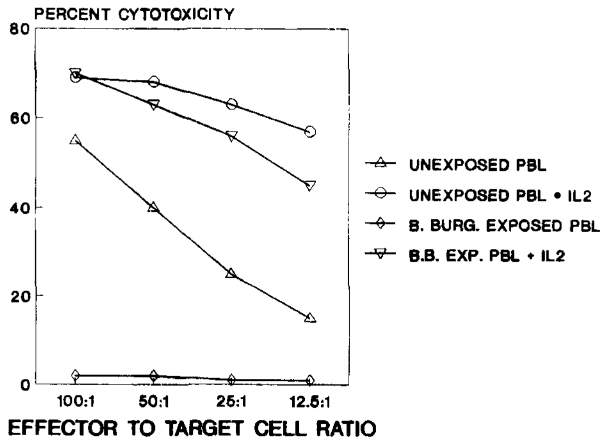

FIGURE 6. Recovery with IL-2 of NK-like activity from PBL cultured with *B. burgdorferi*. Normal healthy PBL were cultured with or without *B. burgdorferi* in BSKII media for 5 days. The cultures were then washed and cultured an additional three days with IL-2. NK activity was then determined; $p < 0.0001$ for *B. burgdorferi* exposed without IL-2 compared to *B. burgdorferi* exposed with IL-2.

burgdorferi. The cytotoxicity exhibited by both IL-2-stimulated populations was significantly ($p < 0.0001$) augmented over that of the control PBL never exposed to spirochetes or IL-2. Whether this is due to an actual recovery of the inhibited NK cells or due to the induction of lymphokine-activated killer (LAK) cells is under investigation and is discussed below.

DISCUSSION

It has been demonstrated that abnormalities in NK function and numbers exist in distinct populations of patients with Lyme borreliosis. Patients with chronic active disease and untreated patients in the early stages of the disease were found to exhibit a highly significant fourfold increase in the peripheral blood of the absolute number of NK cells expressing the CD16 (Leu-11) phenotype. The majority of these $CD16^+$ cells

must also be Leu-7, based on the absolute numbers found. This increase may be the result of lymphokines (*i.e.*, IL-2, interferon) produced or actual bacterial antigens that have been reported to recruit previously inactive pre-NK cells into the cytotoxic NK pool.[17,18,25] However, this does not explain the significantly decreased NK cytotoxic ability of these patients' PBL. It is possible that this inhibition of NK activity is the result of a recruitment of inactive NK cells expressing CD16. However, this is unlikely since it has been reported that the majority of $CD16^+$, $Leu\text{-}7^-$ cells have cytotoxic activity.[26] Alternatively, the decrease in NK activity may be due to a direct effect of the *B. burgdorferi* organism or its products on the NK cell. It has been reported that LPS isolated from *Salmonella* has the ability to inhibit the bacterial augmentation of activated killers while leaving endogenous NK activity intact.[17] Thus, if a similar mechanism is occurring, it must also inhibit endogenous NK. It is also possible that *B. burgdorferi* produces a toxin that is responsible for the inhibition. An adenylate cyclase toxin can be extracted from *Bordetella* that will increase cAMP levels when added to NK cells, resulting in an inhibition of NK activity without killing the NK effector cells.[27]

It is interesting to note that the treated early Lyme and seronegative Lyme patients exhibit normal NK activity. This suggests that proliferating organisms and/or active interaction between *B. burgdorferi* and the immune system is required for inhibition of cytotoxic NK activity. These conditions may not occur in these patients, since treated early Lyme disease patients undergo a limited immune response due to the early eradication of the organism and the seronegative patients most likely have an aborted immune response due to the spirochetes being eliminated from the periphery and localized in immunologically privileged sites.[28]

The inhibition demonstrated *in vivo* was also observed *in vitro*. PBL cultured in the presence of proliferating organisms exhibited a marked diminution of NK activity, while PBL cultured in the presence of nonactively proliferating organisms did not demonstrate this decrease. This suggests that the inhibition may require active spirochetal metabolism. However, the requirement of a critical spirochete number cannot be ruled out since the inhibition occurs after the organisms have proliferated. Preliminary results suggest that the inhibition seen both *in vivo* and *in vitro* is due to an aberration in the lytic recycling ability of the NK cells and not in the actual recognition or lytic events associated with killing.

The *in vitro* inhibition of NK could be abrogated by additional culturing in the presence of interleukin-2. This may be due to actual recovery of the inhibited NK, recruitment of pre-NK cells into the cytotoxic NK pool, or the induction of lymphokine activated killer (LAK) cells. Preliminary evidence suggests that the majority of this "recovery" is due to LAK cells. However, contribution by the other mechanisms cannot be ruled out and is currently under investigation.

In summary, *B. burgdorferi* can induce a severe inhibition both *in vivo* and *in vitro* in NK cell cytotoxic capabilities. This is in contrast to other bacteria that are known to activate NK cells. While the inhibition appears to be directly related to spirochetal proliferation or concentration, the exact mechanism is unclear and is under active investigation. Interleukin-2 stimulation abrogates this inhibitory effect, possibly via the induction of LAK cells, which may be involved secondarily to the inhibited endogenous NK. More studies will be needed to elucidate these interactions and their full significance to the host-microorganism interaction.

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