Immune Evasion by Tickborne and Host-Adapted *Borrelia burgdorferi*

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Immune sera from mice infected with the Lyme disease spirochete, *Borrelia burgdorferi*, have strong biologic activity against spirochetes cultured in vitro. Recent studies with rodents and ticks infected with *B. burgdorferi* indicate that spirochetes undergo major changes in protein expression as they adapt to the diverse environments encountered by a vectorborne pathogen. The purpose of this study was to explore the susceptibility of three different adaptive forms of *B. burgdorferi* (in vitro cultured, host-derived, and tickborne) to immune sera. Passive transfer of immune sera protected mice when they were challenged with spirochetes cultured in vitro. Immune sera did not protect mice from tickborne spirochetes or spirochetes derived from infected mice. These results indicate that spirochetes that have adapted within either the feeding tick or host are relatively invulnerable to the protective effects of immune sera, unlike spirochetes grown in vitro, which are highly susceptible.

Sera from patients and animals with Lyme disease contain antibody reactivity against a number of proteins of the causative spirochete, *Borrelia burgdorferi*. More important, such sera have strong biologic activity [1–3]. Sera from infected mice (immune sera) are borreliacidal to organisms grown in vitro [4]. Passive transfer of remarkably small amounts of immune sera to naive mice protects them against challenge with in vitro–grown spirochetes [2]. Protective activity has likewise been documented in immune sera from naturally infected humans and dogs when passively transferred to laboratory rodents [5–7]. Collectively, these observations underscore the fact that infection with *B. burgdorferi* induces a strong humoral protective immune response in a variety of host species, demonstrable by passive transfer of immune sera to naive recipients, but that immunity is not capable of eliminating infection in the persistently infected serum donor.

Studies to characterize the protective capacity of passively administered immune sera have been carried out by syringe challenge of mice with in vitro–grown spirochetes. Recent studies with *Ixodes dammini* (*Ixodes scapularis*) [8], the tick vector of Lyme disease, indicate that transfer of spirochetes from the tick into the host is not merely a mechanical event. During the process of feeding by the tick, spirochetes within the vector grow in number, invade tick salivary glands, and undergo major changes in protein expression [9–13]. Furthermore, changes in *B. burgdorferi* protein expression are not limited to the vector. *B. burgdorferi* continues to alter its pattern of gene expression within the mammalian host [12, 14–21]. The changes in spirochete gene expression that occur during tick feeding and during infection of the mammalian host may help spirochetes to effectively infect the host and cope with the host’s immune response. Spirochetes grown in vitro that are injected into the host may be especially vulnerable to antibodies in immune serum because they may not reflect the adaptive state of spirochetes entering the host via tick bite. The purpose of this study was to explore the susceptibility of different adaptive forms of *B. burgdorferi* to the protective effects conferred by immune serum.

**Materials and Methods**

*Mice.* Pathogen-free inbred C3H/HeN(Cr) (C3H) mice were purchased from the National Cancer Institute Animal Production Program (Frederick Cancer Research Center, Frederick, MD). Outbred Crl:CD-1(ICR) (CD-1) mice were purchased from Charles River Breeding Laboratories (Wilmington, MA).

*Borrelia burgdorferi.* A cloned isolate of the N40 strain of *B. burgdorferi* was used in these studies [22]. Spirochetes were grown at 33°C in glass screw-cap tubes containing modified BSK II medium [23]. Spirochetes were counted with a bacterial counting chamber (Petroff-Hauser; Baxter Scientific Products, McGaw Park, IL) by darkfield microscopy. Depending on the experiment, passively immunized mice were challenged with three different forms of spirochetes, representing different adaptive states (in vitro–cultured, in vivo–derived, and tickborne).
Mice were challenged with spirochetes cultured in vitro by intradermal injection of $10^4$ spirochetes in 0.1 mL of BSK II medium. Mice were challenged with in vivo–derived spirochetes either by transplantation of small pieces of infected ear tissue or by syringe inoculation with chamber-derived spirochetes. For transplantation challenge, donor mice were inoculated intradermally with $10^4$ cultured *B. burgdorferi* as above. At 2 weeks after inoculation, an interval at which spirochetes have been shown to have disseminated to the ear [24], mice were killed, ears were excised, their surface was sterilized, and then the ears were cut into 2-mm squares. Previous studies have shown that ear pieces of this size contain $1 \times 10^4$ spirochetes (equivalent or less than the syringe challenge dose) at this interval of infection [19]. Ear tissues for transplantation were placed in cold BSK II medium, then immediately transplanted into the subcutis of recipient mice through small cutaneous stab incisions under ketamine-xylazine anesthesia and aseptic technique.

Chamber-derived spirochetes were collected from spirochete-containing chambers implanted into the peritoneal cavity of uninfected mice [25, 26]. Chambers were constructed from 13-mm plastic rings with a side filling hole. Both sides were covered with 0.1-µm-pore membrane filters cemented into place (Millipore, Bedford, MA). Ethylene oxide–sterilized chambers were filled with 0.2 mL of BSK II medium containing $5 \times 10^4$ spirochetes (clone *B. burgdorferi N40*) via the side filling hole with a needle and syringe; then the hole was sealed with a plastic rod. Two chambers were surgically implanted into the peritoneal cavity of each mouse under ketamine-xylazine general anesthesia. On the basis of pilot experiments, we determined that maximal yields of spirochetes occurred at 10 days after implantation (data not shown). Therefore, 10 days after implantation, mice were euthanized and chambers removed. Spirochetes were harvested by aspiration of the chamber with a needle and syringe after flushing with a small amount of BSK II medium. Viable spirochetes were immediately enumerated and used to inoculate mice at a dose equivalent to syringe challenge ($10^4$) with cultured spirochetes.

Mice were challenged with tick-derived spirochetes by allowing 5–8 infected nymphal ticks to attach to and feed on each mouse. Some of the nymphs were removed from the animals 48 h after attachment to determine the distribution of spirochetes within the feeding vector. The remaining nymphs were allowed to feed to repletion and naturally detach from the host.

**Ticks.** *I. dammini* ticks, free of inherited infection, were derived from a colony in its second generation from field-collected adults. Larvae were allowed to engorge on CD-1 mice that had been infected 2 weeks previously by the bites of 3–5 nymphal ticks infected with the N40 strain of *B. burgdorferi*. Engorged larvae were collected, held in mesh-covered plaster of paris–containing vials, and allowed to molt in 95% relative humidity at 21°C. Infected nymphs were held under the same conditions before use in experiments, usually within 2 months of molting. The infection status of fully engorged ticks was determined by immunofluorescence microscopy as previously described [9]. In brief, 10 days after detachment from mice, internal organs were dissected from each tick, smeared on glass slides, and allowed to air dry. Each slide was dipped in acetone for 5 min before staining with the fluoresceinated *B. burgdorferi* antiserum (Kirkegaard & Perry Laboratories, Gaithersburg, MD).

To determine the distribution of spirochetes within feeding ticks, guts and salivary glands were dissected out of unfed and partially fed (48 h) nymphs and examined for spirochetes by confocal scanning laser microscopy as previously described [9]. In brief, the organs were immobilized on glass slides and stained with the fluoresceinated *B. burgdorferi* antiserum. The tissues were imaged on a confocal scanning laser microscope (MRC 600; Bio-Rad Laboratories, Microscience Division, Cambridge, MA) equipped with an argon-krypton laser. When tick organs were examined for spirochetes, the whole organ, including the entire thickness, was evaluated for the presence of spirochetes.

**Preparation of immune and hyperimmune sera.** To generate immune sera for these studies, C3H mice were inoculated intradermally with $10^2$ live *B. burgdorferi* in 0.1 mL of BSK II medium. This input inoculum dose has been shown to be too small to elicit a detectable antibody response without subsequent replication and dissemination of spirochetes in the infected host [2, 19], so that antibody within immune serum reflected antibody developed against *B. burgdorferi* in vivo. We have previously shown that inocula of $<10^4$ spirochetes do not elicit OspA responses in infected mice [2, 19], and we have shown that OspA is not expressed in vivo [10, 19]. Therefore we selected the $10^2$ infecting dose to be certain that immune sera reflected antibody to antigens expressed in vivo during active infection and not as a result of the immunizing effect of the initial inoculum.

Lack of OspA antibody in immune sera was verified by ELISA, using recombinant OspA antigen, and by immunoblot with cultured *B. burgdorferi* lysates (data not shown). At 90 days after inoculation, infection was verified by culture of blood, spleen, and urinary bladder. Sera from multiple mice with verified infection were pooled. Hyperimmune OspA antiserum was prepared by immunizing a rabbit subcutaneously with 40 μg of recombinant OspA–glutathione-S-transferase fusion protein in complete Freund’s adjuvant. After immunization, bleedings were performed at weekly intervals. Serum samples were taken from the rabbits 9 weeks after the initial immunization. OspA antibodies were determined by ELISA with recombinant OspA (2-μg aliquots of OspA were immobilized in 159 wells of an ELISA plate) against a serial two-fold dilution of the rabbit sera. The antibody titer was defined as the reciprocal of the highest dilution that gave an absorbance reading above the mean absorbance of the negative control samples plus 2 standard deviations. Serologically positive sera were used for the preparation of hyperimmune sera.

**Table 1.** Protective capacity of immune serum against different adaptive forms of *B. burgdorferi.*

<table>
<thead>
<tr>
<th>Serum</th>
<th>Time administered relative to challenge* (days)</th>
<th>Adaptive form of challenge spirochete†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Culture</td>
</tr>
<tr>
<td>Normal</td>
<td>0</td>
<td>0/5</td>
</tr>
<tr>
<td>Immune</td>
<td>0</td>
<td>0/5</td>
</tr>
<tr>
<td>Normal</td>
<td>4</td>
<td>5/5</td>
</tr>
<tr>
<td>Immune</td>
<td>4</td>
<td>4/5</td>
</tr>
<tr>
<td>Normal</td>
<td>8</td>
<td>5/5</td>
</tr>
<tr>
<td>Immune</td>
<td>8</td>
<td>4/5</td>
</tr>
</tbody>
</table>

**NOTE.** Data are prevalence of infection (no. infected/total). Mice were checked for infection by culturing blood, urinary bladder, spleen, and skin in BSK II medium 2 weeks after challenge. All sites were culture-positive in all groups of infected mice, with no difference in patterns of organ infection among treatment groups. ND, not done.

* 10 μL of serum was injected subcutaneously into each mouse.
† Mice were challenged with $\sim 10^4$ spirochetes. Challenge spirochetes were derived from culture grown in vitro, tissue transplants from infected animals, or chambers implanted in mice. Chamber challenge groups were slightly smaller ($n = 4$) because of limitations in amount of inocula available for experiment.
administered 1 day before and 3, 7, and 11 days after challenge, or 200 μL of OspA antiserum administered 1 day before challenge.

Evaluation of mice for *B. burgdorferi* infection and disease. Mice were killed with CO₂ gas, followed by exsanguination by cardiocentesis. Blood, urinary bladder, spleen, ear, and inoculation site were collected aseptically and cultured for 2 weeks in BSK II medium, as described previously [21]. An animal was considered infected if at least one of the sites yielded spirochetes. Rear legs (with knees and tibiotarsi) were fixed in neutral buffered formalin, paraffin-embedded, and processed for histology as described previously [21]. Arthritis was scored on a scale of 0 (no arthritis) to 3 (severe). Arthritis was evaluated by blinded examination of both tibiotarsi, after which the score of the most arthritic tibiotarsal joint of each mouse was recorded. The severity of disease among treatment groups was compared by calculating the mean and SD for each group and using Student’s unpaired *t* test.

### Results

As shown previously [2], mice were protected from *B. burgdorferi* infection when 10 μL of immune serum was administered at the time of challenge with in vitro–grown spirochetes, but immune serum administered 4 days after challenge did not eliminate the infection (table 1). The inability of immune serum from infected mice to clear spirochetes 4 days after infection suggested that once in the host, spirochetes may “adapt” to evade borreliacidal antibodies. To further test this hypothesis, skin transplant experiments were carried out. Since spirochetes are consistently recovered from the skin of infected mice, skin transplants are a simple method for transferring host-adapted spirochetes [27]. Naïve mice were infected by transplanting 2-mm² ear pieces from donor mice infected for 2 weeks. On days 0, 4, or 8 relative to the transplant, mice were treated with 10 μL of normal or immune serum. Unlike culture-challenged

#### Table 2. Effect of multiple doses of immune serum on mice infected by skin transplant.

<table>
<thead>
<tr>
<th>Serum</th>
<th>Dose*</th>
<th>Adaptive form of challenge spirochetes</th>
<th>Prevalence of infection</th>
<th>Arthritis severity†</th>
<th>Prevalence of infection (mice)</th>
<th>Partially engorged</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>50 μL (4×)</td>
<td>Culture</td>
<td>5/5</td>
<td>ND</td>
<td>0/10</td>
<td>0/10</td>
</tr>
<tr>
<td>Immune</td>
<td>50 μL (4×)</td>
<td>Transplant</td>
<td>5/5</td>
<td>2.0 ± 0.6</td>
<td>0/10</td>
<td>0/10</td>
</tr>
</tbody>
</table>

NOTE. Mice were checked for infection by culturing blood, urinary bladder, spleen, and skin in BSK II medium 2 weeks after challenge. Arthritis was evaluated in each animal by blinded examination of both tibiotarsi and recording the score of the more arthritic joint.

Freund’s adjuvant. The rabbit was boosted twice at 10-day intervals with 40 μg of fusion protein in incomplete Freund’s adjuvant before the rabbit was bled and sera collected for use in experiments.

Mice were passively immunized with sera as follows. In the experiment to determine the effects of immune serum on in vivo–derived spirochetes (table 1), mice were injected subcutaneously with 10 μL of normal or immune serum 24 h before challenge. Some of the mice challenged by transplant also received a higher dose of serum (table 2), which consisted of 50-μL doses of normal or immune serum administered multiple times on the day before challenge and 4, 8, and 12 days after challenge. Groups of mice challenged by infected tick bite (table 3) received a subcutaneous injection of 10 μL of normal or immune serum (low dose) 1 day before challenge, or 50-μL doses of immune serum (high dose) administered 1 day before and 3, 7, and 11 days after challenge, or 200 μL of OspA antiserum administered 1 day before challenge.

#### Table 3. Protective capacity of immune serum against *B. burgdorferi* delivered by tick bite.

<table>
<thead>
<tr>
<th>Serum*</th>
<th>Adaptive form of challenge spirochetes</th>
<th>Prevalence of infection (mice)</th>
<th>Prevalence of infection (ticks)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>Tickborne</td>
<td>4/4</td>
<td>3/4</td>
</tr>
<tr>
<td>Ospa antiserum</td>
<td>Tickborne</td>
<td>0/4</td>
<td>0/10</td>
</tr>
<tr>
<td>Immune (low dose)</td>
<td>Tickborne</td>
<td>4/4</td>
<td>7/10</td>
</tr>
<tr>
<td>Immune (high dose)</td>
<td>Tickborne</td>
<td>3/4</td>
<td>ND</td>
</tr>
<tr>
<td>Immune (low dose)</td>
<td>Culture</td>
<td>0/4</td>
<td>ND</td>
</tr>
</tbody>
</table>

NOTE. Mice were checked for infection by culturing blood, urinary bladder, spleen, and skin in BSK II medium 2 weeks after challenge. ND, not done.

* Mice were treated with normal mouse serum (10 μL), Ospa antiserum (200 μL), or low-dose immune serum (10 μL) 24 h before tick challenge. Mice receiving high dose of immune serum received 50-μL doses of serum administered 24 h before and at days 3, 7, and 11 after tick challenge.

† Mice were challenged with spirochetes grown in culture or by tick bite.

‡ Salivary glands and guts were dissected out of ticks that had fed for 48 hours (partially engorged) and examined for spirochetes by confocal microscopy. Whole tick smears were prepared from fully engorged ticks and examined for spirochetes by immunofluorescence microscopy.

As shown previously [2], mice were protected from *B. burgdorferi* infection when 10 μL of immune serum was administered at the time of challenge with in vitro–grown spirochetes, but immune serum administered 4 days after challenge did not eliminate the infection (table 1). The inability of immune serum from infected mice to clear spirochetes 4 days after infection suggested that once in the host, spirochetes may “adapt” to evade borreliacidal antibodies. To further test this hypothesis, skin transplant experiments were carried out. Since spirochetes are consistently recovered from the skin of infected mice, skin transplants are a simple method for transferring host-adapted spirochetes [27]. Naïve mice were infected by transplanting 2-mm² ear pieces from donor mice infected for 2 weeks. On days 0, 4, or 8 relative to the transplant, mice were treated with 10 μL of normal or immune serum. Unlike culture-challenged...
mice, no protection was observed against transplant challenge when immune serum was administered at day 0 or on days 4 or 8 (table 1). Transplant-derived spirochetes were able to infect and disseminate within the recipient mice in the presence of passively administered immune serum.

The process of host adaptation may require spirochetes to disseminate and possibly enter certain tissues of the host and/or come into contact with host cells. Alternatively, regardless of the location of the spirochete, interaction of the spirochete with a ubiquitous factor in the host may be sufficient for adaptation. To further characterize the process of host adaptation, experiments were done with spirochetes recovered from chambers implanted in mice. Mice were passively immunized, then challenged with $10^4$ chamber-derived spirochetes intradermally. Unlike spirochetes grown in vitro, which were susceptible to immune serum, equivalent challenge doses of chamber-derived organisms infected mice passively immunized with immune serum (table 1). The process of host adaptation appeared not to be dependent on contact with host cells or dissemination to particular tissues.

We next investigated if host-adapted spirochetes were sensitive to a higher dose of immune serum that was repeatedly administered over the course of the infection. Mice were infected by skin transplant; then, 50-μL doses of immune or control sera were administered at days 0, 4, 8, and 12 after transplant. Despite the presence of the higher and repeated doses of immune sera, which were maintained until necropsy on day 14, the mice infected by transplant developed disseminated spirochete infections (table 2).

We have previously reported that both immunocompetent and immunodeficient mice with established B. burgdorferi infections develop active arthritis, and passive transfer of immune serum reduces the severity of arthritis without eliminating infection, including spirochetaemia [2, 28]. Joints of the above-mentioned mice infected by skin transplant in the presence of higher doses of serum were examined to determine the severity of arthritis (table 2). Transplant-inoculated mice receiving normal mouse serum had more severe arthritis (mean score ± SD, 2.0 ± 0) than did transplant-inoculated mice receiving repeated higher doses of immune serum (mean score, 0.4 ± 0.6; $P \leq 0.001$; table 2). Although passively administered immune serum did not prevent infection and dissemination of host-adapted spirochetes, immune serum modulated the severity of arthritis caused by infection via transplant inoculation.

The above studies indicated that host-adapted spirochetes (transplant- and chamber-derived) were resistant to immune serum, while spirochetes grown in vitro were vulnerable. During natural infection, spirochetes that enter the host are neither host-adapted nor grown in vitro. The relevant question with respect to defining protective immunity using immune sera from infected animals is whether the sera protect mice from tickborne infection. Mice were injected with normal mouse serum (10 μL), B. burgdorferi OspA antiserum (20 μL), low-dose immune sera (10 μL), or high-dose immune sera (50 μL) 24 h before challenge with infected ticks. The mice receiving the high-dose immune sera received multiple 50-μL doses of serum that were administered 24 h before as well as at days 3, 7, and 11 after tick challenge. A control group was challenged with $10^4$ cultured spirochetes by syringe inoculation. As expected, all syringe-challenged mice were protected by immune serum (table 3). In contrast, the majority of mice that were treated with low and high doses of immune sera and challenged by tick bite became infected (table 3). Thus, tickborne spirochetes were similar to host-adapted (transplant- and chamber-derived) spirochetes in their ability to infect mice in the presence of immune serum.

Tickborne spirochetes were unable to infect mice in the presence of OspA antibodies (table 3) [29]. The mechanism of protection afforded by OspA antibodies involves the killing of spirochetes directly within feeding ticks so that transmission from the vector to the host is blocked [10, 29]. Therefore, engorged ticks recovered from mice treated with immune sera were examined to determine if spirochetes in the tick had been killed in a similar manner. Unlike ticks recovered from OspA antibody–treated mice, partially and fully engorged ticks recovered from mice treated with immune sera contained spirochetes (table 3). When salivary glands and guts of partially engorged ticks were examined for spirochetes by confocal microscopy, spirochetes were detected in both salivary glands and guts (table 3). Thus, spirochetes within ticks feeding on mice treated with immune serum were able to survive in the gut, invade the salivary glands, and infect the host.

**Discussion**

In passive transfer studies, relatively small amounts of immune serum from actively infected mice have been shown to be protective against syringe-inoculated cultured spirochetes [2]. We show here that these strong protective effects of immune serum may be circumvented during natural transmission. Mice that were passively immunized with relatively large amounts of immune serum, then challenged by tick bite, became infected. The lack of protection with immune serum was also observed when host-adapted (skin transplant and chamber-grown) spirochetes were used to challenge passively immunized mice.

Our results are divergent from another study in which mice were effectively immunized against tick challenge by administration of large amounts of immune serum [30]. In that study, immune serum was derived by pooling serum from 20 mice that had been infected for 6 months. These pooled sera contained OspA antibodies, indicating that at least some of the donor mice had seroconverted to OspA after 6 months of infection [30]. Previous studies have documented that although laboratory mice infected with low doses of spirochetes do not seroconvert to OspA early in the infection [2, 19], mice do develop OspA antibodies after prolonged infection [22]. Tickborne spirochetes are very sensitive to OspA antibodies, which destroy...
spirochetes within feeding ticks and block transmission to the host [10]. Unlike the sera used by Shih et al. [30], the immune sera used in the current study did not contain OspA antibodies, and this difference may explain the divergent results obtained in the two studies.

The differential effect of immune serum on various adaptive forms of spirochetes requires further study. An infected tick feeding on a rodent requires 48 h to transmit spirochetes to the host [13]. During this 48-h period, spirochetes grow, invade salivary glands of the tick, and undergo changes in protein synthesis [9, 10, 12, 31]. *B. burgdorferi* OspA, which is present on most spirochetes within ticks before feeding, is lost from the majority of spirochetes during tick feeding [10]. In contrast, *B. burgdorferi* OspC synthesis is increased during tick feeding [12]. These changes in spirochetal gene expression in response to tick feeding may reflect a phenomenon that allows the pathogen to more effectively establish an infection in the host.

Changes in antigen expression are not restricted to spirochetes within feeding ticks. Studies that have compared spirochetes in mice with those grown in culture have identified several genes that appear to be selectively expressed only during infection of the host [14–18, 21]. The immune serum used in this study has antibodies against many of the antigens that are selectively expressed in the host, yet tickborne spirochetes and host-adapted spirochetes were resistant to the protective effects of this serum. Recent evidence points to *B. burgdorferi* not only expressing host-specific antigens but also modulating the expression of these antigens over the course of an infection [14, 21]. Thus, the common factor that allows both host-adapted and tickborne spirochetes to survive in the presence of immune serum (or within the infected immunocompetent host) may be their ability to rapidly alter the expression of surface antigens and not the presence of any particular antigen on their surface. *B. burgdorferi* grown in vitro and introduced to the host may be unable to rapidly adapt and alter surface antigens, allowing them to remain vulnerable to immune serum during the first few days after inoculation. This adaptive period has been shown to last for <5 days, during which time spirochetes are vulnerable to immune serum treatment [3].

These factors currently preclude comparative analysis among antigens recognized by immune serum and the antigens expressed by *B. burgdorferi* within the context of flat ticks, feeding ticks, and the host. Indeed, antigen expression by *B. burgdorferi* is also influenced by culture conditions in vitro [12]. The current study used standardized immune conditions, generated in mice inoculated with minimal infectious doses of spirochetes, thereby reflecting antibody responses to antigens expressed by *B. burgdorferi* during the course of infection. This is a critical element in design of such experiments, as high inocula stimulate an artificial (and potentially protective) OspA antibody response, reflective of antigens expressed on the cultured, input spirochetes [2, 19]. We also attempted to equilibrate challenge doses of different adaptive forms of spirochetes, but there is no way to determine the infecting dose of spirochetes delivered by tick feeding. Nevertheless, our data clearly document significant relative differences in the vulnerability of different adaptive states of spirochetes to the protective effects of immune serum from actively infected mice. An important area for future study is the environmental and molecular cues that regulate gene expression in *B. burgdorferi*.

Acknowledgments

We thank Subrata Das for critically reading the manuscript and Debby Beck, Rhonda Bangham, and Paula Katavolos for technical assistance.

References


