Immune Elimination of *Leishmania major* in Mice: Implications for Immune Memory, Vaccination, and Reactivation Disease

Jude E. Uzonna, Guojian Wei, Dean Yurkowski and Peter Bretscher

*J Immunol* 2001; 167:6967-6974; doi: 10.4049/jimmunol.167.12.6967

http://www.jimmunol.org/content/167/12/6967

References

This article cites 42 articles, 13 of which you can access for free at: http://www.jimmunol.org/content/167/12/6967.full#ref-list-1

**Why The JI?** Submit online.

- **Rapid Reviews!** 30 days* from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

*average

**Subscription** Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions** Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts** Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts

"Make your mark. Discover reagents that make your research stand out."

The Journal of Immunology is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852

Copyright © 2001 by The American Association of Immunologists All rights reserved. Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Immune Elimination of *Leishmania major* in Mice: Implications for Immune Memory, Vaccination, and Reactivation Disease¹

Jude E. Uzonna, Guojian Wei, Dean Yurkowski, and Peter Bretscher²

Infection of susceptible BALB/c mice with a large, moderate, or low number of *Leishmania major* parasites respectively results in progressive disease, the formation of substantial but stable lesions, denoted as borderline disease, and the absence of a visible lesion. Infection with a low number of parasites results over the long term in either subclinical infections or an asymptomatic state. Subclinical mice produce a predominant Th1 response and are resistant to challenge, in contrast to their asymptomatic counterparts. Statistical and other evidence suggest that the asymptomatic state can arise from a subclinical state following parasite clearance, with consequent loss of resistance. Cell transfer studies demonstrate unequivocally that immune cells from subclinical mice can protect naive mice against a pathogenic challenge and can clear the parasite, leaving the mice susceptible to a rechallenge infection. This susceptibility is associated with the disappearance of both parasite-specific effector and memory T cells from secondary lymphoid organs. These findings have implications for vaccination, maintenance of memory, and prevention of reactivation disease. *The Journal of Immunology*, 2001, 167: 6967–6974.

Infection of humans by the *Leishmania* species, which is able to cause cutaneous leishmaniasis, results in a spectrum of clinical manifestations, ranging from fatal diffuse disease to self-healing lesions (1, 2). The murine model of cutaneous leishmaniasis is extensively studied because different mouse strains mount immune responses of different Th1/Th2 phenotype upon challenge with a million parasites, associated with different clinical outcomes. The prototypical resistant C57BL/6 and CBA strains mount a predominant Th1 response and contain the infection, while susceptible BALB/c mice mount a predominant Th2 response and suffer progressive disease. The Th1/Th2 nature of the immune response determines resistance/susceptibility (3–5).

Resistance/susceptibility to *L. major* depends, among other factors, upon the number of parasites employed for infection (6–8). Susceptible BALB/c mice resist a low dose infection, and resistant CBA mice develop lesions when infected with very high numbers (8). We undertook a detailed investigation of the relationship between the number of parasites employed for infection and the ensuing pathophysiological state in BALB/c mice. Our incidental observations over a period of several years following infection with low numbers of parasites suggested that immune elimination of *L. major* may occur, contrary to conventional views (9–14). Given the potential importance of this tentative inference for analyzing the requirements for maintenance of immunological memory, for vaccination, and for the control of reactivation disease, we attempted to develop a more incisive and reproducible system to examine whether parasite clearance can occur and to determine what the consequences are of such clearance for immunological resistance and memory.

**Materials and Methods**

**Mice**

BALB/c mice, 8–10 wk of age and bred at the animal facility of the Department of Microbiology and Immunology, University of Saskatchewan, were used. All mice were maintained according to the guidelines of the Canadian Council of Animal Care.

**Parasites and parasite Ags**

Mice were infected with stationary phase promastigotes of *L. major* MHOM (WHO MHOM/IL/80/Friedlin) strain as described previously (7). Soluble leishmanial Ag (SLA)³ was prepared, and protein content was determined by the Bradford method (15). SLA was used at 30 μg/ml for in vitro stimulation of splenocytes and lymph node cells for the detection of *L. major* Ag-specific cytokine-producing cells.

**Measurement of footpad lesions and detection of lymph node (LN) swellings**

The size of footpad lesions was measured with dial calipers (Oditest, Langenmesstechnik, Kroeplin, Germany), using the contralateral uninfected foot as a control. Mice were designated LN positive (LN⁺) if the draining popliteal lymph nodes were enlarged, as assessed by palpation.

**Estimation of parasite burden**

The parasite burden in the footpad, draining popliteal lymph node, spleen, and bone marrow of infected mice was estimated by limiting dilution analysis as previously described (16). To enhance sensitivity, 2-fold dilutions of undiluted samples (up to 1/100) were used. Thereafter, the conventional 10-fold dilutions were used.

**Limiting dilution assay for the frequency of *L. major*-specific cells**

The frequency of *L. major*-specific precursor T cells able to produce IL-2-, IL-4-, and IFN-γ-reactive cells was measured by limiting dilution as previously described (17) with minor modifications. Briefly, single-cell suspensions of spleens and draining popliteal lymph nodes from age-matched mice were infected with stationary phase promastigotes of *L. major* MHOM (WHO MHOM/IL/80/Friedlin) strain (7). Soluble leishmanial Ag (SLA)³ was prepared, and protein content was determined by the Bradford method (15). SLA was used at 30 μg/ml for in vitro stimulation of splenocytes and lymph node cells for the detection of *L. major* Ag-specific cytokine-producing cells.

Department of Microbiology and Immunology, University of Saskatchewan, Saskatchewan, Canada

Received for publication June 25, 2001. Accepted for publication October 9, 2001.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

³ Abbreviations used in this paper: SLA, soluble leishmanial antigen; LN, lymph node.
control mice or mice previously infected with *L. major* and from mice in the asymptomatic or subclinical state were serially diluted 2-fold (1000 to 50 cells/well; 32 replicate wells/dilution) and cultured in round-bottom 96-well culture plates (Nalgene, Nunc International, Naperville, IL.) with $5 \times 10^5$ irradiated (1500 rad from a $^{60}$Co source; Atomic Energy of Canada, Ottawa, Canada) syngeneic spleen cells (APC), rIL-2 (20 WHO U/ml; Genzyme, Mississauga, Canada), and SLA at 30 pg/ml. Negative controls included irradiated spleen cells cultured in the absence of responder cells. To assess spontaneous cytokine release, responder cells were cultured with APC in the absence of SLA. After 14 days, the cultures were washed three times and restimulated with fresh APC ($5 \times 10^5$ cells/well) and SLA in the absence of rIL-2. Supernatant fluids were collected after 48 h and assayed for IL-2, IL-4, and IFN-$\gamma$ production by ELISA. In each assay, wells were considered positive for cytokine production if the absorbance value was $>3$ SD above the mean value obtained from wells lacking responder cells only.

**Assessment of protective capacity by direct challenge or by transfer of lymphocyte populations to irradiated recipients that receive a parasite challenge**

Groups of mice that were previously exposed to a low dose infection of $3 \times 10^3$ *L. major* parasites in the right hind footpad and their age-matched controls were challenged with $10^6$ promastigotes given s.c. into the left hind footpad, and lesion development was monitored. The protective capacity of spleen cells from mice that had been exposed to parasites was sometimes assessed by the adoptive transfer assay (18). Twenty million splenocytes from age-matched control mice or experimental mice were injected i.v. into lightly irradiated (450 rad from a $^{60}$Co source) syngeneic mice. The next day, recipient mice were challenged with $10^6$ promastigotes given s.c. into the footpad and were monitored for lesion development.

**Measurement of parasite-specific IgG1 and IgG2a Abs**

At regular intervals, infected mice were tail bled, and the plasma obtained was used to determine the level of parasite-specific IgG1 and IgG2a Abs by ELISA (6, 8).

**ELISPOT assay for Ag-specific IFN-$\gamma$ and IL-4-secreting cells**

The Ag-specific IFN-$\gamma$ and IL-4-secreting cells in the draining popliteal lymph nodes and spleens of infected mice were enumerated by the ELISPOT assay as previously described (19). Spleen cells were assayed at $5 \times 10^6$ cells/well, while lymph node cells were assayed at $10^5$ cells/well in 200-$\mu$l aliquots in the presence or the absence of SLA (3 pg/well). In all cases the total number of cells per well was made up to $10^6$ by adding $5 \times 10^5$ or $9 \times 10^5$ unimmunized syngeneic splenocytes to the spleen and lymph node cell test cultures, respectively. It has been shown previously that this allows valid enumeration of Ag-specific cytokine-producing cells (19). The data are presented as the number of Ag-dependent spots adjusted to the expected number from $5 \times 10^6$ test cells unless otherwise stated.

**Cytokine determinations**

The levels of IL-2, IL-4, and IFN-$\gamma$ in supernatants of cultures from limiting dilution analysis were determined by routine sandwich ELISA. Paired Abs against murine IL-2, IL-4, and IFN-$\gamma$- and recombinant IL-2, IL-4, and IFN-$\gamma$, used as standards, were purchased from PharMingen (San Diego, CA) and used according to the manufacturer’s suggested protocol. The sensitivities of the ELISA were 3 pg/ml for IL-4 and 7.5 pg/ml for IL-2 and IFN-$\gamma$.

**Longitudinal characterization over many months of mice infected with low numbers of parasites**

Mice infected s.c. in the foot with lower numbers of parasites, which did not often cause progressive disease, were monitored for several months, up to 24 mo in some cases. Mice were individually tagged and were assessed every 2 wk for lesion formation/size and for the palpability of the lymph node draining the site of infection. Blood samples were taken regularly, about every other month. Levels of parasite-specific Ab in the plasma were assessed by ELISA as were the number of parasite-specific IFN-$\gamma$- and IL-4-producing cells in a million peripheral blood cells by the ELISPOT assay. Some mice were also sacrificed so that the splenic responses and parasite burdens in their draining lymph nodes and footpads could be assessed.

**Statistical analysis**

Estimates of the frequency of *L. major*-specific precursor T cells able to give rise to IL-2- and IL-4-, and IFN-$\gamma$-producing cells were obtained by both maximum likelihood and minimum $\chi^2$ methods, based on a Poisson regression between the number of positive wells and the logarithm of negative wells using the PROC GENMOD procedure of the Statistical Analysis System (SAS Institute, Cary, NC). The maximum likelihood results are presented, as similar results were obtained by either method. Significance of differences for ELISPOT data and parasite burdens was determined by ANOVA, while differences in disease outcome were determined by repeated measure ANOVA using StatView software (Abacus Concepts, Berkeley, CA).

**Results**

**Distinct pathophysiological states develop following infection of BALB/c mice with different numbers of *L. major* parasites**

We studied infected mice longitudinally over many months, sometimes up to 24 mo. The level of *L. major*-specific Abs in plasma, the size of the infected footpad, and the palpability of the draining popliteal lymph node were assessed periodically. We defined four pathophysiological states, assessed at $\geq 3$ mo postinfection. 1) The asymptomatic state is characterized by the absence of a visible lesion, the absence of a palpable draining lymph node, and the absence of detectable parasite-specific Abs. 2) No visible cutaneous lesion is evident in the subclinical state, but the draining lymph node is readily palpable, and parasite-specific IgG2a Abs predominate over IgG1 Abs. 3) Footpad lesions (0.5–2 mm) that do not change by $>0.5$ mm/wk and without a consistent trend to increase or decrease over several weeks characterize the borderline state. These mice have readily palpable draining lymph nodes and roughly equal amounts of IgG2a and IgG1 Ab in their plasma. Lesions in a few of these mice spontaneously heal or become progressive. 4) Mice in a progressive state have large palpable lymph nodes and large cutaneous lesions that continuously increase in size, often at a rate $>0.5$ mm/wk. These mice produce substantial parasitic-specific IgG1 Ab, and if mice are not euthanized, the lesions become necrotic with consequent loss of the foot.

We first assessed the size of the infection required to favor the generation of these distinct states. In one of three similar experiments, groups of BALB/c mice (20–54 mice/group) were infected with different numbers ($33–10^6$) of *L. major* parasites and were assessed for lesion development, palpability of the draining lymph node, and nature of the Abs present. The generation of different pathophysiological states depends upon the number of *L. major* parasites employed for infection, as assessed between 3 and 15 mo postinfection (see Table I). Infection with a million parasites inevitably leads to progressive disease, whereas most mice infected with 110 parasites were classified as asymptomatic/subclinical, and over half the mice infected with 3000 parasites developed borderline disease.

**Different pathophysiological states are associated with distinct Th cell responses**

We infected 60 BALB/c mice with $3 \times 10^3$ *L. major* parasites, as this infection leads to the generation of all four pathophysiological states defined above. Four weeks after infection, eight of eight mice had viable parasites in their footpads and draining lymph nodes (data not shown). Starting from 10 wk postinfection, mice were classified as belonging to one of the four states and were sacrificed at different times for enumeration of Ag-specific IFN-$\gamma$- and IL-4-secreting parasite-specific T cells in the spleen and of parasite burden in the footpad and lymph node and for quantitation of parasite-specific IgG1 and IgG2a Abs in the plasma. As shown in Fig. 1 (left), the number of parasites detected in tissue extracts correlates with the different pathophysiological states. Parasites could be detected only in the draining lymph nodes of subclinically infected mice. These mice mounted an IFN-$\gamma$-dominated Th1 response and had a low ratio of IgG1/IgG2a parasite-specific Abs

---

**Results**

**Distinct pathophysiological states develop following infection of BALB/c mice with different numbers of *L. major* parasites**

We studied infected mice longitudinally over many months, sometimes up to 24 mo. The level of *L. major*-specific Abs in plasma, the size of the infected footpad, and the palpability of the draining popliteal lymph node were assessed periodically. We defined four pathophysiological states, assessed at $\geq 3$ mo postinfection. 1) The asymptomatic state is characterized by the absence of a visible lesion, the absence of a palpable draining lymph node, and the absence of detectable parasite-specific Abs. 2) No visible cutaneous lesion is evident in the subclinical state, but the draining lymph node is readily palpable, and parasite-specific IgG2a Abs predominate over IgG1 Abs. 3) Footpad lesions (0.5–2 mm) that do not change by $>0.5$ mm/wk and without a consistent trend to increase or decrease over several weeks characterize the borderline state. These mice have readily palpable draining lymph nodes and roughly equal amounts of IgG2a and IgG1 Ab in their plasma. Lesions in a few of these mice spontaneously heal or become progressive. 4) Mice in a progressive state have large palpable lymph nodes and large cutaneous lesions that continuously increase in size, often at a rate $>0.5$ mm/wk. These mice produce substantial parasitic-specific IgG1 Ab, and if mice are not euthanized, the lesions become necrotic with consequent loss of the foot.

We first assessed the size of the infection required to favor the generation of these distinct states. In one of three similar experiments, groups of BALB/c mice (20–54 mice/group) were infected with different numbers (33–10^6) of *L. major* parasites and were assessed for lesion development, palpability of the draining lymph node, and nature of the Abs present. The generation of different pathophysiological states depends upon the number of *L. major* parasites employed for infection, as assessed between 3 and 15 mo postinfection (see Table I). Infection with a million parasites inevitably leads to progressive disease, whereas most mice infected with 110 parasites were classified as asymptomatic/subclinical, and over half the mice infected with 3000 parasites developed borderline disease.

**Different pathophysiological states are associated with distinct Th cell responses**

We infected 60 BALB/c mice with $3 \times 10^3$ *L. major* parasites, as this infection leads to the generation of all four pathophysiological states defined above. Four weeks after infection, eight of eight mice had viable parasites in their footpads and draining lymph nodes (data not shown). Starting from 10 wk postinfection, mice were classified as belonging to one of the four states and were sacrificed at different times for enumeration of Ag-specific IFN-$\gamma$- and IL-4-secreting parasite-specific T cells in the spleen and of parasite burden in the footpad and lymph node and for quantitation of parasite-specific IgG1 and IgG2a Abs in the plasma. As shown in Fig. 1 (left), the number of parasites detected in tissue extracts correlates with the different pathophysiological states. Parasites could be detected only in the draining lymph nodes of subclinically infected mice. These mice mounted an IFN-$\gamma$-dominated Th1 response and had a low ratio of IgG1/IgG2a parasite-specific Abs.
(Fig. 1). Mice with borderline disease had parasites both in the originally infected footpad and in the draining lymph nodes (Fig. 1). Parasite-specific IFN-ˇgamma and IL-4-producing cells were equally prevalent in the spleen, and the ratio of IgG1 and IgG2a Abs was near unity, indicating a mixed Th1/Th2 response. In contrast, mice with progressive disease mounted a dominant Th2 response, with high numbers of parasites in their footpads and lymph nodes (Fig. 1). Thus, different pathophysiological states are associated with distinct Th responses.

Observations suggesting elimination of leishmania parasites

Some observations led us to infer that infections may have been established and eliminated in some asymptomatic mice. We first characterized, in four separate experiments, the immune state and the presence of parasites in a total of 16 asymptomatic and 16 subclinical mice as well as in eight age-matched control mice at 9 mo postinfection with 1000 parasites. The results from one of these experiments are presented in Fig. 2. Subclinical mice that had palpable lymph nodes (LN†) and parasite-specific Ab (Ab‡) in their plasma always had parasites in their lymph nodes and sometimes in their footpads, but not in spleen and bone marrow (data not shown). However, parasites were undetectable in all asymptomatic mice (Fig. 1). Thus, different pathophysiological states are associated with distinct Th responses.

Table I. Relationship between the number of infecting parasites and the different pathophysiological states of cutaneous leishmaniasis in BALB/c mice as assessed 3–15 mo postinfection

<table>
<thead>
<tr>
<th>No. of Infecting Parasites</th>
<th>No. of Mice/Group</th>
<th>Progressive</th>
<th>Borderline</th>
<th>Subclinical/asymptomatic</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0 × 10⁶</td>
<td>20</td>
<td>100.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3.0 × 10⁶</td>
<td>44</td>
<td>32.0</td>
<td>54.3</td>
<td>13.7</td>
</tr>
<tr>
<td>1.0 × 10⁷</td>
<td>48</td>
<td>18.0</td>
<td>43.2</td>
<td>38.8</td>
</tr>
<tr>
<td>3.3 × 10⁸</td>
<td>54</td>
<td>15.6</td>
<td>39.2</td>
<td>43.2</td>
</tr>
<tr>
<td>1.1 × 10⁹</td>
<td>28</td>
<td>14.0</td>
<td>14.0</td>
<td>72.0</td>
</tr>
<tr>
<td>3.7 × 10⁹</td>
<td>26</td>
<td>7.2</td>
<td>0</td>
<td>92.8</td>
</tr>
</tbody>
</table>

*Some mice were clearly in a progressive state earlier than 3 mo postinfection, in which case they were euthanized. The 3- to 15-mo period of observation refers to the other states. Data are representative of three similar experiments.

We observed in a longitudinal study that some subclinically infected mice lost their parasite-specific Ab, i.e., serodeconverted (became Ab−), and at roughly the same time lymph node swelling regressed (i.e., became LN−). These changes occurred approximately 9 mo after infection with 330 or 1000 parasites and in one study occurred in 14% (7 of 51) of seropositive, subclinically infected mice at 12 mo postinfection. We examined whether this loss of parasite-specific Ab was associated with the absence of parasites in the draining lymph node and of resistance. In each of two separate experiments, two such mice were sacrificed between 12 and 18 mo postinfection to determine the parasite burden in their footpads and draining lymph nodes and to enumerate the L. major-specific effector T cells in the spleen and lymph node. We also employed the adoptive transfer assay to assess the protective capacity of the splenocytes from these mice. In contrast to mice in a subclinical state (Ab−/LN−), those that had serodeconverted (Ab−) had no parasites detectable in either footpad or draining

FIGURE 1. Distinct pathophysiological states of L. major infection in BALB/c mice are associated with distinct immune responses. L. major-infected BALB/c mice presenting different pathophysiological states were sacrificed, and parasite burden in the footpad (A) and draining popliteal lymph nodes (B) was determined (A). Splenocytes from infected mice were also assessed for the presence of L. major-specific IL-4-producing (C) and IFN-γ-producing (D) cells (B). Similar results were obtained using cells from the draining popliteal lymph node. The levels of parasite-specific IgG1 (E) and IgG2a (F) Abs in the plasma were also assessed (C). Results are means ± SD of data from seven to nine mice per group. Observations are from one of three similar experiments. *The parasite burden was >1200 × 10⁵; Subcl., subclinical disease; Bord., borderline disease; Prog., progressive disease.
lymph nodes. Moreover, no parasite-specific effector Th cells were detectable in either spleen or draining lymph node (see Fig. 3A). The transfer of these cells to lightly irradiated naive recipients also failed to protect the recipients from a normally lethal challenge of a million L. major parasites (Fig. 3B). In addition, the three remaining serodeverted mice were shown to be susceptible by direct challenge. These observations indicate that an L. major infection may be completely eliminated even in highly susceptible mice, contrary to common belief. Secondly, they imply, if this primary inference is correct, that maintenance of active resistance and immunological memory requires the presence of L. major parasites.

Probability arguments also support the idea that parasites can be eliminated. In one experiment, five of five mice from a group infected with 330 parasites had demonstrable parasites 5 wk after infection, and after 9 mo half these mice were Ab+/LN− and the other half were (Ab−/LN−). The presence of parasites in 50% of the mice at 9 mo postinfection could reflect the extreme possibilities that parasite infections were established in only half the mice or that they were established in all the mice, but half the infections were cleared. The first extreme seems unlikely on probabilities grounds. In this case the probability of finding that all five of the five mice examined contained parasites at 5 wk postinfection would be (1/2)⁵ or 1/64, which

---

**FIGURE 2.** Presence of parasite-specific Abs and palpable popliteal lymph nodes and resistance to parasite challenge distinguish subclinical from asymptomatic mice and are associated with a dominant Th1 response. BALB/c mice were infected with 1000 L. major parasites and were screened 9 mo later for the presence or the absence of parasite-specific Abs (Ab− or Ab+) and for the palpability of the draining popliteal lymph node (LN+ or LN−). Four mice each characterized as Ab+/LN− and Ab−/LN− and two age-matched control mice were killed, and their spleens (A) and draining lymph nodes (B) were assessed for parasite-specific IFN-γ-producing (■) and IL-4-producing (□) cells. Other Ab+/LN− and Ab+/LN− mice (five mice per group) were challenged with a usually pathogenic dose (10⁶) of L. major, and the kinetics of footpad swelling were monitored (C). Spleen cells from Ab+/LN− and Ab+/LN− mice were also adoptively transferred to lightly irradiated, naive, syngeneic mice (2 x 10⁶ cells/mouse), which were then challenged with 10⁶ L. major organisms the next day (D). The data presented are from one of four similar experiments.

---

**FIGURE 3.** Loss of parasite-specific Abs is associated with loss of parasite-specific cytokine-producing cells and of protective capacity of spleen cells. BALB/c mice previously infected with 330 L. major parasites were monitored periodically for the presence of parasite-specific Abs. After 12 mo, a few mice that had been Ab− had become Ab+, i.e., serodeconverted. Two uninfected age-matched control mice, two Ab− mice, and two mice that had serodeverted were killed, and their spleen cells were assayed for L. major-specific IFN-γ-producing (■) and IL-4-producing (□) cells (A) and for their ability to provide protection in the adoptive transfer assay (B). Data are means ± SD of two mice (A) or of four recipients (B) and are from one of two similar experiments.
is unlikely. Such statistical arguments support the possibility that parasite elimination occurs, and that serodeconversion is a consequence of such elimination.

Complete elimination of L. major in lightly irradiated mice made resistant by the transfer of immune cells from subclinically infected mice results in loss of resistance

We infer from the above observations that established parasite infections may be eliminated. A direct demonstration of such elimination is problematical, as a mouse must be killed to establish unequivocally that it is infected, and it is then impossible to assess whether the infection would have been eliminated. We know that infections are established with great reliability (>99%) upon infecting normal BALB/c mice with a million parasites. We attempted to examine the relationship between parasite clearance and loss of resistance in a more controlled setting involving such a challenge. We transferred spleen cells from mice with a subclinical infection (Ab+/LN−) or from age-matched control mice to irradiated, naive, syngeneic recipients. One day after the transfer these mice were challenged with a million parasites. All the mice that received cells from subclinically infected mice had palpable lymph nodes 4 wk postinfection, but none developed any visible cutaneous lesion. Spleen and lymph node cells were harvested from four of these recipient mice at both 4 and 18 wk postchallenge. The mice had predominant Th1 responses, and parasites were detectable in the infected draining lymph nodes (Table II). However, lymph node swelling regressed in five of the remaining 12 mice approximately 36 wk postchallenge, and at the same time no parasite-specific Ab could be detected. Two LN− and three LN+ mice were sacrificed, and their spleen and lymph node cells were assessed for L. major–specific IL-4– and IFN-γ–producing cells and parasite burden. Their spleen cells were also adoptively transferred to another set of lightly irradiated mice that was later challenged with a normally pathogenic dose of a million L. major parasites the next day. The remaining mice (three Ab+/LN− and four Ab+/LN+) were again challenged with 2 × 10^6 L. major promastigotes in the opposite hind footpad. In contrast to recipients that remained in the subclinical state (LN−), parasite-specific IFN-γ– and IL-4–producing cells were undetectable in the spleen and lymph nodes of the Ab+/LN− mice (Figs. 4, A and B), and no parasites could be detected in the draining lymph nodes (Table II). Most importantly, spleen cells from LN− mice failed to protect a new set of naive recipients from a high dose challenge of L. major (see Fig. 4C). Similar results were seen in intact mice. Upon challenge with a normally pathogenic dose of L. major, three previously protected LN− mice were susceptible, in contrast to four LN+ mice, which displayed a resistant phenotype (Fig. 4E). These results demonstrate unequivocally that elimination of L. major by a dominant Th1 response results in loss of protection and suggests that the maintenance at least of parasite-specific effector cells requires the continual presence of live parasites in the animal.

Memory T cells require longer antigen stimulation than effector T cells to proliferate and differentiate into cytokine-producing cells (20–22). Because the ELISPOT assay measures effector cell populations, we wondered whether our inability to detect L. major–specific cytokine-producing cells in the spleens and lymph nodes of asymptomatic mice was due to the presence of predominantly L. major–specific memory cells in these mice. Therefore, we also sought to determine, employing limiting dilution analysis, the preponderance of L. major–specific memory T cells in spleens and draining lymph nodes of these LN+ and LN− mice and their age-matched normal counterparts. The frequencies of L. major–specific IL-2, IL-4, and IFN-γ precursor T cells in the spleens and draining popliteal lymph nodes of control mice and of these LN− and LN+ mice, which had been previously protected from a normally lethal challenge of parasites by the transfer of spleen cells from subclinically infected mice, are shown in Table III. There is no significant difference in the frequencies of L. major–specific reactive cells in either the spleen or draining lymph node of LN− recipients and their uninfected age-matched counterparts. In contrast, the frequencies of these cells were considerably higher in LN+ mice.

Discussion

We report here four main findings. Firstly, different kinds of pathophysiological states can be generated in genetically identical mice following infection with different numbers of L. major parasites. Secondly, these distinct pathophysiological states are associated with distinct immune responses with characteristic Th1/Th2 phenotypes. Thirdly, parasite infections can be established and cleared by an appropriate immune response in susceptible BALB/c mice. Finally, such parasite clearance results in loss of resistance to the parasite and of immunological memory.

The observations on clearance of parasites and its effect on resistance have important implications for strategies of vaccination and preventing reactivation leishmaniasis. It is widely believed that following healing of leishmania infections, parasites persist in tissues, resulting in a subclinical carrier state (11, 13, 14). However, immune individuals who live in a region endemic for malaria and then move to a nonendemic location for some time can become ill, sometimes fatally, upon returning to the endemic region (23–25). This suggests that such an exposed individual has immunity that can eliminate the parasite and that resistance then decays in the absence of reinfection (R. Sanderson, personal communication). The mice in this study with a subclinical infection appear resistant indefinitely unless this infection is cleared, in which case resistance is lost.

Table II. Elimination of L. major in lightly irradiated mice made resistant by the transfer of immune cells from subclinically infected mice results in loss of parasite-specific IL-4 and IFN-γ responses

<table>
<thead>
<tr>
<th>Time After Infection (wk)</th>
<th>Lymph Nodea</th>
<th>Parasite Burden (×10^3) ± SD</th>
<th>L. major-Specific Spots (per 10^6 cells) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lymph node</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>IL-4</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>80.0 ± 31</td>
<td>206 ± 76</td>
</tr>
<tr>
<td>18</td>
<td>+</td>
<td>36.0 ± 13</td>
<td>93 ± 13</td>
</tr>
<tr>
<td>36</td>
<td>+</td>
<td>12.4 ± 3.5</td>
<td>50 ± 11</td>
</tr>
<tr>
<td>36</td>
<td>−</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

a Two to four mice were assessed for parasite burden in the draining LN and Ag-specific cytokine secretion at each time point.

b + and − refer to palpable and nonpalpable draining popliteal LN, respectively.

c Parasite burden in the draining popliteal LN. No parasites were detected in the infected foot pads from 18 wk postchallenge.
It has been shown in other systems that IL-12 is required for the maintenance of resistance to *L. major* as IL-12p40−/− mice, made resistant by administration of rIL-12 during the time of primary infection, develop progressive disease either spontaneously or several weeks following rechallenge (26). However, in this report, mice were infected with very high numbers of *L. major* parasites that normally cause fatal progressive disease and result, if the response is modulated to achieve containment, in substantial numbers of parasites in the healed foot pad and draining popliteal lymph nodes (11, 13, 14). Furthermore, it had previously been reported that IL-12 is not important in the maintenance of resistance to *L. major*, as healed C3H mice reinfected with *L. major* parasites...
maintained resistance after administration of anti-IL-12 Abs at a dose that ablates resistance during primary infection of naive mice (27). Another report shows that the induction and maintenance of immunity in mice immunized with recombinant LACK protein are IL-12 dependent (28). Interestingly, it was shown in other studies by this group that the duration of immunity to L. major infection in BALB/c mice induced by recombinant LACK protein is shorter than that induced by a plasmid DNA encoding LACK protein (29).

It was proposed in these studies that the superior protection achieved by DNA immunization is because LACK DNA persists longer than rLACK protein, thereby acting as a source of the continuous antigenic restimulation of LACK-specific memory T cells (29). Our results are in agreement with this explanation and support the proposal that the presence of a few viable L. major organisms might be required to maintain protective immunity to re-infection (10, 11, 30).

Rapid loss of memory in the absence of Ag is not unique to parasite infections. Recently, it has been shown that HIV-specific CD4+ memory T cells are rapidly lost following chemotherapy (31), probably due to a reduced viral load. Following viral infections, a state of active memory can probably be maintained by incomplete elimination of viruses (32, 33). In the late 1960s the term infectious immunity was used to describe the persistence of delayed-type hypersensitivity responses to pathogens that induce granulomas characteristic of tuberculosis, brucellosis, leprosy, and histoplasmosis (34). Recently, the question of whether Ag persistence is required for the maintenance of memory has drawn considerable attention (35–39). We suggest that a positive demonstration of a requirement for Ag to maintain memory is more likely to be valid, as maintenance of memory in the absence of the test Ag can always be explained by the presence of environmental Ags cross-reacting with the test Ag. In addition, recent investigations have led to an appreciation of homeostatic regulatory mechanisms that might complicate some analyses. For example, naive T cells placed in an in vivo lymphopenic environment, models often used to test for the requirement of Ag for the maintenance of memory T cells, acquire an activated phenotype even in the absence of Ag (40–42). Our observations for the most part are made in unmanipulated animals or in animals in which much time has elapsed after artificial conditions existed. In the latter situation the pertinent homeostatic mechanisms operating are likely to reflect those in a normal healthy individual, rather than those reflecting stress, such as might occur in lymphopenic and/or gene knockout animals.

Our findings have implications for achieving effective vaccination against leishmaniasis and other chronic infectious diseases caused by intracellular pathogens. Firstly, mice with a subclinical infection, but not asymptomatic mice, are resistant over the long term, but are likely to be susceptible, under adverse conditions, to reactivation leishmaniasis (11). For instance, inhibition of inducible NO synthase enzyme in mice in the subclinical state several months after clinical cure results in reactivation of latent leishmaniasis (13). Indeed, reactivation leishmaniasis is now common in parts of Africa where AIDS is prevalent (43, 44).

Should we strive to generate, by vaccination, a state leading to a subclinical infection or to parasite elimination? The observations discussed on susceptibility to malaria suggest that a response able to eliminate the parasite is effective as long as the immune system is continually and sufficiently stimulated by the pathogen, or cross-reacting Ags, to maintain memory and resistance. Similarly, in tuberculosis it is anticipated that exposure to environmental mycobacteria may be sufficient to maintain an effective Th1 imprint, which is believed to be required to contain Mycobacterium tuberculosis (45–47). If exposure to M. tuberculosis is relatively rare compared with exposure to environmental mycobacteria, as we suspect, a response that is able to eliminate the mycobacteria would have the advantage of minimizing the occurrence of reactivation disease. All in all, the generation of a response that can eliminate the pathogen or the attenuated pathogen employed for vaccination would seem desirable, with the maintenance of the appropriate resistant, pathogen-eliminating state by natural exposure to parasites and micro-organisms or, where necessary, by deliberate booster immunizations.

Acknowledgments

We thank Dr. Poonam Fakhw, Department of Agricultural Medicine, University of Saskatchewan, for help with statistical analysis, and Tara Strutt and Drs. Chris Hunter and Jay Farrell, for critically reading the manuscript and making helpful suggestions.

References

23. Perignon, J. L., and P. Druilhe. 1994. Immune mechanisms underlying the pre-
26. Park, A. Y., B. D. Hondowicz, and P. Scott. 2000. IL-12 is required to maintain
a Th1 response during *L. major* infection. *J. Immunol.* 165:896.
27. Constantinescu, C. S., B. D. Hondowicz, M. M. Elloso, M. Wysecka,
G. Trinchieri, and P. Scott. 1998. The role of IL-12 in the maintenance of an
established Th1 immune response in experimental leishmaniasis. *Eur. J. Immu-
nol.* 22:2227.
28. Muller, I. 1992. Role of T cell subsets during the recall of immunologic memory
and L. J. Picker. 1999. HIV-1 specific CD4+ T cells are detectable in most
individuals with active HIV-1 infection, but decline with prolonged viral sup-
and R. A. Seder. 2000. The role of antigen and IL-12 in sustaining Th1 memory cells
in vivo: IL-12 is required to maintain memory/effector Th1 cells sufficient to
Induction of long-lived germinal centers associated with persisting antigen after
34. Bretscher, P. A. 1996. Quantitative considerations in the design of vaccination
strategies against pathogens uniquely susceptible to cell-mediated attack. In *Con-
infection, reactivation and latent infection: impact of the CD4+ T-lymphocyte