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NOTES

Listeria monocytogenes Infection in β2 Microglobulin-Deficient Mice

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β2 microglobulin-deficient mice, in which the β2m gene has been disrupted by homologous recombination, lack functional CD8 T cells and are able to contain but not resolve an intravenous immunizing inoculum of *Listeria monocytogenes*. We present evidence that compensatory immunity in such immunodeficient mice was mediated by a population of γδ T-cell receptor-positive cells; in contrast, neither CD4 cells nor natural killer cells appeared to play any part in this process. These data further support the emerging hypothesis that immune cells other than those bearing the αβ T-cell receptor type can also play an important role in acquired resistance to listeriosis.

The pivotal observations of Lane and Unanue (16), and of North (19, 20) that cell-mediated immunity to the facultative intracellular bacterial parasite *Listeria monocytogenes* is mediated by Thy-1$^+$ cells led naturally to the hypothesis that specific resistance to this infection was mediated by T cells. Further data from a number of laboratories (1, 3, 6, 7, 9, 18, 22) have more recently provided the consensus that CD8 T cells, rather than CD4 cells, are the key T-cell subset involved in protective immunity.

It is now apparent, however, that this hypothesis needs amending in two important aspects. First, it is clear that an additional Thy-1$^+$ CD3$^+$ population of γδ T-cell receptor-positive cells may play a role in immunity to *L. monocytogenes* infection (21), whereas an important role for Thy-1$^+$ CD3$^-$ natural killer (NK) cells was also recently proposed (10). Second, given the observation (11) that depletion of both CD4 and CD8 cells does not result in lethal infection in mice given an immunizing inoculum of *L. monocytogenes*, the concept has emerged that the Thy-1$^+$ αβ T-cell receptor-negative subsets of leukocytes, rather than playing an ancilliary role to αβ$^+$ cells, may in fact play a more important role than previously appreciated in responding to this bacterial infection.

We present here data from a new mouse model of listeriosis that further support the role of Thy-1$^+$ αβ$^-$ cells. Mice in which expression of the β2-microglobulin gene was inactivated by gene targetting were used; the practical outcome of this inactivation is a failure to express class I major histocompatibility complex gene products and an almost total absence of functional CD8 T cells (15, 24). The results of this study show that such mice are able to contain but not resolve an intravenous immunizing inoculum of *L. monocytogenes*. Furthermore, treatment of such mice in vivo with antibody to the γδ TCR indicated that the residual immunity in these mice was mediated by a population of γδ T cells.

Female C57BL/6J mice were obtained from the Jackson Laboratories, Bar Harbor, Maine. C57BL/6 mice in which the β2-microglobulin gene had been inactivated by gene targetting were obtained from B. Koller, Chapel Hill, N.C. *L. monocytogenes* EDG was grown in tryptic soy broth to the midlog phase. Mice were injected intravenously with 5×10^3 bacteria suspended in 0.2 ml of sterile saline via a lateral tail vein. The course of bacterial infections over time was monitored by plating serial dilutions of individual whole organ homogenates on tryptic soy agar and counting colony formation after incubation for 24 h at 37°C in humidified air.

Spleen cells from infected mice were purified by passing spleens through fine-mesh stainless steel screens and washing once (centrifugation at 200 × *g* for 5 min). Media consisted of RPMI 1640 that was deficient in phenol red and biotin but was supplemented with 1% bovine serum albumin and 1% *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer and adjusted to pH 7.4. Splenic leukocyte numbers were counted with a Coulter counter (Coulter Electronics, Hialeah, Fla.) fitted with a 100-μm aperture and with the current, theshold, and amplification settings at an equivalent diameter of 3.2 μm or greater.

To determine T-cell subset numbers, harvested spleen cells were incubated on 150-mm plastic tissue culture plates (Falcon 3025; Becton Dickenson, Lincoln Park, N.J.) for 1 h at 37°C; then nonadherent cells were removed and washed twice. Erythrocytes were lysed by incubating samples for 5 min at room temperature in a solution of 0.155 M ammonium chloride and 0.01 M potassium bicarbonate (5.0 ml per spleen). The remaining cells were then washed, resuspended in 15-ml tubes containing anti-J11d.2 monoclonal antibody (4) supernatant and 1 ml of Low-Tox rabbit complement (Accurate, Westbury, N.Y.), and incubated for 1 h at 37°C to lyse B cells and immature thymocytes.

Numbers of CD4, CD8, and γδ cells were determined by flow cytometry with a Coulter Epic II flow cytometer. All antibodies used were obtained from Pharmingen, San Diego, Calif. Anti-CD3 (clone 145-2C11) was directly conjugated to phycoerythrin. Anti-CD44 (clone IM7), anti-CD45RB (clone 16A), anti-CD4 (clone RM4-5), anti-CD8 (clone 53-6.7), and anti-γδ T-cell receptor (clone GL3) were directly conjugated to fluorescein isothiocyanate (FITC). Controls consisted of cells treated with staining medium alone and appropriately tagged isotype-matched antibodies to irrelevant peptides. Harvested cells were washed for 5 min at 200 × *g* and 4°C, stained with anti-CD3 phycoerythrin and a single FITC-conjugated secondary stain, and then incubated for 20 to 30

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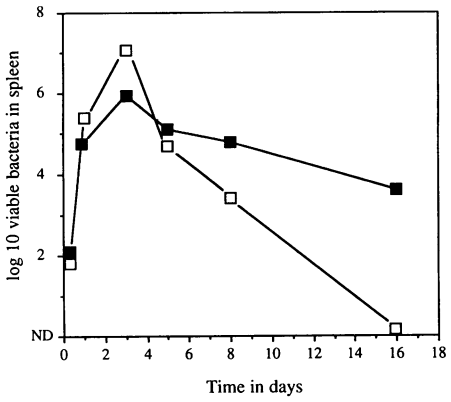

FIG. 1. Course of *L. monocytogenes* infection in C57BL/6 mice (□) and β2m$^-$ mice (■). Data are expressed as the mean numbers of bacteria recovered from groups of four mice (the standard error of the mean is omitted; it did not exceed 0.3). Note the initial increased resistance of the β2m$^-$ mice to the infection.

min at 4°C in the dark. Propidium iodide (PI; 50 μl) at a concentration of 2 mg/ml was added during the last 2 min of incubation. Cells were washed three times and suspended in 100 μl of staining medium per 2 × 10^6 cells. During each test, 20,000 viable CD3$^+$ lymphocytes were gated on the basis of phycoerythrin fluorescence and PI exclusion.

To observe potential changes in the important T-cell activation and homing markers CD44 and CD45 on CD8 cells in infected mice (17), single-parameter histograms of FITC fluorescence were generated and analyzed with the MKFLOW data analysis package (a kind gift of John Kappler, Denver, Colo.). With this program, 10 iterations of a three-point curve-smoothing routine were applied to more clearly reveal changes in relative fluorescence on cells bearing these markers.

Spleen cells were enriched for passive cell transfer by adherence and anti-J11d.2 monoclonal antibody treatment as described above. Further enrichment was achieved by negative selection on petri dishes coated with 1 μg each of anti-CD4 (clone GK1.5) or anti-CD8 (clone Lyt-2.43) per ml plus a mixture of anti-γδ (clone GL3) and anti-NK (clone PK136). Purity of eluted cells was assessed by flow cytometry as described above. Normal recipient C57BL/6 mice were infused intravenously with one spleen equivalent (chosen because the yields of CD8 cells from β2m$^-$ mice was very low) of selected cells and then rechallenged with 10^5 *L. monocytogenes* cells. Bacterial numbers in the spleens of recipients were determined 48 h later.

For in vivo depletion experiments, mice were injected intraperitoneally on days 0 and 2 with 250 μg of anti-γδ (clone 403.A10.2) or 50 μl of anti-NK (polyclonal anti-asialo-GM; WAKO Pharmaceuticals, Dallas, Tex.). Control mice received normal hamster serum or normal rabbit serum, respectively. These treatments had no effect on bacterial growth (data not shown). Flow cytometric analysis indicated that depletion reduced the numbers of target cells in the spleen (5 to 8% γδ cells and 1.8 to 2.2% NK cells) to below detectable levels.

The course of a sublethal infection with *L. monocytogenes* in control and β2m$^-$ mice is shown in Fig. 1. Over the first 2 days, the numbers of bacteria in the β2m$^-$ mice was actually lower than that in control mice, but after this period the infection was progressively cleared in these latter mice, while remaining at high numbers in the β2m$^-$ animals.

Changes in cell populations in infected mice are shown in Fig. 2. An increase of approximately 10-fold in overall leukocyte numbers was observed in both groups of mice over the first 9 days of the infection. Small rises were observed in both CD4 and CD8 populations, although nothing above a threefold increase was seen in two separate experiments. In β2m$^-$ mice, however, very low levels of CD8-staining cells were found (92 to 95% less than the level in controls) and no changes could be detected during the course of the infection. (The possibility that these residual cells could still influence resistance is deemed unlikely, given the fact that all studies to date have found CD8 cells in β2m$^-$ mice to be completely dysfunctional [24].) Finally, in C57BL/6 mice, a three- to fourfold increase in the number of γδ cells was observed over the initial course of the infection, whereas approximately twice this level of response was observed in β2m$^-$ mice.

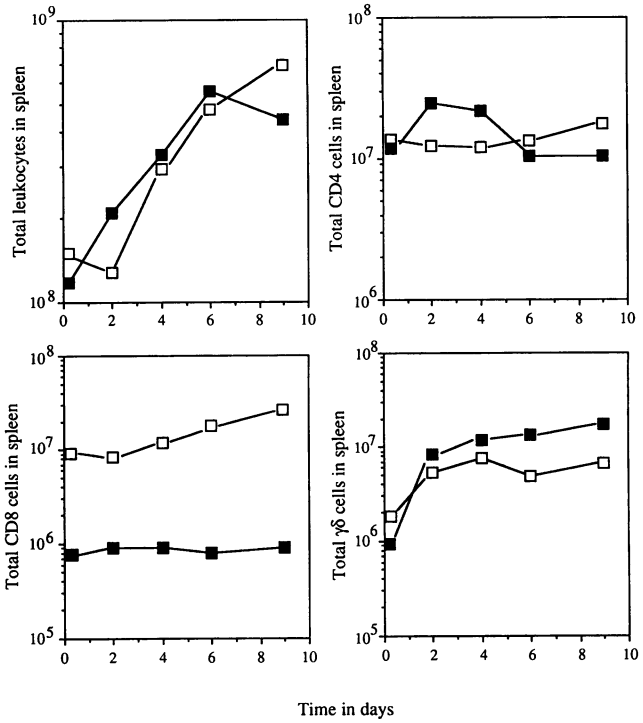

FIG. 2. Changes in cell populations in C57BL/6 mice (□) and β2m$^-$ mice (■) infected with *L. monocytogenes*. Cell numbers were determined by automated cell counting (leukocytes) and by flow cytometry on groups of three mice per time point. Values for each individual animal were determined; the data are expressed as the means of these data. Standard errors of the mean are omitted; for the leukocyte counts they did not exceed 0.21 log unit, and for the subset numbers of the variances did not exceed 15% of the mean values. Further statistical analysis of the counting technique is given elsewhere (13).

Flow cytometric analysis of changes in cell activation markers is shown in Fig. 3. In C57BL/6 mice there was a time-dependent shift in expression of CD44 (up-regulation) and CD45 (down-regulation) on CD8 T cells, indicating activation of cells within this population. (Unfortunately, we were unable to collect sufficient CD8 from β2m$^-$ mice for a similar analysis.)

To test whether β2m$^-$ mice compensated for lack of CD8 cells by generating CD4 protective T cells, cell samples highly enriched with CD4 and CD8 cells from such mice were transferred (in one-spleen equivalents because of the

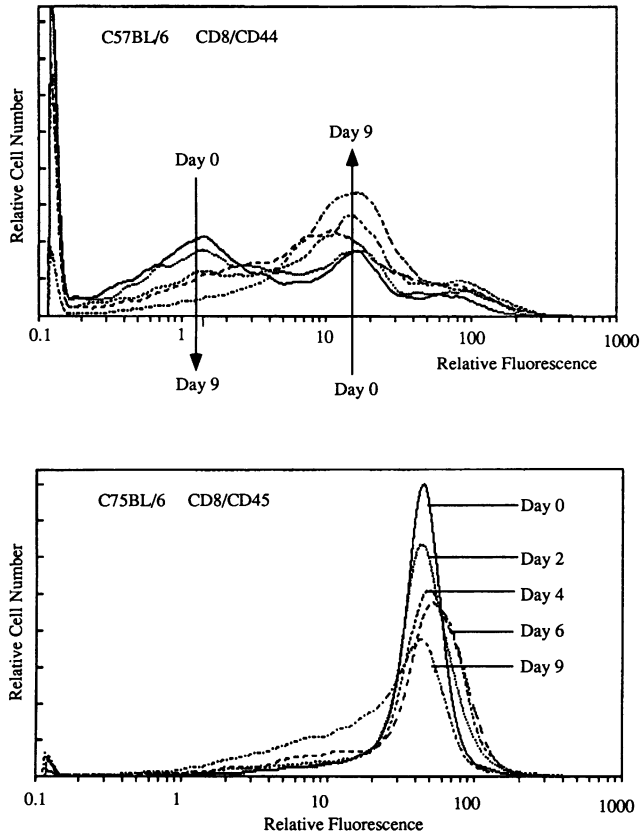

FIG. 3. Flow cytometric analysis of CD8 T cells harvested from C57BL/6 mice infected with *L. monocytogenes*. Cells were double stained with anti-CD8 plus either anti-CD44 or anti-CD45RB. The flow cytometer was then gated on $CD8^+$ cells, and single-parameter histograms for the expression of CD44 or CD45RB on these cells were generated. The data were then smoothed by using the MK-FLOW program. Note the time-dependent increased expression on CD8 cells of CD44 (the data lines were generated on cells harvested on days 0, 2, 4, 6, and 9 of the infection) and a reciprocal down-regulation of CD45RB over the same time period.

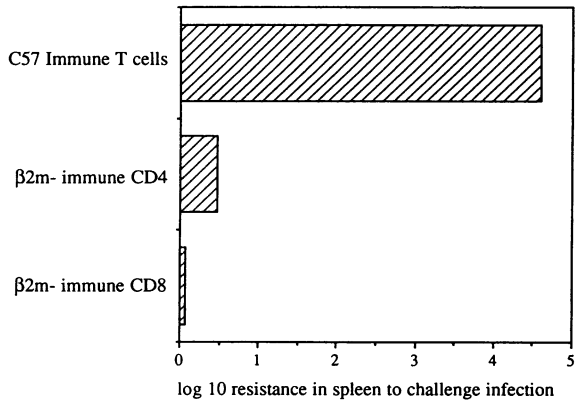

FIG. 4. Evidence that one-spleen equivalents of CD4 or CD8 cells from $\beta2m^-$ mice fail to adoptively protect normal C57BL/6 recipients from a lethal challenge dose (2×10^5 cells, given intravenously) of *L. monocytogenes*, whereas a similar inoculum of T cells from C57BL/6 mice transferred over 4 log units of protection. Mice were infused with indicated cells 2 h before bacterial challenge. Bacterial numbers in the spleen were then determined 48 h later. The data are expressed as the $\log_{10}$ means between groups of four mice receiving normal T cells and those receiving the cells indicated.

very low number of CD8 cells) into normal C57BL/6 controls, which were then challenged with a lethal dose of *L. monocytogenes*. Neither CD4 or CD8 cells harvested from $\beta2m^-$ mice had any protective capacity (Fig. 4).

In view of the above result that indicated a lack of activity of $\alpha\beta$ T cells, $\beta2m^-$ mice were depleted in vivo of $\gamma\delta$ or NK cells by monoclonal antibody administration. Depletion of $\gamma\delta$ cells caused a significant difference ($P < 0.01$ for both spleen and liver; Student's t test) in bacterial numbers, whereas treatment with anti-NK cell antibody did not (Fig. 5).

It has been observed that nude mice (5, 12), TXB mice (19), *scid* mice (2), or mice depleted of T cells by in vivo administration of monoclonal antibodies (8, 11) are capable of containing but not efficiently resolving an immunizing inoculum of *L. monocytogenes*. In view of this, Dunn and North (11) recently proposed that CD4 and CD8 cells play only a limited role in immunity to *L. monocytogenes* and that other $\alpha\beta^-$ leukocyte populations, until recently disregarded or undiscovered, may also make an important contribution to the response to this infection.

The results of the present study support the above hypothesis by showing that $\beta2m^-$ mice, which lack functional CD8 cells, behave similarly to the earlier models in that they, too, are incapable of efficiently resolving a *L. monocytogenes* infection. In such mice, we observed a large accumulation of $\gamma\delta$ cells, representing an 8- to 10-fold increase in numbers compared with those seen before infection, which is reminiscent of our previous observations in immunocompetent mice infected with *Mycobacterium tuberculosis* (13). Subsequent experiments, in which mice were depleted intravenously of NK or $\gamma\delta$ cells by monoclonal antibodies, revealed that depletion of the $\gamma\delta$ population in $\beta2m^-$ mice further exacerbated growth of the bacterial infection. These observations are therefore consistent with the studies of Ohga and colleagues (21), who observed an early accumulation of $\gamma\delta$ cells in the peritoneal cavities of mice infected with *L. monocytogenes* and hence concluded that such cells might contribute to subsequent antimicrobial resistance, and they are highly consistent with the observations of Hiromatsu et

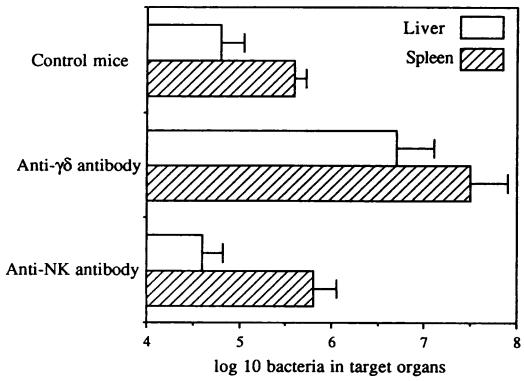

FIG. 5. Demonstration that in vivo administration of anti-$\gamma\delta$ antibody, but not anti-NK antibody, worsened the course of disease in mice infected with *L. monocytogenes*. Mice were given antibodies on day 0 and 2 of the infection, and bacterial numbers were determined on day 4. The data are expressed as mean values ± standard errors of the means ($n = 4$).

al. (14), who found that depletion of γδ cells in normal C3H mice exacerbated the course of *L. monocytogenes* infection in the spleen after intravenous inoculation. In the experiment of Hiromatsu et al., the otherwise immunocompetent mice resolved the infection, whereas in the present study the CD8-defective β2m$^-$ mice did not.

In this regard, the time-dependent changes in CD44 and CD45 expression on CD8 cells seen here, which are well-established markers of T-cell activation (17), are consistent with an important role for CD8 cells in protection against *L. monocytogenes* infection. Moreover, the present findings indicate that γδ T cells also make an important contribution to specific resistance to this bacterial parasite. In the absence of CD8 T cells, which occurs in the β2m$^-$ mice, an enhanced γδ response may ensue as a compensatory device in an attempt to contain the infection. It is quite possible, of course, that the reverse is true, in that the actual role of CD8 cells over these first 2 to 3 days of infection may be minor, as suggested elsewhere (11). However, the subsequent inability to quickly clear the infection that ensues after this time if CD8 cells are absent strongly indicates that this T-cell subset plays a major role in the resolution of the infection, if not in its initial containment.

We note, however, that it has been suggested elsewhere (9) that CD8 cells are incapable of mediating an infection resolving role because such cells are unable to interact with granulomas forming in infected mice 24 h after inoculation. In our hands (23), however, such lesions tend to be predominantly occupied by granulocytes at this time, with obvious mononuclear cell granulomas only being evident 5 to 6 days later. Thus, we feel that it is quite likely that CD8 T cells should be able to migrate into and clonally expand within such structures by that time.

Another issue is the role of CD4 T cells in listeriosis. In the present model, we saw no evidence to suggest that CD4 cells could compensate in the mutant mice. However, this does not rule out an ancilliary role for these cells in immunocompetent mice, and it should be noted that depletion of CD4 cells in such mice clearly does modulate the subsequent resistance of the animal to listeriosis (7, 8). In addition, once the infection has been resolved, the CD4 population may play a role in memory immunity (22).

In *scid* mice, nude mice, or other models in which total T-cell numbers are zero or very low, the ability of these animals to at least initially contain a *L. monocytogenes* infection is indicative that other cell populations are also able to compensate for the absence of such cells. Whereas the data herein suggest a major role for γδ cells under such circumstances, recent evidence (10) also indicates that other cell populations, notably Thy-1$^+$ NK cells, may also contribute to early resistance to listeriosis if the infection is delivered by the subcutaneous route. Why such differences occur on the basis of the route of inoculation is not known.

Breeding pairs of the β2m$^-$ mice used in this study were a kind gift of Beverly H. Koller, University of North Carolina.

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