INFECTION AND IMMUNITY, Apr. 1989, p. 1011–1017
0019-9567/89/041011-07$02.00/0

Vol. 57, No. 4

Resistance to Infection in Murine β-Thalassemia

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Received 26 September 1988/Accepted 11 December 1988

Clinical evidence suggests that individuals with chronic iron overload may be at increased risk of bacterial infection. We studied this question by using a unique model in which mice homozygous for a deletion in the gene encoding for the β-major globin develop moderate anemia, splenomegaly, and tissue iron overload, a syndrome similar to β-thalassemia in humans. Mice heterozygous for the gene deletion were phenotypically normal. Homozygous mice were significantly more susceptible to infection with *Listeria monocytogenes* than were heterozygous mice ($P < 0.01$). This increased susceptibility was associated with a greater number of organisms in the liver and spleen than was found in heterozygous mice ($P < 0.05$). However, histologic studies demonstrated similar inflammatory responses within these organs in homozygous and heterozygous mice. The increased susceptibility of homozygous mice to infection with *L. monocytogenes* was not seen when homozygotes were immunized with a low dose of *L. monocytogenes*. Although the results were not as striking as with *L. monocytogenes*, homozygous mice were also found to be more susceptible to infection with *Salmonella typhimurium* than were heterozygous mice ($P < 0.05$). Splenic mononuclear cells from homozygous mice demonstrated less responsiveness in vitro to the mitogens concanavalin A and phytohemagglutinin than did those from heterozygotes ($P < 0.05$). These data suggest that there is a generalized defect in innate immunity in homozygous mice which makes them more susceptible to infection by *L. monocytogenes* and *S. typhimurium*. The site of this immunological defect is not known but is most likely in the mononuclear phagocyte and may be due to tissue iron overload.

Clinical evidence has long suggested that conditions associated with chronic iron excess lead to an increased risk of infection (3, 4, 6). More recently, reports have been published indicating that patients with chronic iron overload appear to be at higher risk for developing infection caused by the microorganism *Listeria monocytogenes* (18, 29). Furthermore, clinical iron overload has been implicated as a cause of defective monocyte function in patients with thalassemia major (2). However, it is not universally agreed that chronic iron overload is associated with an increased risk of infection (9), and the mechanisms of that increased risk, if they exist, are not established.

To explore this question, we used a recently developed murine model of β-thalassemia (8, 27). In this model, an inheritable deletion in chromosome 7 results in a lack of β-major globin production, the normal adult β-globin. In mice heterozygous for this deletion, there is compensatory production of β-minor globin, with the result that these mice are phenotypically normal. In homozygous mice, on the other hand, there is incomplete β-minor globin compensation, and these animals develop anemia, shortened erythrocyte survival, and splenomegaly, characteristics similar to those of human β-thalassemia. In conjunction with these characteristics, mice homozygous for the β-major globin gene deletion have a marked increase in the iron content of the liver, spleen, and bone marrow, and plasma iron turnover is threefold greater than in heterozygous mice (32).

In this study, we examined the resistance of mice homozygous and heterozygous for the β-major globin gene deletion to infection with *L. monocytogenes*. Our findings indicate that homozygous mice have a marked defect in resistance to this microorganism as well as to *Salmonella typhimurium*, which suggests a generalized immunologic defect.

(Part of this research was presented at the 27th and 28th Interscience Conferences on Antimicrobial Agents and Chemotherapy [4 through 7 October 1987, New York, N.Y., and 23 through 26 October 1988, Los Angeles, Calif., respectively].)

MATERIALS AND METHODS

Mice. The mice used in these experiments were as previously described (32). The original isolation of the mutation resulting in a deficiency in β-major globin production is described by Skow and co-workers (27). Briefly, homozygous mice were the offspring of homozygous breeding pairs taken from descendants of the original proband male DBA/2 mouse that had been backcrossed onto the C57BL/6J background for at least five generations. Heterozygous mice were the offspring of female homozygous mice crossed with male C57BL/6J mice (Jackson Laboratory, Bar Harbor, Maine). Female C57BL/6J mice used in some of the experiments were purchased from Jackson Laboratory. The homozygous breeding pairs were maintained by R. A. Popp at the Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tenn. The production colony of homozygous and heterozygous mice was raised and housed in the Animal Research Facility at the Tucson Veterans Administration Medical Center. The colony was routinely screened for infection with Sendai virus. Female mice were used in all experiments and, to allow for steady-state iron accumulation, were between 90 and 140 days old. Mice were fed and watered ad libitum.

L. monocytogenes. The EGD stain of *L. monocytogenes* was originally obtained from Frank Collins, Trudeau Institute, Saranac Lake, N.Y., and was donated to our laboratory by Edward Wing, University of Pittsburgh, Pittsburgh, Pa. The organism was handled as previously described (7).

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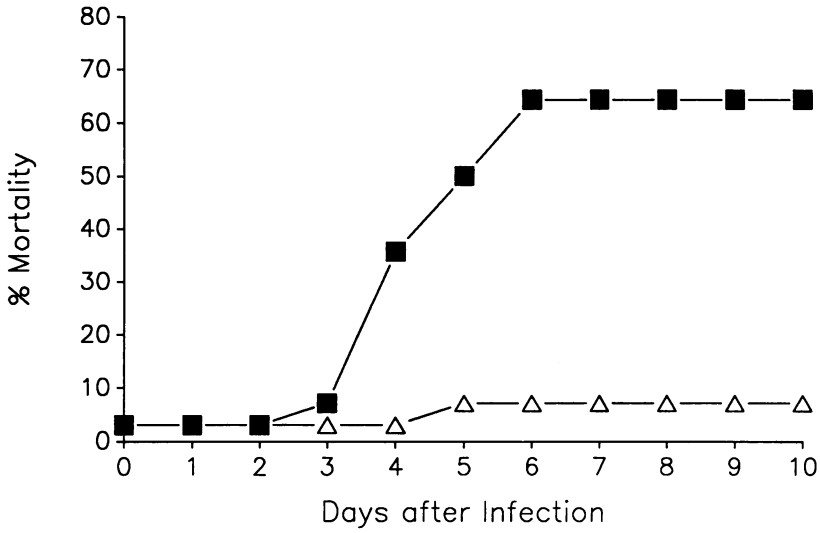

FIG. 1. Cumulative mortality over time of mice homozygous for β-thalassemia (■) and of heterozygous mice (△) after infection with a mean of 6.1×10^4 CFU of *L. monocytogenes*. There were 14 mice in each group; data are from three consecutive experiments. Mortality was significantly greater among homozygous mice ($P < 0.01$).

Virulence of the strain was maintained by injecting the organism intravenously into mice and harvesting it from the spleens 72 h later. Stock suspensions were prepared by inoculating the organism into tryptic soy broth and incubating the culture at 37°C for 8 h to achieve log-phase growth. The organisms were washed and stored at −70°C. Mice were injected with 0.1 ml of a suspension of organisms in sterile saline through the lateral tail vein. Quantitation of the inoculum was determined by subculture on tryptic soy agar.

S. typhimurium. The C5 strain of *S. typhimurium* was donated by Bruce Stocker, Stanford University, Stanford, Calif. It was maintained and handled in the manner described above for *L. monocytogenes*.

Survival. The 50% lethal dose (LD_{50}) of *L. monocytogenes* and *S. typhimurium* for the mice were determined by the method of Reed and Muench (24). Mortality was assessed by inoculating mice intravenously with a specific concentration of these microorganisms and observing the mice once to twice daily for up to 21 days.

Quantitation of *L. monocytogenes* in livers and spleens. The quantitation method described by Wing and co-workers was used (34). Mice were sacrificed by cervical dislocation at various times after injection of *L. monocytogenes*. Livers and spleens were aseptically removed and placed in 3.0 ml of sterile, iron-free water. Organs were then homogenized, and the resulting suspension was serially diluted in water and plated on tryptic soy agar. The number of CFU was determined after overnight incubation at 37°C. From this number, we determined the logarithmic mean number of organisms per organ.

Immunization of mice with *L. monocytogenes*. Homozygous and heterozygous mice were immunized by intravenous injection of 9.0×10^2 CFU of live *L. monocytogenes* in saline. Control mice were injected with the same volume of sterile saline alone. To assess the effect of immunization, mice were rechallenged with 1.9×10^5 CFU of live *L. monocytogenes*, and survival was determined.

Measurement of hematocrit, plasma iron, total iron-binding capacity, and tissue iron. We determined the hematocrit by centrifuging a heparinized capillary tube and determining the ratio of plasma to packed erythrocytes. The plasma iron concentration was measured by a coulometric method, using a Ferrochem II analyzer (Environmental Sciences Associates, Bedford, Mass.). Total iron-binding capacity was determined by a proprietary resin exchange method as previously described (32).

Iron contents of the liver and spleen was assessed by a method recently described (31). Equal portions of liver and spleen suspensions were dried, combined with 1.0 ml each of concentrated HNO_3 and H_2O_2, digested in closed Teflon PFA vessels by using an MD-81D microwave digestion system (CEM Corp., Indian Trial, N.C.), and diluted in deionized water; the actual iron content was determined by using an atomic absorption spectrometer (model 603; The Perkin-Elmer Corp., Norwalk, Conn.). Results were expressed as micrograms per milligram of dry weight.

Lymphocyte transformation assay. The lymphocyte transformation assay was performed by a standard procedure

TABLE 1. Relationship between time after a mean intravenous injection of 3.5×10^4 CFU of *L. monocytogenes* into homozygotes and heterozygotes and hematocrit, plasma iron, and liver and spleen tissue iron[a]

Time (h) after infection	Mouse group[b]	Hematocrit (%)	Plasma iron (μg/ml)	Iron content (μg/mg)	
				Liver	Spleen
0[c]	HM	38 ± 3[d]	153 ± 27	0.87 ± 0.08[d]	6.53 ± 0.45[d]
	HT	51 ± 1	174 ± 21	0.44 ± 0.01	2.17 ± 0.57
24	HM	35 ± 4[e]	160 ± 60	1.29 ± 0.13[d]	5.79 ± 0.80[e]
	HT	52 ± 1	110 ± 11	0.49 ± 0.06	1.77 ± 0.54
48	HM	30 ± 2[d]	31 ± 9[f]	1.03 ± 0.12[e]	5.20 ± 0.63[e]
	HT	46 ± 1	63 ± 12	0.45 ± 0.07	1.64 ± 0.41
72	HM	26 ± 4[e]	ND[g]	1.31 ± 0.07[d]	4.82 ± 0.53[d]
	HT	45 ± 2	64 ± 12	0.56 ± 0.06	1.12 ± 0.08

[a] Values represent means ± standard deviations obtained from three mice, using different groups of mice for each time point. Hematocrit changed significantly over time for both homozygotes and heterozygotes ($P < 0.05$), whereas liver and spleen iron contents changed significantly over time for homozygotes only ($P < 0.05$).

[b] Measurements done without injection of *L. monocytogenes*.

[c] HM, Homozygote; HT, heterozygote.

[d] $P < 0.001$ by the Student *t* test for difference from heterozygote value.

[e] $P < 0.01$ by the Student *t* test for difference from heterozygote value.

[f] $P < 0.05$ by the Student *t* test for difference from heterozygote value.

[g] ND, Not done.

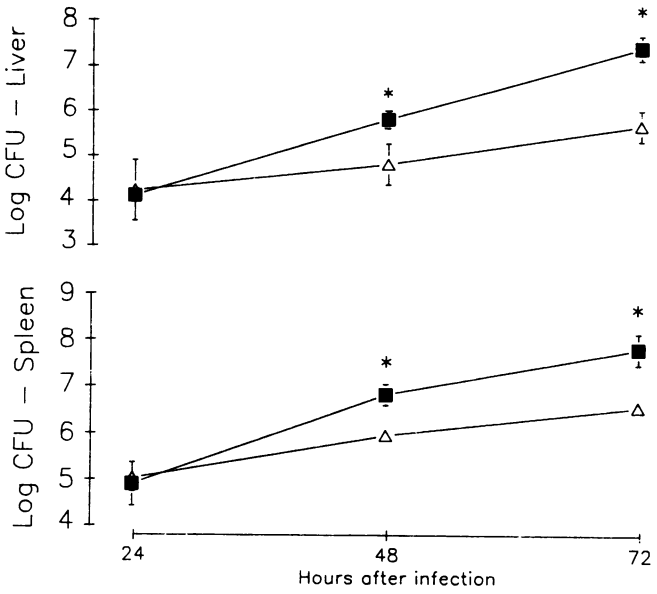

FIG. 2. Log CFU of *L. monocytogenes* over time in livers and spleens from homozygous (■) and heterozygous (△) mice. Data represent mean ± standard deviation. Each time point represents a separate experiment with three mice in each group. Differences between homozygous and heterozygous mice at 48 and 72 h after infection for both liver and spleen are significant ($P < 0.05$).

(22). Spleens were aseptically removed and gently homogenized, using a sterile glass homogenizer tube. We placed 10^5 nucleated splenic cells into 6-mm wells in RPMI cell culture media (GIBCO Laboratories, Grand Island, N.Y.) with 10% fetal calf serum (GIBCO) and added various concentrations of the mitogens phytohemagglutinin (PHA) (Sigma Chemical Co., St. Louis, Mo.) and concanavalin A (ConA) (Sigma). Each concentration was represented by three wells. Wells were incubated for 48 h, labeled with 1.25 μCi of [^{3}H]thymidine (Dupont, NEN Research Products, Boston, Mass.), incubated for an additional 24 h, harvested, and counted by scintillation spectrophotometry. Data were analyzed as counts per minute.

Experimental design and statistical analysis. All experiments were performed on at least three separate occasions. Data that were parametric were analyzed by the Student *t* test or by analysis of variance. The Mann-Whitney rank test was used for analysis of survival studies. A *P* value of <0.05 was considered significant.

RESULTS

Resistance to infection with *L. monocytogenes*. In preliminary experiments, we found the LD_{50} of *L. monocytogenes* to be approximately 2.8×10^4 CFU when the organism was administered to mice homozygous for the gene deletion resulting in β-thalassemia. When homozygous and heterozygous mice were simultaneously challenged intravenously with a mean of 6.1×10^4 CFU of *L. monocytogenes*, we

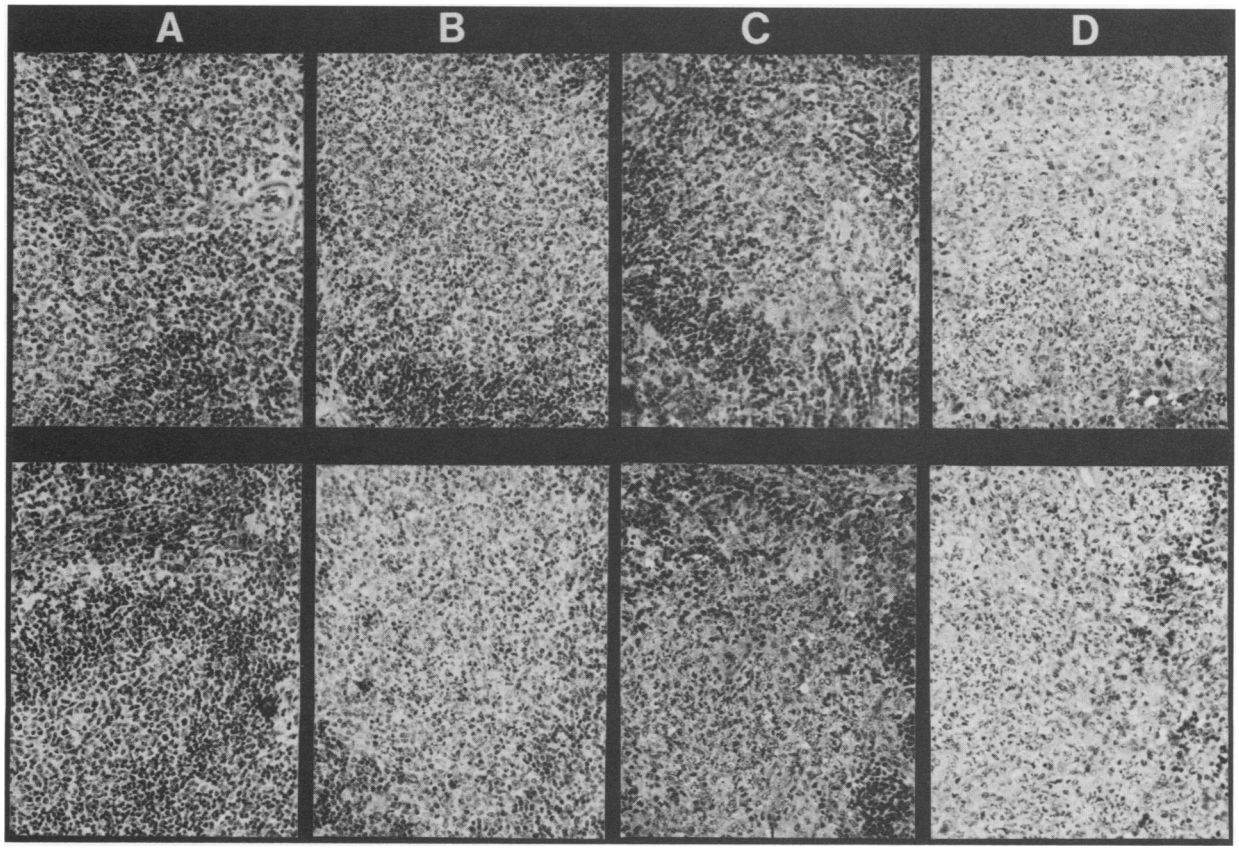

FIG. 3. Comparison of histologic findings from spleens of homozygous (upper row) and heterozygous (lower row) mice after infection with *L. monocytogenes* (magnification, ×350; hematoxylin-eosin stain). Letters indicate times after infection: A (control), before infection; B, 24 h; C, 48 h; D, 72 h. Spleens from both homozygous and heterozygous mice demonstrate marked influx of inflammatory cells over time, with destruction and loss of splenic architecture.

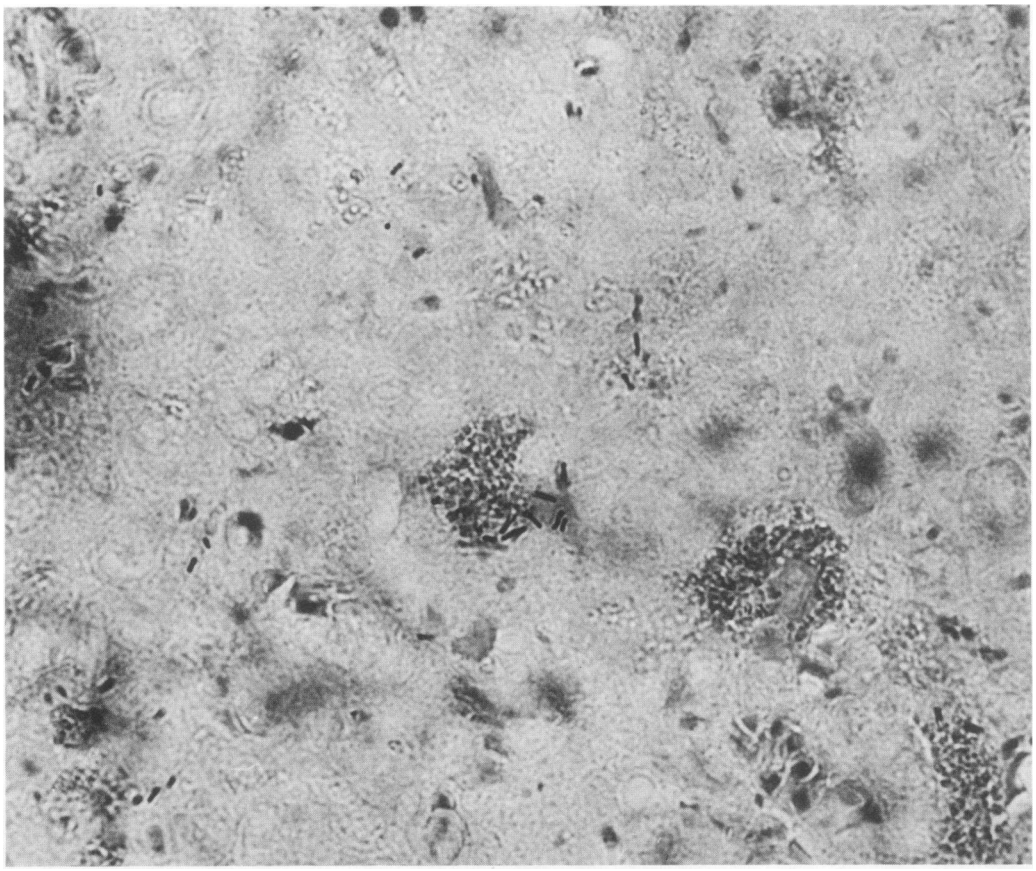

FIG. 4. Gram stain of the spleen of a homozygous mouse 72 h after infection with *L. monocytogenes* (magnification, ×1,000). Microorganisms are clearly associated with the macrophage in the center.

observed a 64% mortality in homozygotes, compared with a 7% mortality in heterozygotes (Fig. 1). As well, deaths occurred sooner in the homozygous the heterozygous mice than in ($P < 0.01$).

In a series of consecutive experiments, homozygous, heterozygous, and background C57BL/6J mice were injected intravenously with a mean of 3.5×10^4 CFU of *L. monocytogenes* and sacrificed after 24, 48, and 72 h. Zero-hour data were generated by sacrificing animals that were not inoculated with *L. monocytogenes*. Several parameters were measured during each time point.

Table 1 demonstrates the changes in hematocrit, plasma iron concentration, and liver and spleen iron contents during the course of infection. Before and throughout infection, homozygotes demonstrated significantly lower hematocrit and significantly higher liver and spleen iron contents than did heterozygotes. Homozygotes also demonstrated a significant fall in both hematocrit and spleen iron content and a significant rise in liver iron content during the course of infection ($P < 0.05$). Similar trends occurred in heterozygotes but were significant only for the decrease in hematocrit over time. Plasma iron concentration was slightly but significantly lower in homozygotes than in heterozygotes after 48 h of infection. Total iron-binding capacity was measured during the first 48 h after infection and was not significantly different between homozygous and heterozygous mice (data not shown). We were unable to measure plasma iron and total iron-binding capacity in homozygous mice by 72 h after infection because the mice were too ill. In all of these experiments, the results for C57BL/6J mice were not different from those for heterozygous mice (data not shown).

In association with the increased mortality that homozygous mice demonstrated after infection with *L. monocytogenes* compared with heterozygotes, there was increased growth of *L. monocytogenes* in the livers and spleens of the homozygous mice. By 48 and 72 h after infection, the mean CFU titer of *L. monocytogenes* was more than 10-fold higher in both the liver and spleen in homozygous mice in comparison with liver and spleen counts in heterozygous mice (for each, $P < 0.05$) (Fig. 2). We were unable to follow growth of listeria in organs beyond this time because of the high mortality of the homozygous mice at this inoculum of *L. monocytogenes*.

Spleen and liver histologic findings after *Listeria* infection. Histologic changes in the spleens during listeria infection in homozygous and heterozygous mice are demonstrated in Fig. 3. Both groups of mice demonstrated marked influx of inflammatory cells beginning as early as 24 h after infection. By 72 h, there was marked disruption of splenic architecture, with necrosis in both animal groups. Similar but less striking changes were seen in the livers from both groups of animals (not shown). When examined under oil with a Gram stain, organisms were readily apparent in the spleen tissues of the homozygous mice; these organisms appeared to be associated with macrophages (Fig. 4). Fewer organisms were identified in the spleens of homozygous mice, and these were not clearly macrophage associated (not shown).

Effect of *Listeria* immunization. Nine days after intrave-

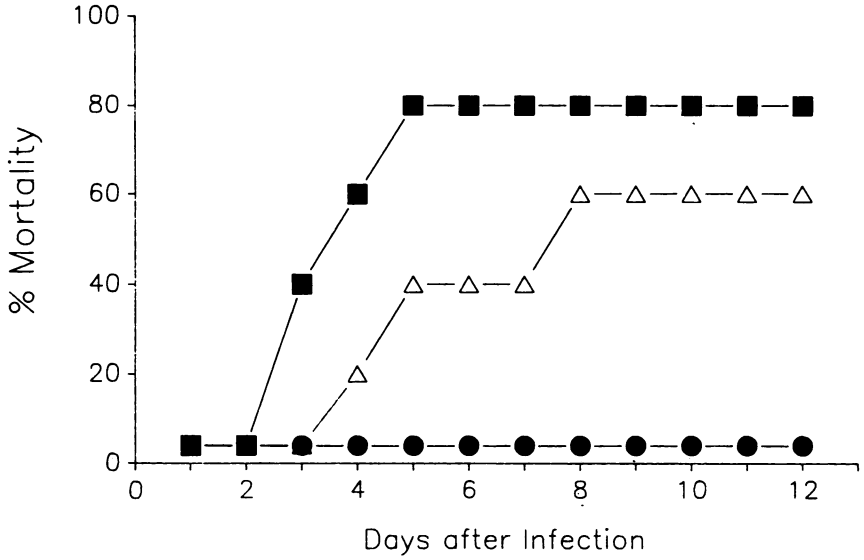

FIG. 5. Cumulative mortality of homozygous and heterozygous mice challenged intravenously with 1.9×10^5 CFU of live *L. monocytogenes* 9 days after intravenous immunization with 9×10^2 CFU of live *L. monocytogenes*. Control nonimmune mice were given saline rather than *L. monocytogenes* at the time of immunization. Each group contained five mice. Immunized homozygotes (●) were significantly less susceptible to infection than were either nonimmunized homozygous (■) or heterozygous (△) mice ($P < 0.001$).

nous injection of a low inoculum of live *L. monocytogenes* to induce immunity, mice were rechallenged with approximately seven times the LD_{50} of *L. monocytogenes* for homozygous mice, and survival was assessed. Immunized homozygous mice were significantly less susceptible to listeria infection than were either the nonimmunized control homozygous or heterozygotes (for each, $P < 0.001$) (Fig. 5). At the inoculum injected, none of the immunized homozygous or heterozygous mice died.

Resistance to *Salmonella* infection. To determine whether the defect in immunity was specific to *L. monocytogenes* or represented a broader susceptibility to bacterial infection, survival of homozygotes and heterozygotes was assessed after intravenous infection with *S. typhimurium* C5. We determined the LD_{50} of *S. typhimurium* for C57BL/6J mice, the background mouse strain for both heterozygous and homozygous mice, to be approximately 3.5×10^3 CFU; for DBA/2 mice, the proband strain for the original gene deletion, the LD_{50} of *S. typhimurium* was $>1.7 \times 10^5$. After intravenous infection of homozygous mice with a mean of 5.1×10^3 of *S. typhimurium*, the cumulative mortality was found to be 29%. This mortality was higher and occurred sooner than in heterozygotes, which had a cumulative mortality of 21% ($P < 0.05$) (Fig. 6).

Lymphocyte transformation. We measured in vitro lymphocyte transformation as a general assessment of cell-mediated immunity. At 2.5 μg of PHA per ml and all concentrations of ConA tested, splenic mononuclear cells

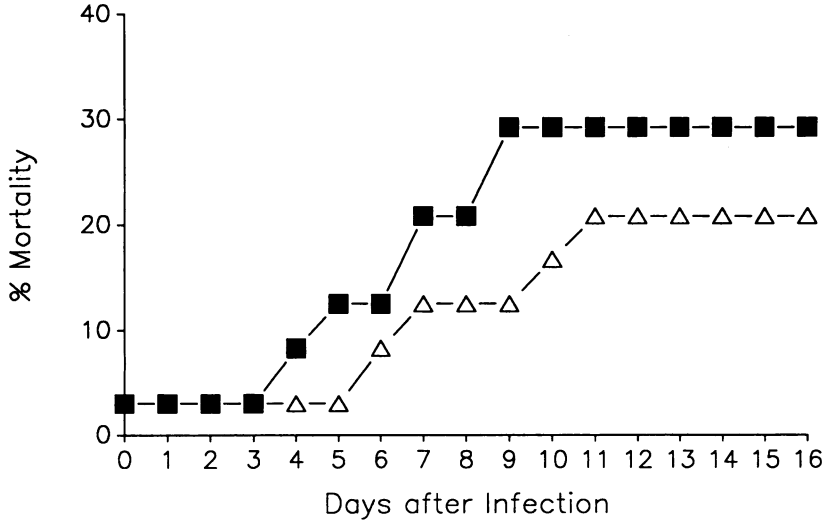

FIG. 6. Cumulative mortality of homozygous (■) and heterozygous (△) mice after infection with a mean of 5.1×10^3 CFU of *S. typhimurium* C5. There were 24 mice in each group; data are from four consecutive experiments. Mortality was significantly greater in homozygous than in heterozygous mice ($P < 0.05$).

TABLE 2. Results of the lymphocyte transformation assay using PHA and ConA

Group	cpm at given concn (μg/ml) of[a]:						
	Control	PHA			ConA		
		0.1	1.0	2.5	0.5	2.5	5.0
Homozygote							
Mean	6,607	10,042	16,870	18,232	7,390	53,209	98,155
±SEM	646	2,262	4,085	3,707	1,178	27,865	15,870
Heterozygote							
Mean	5,350	10,806	38,100	64,590	22,886	228,964	237,521
±SEM	378	3,430	13,905	11,230	4,198	34,885	30,327
P value	NS	NS	NS	<0.02	<0.02	<0.02	<0.02

[a] Data are from three consecutive experiments. NS, Not significant.

from homozygous mice were significantly less responsive than were those from heterozygous mice ($P < 0.02$) (Table 2).

DISCUSSION

Our findings demonstrate that mice homozygous for the deletion of the β-major globin gene, resulting in β-thalassemia, are more susceptible to infection with *L. monocytogenes* than are phenotypically normal heterozygous mice. These results support clinical data indicating that patients with β-thalassemia (6) and other anemias (3), as well as other iron-loading conditions (4, 18, 29), are at increased risk of infection.

Resistance to listeriosis in mice has been shown to depend on cell-mediated immunity (1, 15) and has been divided into three phases (17, 19). The first two phases, termed natural or innate resistance, do not depend on specific immunologic mechanisms and are characterized by a marked influx of mononuclear phagocytes into the livers and spleens of infected animals (12, 17). During the third phase of response, which begins approximately 3 days after infection, the mononuclear phagocytes become activated in the presence of specifically immune T lymphocytes and then begin to suppress growth of *L. monocytogenes* (12, 16). This third phase represents acquired cellular resistant (12). The site of the resistance defect in β-thalassemia mice appears to be in early, innate immunity. This view is supported by the findings of early death among homozygous mice after listeria infection and by the increased growth of *L. monocytogenes* in the livers and spleens of homozygous mice by 48 h after infection. It is also supported by the increased resistance observed in homozygotes after immunization. However, the development of resistance seen in the homozygous mice may have been only relative. Even higher inocula of *L. monocytogenes* must be given to assess whether the defect in host resistance seen in homozygotes was truly abrogated by immunization.

The defect in innate immunity observed was not restricted by *L. monocytogenes* but was also demonstrated for *S. typhimurium*. However, this difference in resistance between homozygous and heterozygous mice was less striking for *S. typhimurium* than for *L. monocytogenes*. Resistance to *S. typhimurium* appears to occur in stages similar to those for resistance to *L. monocytogenes* (21). However, although natural resistance to both *L. monocytogenes* and *S. typhimurium* is under genetic control, these controls are different for each organism (10, 12, 20, 26). C57BL/6J mice, the background strain for both the homozygous and heterozygous mice, are relatively resistant to *L. monocytogenes* but are susceptible to infection with *S. typhimurium* (12, 26). Conversely, DBA/2 mice, the original proband strain for the β-major globin gene deletion, are relatively resistant to *S. typhimurium* but susceptible to *L. monocytogenes* (12, 26). The fact that homozygous mice are more susceptible to both microorganisms indicates that the increased susceptibility is not simply due to a genetic resistance pattern. Additional support that the defect is global is the finding of lower responsiveness of splenic mononuclear cells from homozygous mice to the mitogens PHA and ConA than of cells from heterozygous animals.

The most likely site for the defect in resistance is the mononuclear phagocyte. One explanation for the defect is that there is defective influx of mononuclear phagocytes from the bone marrow to the site of infection in β-thalassemia mice. However, the inflammatory response within the livers and spleens of homozygous mice was comparable histologically to that seen in heterozygotes. A second, more likely explanation is that individual mononuclear phagocytes in the homozygous mice were unable to effectively control the infection. Mononuclear phagocytes are known to store iron intracellularly under conditions of iron excess (30), and this may lead to an impairment of function (2, 29, 33). Kaye and co-workers (11) have shown that erythrophagocytosis by mouse macrophages interferes with the ability of the macrophages to kill *S. typhimurium*. A similar mechanism may act in mice homozygous for β-thalassemia. It is also possible that the excess tissue iron acts as a virulence factor for *L. monocytogenes* and *S. typhimurium*, both of which display increased growth under conditions of excess iron (5, 23, 25, 28). Excess tissue iron stores have been shown to enhance the pathogenicity of other intracellular pathogens (13, 14). However, the exact mechanism for the defect in resistance to bacterial infection in mice homozygous for the deletion in the β-major globin gene remains to be determined and may be due to factors other than these.

ACKNOWLEDGMENTS

We thank Edward Wing and Mitch Magee for support and advice during this project and Bruce Stocker for his donation of *S. typhimurium*.

This work was supported by a Flinn Foundation award from the University of Arizona (N.M.A.), by a merit review award from the Veterans Administration (D.B.V.W.), and by Public Health Service grant HL-37056 from the National Institutes of Health and Department of Energy Contract AC05-840R21400 with Martin Marietta Energy Systems, Inc. (R.A.P.).

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