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Significant Contribution of Spleen Cells in Mediating the Lethal Effects of Endotoxin In Vivo

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Two closely related, histocompatible mouse strains that have marked differences in both in vitro and in vivo responses to endotoxin were used to evaluate the contribution of lymphoid cells to the lethal effect of endotoxin. C3H/HeJ mice are endotoxin resistant, whereas C3H/HeN mice are endotoxin sensitive. In vitro spleen cell mitogenic responses to endotoxin were similar in untreated mice and in mice that received sublethal irradiation (450 R) followed by reconstitution with autologous spleen cells. Reconstitution with spleen cells from the related strain produced chimeric animals with spleen cell mitogenic activity like that of the donor strain. When chimeric animals were subjected to a lethal challenge of endotoxin, their response was markedly altered by the transferred lymphoid cells. C3H/HeJ animals reconstituted with C3H/HeN cells became more endotoxin sensitive, whereas C3H/HeN animals reconstituted with C3H/HeJ cells became more endotoxin resistant. These results indicate that spleen cells play a significant, detrimental role in endotoxin-induced lethality.

In spite of intensive investigation, the biological events that lead to death in animals exposed to lethal doses of endotoxin remain unclear. Although some of the deleterious effects of endotoxin such as sensitizing the pulmonary vasculature to acetylcholine may be direct (19), many of the phenomena leading to death are probably induced by vasoactive substances released during the course of endotoxicosis. Since lymphocytes are an important source of biologically active molecules including prostaglandins (7, 8) and are directly activated by endotoxin (2), it seemed possible that lymphoid cells might play a major role in producing the lethal effects of endotoxin in vivo. To examine this possibility, we performed spleen cell transfer experiments between the endotoxin-resistant C3H/HeJ mouse strain and the closely related, fully histocompatible C3H/HeN strain, which has normal sensitivity to endotoxin. Previous work in this laboratory had demonstrated that spleen cells could be adoptively transferred between these two strains to create chimeric animals (17). The chimeric mice then exhibited the B-cell proliferative responses of the donor to endotoxin; that is, either strain, when reconstituted with C3H/HeJ strain spleen cells, was unresponsive to the mitogenic properties of endotoxin in vitro and, when reconstituted with C3H/HeN strain spleen cells, was sensitive to this effect. In the present paper, we report that spleen cells also transfer resistance or sensitivity to the lethal in vivo effects of endotoxin. Endotoxin-resistant C3H/HeJ mice were sensitized to these lethal endotoxin effects by C3H/HeN spleen cells, whereas endotoxin-sensitive C3H/HeN mice were rendered more resistant to endotoxin lethality by C3H/HeJ spleen cells. The findings clearly demonstrate a significant contribution by spleen cells to the lethality of endotoxicosis.

MATERIALS AND METHODS

Animals. Normal 6- to 8-week-old female C3H/HeJ mice were obtained from Jackson Laboratories, Bar Harbor, Me. Normal 6- to 8-week-old female C3H/HeN mice were obtained from the Division of Research Services, National Institutes of Health, Bethesda, Md. When used, mice weighed 20 to 30 g.

Endotoxin. Endotoxin from *Escherichia coli* K-235, kindly provided by William Sievert, Abbott Laboratories, North Chicago, Ill., and Floyd McIntire, University of Colorado, Denver, was prepared as previously described (14). Endotoxin from *Salmonella typhimurium* (Westphal phenol-water preparation) was obtained from Difco Laboratories, Detroit, Mich.

Adoptive transfer of spleen cells. Animals received 450 R of X irradiation and were reconstituted within 6 h by tail vein injection with 3×10^7 spleen cells suspended in RPMI 1640 media (Grand Island Biological, Grand Island, N.Y.). Tests of the completeness of transfer were made by analysis of the in

vitro proliferative response to endotoxin in control animals as previously described (17).

Lethality testing. Chimeric animals were housed three to eight per cage at 25°C until tested. Varying doses of endotoxin were injected intraperitoneally in 0.5 ml of RPMI 1640. Deaths were recorded daily for 3 days, after which no further deaths were observed. Fifty percent lethal dose (LD_{50}) values were calculated by the Reed-Muench method (16), and statistical analysis of composite LD_{50} data was made by Student's *t* test (20).

RESULTS

The effect of adoptively transferred spleen cells on resistance to the lethal effects of endotoxin was studied. Figure 1 shows the results of the first experiment, performed with *E. coli* K-235 endotoxin 6 weeks after irradiation and reconstitution. All of the C3H/HeN mice reconstituted with autologous sensitive cells died when given 0.25 mg or more of endotoxin, whereas those C3H/HeN mice reconstituted with resistant C3H/HeJ spleen cells all survived the 0.25-mg dose and 50% survived a 0.5-mg dose of endotoxin. Similarly, C3H/HeJ mice reconstituted with sensitive C3H/HeN spleen cells experienced 100% lethality with a 3-mg dose of endotoxin and 20% lethality at 1.5 mg, but survived all doses tested when reconstituted with autologous resistant spleen cells. Thus, adoptively transferred spleen cells, though unable to completely change the in vivo resistance to endotoxin, caused significant changes in the survival curves.

Two further experiments using this approach were performed (Table 1). In experiment 2, C3H/HeN mice experienced no mortality at 0.25 mg when reconstituted with C3H/HeJ spleen cells versus 50% mortality when reconstituted with autologous cells. C3H/HeJ recipients were all killed with 2 mg if donor cells were from the endotoxin-sensitive C3H/HeN strain, but none died when reconstituted with unresponsive C3H/HeJ spleen cells. Experiment 3 shows similar results, but was performed with *S. typhimurium* endotoxin 6 months after irradiation and reconstitution. Thus, the effect of adoptively transferred cells is long-lived and not restricted to a single endotoxin preparation.

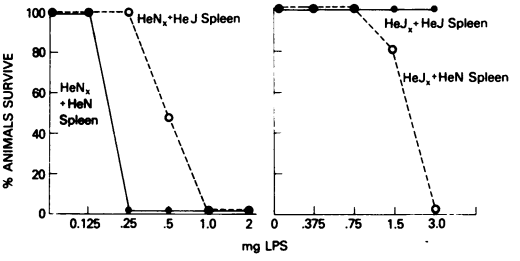

FIG. 1. *Dose response curves of chimeric mice to endotoxin-induced lethality. Each point represents the percent survival of four to five mice that were irradiated (C3H/HeN$_x$ or C3H/HeJ$_x$) and reconstituted with autologous or heterologous cells. C3H/HeN$_x$ mice became more resistant to the lethal effects of endotoxin with adoptively transferred C3H/HeJ (unresponsive) spleen cells. C3H/HeJ$_x$ mice were sensitized to endotoxin lethality by C3H/HeN (responsive) spleen cells.*

TABLE 1. *Influence of adoptively transferred spleen cells on the lethal effect of endotoxin in vivo*

Endotoxin dose (mg)	Endotoxin-induced lethality (no. killed/no. injected)							
	Expt 2[a]				Expt 3[b]			
	N_x + N[c]	N_x + J	J_x + J	J_x + N	N_x + N	N_x + J	J_x + J	J_x + N
6							1/4	4/4
4			2/4	4/4				
3							2/4	5/5
2			0/4	4/4				
1.5		4/4	0/4	1/4		3/3	2/4	3/5
1.0	4/4	4/4	0/4	0/3				
0.75	4/4	3/4				3/3		1/4
0.5	4/4	4/4						
0.38					3/3	1/4		1/4
0.25	2/4	0/3						
0.19					3/3	0/3		
0.12	0/3							
0.09					1/3			

[a] *E. coli* K-235 endotoxin; 5-week chimeras.

[b] *S. typhimurium* endotoxin; 6-month chimeras.

[c] First symbol indicates recipient mouse strain; second symbol indicates reconstituting cells. Key: N_x, C3H/HeN mouse irradiated with 450 R before reconstitution; N, 3×10^7 C3H/HeN spleen cells; J, 3×10^7 C3H/HeJ spleen cells; J_x, C3H/HeJ mouse irradiated with 450 R before reconstitution.

The composite LD_{50} values obtained from the three experiments are shown in Table 2. The mean LD_{50} for animals from either strain reconstituted with endotoxin-resistant C3H/HeJ spleen cells was significantly ($P < 0.01$) higher than that for animals reconstituted with endotoxin-sensitive C3H/HeN spleen cells. The ratios of the LD_{50} values (i.e., 0.46/0.18 = 2.6 for $C3H/HeN_x$ recipients and 4.0/1.6 = 2.5 for $C3H/HeJ_x$ recipients) were nearly identical, suggesting a proportional response to the spleen cell manipulations in the recipient strains.

To document the chimeric state of the mice used in the above experiments 5 weeks to 6 months after irradiation and reconstitution with spleen cells, animals of each type were sacrificed and their in vitro response to endotoxin was examined. The maximum lymphocyte proliferation as measured by the incorporation of tritiated thymidine in response to dilutions of 1.0 to 100 μg of endotoxin per ml in culture with 8×10^5 spleen cells for 48 h is shown in Table 3. These results show that B cells in the spleens of recipients retained the characteristic mitogenic reactivity of the donors for at least 6 months. They further confirm the previously demonstrated histocompatibility of the two mouse strains (17) and make it unlikely that graft-versus-host or rejection reactions played any role in the modification of the lethality curves.

DISCUSSION

The data reported in this paper strongly suggest that spleen cells play a major role in mediating the lethality of endotoxin in vivo. The cellular mechanism by which spleen cells exert their harmful effect on the host, however, remains unclear. Since a large proportion of labeled endotoxin is rapidly removed from the circulation by the liver, spleen, and lymph nodes, these lymphoid organs are preferred sites for endotoxin interaction with cells (3, 5). Previous studies have shown that both liver and spleen are also capable of metabolically

TABLE 2. *Influence of spleen cells on LD_{50} values for endotoxin in mice*[a]

Recipient strain	Reconstituting cells	LD_{50} (mg)				
		Expt			Mean ± SE[b]	P[c]
		1	2	3		
$C3H/HeN_x$	C3H/HeJ	0.50	0.40	0.47	0.46 ± 0.03	<0.01
	C3H/HeN	0.18[d]	0.25	0.11	0.18 ± 0.04	
$C3H/HeJ_x$	C3H/HeJ	4.2[e]	4.0	3.8	4.0 ± 0.12	<0.01
	C3H/HeN	2.0	1.6	1.1	1.6 ± 0.26	

[a] Recipient animals$_x$ received 450 R of X irradiation before reconstitution with 3×10^7 spleen cells.
[b] SE, Standard error.
[c] Probability value calculated by Student's *t* test.
[d] LD_{50} in milligrams per mouse determined by the Reed-Muench method (16).
[e] Minimum estimate of LD_{50} assuming that all animals would have died at the next highest dose of endotoxin (6 mg).

TABLE 3. *Assay of chimeric state by in vitro lymphocyte response to endotoxin*

Recipient strain	Reconstituting cells	Maximum lymphocyte proliferative response[a]			
		5 Weeks[b]		6 Months[b]	
		Control	+LPS[c]	Control	+LPS[c]
C3H/HeN[d]	None	257	10,120	3,178	28,474
$C3H/HeN_x$[e]	C3H/HeN	237	14,010	2,543	26,077
	C3H/HeJ	176	2,721	1,056	2,958
C3H/HeJ[d]	None	119	521	826	1,034
$C3H/HeJ_x$[e]	C3H/HeN	230	20,0073	1,823	23,148
	C3H/HeJ	100	201	722	851

[a] Expressed as counts per minute of [^{3}H]thymidine incorporated per 8×10^5 cells.
[b] Time after irradiation and reconstitution.
[c] Maximum response to *E. coli* K-235 endotoxin (lipopolysaccharide [LPS]) tested in a dose range from 1.0 to 100 μg/ml.
[d] Normal unirradiated mouse.
[e] Animals received 450 R of X irradiation before reconstitution with 3×10^7 spleen cells.

degrading endotoxin, and thus it has been felt that the reticuloendothelial system, by trapping and degrading endotoxin, acts primarily to defend the animal against the toxic and lethal sequelae of endotoxicosis (4, 6, 9, 18).

The first evidence that cells from the spleen might also contribute to lethality was provided by Agarwal et al. (1). In attempting to show the relative unimportance of the spleen as compared with the liver in protecting the host, they performed lethality experiments in splenectomized mice. Splenectomy had no effect on the clearance rate of endotoxin, nor did it prevent the development of tolerance induced by endotoxin pretreatment, indicating that the spleen was not crucial to survival in this species. An unexpected and unexplained finding in that study, however, was that splenectomized animals were actually more resistant to a lethal dose of endotoxin. It is now evident that one reason for the change in sensitivity of the splenectomized animal may have been a decrease in the number of endotoxin-sensitive lymphoid cells.

Recent studies in several laboratories provide insight into possible ways in which endotoxin-sensitive lymphoid cells may contribute to lethality. Prostaglandins are now known to be one of the mediators that are synthesized by lymphocytes treated in vitro with mitogens (7). Furthermore, prostaglandin synthesis by the intact isolated spleen has also been documented (8), and elevated prostaglandin-like material has been found in the plasma of dogs with endotoxin shock (13). The importance of such vasoactive agents in endotoxicosis has been evident for some time (17), but the possibility that prostaglandins per se play a central role is suggested by more recent experiments showing protection of animals pretreated with salicylates or indomethacin, potent blockers of prostaglandin synthesis (12, 15). Thus, an animal with endotoxin-sensitive lymphoid cells may have a large in vivo source for at least one of the known mediators that produce adverse cardiovascular effects. Our data support this hypothesis. C3H/HeJ B cells have been shown to be unresponsive to the mitogenic effects of endotoxin, whereas C3H/HeN B cells are normally responsive (10). Therefore, animals reconstituted with C3H/HeN spleen cells contained an endotoxin-responsive population of B cells that were a potential source of harmful vasoactive agents. On the other hand, when the C3H/HeJ strain was used as a source of reconstituting spleen cells, there were many fewer endotoxin-responsive cells as evidenced by the absence of a mitogen effect, and this was associated with a significantly higher LD_{50}.

Although this model provides one explanation for the lethal effects of endotoxin, it clearly does not explain the entire picture of endotoxin-induced lethality. We were, for example, unable to make chimeric mice that were either fully resistant or fully sensitive to the lethal effects of endotoxin by the mere transfer of spleen cells. One explanation for this may be that a sublethal dose of irradiation was used to prepare recipient animals and there were thus stem cells that survived and contributed normal recipient cells to the lymphoid organs. This phenomenon is evident in Table 3, where it can be seen that C3H/HeN mice reconstituted with C3H/HeJ spleen cells had higher mitogenic responses than did normal C3H/HeJ mice or C3H/HeJ mice reconstituted with their own cells. It is possible that much more pronounced effects on lethality would have been observed if larger numbers of spleen cells or precursor bone marrow cells had been used in reconstitution since there would presumably have been less expansion of the surviving recipient stem cells. It should also be pointed out that radiation at this dose has little effect on the recipient macrophages, so that these cells remain viable in the recipient animal and may also exert an effect. Finally, endotoxin produces diverse effects on a large number of sensitive or resistant nonlymphoid cells in the recipient animals, and these effects may also contribute to the gross end point of death.

The experiments reported here provide an important model for the study of endotoxin effects in vivo at the cellular level. It is now evident that there may be a relationship between lymphoid cell activation (for example, B-cell mitogenesis) and endotoxin lethality. This relationship may be further studied by searching for circulating lymphocyte mediators in the serum of chimeric animals as well as in vitro cultures of spleen cells. If mediators such as prostaglandins are found, the potential for specific therapeutic intervention, as with indomethacin or salicylates, may likewise be studied at a cellular level in vivo. Finally, the availability of a fully histocompatible mouse strain pair differing only in endotoxin sensitivity should allow definition of other cellular contributions to the diverse biological and toxic effects of endotoxin.

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