

Induction of Early-Phase Endotoxin Tolerance in Athymic (Nude) Mice, B-Cell-Deficient (*xid*) Mice, and Splenectomized Mice

GARY S. MADONNA AND STEFANIE N. VOGEL*

Department of Microbiology, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814

Received 5 February 1986/Accepted 19 May 1986

Early-phase endotoxin tolerance was inducible in mice which were T cell deficient (nude), B cell deficient (*xid*), or asplenic, which suggests that these lymphoid cell subsets and the spleen do not contribute significantly to the induction of acquired lipopolysaccharide hyporesponsiveness. C3H/HeJ mice did not exhibit the hematopoietic changes observed in mice made endotoxin tolerant, which suggests that multiple mechanisms may underlie lipopolysaccharide hyporesponsiveness.

Much of our understanding of the cellular mechanisms which contribute to lipopolysaccharide (LPS) sensitivity has been derived from studies which involved the use of C3H/HeJ (*Lps^d*) mice which, because of a single gene defect, are highly refractory to the effects of LPS both in vivo and in vitro (for a review, see reference 19). In conjunction with syngeneic, fully LPS-responsive (*Lpsⁿ*) strains, strong evidence has been put forth that many of the physiologic changes which result from LPS administration are mediated indirectly by a number of macrophage-derived immunoregulatory factors (2-4, 12, 14, 18, 22, 27).

A second model of murine endotoxin hyporesponsiveness is a state of tolerance toward endotoxin which is acquired after exposure of a normally sensitive animal to a sublethal injection of endotoxin. An early phase of endotoxin tolerance occurs within the first few days after exposure to LPS, is transient, and is not O-antigen specific (8). Studies of early-phase endotoxin tolerance have also indicated a central role for macrophages. Several groups have demonstrated that macrophages from animals made endotoxin tolerant are refractory to LPS in vitro (5, 23, 28, 30). In a recent study (11), we used the early-phase endotoxin tolerance system established by Williams et al. (30), in which serum colony-stimulating factor (CSF) was measured as an indicator of LPS responsiveness. The induction and maintenance of early-phase endotoxin tolerance in this system (as measured by suppression of CSF activity) were optimal 3 to 4 days after initial exposure to LPS and correlated temporally with a marked increase in bone marrow-derived macrophage progenitor cells (macrophage colony-forming units [M-CFU]). Cell-sizing profiles of the bone marrow cells from mice made endotoxin tolerant indicated an enrichment for a denser population of cells which contained the increased numbers of macrophage progenitors. This suggested that early-phase endotoxin tolerance might be related to an overabundance of immature monocytic precursors in the bone marrow which could, in turn, result in reduced availability of mature (and more LPS-responsive) monocytic cells in the periphery. This study extends these findings to an analysis of early-phase endotoxin tolerance induction in mice with defects in specific lymphoid compartments.

(This research was conducted by G. S. Madonna in partial fulfillment of the requirements for the Ph.D. degree from the Uniformed Services University of the Health Sciences, Bethesda, Md., 1986.)

Homozygous expression of the nude allele (*nu/nu*) results in congenitally athymic mice that possess extremely low levels of functional T cells (6, 21). Since there have been several recent reports which suggest the existence of LPS-responsive T cells (9, 13, 15, 25, 29), nude mice were used to determine whether T cells were involved in the induction of early-phase endotoxin tolerance. BALB/c athymic nude (*nu/nu*) mice and their euthymic (*nu/+*) littermates (Harlan Sprague-Dawley, Indianapolis, Ind.) were injected intraperitoneally with either saline or phenol-water-extracted *Escherichia coli* K235 LPS (25 µg per mouse) on day 0 and were challenged 3 days later with *E. coli* LPS (25 µg per mouse). Mice were bled 6 h after challenge, and sera were tested for CSF activity as described elsewhere (11). BALB/c control and athymic mice injected with saline on day 0 responded comparably to an LPS challenge on day 3 with the production of serum CSF (Table 1). Exposure to LPS on day 0 induced tolerance in both athymic and euthymic mice comparably, as evidenced by a greatly reduced production of serum CSF after challenge with LPS on day 3. These findings suggest that T cells do not contribute significantly to either the induction of CSF by LPS or the induction of early-phase endotoxin tolerance.

The *xid* mutation, originally described in CBA/N mice, is an X-linked defect which interferes with the development of functional B cells that bear the Lyb 5 surface antigen (1). Mice which express this defect are unable to respond to a number of B cell mitogens, including LPS (26). Although spleen cells from CBA/N mice are deficient with respect to LPS-induced mitogenesis (7), these mice are fully responsive to the toxic effects of LPS and bear macrophages which respond normally to LPS in vitro to produce interleukin 1 (24). Recently, Mond et al. (17) showed that spleen cells from C3.CBA/N mice, which are an *xid* congenic strain, also failed to respond in vitro to B cell mitogens, including LPS. These mice were used to probe whether the population of LPS-responsive B cells which are deficient in these mice were involved in the production of CSF or the induction of early-phase endotoxin tolerance. Female C3.CBA/N mice, homozygous for the *xid* defect (*xid/xid*), were the kind gift of J. J. Kenny, Uniformed Services University of the Health Sciences. These mice were age matched with congenic female C3H/HeN mice (Harlan Sprague-Dawley) and were subjected to the standard endotoxin tolerance regimen. The results (Table 1) indicate that the *xid* defect of C3.CBA/N mice had no effect on the induction of serum CSF after LPS challenge of mice injected with saline on day 0. In addition,

* Corresponding author.

TABLE 1. Effect of an early-phase endotoxin tolerance induction protocol on induction of CSF activity and bone marrow precursor numbers in control and defective mice

Mouse strain or treatment	CSF (CFU/ml) response to the following sequence of treatments ^a :		Day 3 M-CFU/10 ⁵ bone marrow cells from mice injected on day 0 with ^b :	
	Saline on day 0, LPS on day 3	LPS on day 0, LPS on day 3	Saline	LPS
BALB/c (nu/+) ^c	9,530 ± 588	1,330 ± 136	NT ^d	NT
BALB/c (nu/nu) ^c	9,970 ± 884	1,260 ± 220	NT	NT
C3H/HeN (+/+)	2,100 ± 140	250 ± 155	78 ± 9	240 ± 2
C3.CBA/N (xid/xid)	2,080 ± 185	220 ± 56	85 ± 7	255 ± 4
Untreated ^e	4,048 ± 555	856 ± 254	NT	NT
Sham splenectomized	4,336 ± 491	816 ± 213	98 ± 4	234 ± 21
Splenectomized	3,800 ± 317	608 ± 118	110 ± 3	192 ± 9
C3H/OuJ (Lps ^o)	7,000 ± 250	1,300 ± 110	107 ± 1	233 ± 10
C3H/HeJ (Lps ^d)	0	0	82 ± 6	102 ± 3

^a CSF levels were determined with pooled serum samples collected 6 h after the second injection (five mice per treatment group). Results represent the arithmetic mean ± standard deviation of duplicate determinations. No CSF was detectable in serum samples collected from mice injected with saline on both days.

^b Five mice per treatment group. The number of bone marrow macrophage progenitor cells was determined by plating bone marrow cells in an excess of CSF as described elsewhere (11). Results represent the arithmetic mean ± standard deviation of duplicate determinations.

^c By FACS analysis of individual spleen suspensions (16), the BALB/c (nu/+) and BALB/c (nu/nu) spleens were found to contain 24.4 ± 1.5 and 3.0 ± 0.7 Thy 1.2-positive cells, respectively.

^d NT, Not tested.

^e Outbred HSD(ICR)BR mice were used in this series of experiments.

preexposure to LPS on day 0 resulted in early-phase endotoxin tolerance, as assessed by a reduction in serum CSF after LPS challenge on day 3.

Bone marrow cell-sizing profiles were carried out 3 days after injection of saline or LPS. Control (saline-injected) C3.CBA/N and C3H/HeN mice showed a bimodal distribution of cells by size (Fig. 1) which was similar to that reported for outbred mice (11). This picture was altered in both the normal and defective (*xid*) mice by prior exposure to LPS, as evidenced by a decrease in the number of cells in the peak of smaller cells (peak 1) with an increase in both cell number (C3H/HeN, 18% increase; C3.CBA/N, 19% increase) and average cell size in peak 2. Both strains also responded to prior LPS exposure with an increase (approximately threefold) in the number of bone marrow-derived macrophage progenitor cells (M-CFU; Table 1). These results support the notion that the population of LPS-responsive B cells deficient in *xid* mice does not play an essential role in either LPS sensitivity or the induction of early-phase endotoxin tolerance.

In the study of Williams et al. (30), it was demonstrated that early-phase endotoxin tolerance induced in outbred mice could be reversed by injecting endotoxin-tolerant mice with spleen cells from control mice 24 h before LPS challenge. These investigators, therefore, suggested that spleen cells contribute significantly to the production of circulating CSF in response to LPS. To assess the contribution of the

spleen in the production of CSF and the induction of early-phase endotoxin tolerance, outbred HSD(ICR)BR mice (Harlan Sprague-Dawley) were divided into three experimental groups: untreated (no surgery), sham splenectomized, and splenectomized. Mice were allowed to recover from surgery and then were subjected to the tolerance induction protocol. The results (Table 1) illustrate the following. (i) High levels of LPS-induced serum CSF were found in all three saline-pretreated groups, regardless of the presence or absence of the spleen. (ii) All three groups (i.e., untreated, sham splenectomized, and splenectomized) were made tolerant comparably by preexposure to LPS, as evidenced by their reduced capacity to produce CSF in response to a second injection of LPS.

The effect of the tolerance induction regimen on bone marrow cell-sizing profiles and M-CFU numbers was also assessed. Bone marrow cell-sizing profiles from splenectomized and sham-splenectomized mice were obtained 3 days after injection of saline or LPS. Splenectomy had little effect on the LPS-induced alterations in bone marrow cell-sizing profile; i.e., both splenectomized and sham-splenectomized bone marrow cells exhibited a reduction in cell numbers in peak 1 and an increase in median cell size and numbers in

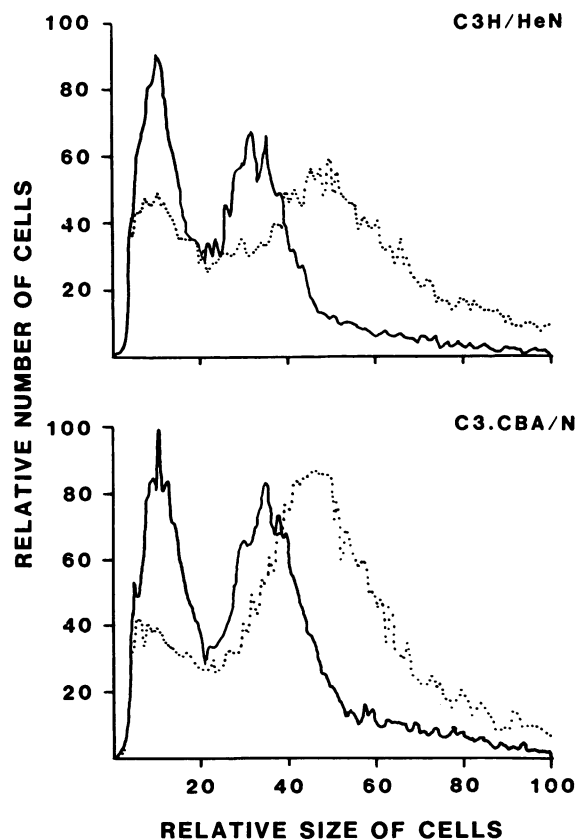


FIG. 1. Cell-sizing profiles from C3.CBA/N and C3H/HeN mice. On day 0, C3.CBA/N or C3H/HeN mice (five mice per group) were injected with saline or *E. coli* LPS (25 µg per mouse). Three days later, the mice were sacrificed, the bone marrow cells were obtained, and the cell-sizing profiles were determined with a Coulter Channelyzer. —, Saline treated; . . . , LPS treated. In C3H/HeN mice, pretreatment with LPS resulted in an increase in the numbers of cells in peak 2 from 59 to 77% of the cells analyzed. In C3.CBA/N mice, pretreatment with LPS increased the numbers of cells in peak 2 from 65 to 84% of the cells analyzed.

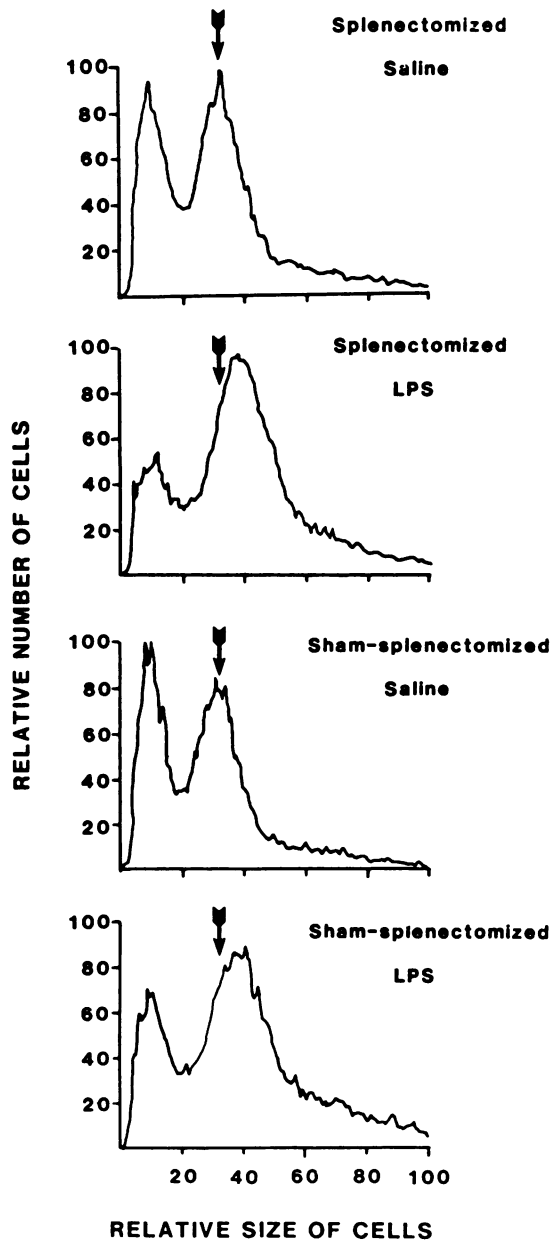


FIG. 2. Bone marrow cell-sizing profiles from splenectomized and sham-splenectomized HSD(ICR)BR mice after saline or LPS administration. On day 0, splenectomized or sham-splenectomized HSD(ICR)BR mice (five mice per group) were injected with saline or *E. coli* LPS (25 μ g per mouse). Three days later, the mice were sacrificed, the bone marrow cells were obtained, and the cell-sizing profiles were determined with a Coulter Channelyzer. The arrows indicate the relative positions of peak 2 bone marrow cells from control (saline-injected) mice. In splenectomized mice, pretreatment with LPS resulted in an increase in the numbers of cells in peak 2 from 65 to 82% of the cells analyzed. In sham-splenectomized mice, pretreatment with LPS increased the numbers of cells in peak 2 from 64 to 80% of the cells analyzed.

peak 2 after injection with LPS, as compared with the profiles obtained from saline-treated mice (splenectomized, 17% increase; sham splenectomized, 16% increase) (Fig. 2). The number of bone marrow M-CFU was also found to be approximately doubled in both groups 3 days after adminis-

tration of LPS (Table 1). Thus, spleen cells cannot be considered to be a major source of LPS-induced CSF, as suggested by Williams et al. (30); this supports the alternative suggestion by these investigators that other organs, such as lungs, or resident peritoneal macrophages, may contribute significantly to the production of LPS-induced CSF. Changes in M-CFU numbers and alterations in bone marrow cell-sizing profiles in endotoxin-tolerant, splenectomized mice were also consistent with the notion that the spleen plays little role, if any, in these manifestations of LPS-induced tolerance.

We next sought to determine whether any of the hematopoietic changes observed in endotoxin-tolerant mice were present in the genetically LPS-hyporesponsive, C3H/HeJ strain. To do this, C3H/HeJ (*Lps^d*) mice and syngeneic, fully LPS-responsive C3H/OuJ (*Lpsⁿ*) mice (Jackson Laboratory, Bar Harbor, Maine) were subjected to the same endotoxin-tolerance regimen which was established for outbred mice. C3H/OuJ mice responded to LPS challenge in a manner similar to that of outbred mice, i.e., a dramatic increase in serum CSF from control (saline-pretreated) mice and a mitigated CSF response in endotoxin-tolerant mice (i.e., those pretreated with LPS) (Table 1). In contrast, C3H/HeJ mice did not respond with detectable levels of serum CSF after either treatment. Bone marrow cells from LPS-responsive (C3H/OuJ) and LPS-hyporesponsive mice injected on day 0 with either saline or *E. coli* LPS were cultured 3 days postinjection for determination of M-CFU numbers. As had been observed in LPS-responsive, outbred mice previously (11) (Table 1), the M-CFU numbers were also found to be greatly increased in bone marrow cells derived from C3H/OuJ mice exposed on day 0 to LPS, whereas the increase observed in M-CFU numbers from LPS-treated C3H/HeJ mice was minimal (Table 1). These results indicate that expression of the *Lps^d* gene defect results in: (i) an inability to respond to LPS by producing serum CSF (as had been reported previously [2]) and (ii) no alteration in either bone marrow M-CFU numbers (Table 1) or bone marrow cell-sizing profiles (11). If the mechanisms by which C3H/HeJ and endotoxin-tolerant mice failed to respond to LPS were the same, one might expect to have observed both a shifted bone marrow cell-sizing profile and increased M-CFU numbers in untreated C3H/HeJ mice. Neither was observed in this study or in our previous study (11). However, one report has suggested that M-CFU numbers are increased in C3H/HeJ mice when compared with C3HeB/FeJ (*Lpsⁿ*) mice (10). Further studies will be required to confirm these observations in light of recent findings that C3HeB/FeJ mice express other gene defects (distinct from *Lps^d*) which may compromise the functioning of their macrophages (20).

In summary, the cellular mechanisms which underlie an early state of induced LPS hyporesponsiveness were investigated by using various murine models to assess the relative contribution of certain lymphoid cell subsets. Mice that were T cell deficient, deficient in a population of LPS-responsive B cells, or splenectomized were all capable of being rendered fully tolerant toward endotoxin. These findings suggest that these lymphoid cell subsets and the spleen do not contribute significantly to the induced state of early-phase endotoxin tolerance.

We thank Marion Fultz for her critical review of this manuscript and assistance in fluorescence-activated cell sorter (FACS) analysis of spleen cells, Mary Mills for excellent secretarial assistance, and Eric Kaufman for helpful technical assistance.

This work was supported by Uniformed Services University of the Health Sciences Protocol no. RO7338 and GO7364.

LITERATURE CITED

- Ahmed, A., I. Scher, S. O. Sharrow, A. H. Smith, W. E. Paul, D. H. Sachs, and K. W. Sell. 1977. B lymphocyte heterogeneity: development and characterization of an alloantiserum which distinguishes B-lymphocyte differentiation alloantigens. *J. Exp. Med.* **145**:101-110.
- Apte, R. N., C. Galanos, and D. H. Pluznik. 1976. Lipid A, the active part of bacterial endotoxin in inducing serum colony stimulating activity and proliferation of splenic granulocyte-macrophage progenitor cells. *J. Cell. Physiol.* **87**:71-77.
- Apte, R. N., and D. H. Pluznik. 1976. Control mechanisms of endotoxin and particulate material stimulation of hemopoietic colony forming cell differentiation. *Exp. Hematol.* **4**:10-18.
- Atkins, E., P. Bodel, and L. Francis. 1967. Release of an endogenous pyrogen *in vitro* from rabbit mononuclear cells. *J. Exp. Med.* **126**:357-383.
- Dinareello, C. A., P. T. Bodel, and E. Atkins. 1968. The role of the liver in the production of fever and in pyrogenic tolerance. *Trans. Assoc. Am. Physicians* **81**:334-344.
- Flanagan, S. P. 1966. "Nude"—a new hairless gene with pleiotropic effects in the mouse. *Genet. Res.* **8**:295-309.
- Glode, L. M., and D. L. Rosenstreich. 1976. Genetic control of B cell activation by bacterial lipopolysaccharide is mediated by multiple distinct genes or alleles. *J. Immunol.* **117**:2061-2066.
- Greisman, S. E., and R. B. Hornick. 1976. Endotoxin tolerance, p. 43-50. *In* R. F. Beers and E. G. Basset (ed.), *The role of immunologic factors in infectious, allergic, and autoimmune processes*. Raven Press, New York.
- Koenig, S., M. K. Hoffmann, and L. Thomas. 1977. Induction of phenotypic lymphocyte differentiation in LPS unresponsive mice by an LPS-induced serum factor and by lipid-A-associated protein. *J. Immunol.* **118**:1910-1911.
- MacVittie, T. J., and S. R. Weinberg. 1980. An LPS responsive cell in C3H/HeJ mice: the peritoneal exudate-derived macrophage colony-forming cell (M-CFC), p. 511-518. *In* E. E. Skamene, P. Kongshavn, and M. Landy (ed.), *Genetic control of natural resistance to infection and malignancy*. Academic Press, Inc., New York.
- Madonna, G. S., and S. N. Vogel. 1985. Early endotoxin tolerance is associated with alterations in bone marrow-derived macrophage precursor pools. *J. Immunol.* **135**:3763-3771.
- Mannel, D. N., R. N. Moore, and S. E. Mergenhagen. 1980. Endotoxin-induced tumor cytotoxic factor, p. 141-143. *In* D. Schlessinger (ed.), *Microbiology—1980*. American Society for Microbiology, Washington, D.C.
- McGhee, J. R., J. J. Farrar, S. M. Michalek, S. E. Mergenhagen, and D. L. Rosenstreich. 1979. Cellular requirements for lipopolysaccharide adjuvanticity. *J. Exp. Med.* **149**:793-807.
- Metcalf, D. 1971. Acute antigen-induced elevation of serum colony stimulating factor (CSF) levels. *Immunology* **21**:427-436.
- Mita, A., O. Hiedazu, and T. Mita. 1982. Induction of splenic T cell proliferation by lipid A in mice immunized with sheep red blood cells. *J. Immunol.* **128**:1709-1711.
- Mond, J. J., S. Kessler, F. D. Finkleman, W. E. Paul, and I. Scher. 1980. Heterogeneity of Ia expression on normal B cells, neonatal B cells, and on cells from B cell-defective CBA/N mice. *J. Immunol.* **124**:1675-1682.
- Mond, J. J., G. Norton, W. E. Paul, I. Scher, F. D. Finkleman, S. House, M. Schaefer, P. K. A. Mongini, C. Hansen, and C. Bona. 1983. Establishment of an inbred line of mice which express a synergistic immune defect precluding *in vitro* responses to type 1 and type 2 antigen, B mitogens and a number of T cell-derived helper factors. *J. Exp. Med.* **158**:1401-1414.
- Moore, R. N., K. J. Goodrum, and L. J. Berry. 1976. Mediation of an endotoxin effect by macrophages. *RES J. Reticuloendothel. Soc.* **19**:187-197.
- Morrison, D. C., and J. L. Ryan. 1979. Bacterial endotoxins and host immune responses. *Adv. Immunol.* **28**:293-450.
- O'Brien, A. D., and D. L. Rosenstreich. 1983. Genetic control of the susceptibility of C3HeB/FeJ mice to *Salmonella typhimurium* is regulated by a locus distinct from known *Salmonella* response genes. *Immunology* **131**:2613-2615.
- Pantelouris, E. M. 1968. Absence of thymus in a mouse mutant. *Nature (London)* **217**:370-371.
- Quesenberry, P. J., A. Morley, F. Stohlman, Jr., K. Richard, D. Howard, and M. Smith. 1972. Effect of endotoxin on granulopoiesis and colony stimulating factor. *N. Engl. J. Med.* **286**:227-232.
- Rietschel, E. T., U. Schade, M. Jensen, H. W. Wollenweber, O. Luderitz, and S. E. Greisman. 1982. Bacterial endotoxins: chemical structure, biological activity and role in septicemia. *Scand. J. Infect. Dis.* **31**(Suppl.):8-21.
- Rosenstreich, D. L., S. N. Vogel, A. Jacques, L. M. Wahl, I. Scher, and S. E. Mergenhagen. 1978. Differential endotoxin sensitivity of lymphocytes and macrophages from mice with an X-linked defect in B cell maturation. *J. Immunol.* **121**:685-690.
- Scheid, M. P., M. K. Hoffmann, K. Komuro, U. Hammerling, J. Abbott, E. A. Boyse, G. H. Cohen, J. A. Hooper, R. S. Schulof, and A. L. Goldstein. 1973. Differentiation of T cells induced by preparations from thymus and by nonthymic agents. *J. Exp. Med.* **138**:1027-1032.
- Scher, I. 1982. The CBA/N mouse strain: an experimental model illustrating the influence of the X-chromosome on immunity. *Adv. Immunol.* **33**:1-71.
- Skarnes, R. C., and M. J. K. Harper. 1972. Relationship between endotoxin-induced abortion and the synthesis of prostaglandin F. *Prostaglandins* **1**:191-203.
- Sullivan, R., P. J. Gans, and L. A. McCarroll. 1983. The synthesis and secretion of granulocyte-macrophage colony-stimulating activity (CSA) by isolated monocytes: kinetics of the response to bacterial endotoxin. *J. Immunol.* **130**:800-807.
- Vogel, S. N., M. L. Hilfiker, and M. J. Caulfield. 1983. Endotoxin-induced T lymphocyte proliferation. *J. Immunol.* **130**:1774-1779.
- Williams, Z., C. F. Hertogs, and D. H. Pluznik. 1983. Use of mice tolerant to lipopolysaccharide to demonstrate requirement of cooperation between macrophages and lymphocytes to generate lipopolysaccharide-induced colony-stimulating factor *in vivo*. *Infect. Immun.* **41**:1-5.