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LIPID A-ASSOCIATED PROTEINS PROVIDE AN ALTERNATE "SECOND SIGNAL" IN THE ACTIVATION OF RECOMBINANT INTERFERON-γ-PRIMED, C3H/HeJ MACROPHAGES TO A FULLY TUMORICIDAL STATE[1]

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Previous studies have shown that the activation of murine macrophages to a fully tumoricidal state requires that specific environmental signals be delivered to the macrophage in a stepwise manner: a "priming" signal first renders the macrophage responsive to a second or "trigger" signal. One potent "priming" signal has been identified as the T cell-derived lymphokine, interferon-γ (IFN-γ) and one often used "trigger" signal is lipopolysaccharide (LPS), the endotoxin derived from Gram-negative bacteria. In these studies, endotoxin-responsive C3H/OuJ (*Lps*^{n}) and endotoxin-hyporesponsive C3H/HeJ (*Lps*^{d}) macrophages were exposed in vitro to recombinant IFN-γ (rIFN-γ) and various preparations of endotoxin or purified lipid A-associated proteins (LAP). The resultant tumoricidal responses were evaluated to define the activation requirements of murine macrophages and to examine further the LPS defect exhibited by C3H/HeJ mice. The findings presented herein demonstrate that C3H/OuJ macrophages primed by rIFN-γ respond to protein-free LPS (phenol-water extracted LPS), protein-rich LPS (butanol-extracted LPS), or purified LAP. In contrast, rIFN-γ-primed C3H/HeJ macrophages failed to become cytolytic with phenol-water extracted LPS, but could be rendered fully tumoricidal if either butanol-extracted LPS or LAP were used as "second signals." These data indicate that C3H/HeJ macrophages are fully responsive to the priming effects of IFN-γ, but remain restricted in their capacity to recognize protein-free LPS as a second signal. Alternate second signals, such as LAP, may provide a compensatory pathway by which these macrophages are rendered fully tumoricidal.

Endotoxin, the ubiquitous lipopolysaccharide (LPS) component derived from Gram-negative bacterial membranes, has been shown to invoke a spectrum of physiologic responses in experimental animals (reviewed in Reference 1 and 2). These include toxic manifestations (e.g., diarrhea, ruffled fur, weight loss, hypoglycemia, hypothermia, fever, abortion, shock, or death), as well as responses which are beneficial to the host (e.g., increased resistance to infection and malignancy, radioprotection, adjuvanticity, and normal development of lymphoid organs). Some time between 1960 and 1965, a spontaneous mutation occurred within the C3H/HeJ mouse strain, which rendered it highly refractory to LPS, and specifically, to the lipid A moiety of this molecule (3, 4). The genetic locus associated with endotoxin hyporesponsiveness (*Lps*^{d}) has been mapped to the fourth chromosome (5) and is reflected in all cell types of the C3H/HeJ mouse strain in vitro (reviewed in Reference 6). In contrast to protein-free preparations of LPS prepared by phenol water-extraction procedures (PW-LPS),[3] butanol-extracted LPS (But-LPS) preparations retain significant levels of lipid A-associated proteins (LAP) and are mitogenic for C3H/HeJ splenocytes (7, 8). In addition, But-LPS has been shown to stimulate factor release by C3H/HeJ macrophages in vitro (9, 10). When LAP was purified from butanol-extracted LPS/LAP mixtures, it was shown to be the component responsible for stimulating proliferation of C3H/HeJ spleen cells, whereas the separated, protein-free LPS component of the mixture was nonmitogenic (8). At this time, the nature of the C3H/HeJ endotoxin hyporesponsiveness is not understood; however, it has been proposed that expression of the mutant allele results in altered expression of a putative "triggering" element within the "LPS receptor" (11) or that an inability to process the LPS molecule into a stimulatory form (12) underlies the failure of mice carrying this mutant allele to respond to LPS.

In 1977, Hibbs and his colleagues (13) demonstrated that specific environmental signals acted sequentially to activate macrophages to a fully tumoricidal state. They proposed that "macrophage-activating factor" provided a "priming" signal to the macrophage, which alone did not render the macrophage tumoricidal (14). Rather, the "primed" macrophage was rendered receptive to a "second signal" which "triggered" full tumoricidal capacity. One potent second signal was shown to be LPS. In an elegant series of studies, Pace and her co-workers (15) and Schreiber et al. (16) demonstrated that interferon-γ (IFN-γ) was a principal species of macrophage-activating factor in lymphokine supernatants. Moreover, Pace et al.

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[3] Abbreviations used in this paper: PW-LPS, phenol water-extracted lipopolysaccharide; IFN-γ, interferon-γ; rIFN-γ, recombinant interferon-γ; But-LPS, butanol-extracted lipopolysaccharide; LAP, lipid A-associated protein; TNF, tumor necrosis factor.

(17) showed that LPS-responsive (*Lps*^{n}), C3H/HeN macrophages, but not C3H/HeJ macrophages, could be rendered cytolytic by treatment with recombinant interferon-γ (rIFN-γ) (as a "priming" signal) and protein-free, PW-LPS (as a "second signal"). However, if an alternate (non-LPS), second signal were provided (e.g., heat-killed *Listeria monocytogenes*), rIFN-γ-primed C3H/HeJ macrophages could be rendered fully activated to kill tumor cells in vitro.

To identify structural components associated with LPS that could potentially be used as "second signals" to stimulate rIFN-γ-primed C3H/HeJ macrophages to a fully tumoricidal state, various preparations of LPS were compared in a modification of the two-signal tumoricidal system established by Pace et al. (17). Preparations shown previously to be mitogenic for C3H/HeJ splenocytes were tested for their efficacy as "second signals" for rIFN-γ-primed, LPS-responsive and LPS-hyporesponsive macrophages: protein-rich, But-LPS, which is a mixture of LPS and LAP, as well as purified LAP, were compared with PW-LPS. The findings presented in this report demonstrate that although PW-LPS failed to trigger tumoricidal activity in rIFN-γ-primed, C3H/HeJ macrophages, both But-LPS, and more specifically, LAP, could serve as compensatory, functional second signals.

MATERIALS AND METHODS

Mice. C3H/HeJ and C3H/OuJ mice (female, 5 to 6 wk old) were obtained from The Jackson Laboratory (Bar Harbor, ME) and were used within 1 wk of receipt. Mice were housed in a laminar flow hood in cages fitted with polyester filter hoods and fed standard lab chow and acid water ad libitum.

Reagents. Highly purified, murine rIFN-γ (1.3×10^7 U/mg), cloned in *Escherichia coli*, was kindly provided by Genentech, Inc. (South San Francisco, CA), and was used in all of the experiments described in this paper.

Protein-free (≤0.008% protein by Lowry), PW-LPS was prepared from *E. coli* K235 by the extraction procedure of McIntire et al. (18). Protein-rich (18% protein by Lowry), But-LPS was prepared from *E. coli* K235, according to the method of Morrison and Lieve (19). The LAP preparation was isolated from *Salmonella typhimurium* LT2 by butanol extraction followed by phenol-water extraction in which the LAP was isolated from the phenol phase (19) (J. Killion, personal communication). This preparation was kindly provided by Dr. David Morrison and Mr. J. Killion (University of Kansas Medical Center, Kansas City, KA) and at the highest concentration tested in this study (5 μg/ml) contained <0.1 ng/ml LPS, as assessed by Limulus amebocyte assay.

Macrophage induction and culture. To induce peritoneal exudates, mice were injected i.p. with 3 ml of 3% fluid thioglycollate (BBL, Cockeysville, MD), and 4 days later, macrophage-rich (>85%) exudates were collected by peritoneal lavage. The peritoneal exudates were washed and resuspended in RPMI 1640 (M.A. Bioproducts, Walkersville, MD) supplemented with 2 mM glutamine, 30 mM HEPES (pH 7.2), 3 mg/ml sodium bicarbonate, penicillin/streptomycin (100 IU/ml and 100 mg/ml, respectively), and 10% fetal calf serum (M. A. Bioproducts). All tissue culture reagents were purchased as "endotoxin-free" lots (≤0.01 ng/ml) and confirmed as such in independent Limulus amebocyte lysate assays. Macrophages were incubated in flat-bottom 96-well plates (Falcon Plastics, Oxnard, CA) at 2×10^5/well. After an adherence incubation of approximately 4 hr, nonadherent cell types were removed by washing with complete medium. Macrophages were treated with the indicated concentrations of rIFN-γ, PW-LPS, But-LPS, or LAP in a final volume of 200 μl.

Assay for macrophage-mediated cytolytic activity. Killing of ^{51}Cr-labeled P815 mastocytoma cells was measured by using a 16-hr ^{51}Cr release assay as described elsewhere (20). P815 target cells (kindly provided by Dr. J. Pace, University of Florida, Gainesville, FL) were labeled for 1 to 3 hr at 37° C with 500 μCi of ^{51}Cr/5×10^6 cells (sodium chromate; ICN Biomedicals, Inc., Irvine, CA; specific activity = 500 mCi/mg), washed once by centrifugation, and allowed to "leak" for 1 hr at 37°C in complete RPMI + 10% fetal calf serum and washed again just prior to addition to macrophage cultures. After the macrophages had been treated for 24 hr, ^{51}Cr-labeled tumor cells (1×10^4) were added to the contents of each well in a final volume of 25 μl. Each sample was assayed in triplicate. After 16 hr of incubation at 37°C, the uppermost 0.1 ml of supernatant was removed and assayed for radioactivity in an automatic gamma spectrometer. Results are expressed as percent specific ^{51}Cr release (percent cytotoxicity) as calculated by the following formula:

$$\text{Percent specific cytotoxicity} = \frac{\text{experimental cpm} - \text{spontaneous cpm}}{\text{total cpm} - \text{spontaneous cpm}} \times 100$$

Spontaneous release was determined from untreated macrophage monolayers incubated with ^{51}Cr-labeled P815 cells. Total cpm were obtained from incubation of ^{51}Cr-labeled P815 with 200 μl of 0.5% sodium dodecyl sulfate. Spontaneous release from unstimulated monolayers was approximately 30% and always less than or equal to the release from targets incubated in medium alone.

RESULTS

Comparison of the effects of rIFN-γ and PW-LPS treatment on C3H/OuJ and C3H/HeJ macrophage tumoricidal capacity. C3H/OuJ and C3H/HeJ macrophage cultures were treated with varying concentrations of rIFN-γ and/or PW-LPS. After a 24-hr incubation period, ^{51}Cr-labeled, P815 mastocytoma cells were added to the assay for an additional 16 hr. At that time, supernatants were removed and macrophage-mediated cytotoxicity was measured by the release of ^{51}Cr into the supernatants. Figure 1*A* illustrates the cytotoxic levels achieved by C3H/OuJ macrophages at each concentration of stimuli. Treatment of C3H/OuJ macrophages with either rIFN-γ alone (indicated on the graph as "medium") or PW-LPS (up to 5 μg/ml), resulted in very low levels of cytotoxicity (<20%). By contrast, the combination of rIFN-γ and PW-LPS induced high levels of tumor cell killing. C3H/OuJ macrophages were efficiently triggered with as little as 0.5 ng/ml PW-LPS to significant but suboptimal levels of target lysis in the presence of 5 to 10 U/ml rIFN-γ (data not shown). Figure 1*B* depicts the effects of rIFN-γ and PW-LPS on the ability of C3H/HeJ macrophages to lyse tumor targets. In contrast to the synergistic effect of rIFN-γ and PW-LPS observed in C3H/OuJ macrophages, C3H/HeJ macrophages failed to be rendered tumoricidal by any combination of experimental treatments. These studies confirm and extend the results reported originally by Pace et al. (17) who showed that proteose peptone-elicited, C3H/HeN macrophages, but not C3H/HeJ macrophages, could be induced to an efficient tumoricidal state by PW-LPS in the presence of varying concentrations of rIFN-γ. In addition, these studies show that C3H/HeJ macrophages remain refractory to PW-LPS at concentrations up to 1700-times greater than those used by Pace et al. (17).

Comparison of the effects of rIFN-γ and But-LPS on C3H/OuJ and C3H/HeJ macrophage tumoricidal capacity. Rigorous phenol water extraction of LPS (19) results in preparations which are free of contaminating proteins. In contrast, butanol extraction of LPS (8) results in preparations (But-LPS) which are a mixture of LPS and LAP. Previous studies have shown that But-LPS, but not PW-LPS, is capable of stimulating C3H/HeJ B cells to proliferate. But-LPS, but not PW-LPS, will also induce production of interleukin 1 and prostaglandin in C3H/HeJ macrophages (9, 10). When But-LPS was used as a "second signal" in combination with priming concentrations of rIFN-γ, significant levels of tumoricidal activity were observed in both C3H/OuJ and C3H/HeJ macro-

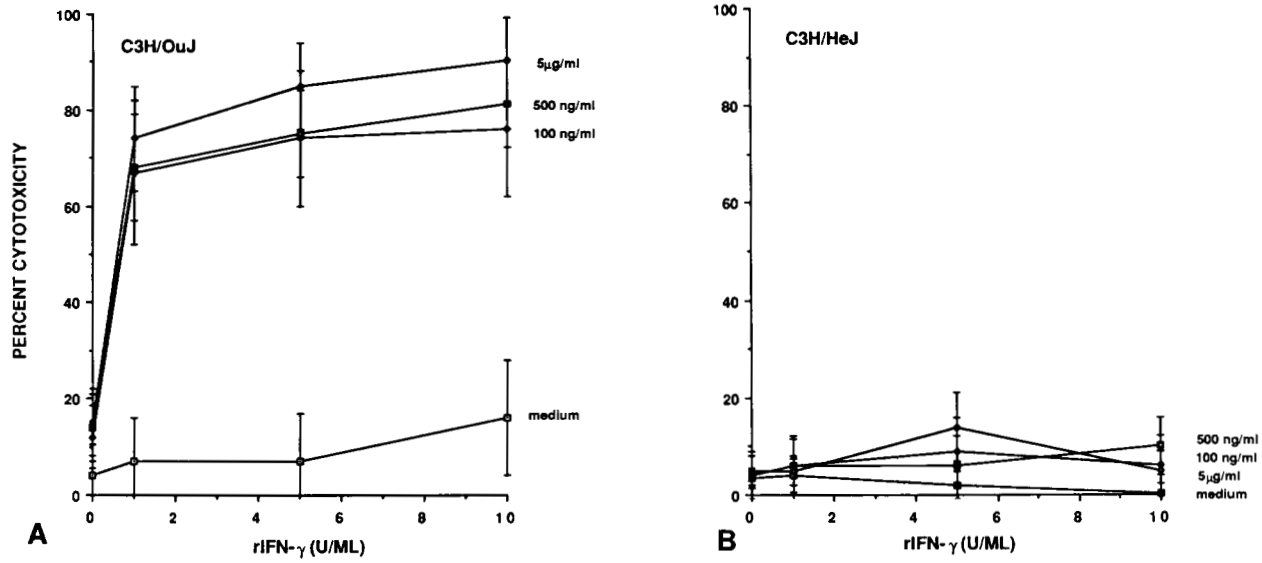

Figure 1. Comparison of the effects of rIFN-γ and PW-LPS treatment of C3H/OuJ and C3H/HeJ macrophage tumoricidal capacity. Macrophage cultures (2×10^5 cells/well) were treated with the indicated concentrations of rIFN-γ and PW-LPS for 24 hr. ^{51}Cr-Labeled P815 tumor cells (1×10^4) were then added to the cultures for 16 hr and the percent cytotoxicity was assessed. Macrophages incubated with dilutions of rIFN-γ alone are indicated on the graph as "medium." Macrophages incubated without PW-LPS or rIFN-γ are plotted at the origin. *A*, C3H/OuJ (*Lps*^{n}) cultures; *B*, C3H/HeJ (*Lps*^{d}) cultures.

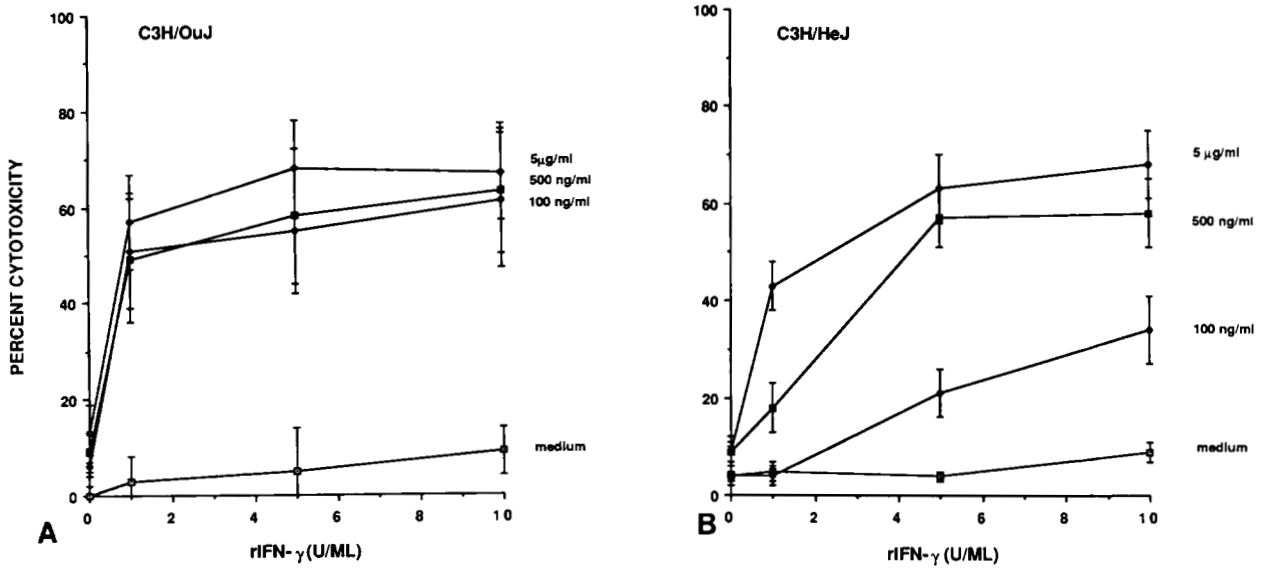

Figure 2. Comparison of the effects of rIFN-γ and But-LPS on C3H/OuJ and C3H/HeJ macrophage tumoricidal capacity. Macrophage cultures (2×10^5 cells/well) were treated with the indicated concentrations of rIFN-γ and But-LPS for 24 hr. ^{51}Cr-Labeled P815 tumor cells (1×10^4) were then added to the cultures for 16 hr and the percent cytotoxicity was assessed. Macrophages incubated with dilutions of rIFN-γ alone are indicated on the graph as "medium." Macrophages incubated without But-LPS or rIFN-γ are plotted at the origin. *A*, C3H/OuJ (*Lps*^{n}) cultures; *B*, C3H/HeJ (*Lps*^{d}) cultures.

phage cultures (Fig. 2, *A* and *B*). However, significantly more But-LPS was required to activate C3H/HeJ macrophage cultures to the same levels exhibited by C3H/OuJ macrophages. This differential response could be the result of 1) a diminished sensitivity of C3H/HeJ macrophages to respond to rIFN-γ as a priming signal, or 2) the ability of C3H/OuJ macrophages to recognize and respond to both LPS and LAP in the But-LPS preparation.

Comparison of the effects of rIFN-γ and LAP on C3H/OuJ and C3H/HeJ macrophage tumoricidal capacity. To test the hypothesis that LAP was the component within the But-LPS preparation that triggered rIFN-γ primed C3H/HeJ macrophages to become tumoricidal, purified LAP was used as a "second signal" for rIFN-γ primed C3H/OuJ and C3H/HeJ macrophages. As shown in Figure 3, *A* and *B*, equivalent levels of cytotoxicity were achieved by macrophages of both strains of mice under identical conditions of priming and secondary signalling. LAP alone did not induce significant levels of cytotoxicity in macrophages derived from either mouse strain (<20%). These findings indicate that C3H/HeJ macrophages do not have a defect in their capacity to be primed with rIFN-γ. In addition, the ability of LAP to trigger primed macrophages to become tumoricidal was not inhibited by polymyxin B sulfate, an agent shown previously to bind the lipid A region of LPS and inactivate it (21), even under conditions where polymyxin B reduced significantly the ability of PW-LPS to trigger primed C3H/OuJ macrophages (data not shown).

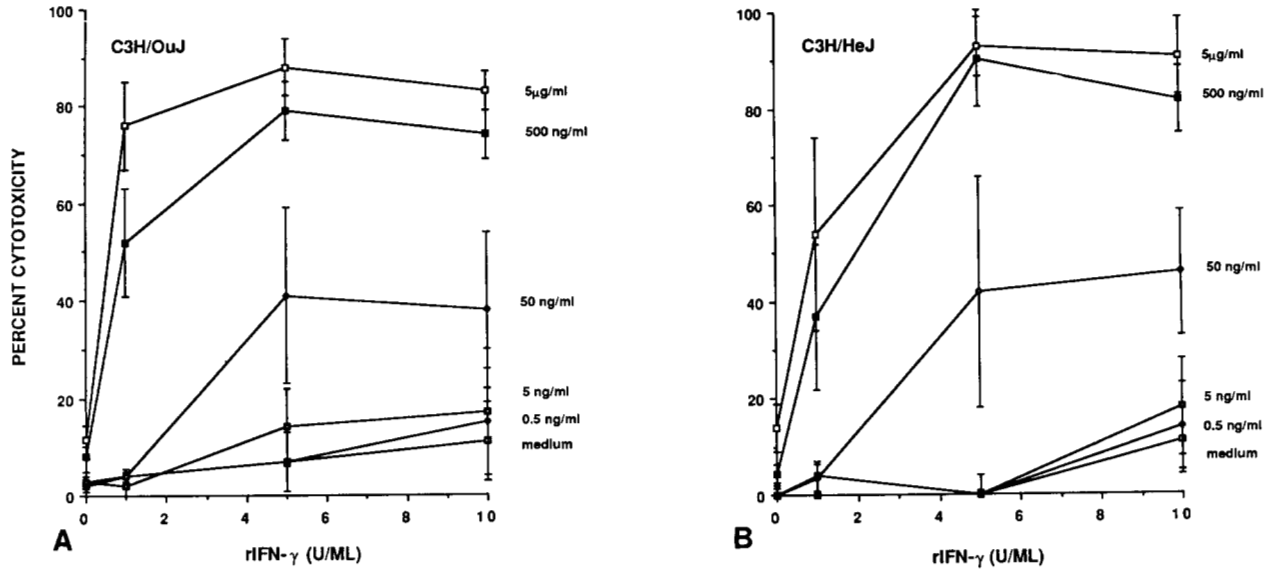

Figure 3. Comparison of the effects of rIFN-γ and LAP on C3H/OuJ and C3H/HeJ macrophage tumoricidal capacity. Macrophage cultures (2×10^5 cells/well) were treated with the indicated concentrations of rIFN-γ and LAP for 24 hr. ^{51}Cr-Labeled P815 tumor cells (1×10^4) were then added to the cultures for 16 hr and the percent cytotoxicity was assessed. Macrophages incubated with dilutions of rIFN-γ alone are indicated on the graph as "medium." Macrophages incubated without LAP or rIFN-γ are plotted at the origin. *A*, C3H/OuJ (*Lps*^{n}) cultures; *B*, C3H/HeJ (*Lps*^{d}) cultures.

DISCUSSION

In 1968, Sultzer (4) described a mutant C3H mouse, the C3H/HeJ strain, which appeared to be resistant to the effects of LPS. This refractory nature was later ascribed to a single gene carried on chromosome 4 (5), which is expressed codominantly in F_1 progeny of crosses between fully LPS-responsive (*Lps*^{n}) strains and the LPS-hyporesponsive (*Lps*^{d}), C3H/HeJ strain (22). Thus, cells derived from F_1 animals show a reduced capacity to respond to LPS in vitro (9). In 1975, Skidmore et al. (8) showed that the C3H/HeJ splenocytes could respond mitogenically to LPS, but only to preparations obtained by selected extraction procedures. LPS isolated by mild extraction techniques, such as those which employ trichloroacetic acid or butanol, were found to be mitogenic for C3H/HeJ lymphocytes, as well as for lymphocytes derived from fully responsive strains. In 1976, Sultzer and Goodman (7) showed that the mitogenic response elicited by these preparations of LPS was associated with the presence of a low m.w. polypeptide (m.w. = 10,000 to 12,000) which appeared to be associated with the lipid A region of the LPS molecule in an undefined manner. When purified and used in culture with C3H/HeJ lymphocytes, this polypeptide was found to be a B cell mitogen and was referred to as "endotoxin protein" (7) or LAP (23). LAP is lost when LPS is prepared by using more rigorous extraction procedures, such as the phenol water extraction method (18), and is retained in the milder procedures. LAP appears to be identical with a low m.w. protein associated with *E. coli* 0111:B4 described previously by Wu and Heath (24) and Lieve et al. (25).

Although the two signal model of tumoricidal activation was confirmed by using rIFN-γ and PW-LPS in macrophages derived from *Lps*^{n} mice, an alternate second signal, heat-killed *L. monocytogenes*, was shown to "trigger" rIFN-γ-primed, C3H/HeJ macrophages to achieve tumor cell lysis (15). Thus, Pace et al. (15) showed that C3H/HeJ macrophages could effect tumor lysis of an equivalent magnitude as *Lps*^{n} macrophages when provided with an appropriate sequence of signals. In light of the historical evidence that C3H/HeJ macrophages would respond to selected preparations of LPS that retained LAP, these preparations warranted investigation as potential, naturally occurring, second signals for C3H/HeJ macrophage tumoricidal activation.

In the present studies, PW-LPS and But-LPS, as well as purified LAP, were compared as potential second signals. C3H/HeJ macrophages failed to respond to any combination of rIFN-γ and PW-LPS (Fig. 1*B*), even at combinations that significantly exceeded the dosages previously examined by Pace and her colleagues (15). In contrast, C3H/OuJ macrophages were able to kill tumor targets with priming concentrations of 1 U/ml rIFN-γ and triggering concentrations as low as 0.5 ng/ml PW-LPS, thus confirming and extending the findings of Pace et al. (15). In contrast to the studies with PW-LPS as a trigger signal, But-LPS, known to contain both LPS and LAP, triggered both C3H/OuJ and C3H/HeJ macrophages to lyse tumor targets (Fig. 2, *A* and *B*). C3H/OuJ macrophages responded to lower combined concentrations of rIFN-γ and But-LPS than C3H/HeJ macrophages. For example, combined treatment of C3H/OuJ macrophages with 1 U/ml rIFN-γ and 100 ng/ml But-LPS stimulated C3H/OuJ macrophages to a cytotoxicity level of 51 ± 12%, whereas the same combination elicited only 4 ± 2% cytotoxicity in C3H/HeJ cultures. At higher dosage combinations, equivalent levels of cytotoxicity were achieved. This differential responsiveness could be due to 1) a diminished capacity of C3H/HeJ macrophages to be "primed" by rIFN-γ, or 2) responsiveness of primed, C3H/OuJ macrophages to both LPS and LAP contained within the But-LPS. To test the hypothesis that LAP was the active component within the But-LPS that was serving as a second signal to primed C3H/HeJ macrophages, purified LAP was tested and was found to evoke identical responses in rIFN-γ primed C3H/OuJ and C3H/HeJ macrophages. These findings suggest that C3H/HeJ macrophages do not have a diminished capacity to be primed by rIFN-γ, and that the differential sensitivity of primed

C3H/OuJ macrophages to But-LPS is most likely due to the availability of both LPS and LAP within the But-LPS preparation. Although some investigators have reported that cultured macrophages require only one signal to be activated to a tumoricidal state (26–29), this discrepancy has been shown to be tumor target-dependent (30), mouse strain-dependent (31), dependent upon the amount of contaminating LPS in the tissue culture reagents (14), and, as suggested by our findings, may be related to the amount of contaminating LAP present in the LPS preparations.

The finding that C3H/HeJ macrophages failed to respond to high concentration (10 μg/ml) of PW-LPS, even in the presence of high concentrations of rIFN-γ (10 U/ml), suggests that, in this system, rIFN-γ fails to correct the C3H/HeJ defect, as has been recently reported for the induction of tumor necrosis factor-α (TNFα) (32). This suggests several possibilities: 1) the sensitivity of the tumor cytotoxicity assay may be significantly less than that of measuring TNFα-specific RNA or secreted TNFα by immunoblot; 2) the C3H/HeJ defect is actually encoded for by multiple, closely linked genes which are independently regulated by IFN-γ, or 3) the LPS or rIFN-γ preparations used in this TNF study contained significant LAP contamination. With a standard, actinomycin D-treated, L929 fibroblast assay for the detection of TNFα (33), we have never been able to detect selected TNFα in supernatants of C3H/HeJ macrophages treated with rIFN-γ and PW-LPS even at the highest dose combinations, whereas treatment of C3H/HeJ macrophages with rIFN-γ and But-LPS or LAP results in significant production of TNFα (M. M. Hogan and S. N. Vogel, manuscript in preparation). Further studies will be required to test these possibilities.

There is little data to suggest that C3H/HeJ mice have a significantly higher incidence of spontaneous tumors than *Lps*^{n} mice, although the incidence of spontaneous mammary tumors in retired breeders has been reported to be significantly greater in female C3H/HeJ mice than in their endotoxin-responsive counterparts (34). This may imply that, in vivo, C3H/HeJ macrophages can be fully activated via alternate second signals, such as LAP. Alternatively, other tumoricidal mechanisms which are independent of LPS recognition may be selectively or compensatorily operative in these animals.

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