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Bacterial Lipopolysaccharide Induction of Leukocyte-Derived Corticotropin and Endorphins

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Previous reports have shown that there is an endogenous opioid component associated with pathophysiological responses to endotoxin. It has been shown that these responses are alleviated by naloxone, a specific opiate antagonist. Results of another study have indicated that leukocytes may mediate some of those responses since leukocyte depletion alleviated the effects of lipopolysaccharide. In view of the above reports as well as the finding that leukocytes produce immunoreactive (ir-) endorphins and corticotropin (ACTH) when stimulated with Newcastle disease virus or ACTH-releasing factor, we postulated that leukocytes may serve as an extrapituitary source of endorphins produced in response to bacterial endotoxin. To test this hypothesis, human peripheral blood leukocytes as well as mouse spleen cells were cultured in vitro with *Escherichia coli* lipopolysaccharide for 48 h. The lipopolysaccharide (i.e., endotoxin) was shown to induce de novo synthesis of ir-ACTH and ir-endorphins. The leukocyte-derived ir-ACTH had a molecular weight of approximately 2,900 and demonstrated a bioactivity similar to that of pituitary-derived ACTH. The lymphocyte-derived ir-endorphin comigrated with α- and γ-endorphin at approximately 1,800 daltons and was shown to bind to brain opiate receptors. These findings imply that leukocyte-derived endorphins may be involved in the pathophysiological response to endotoxin.

Endogenous opioids or endorphins have been implicated as possible mediators of some of the pathophysiological changes induced during endotoxic shock and gram-negative sepsis (9, 10). This implication was a result of the ability of a potent opiate antagonist, naloxone, to alleviate endotoxin-induced hypotension and hyperthermia by apparently blocking the effector molecule (8, 15). Additionally, naloxone treatment improved survival rates (17), and plasma endorphin levels have been shown to rise significantly in sheep injected with *Escherichia coli* endotoxin (lipopolysaccharide [LPS]) (5, 23). Results of depletion studies have indicated that leukocytes may also mediate some of the aforementioned endotoxin effects (3, 22). The mitogenic and lymphokine-inducing effects of LPS on leukocytes are well known (for a review, see reference 16) and, together with the above findings, suggest that endorphins, leukocytes, and their products are all components of endotoxin shock.

Classically, the major source of endorphins was thought to be the pituitary gland, but this laboratory has recently described a molecule produced by virus-infected (2, 20) or corticotropin (ACTH)-releasing factor-treated leukocytes [E. M. Smith, A. M. Morrill, W. J. Meyer III, and J. E. Blalock, Nature (London) in press] that appears structurally and functionally identical to an endorphin. Lolait and coworkers have reported a similar immunoreactive (ir-) endorphin present in mouse spleen macrophages (14). Since both leukocytes and pituitary cells are apparently sources of endorphins, it seems that cells of the immune system would be the more likely source for this production in response to LPS. Therefore, we have postulated that leukocytes may serve as an extrapituitary source of endorphin-like molecules that are produced in response to bacterial endotoxin. To test this hypothesis, leukocytes were stimulated in vitro with bacterial endotoxin and monitored for the production of endorphin-related molecules. Because endorphins and ACTH are derived from the same larger-molecular-weight precursor (pro-opiomelanocortin [POMC]), we also looked for ACTH production. In this report, we show that LPS-treated mononuclear cells make molecules that are antigenically, structurally, and functionally similar to the POMC-derived peptides, ACTH and γ-endorphin. These data provide evidence for the idea that the pathophysiology that is associated with LPS and gram-negative sepsis may in part be mediated through leukocyte-derived endorphins.

MATERIALS AND METHODS

Cell cultures. Human peripheral blood mononuclear cells were prepared by Ficoll-Hypaque density gradient centrifugation as described previously (4), washed twice in Hanks basal salt solution, and suspended in RPMI 1640 (supplemented with 10% fetal calf serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml) at a concentration of 10^7 cells per ml. Two 10-ml cultures were radiolabeled for 48 h with 10 μCi of L-^{3}H-amino acid mixture (ICN Radioisotope Division, Irvine, Calif.), in the presence or absence of 50 μg of bacterial LPS (*E. coli* O127:B8, Difco Laboratories, Detroit, Mich.) at 37°C under 5% CO_2. Mouse spleen cell suspensions were prepared from ICR outbred mice. Mice were sacrificed by cervical dislocation, the spleens were aseptically removed and dissociated, and each spleen cell preparation was individually cultured and induced in an identical fashion to the human peripheral blood mononuclear cells.

Antibody affinity chromatographic purification. Antibody affinity columns were prepared with rabbit antibody against synthetic γ-endorphin and synthetic ACTH (1-13) (UCB Bioproducts Brussels, distributed by Accurate Chemicals, N.Y.). The antibodies were affinity purified and coupled to cyanogen bromide (CNBr)-activated Sepharose 4B as previously described (6). The 48-h culture supernatant fluids were first passed over the anti-γ-endorphin antibody Sepharose affinity column. The effluent was collected as a pool, and the bound portion was eluted with 0.1 M glycine (pH 2). The eluate was restored to neutral pH, lyophilized, and reconsti-

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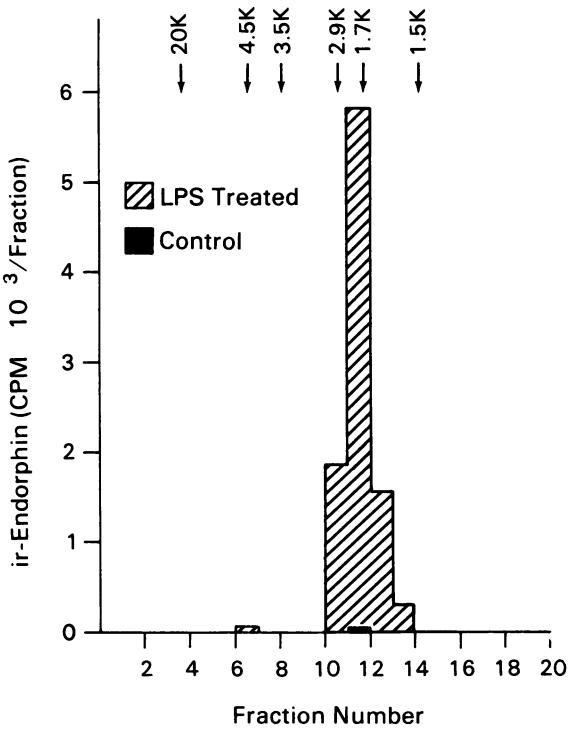

FIG. 1. Gel filtration of human leukocyte-derived radiolabeled ir-endorphin. Each 1-ml fraction of radiolabeled LPS- or mock-induced ir-endorphin from the anti-γ-endorphin antibody affinity column was sized on a P-10 gel filtration column and monitored for counts per minute. Molecular weight markers were blue dextran (20K), ACTH (1-39) (4.5K), β-endorphin (3.5K), ACTH (1-24) (2.9K), γ-endorphin (1.7K), and phenol red (1.5K).

tuted in phosphate-buffered saline. This material was referred to as ir-endorphin. The material not bound to the anti-γ-endorphin antibody affinity column was then passed over the monospecific anti-ACTH (1-13) antibody affinity column. The material that bound to this column was eluted, neutralized, lyophilized, and reconstituted as described above. This material is referred to as ir-ACTH.

Gel filtration. The molecular weights of the radiolabeled ir-endorphin and ACTH were determined by gel filtration on a P-10 gel filtration column (0.5 by 20 cm; Bio-Rad Laboratories, Richmond, Calif.) equilibrated with phosphate-buffered saline. The column was calibrated with ACTH (1-39) (molecular weight, 4,500 [4.5K]), β-endorphin (molecular weight, 3.5K), ACTH (1-24) (molecular weight, 2.9K), and γ-endorphin (molecular weight, 1.8K) as molecular weight markers. Fractions (1 ml) were collected, and samples from these fractions were assayed by standard liquid scintillation spectroscopy techniques.

Opiate receptor binding assay. Binding of nonlabeled leukocyte-derived ir-endorphin (prepared identically to the radiolabeled material) to opiate receptors was measured by inhibition of specific [^{3}H]dihydromorphine binding to mouse brain opiate receptors as previously described (19). The particulate fraction of homogenized ICR mouse brain was suspended at 2% (wt/vol) in 50 mM Tris buffer (pH 7.4) and incubated at 37°C for 5 min with 0.1 ml of the competing substance, which consisted of pooled P-10 fractions from either the mock- or LPS-treated material which corresponded to the molecular weight of α- or γ-endorphin. [*N*-methyl-^{3}H]dihydromorphine (0.1 ml; 84 Ci/mmol; 4 $\times 10^{-9}$ M) was then incubated with the brain fraction for 15 min at 37°C. This mixture was collected on Gelman glass fiber filters, washed, and counted by standard liquid scintillation spectroscopy techniques.

Corticosterone assay. P-10 column fractions of ir-ACTH were assayed for their ability to induce steroidogenesis in mouse adrenal tumor cell (Y-1) cultures (21). Briefly, Y-1 cells were seeded at 5×10^4 cells per well and grown to confluency in 96-well microtiter plates. Cell cultures were incubated overnight with dilutions of P-10 column fractions of ir-ACTH. After freezing and thawing, the culture fluids plus cell lysates were assayed for corticosterone production in a standard radioimmunoassay procedure with an antibody to cortisol (21).

RESULTS

We initially determined whether human mononuclear cells were able to synthesize ir-endorphin in response to LPS. Supernatant fluids from radiolabeled leukocyte cultures were first passed over the anti-γ-endorphin antibody affinity column. Acid-precipitable radiolabeled material from LPS-treated cultures but not from control cultures bound to this affinity column (data not shown). Subsequent sizing of the LPS-induced radiolabeled ir-endorphin by gel filtration showed a peak of radioactivity which comigrated with γ-endorphin at approximately 1,800 daltons (Fig. 1). A similar radiolabeled peak was not observed from control cultures. Therefore, LPS seemed to induce human mononuclear cell production of a molecule that is antigenically and structurally related to γ-endorphin. The fact that the ir-endorphin was intrinsically radiolabeled strongly suggests that it was newly synthesized. As a means to assess its potential biological activity, the ir-endorphin was tested for the ability to bind to opiate receptors. In an in vitro opiate receptor-binding assay (19), LPS-induced ir-endorphin specifically inhibited [^{3}H]dihydromorphine binding to opiate receptors from mouse brain tissue (Table 1). Material from control cultures did not inhibit [^{3}H]dihydromorphine binding to the

TABLE 1. In vitro binding of leukocyte-derived ir-endorphin to opiate receptors[a]

Sample	Dilution or concn (M)	Specifically bound [^{3}H]dihydromorphine (cpm ± SD)	% Inhibition of specific [^{3}H]dihydromorphine binding
LPS treated	1:3	0 ± 145	100
	1:9	183 ± 47	79
	1:27	437 ± 39	49
	1:81	524 ± 89	39
Nontreated	1:3	1,309 ± 174	0
	1:9	945 ± 70	0
	1:27	1,070 ± 193	0
γ-Endorphin	10^{-6}	418 ± 48	51
	10^{-7}	575 ± 8	33
	10^{-8}	722 ± 19	16
	10^{-9}	871 ± 1	2

[a] Mock- and LPS-treated nonlabeled culture fluids were prepared and purified as described in the text. P-10 column fractions corresponding to the radiolabeled peak in Fig. 1 were pooled and used as the competing substance in an opiate radioreceptor assay. In each assay, approximately 1,568 cpm bound nonspecifically as determined by the amount of [^{3}H]dihydromorphine bound in the presence of 10^{-4} M naloxone. The total binding to the brain homogenate was approximately 2,500 cpm, of which 932 was specifically bound.

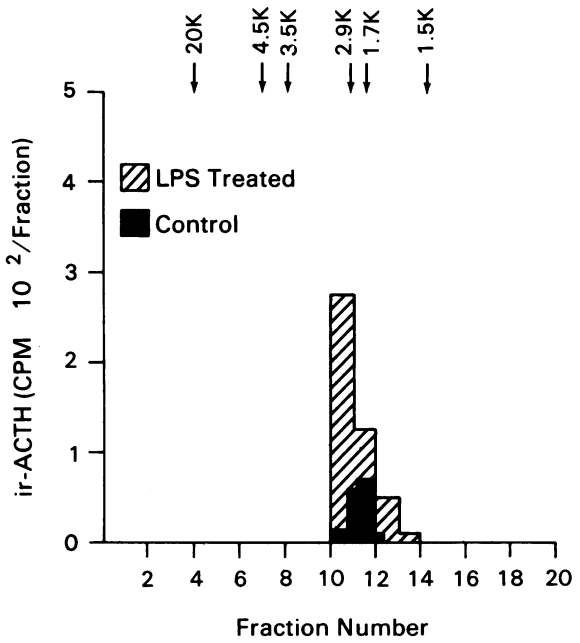

FIG. 2. LPS induction of human leukocyte-derived ir-ACTH. Each 1-ml fraction of radiolabeled LPS- or mock-induced and affinity-purified human mononuclear cell-derived ir-ACTH was sized on a P-10 gel filtration column and monitored for counts per minute. Molecular weight markers were as described in the legend to Fig. 1.

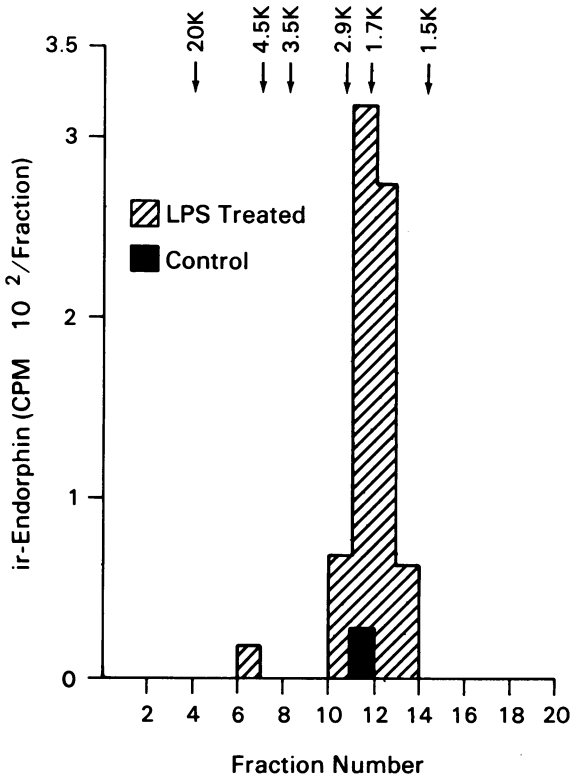

FIG. 4. Gel filtration of mouse spleen cell-derived radiolabeled ir-γ-endorphin. Each 1-ml fraction of radiolabeled LPS- or mock-induced ir-endorphin from the anti-γ-endorphin antibody affinity column was sized on a P-10 gel filtration column and monitored for counts per minute. Molecular weight markers were as described in the legend to Fig. 1.

opiate receptors. Thus, in addition to a similar molecular weight, the LPS-induced ir-endorphin has a biological activity similar to that of γ-endorphin.

As previously mentioned, in the pituitary gland, endorphins are derived from a large-molecular-weight precursor, POMC, which also contains ACTH. To determine whether leukocytes produced the endorphin-like molecules in a manner similar to that observed in the pituitary gland, we also monitored the mononuclear cell preparations for ACTH production. The material that did not bind to the anti-γ-endorphin antibody affinity column was passed over an anti-ACTH (1-13) antibody affinity column and assayed for radioactivity. Acid-precipitable, radiolabeled material was bound from the LPS-treated cultures but not from the control cultures. This material was sized on the P-10 gel filtration column and was shown to comigrate with ACTH (1-24) at approximately 2,900 daltons (Fig. 2). Bioactivity of this LPS-induced ir-ACTH was determined by assaying the gel filtration column fractions for induction of steroidogenesis in mouse Y-1 cells. The fractions inducing the highest corticosterone levels corresponded to the peak radioactive fractions (Fig. 3) found in the LPS-treated material. This indicated that, like the ir-endorphin, the mononuclear cell-derived ir-ACTH was biologically active. The immunoreactive material from control cultures elicited a mild steroidogenic effect in a fraction corresponding to a molecular weight of 4,500, which is the molecular weight of ACTH (1-39). This mild steroidogenic activity, however, did not correspond to a radiolabeled fraction and, thus, was apparently not synthesized de novo. This indicates that it was perhaps already present in the cell and was merely released from the cell after treatment with LPS. Therefore, we concluded that LPS caused the de novo and concomitant synthesis of both biologically active ir-ACTH and endorphin. Thus, mononu-

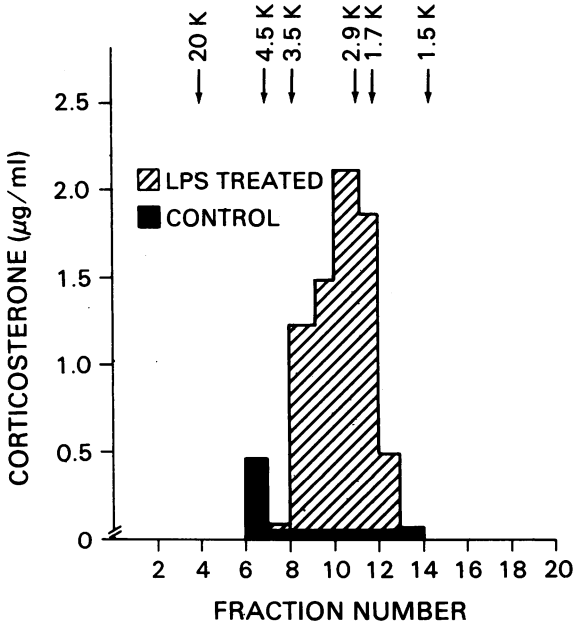

FIG. 3. Biological activity of human leukocyte-derived ir-ACTH. P-10 gel filtration fractions from Fig. 2 were diluted 1:2 and placed on confluent cultures of mouse adrenal tumor (Y-1) cells as described in the text, and steroidogenesis was measured by radioimmunoassay (21). Molecular weight markers were as described in the legend to Fig. 1.

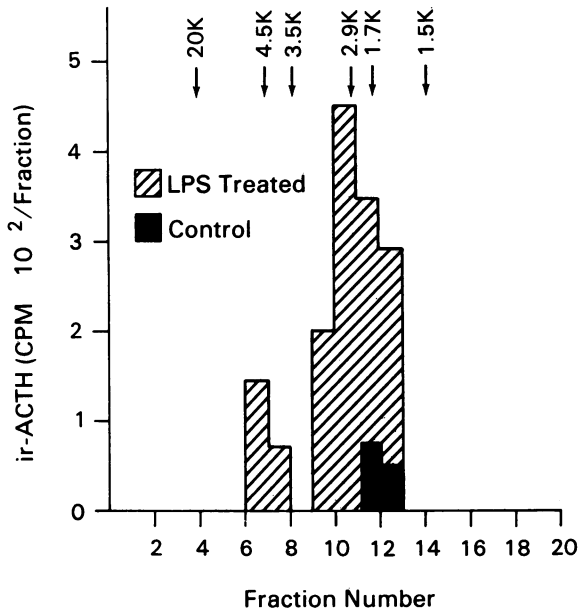

FIG. 5. Mouse spleen cell production of ir-ACTH. Each 1-ml fraction of radiolabeled LPS- or mock-induced and affinity-purified, mouse spleen cell-derived ir-ACTH was sized on a P-10 gel filtration column and monitored for counts per minute. Molecular weight markers were as described in the legend to Fig. 1.

clear cell production of these peptides seems somewhat analogous to that observed in the pituitary gland.

To determine whether the de novo synthesis of these peptide hormones was unique to human cells or was a more general phenomenon, we performed similar experiments with mouse spleen cells. Mouse spleen cells, when stimulated with LPS, were capable of de novo synthesis of a radiolabeled ir-endorphin (Fig. 4). The spleen cell-derived ir-endorphin was also biologically active (data not shown). These cells also produced a radiolabeled ir-ACTH (Fig. 5). The material in the gel filtration fractions which corresponded to the peak radioactivity also possessed steroidogenic activity (data not shown).

DISCUSSION

The studies described above show that LPS-stimulated human mononuclear and mouse spleen cells produce a molecule that is similar if not identical to γ-endorphin with regard to antigenicity, bioactivity, and molecular size. These findings suggest that leukocyte-derived endorphins may be involved in LPS shock and gram-negative sepsis. As mentioned, several reports have demonstrated that lymphocyte depletion of sheep and subsequent injection of *E. coli* endotoxin results in an abrogation of parts of the resulting pathophysiological response (3, 22, 23). This abrogation is similar to that seen after naloxone administration to intact animals (8, 9). Our data tend to meld these two observations in that leukocytes synthesize and release endorphins in response to LPS. As such, deletion of a leukocyte component would remove a source of endorphin-like activity that mediates hypotension and hyperthermia, whereas naloxone would block the action of the leukocyte-derived effector molecule. LPS-sensitive and -resistant mice are currently being tested to further clarify the role that leukocyte-derived endorphins may play in gram-negative sepsis and endotoxic shock.

A further interesting finding from this research is the suggestion of a possible unique leukocyte-processing mechanism for POMC. The evidence for this postulate is as follows. The 31,000-molecular-weight POMC precursor and its cleavage products have been shown to be processed differently, depending on the cell type performing the processing (7). In the cells of the anterior pituitary gland, the major cleavage products are ACTH (1-39) and β-lipotropin. There is alternative processing of these peptides in intermediate lobe pituitary cells in that ACTH (1-39) is further cleaved into ACTH (1-13) and β-lipotropin is processed to β-endorphin (for a review, see reference 13). In the brain and hypothalamus, POMC is processed in a manner similar to that observed in the intermediate lobe of the pituitary gland with the exception that peptides such as ACTH (1-13) are not acetylated. The POMC precursor molecule has also been demonstrated in extrapituitary tissues such as the placenta and gastrointestinal tract. These tissues process the POMC precursor in a fashion analogous to that observed in the intermediate lobe of the pituitary gland. The alternative processing of the same precursor molecule has led researchers to postulate that different enzymes may be responsible for such differential processing (for a review, see reference 12).

Based on the results presented here and elsewhere, it appears that leukocytes synthesize and process a POMC-like molecule (14, 20). Interestingly, although ACTH-releasing factor and Newcastle disease virus elicited the production of peptide hormones with the molecular weight of ACTH (1-39) and β-endorphin, LPS caused the synthesis of similar peptides with the molecular weight of ACTH (1-24) and α- or γ-endorphin. Thus, in response to ACTH-releasing factor and Newcastle disease virus, leukocytes seem to process POMC in a fashion that is analogous to a partial composite of the anterior and intermediate lobe of the pituitary gland. In contrast, LPS seems to cause a completely novel processing pattern which results in peptides with the molecular weight of ACTH (1-24) and α- or γ-endorphin. One possible explanation for such alternative processing of the precursor by leukocytes is that the LPS-treated cells process ACTH (1-24) and α- or γ-endorphin from ACTH (1-39) and β-endorphin, respectively. Alternatively, POMC, ACTH (1-39), or β-endorphin might be cleaved extracellularly by endopeptidases, perhaps from macrophages, that are secreted in response to LPS. Regardless of the site of cleavage, it is tempting to speculate that this phenomenon results from LPS-induced production or release of a proteolytic enzyme(s) that further cleaves the ACTH (1-39) into ACTH (1-24) and the β-endorphin into α- or γ-endorphin.

On a more general note, these data further substantiate the hypothesis that the immune system and the neuroendocrine system are linked via common peptide hormones and receptors (1). These systems are capable of recognizing their own unique stimuli but can respond by producing similar hormones that signal either the other system or components of the same system (11). With regard to the data presented here, it appears that cells of the immune system recognize a bacterial stimulus (LPS) and respond by synthesizing and secreting an endorphin-like molecule. This leukocyte-derived endorphin molecule, in turn, may communicate with and influence the hypothalamus and vascular system to alter body temperature or blood pressure or both, thereby resulting in some of the detrimental effects seen in endotoxic shock. A possible pathophysiological role for this leukocyte-derived peptide seems a distinct possibility when one considers the levels of ir-endorphin that are produced and released by LPS-treated spleen cells. Analysis by an enzyme-

linked immunosorbent assay with monospecific antiserum against γ-endorphin shows extracellular levels of approximately 60 ng/10^8 spleen cells of ir-γ-endorphin. When one considers that there are approximately 1×10^8 to 2×10^8 mononuclear cells per mouse spleen, then the total capacity of this organ would be 60 to 120 ng. Since the blood volume of a mouse is about 3 ml, only 10 to 20% of the total would need to be released to achieve the level of 4 ng of β-endorphin per ml that is observed in rats that are stressed by electric shock (18). Thus, this one lymphoid organ apparently has the capacity to produce enough endorphin to have physiological or pathophysiological consequences. The role, if any, that the mononuclear cell-derived ir-γ-endorphin or the ir-ACTH (1-24) plays in specific or nonspecific modulation of immune responses remains to be determined. To our knowledge, ACTH (1-24) has not been shown in vivo, and when the source of its in vitro production is considered, its possible role in immunomodulation becomes even more intriguing.

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LITERATURE CITED

1. **Blalock, J. E.** 1984. The immune system as a sensory organ. J. Immunol. **132**:1067.
2. **Blalock, J. E., and E. M. Smith.** 1981. Human leukocyte IFN (HuIFN-α): potent endorphin-like opioid activity. Biochem. Biophys. Res. Commun. **101**:422–428.
3. **Bohs, C. T., J. C. Fish, T. H. Miller, and D. L. Traber.** 1979. Pulmonary vascular response to endotoxin in normal and lymphocyte depleted sheep. Circ. Shock **6**:13–21.
4. **Boyum, A.** 1968. Isolation of leukocytes from human blood. Scand. J. Clin. Lab. Invest. Suppl. **97**:31–50.
5. **Carr, D. B., R. Bergland, A. Hamilton, H. Blume, N. Kasting, M. Arnold, J. B. Martin, and M. Rosenblatt.** 1982. Endotoxin-stimulated opioid peptide secretion: two secretory pools and feedback control in vivo. Science **217**:845–848.
6. **Cuatrecasas, P., M. Wilchek, and C. B. Anfinsen.** 1968. Selective enzyme purification by affinity chromatography. Proc. Natl. Acad. Sci. U.S.A. **61**:636–643.
7. **Eipper, B. A., and R. E. Mains.** 1980. Structure and biosynthesis of pro-adrenocorticotropin/endorphin and related peptides. Endocr. Rev. **1**:1–27.
8. **Faden, A. I., and J. W. Holaday.** 1979. Naloxone treatment of endotoxin shock: stereospecificity of physiologic and pharmacologic effects in the rat. J. Pharmacol. Exp. Ther. **212**:441–447.
9. **Faden, A. I., and J. W. Holaday.** 1979. Opiate antagonists: a role in the treatment of hypovolemic shock. Science **205**:317–318.
10. **Holaday, J. W., and A. I. Faden.** 1978. Naloxone reversal of endotoxin hypotension suggests role of endorphins in shock. Nature (London) **275**:450–451.
11. **Johnson, H. M., E. M. Smith, B. A. Torres, and J. E. Blalock.** 1982. Regulation of the *in vitro* antibody response by neuroendocrine hormones. Proc. Natl. Acad. Sci. U.S.A. **79**:4171–4174.
12. **Krieger, D. T.** 1983. Brain peptides: what, where, and why? Science **222**:975–985.
13. **Krieger, D. T., and A. S. Liotta.** 1979. Pituitary hormones in the brain: where, how and why? Science **205**:366–372.
14. **Lolait, S. J., A. T. W. Lim, B. H. Toh, and J. W. Funder.** 1984. Immunoreactive B-endorphin in a subpopulation of mouse spleen macrophages. J. Clin. Invest. **73**:277–280.
15. **Mamazza, J., E. J. Hinckey, and R. Chu-Jeng Chiu.** 1984. The pulmonary effects of opiate blockade in septic shock. J. Surg. Res. **36**:625–630.
16. **Morrison, C., and J. L. Rifan.** 1979. Bacterial endotoxins and host immune responses. Adv. Immunol. **28**:293–450.
17. **Reynolds, D. G., N. V. Gwill, T. Vargish, R. B. Lechner, A. I. Faden, and J. W. Holaday.** 1980. Blockade of opiate receptors with naloxone improves survival and cardiac performance in canine endotoxic shock. Circ. Shock **7**:39–48.
18. **Rossier, J., E. D. French, C. Rivier, N. Ling, R. Guillemin, and F. E. Bloom.** 1977. Foot-shock induced stress increases β-endorphin levels in blood but not brain. Nature (London) **270**:618–620.
19. **Simon, E. J., J. M. Hiller, and I. Edelman.** 1973. Stereospecific binding of the potent narcotic analgesic (^{3}H)-etorphine to rat brain homogenate. Proc. Natl. Acad. Sci. U.S.A. **70**:1947–1949.
20. **Smith, E. M., and J. E. Blalock.** 1981. Human lymphocyte production of corticotropin and endorphin-like substances: association with leukocyte interferon. Proc. Natl. Acad. Sci. U.S.A. **78**:7530–7534.
21. **Smith, E. M., W. J. Meyer, and J. E. Blalock.** 1982. Virus induced corticosterone in hypophysectomized mice: a possible lymphoid adrenal axis. Science **218**:1311–1312.
22. **Traber, D. L., T. Jinkins, K. Rice, L. Eziebert, T. Adams, Jr., N. Henriksen, L. D. Traber, and P. D. Thompson.** 1983. Role of lymphocytes in the ovine response to endotoxin. Adv. Shock Res. **9**:257–263.
23. **Traber, D. L., P. D. Thompson, J. E. Blalock, E. M. Smith, T. Adams, and L. A. Sziebert.** 1983. Action of an opiate receptor blocker on ovine cardiopulmonary responses to endotoxin. Am. J. Physiol. **245**:H189–H193.