*Proc. Natl. Acad. Sci. USA*
Vol. 79, pp. 4226–4230, July 1982
Neurobiology

# β-Endorphin enhances lymphocyte proliferative responses

(neuropeptides/mitogens/lymphocyte activation/opiates/stress)

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*Contributed by Floyd E. Bloom, April 1, 1982*

**ABSTRACT** **The opioid peptides α- and β-endorphin and [D-Ala², Met⁵]enkephalin were investigated for their effect on the proliferation of resting and activated rat splenic lymphocytes *in vitro*. β-Endorphin enhanced the proliferative response of spleen cells to the T cell mitogens concanavalin A and phytohemagglutinin. The effect of β-endorphin was dose dependent and occurred at peptide concentrations similar to those found in rat plasma. α-Endorphin and [D-Ala², Met⁵]enkephalin did not affect the proliferative responses to any mitogen tested. Furthermore, the potentiating effect of β-endorphin was not reversed by treatment with 10 μM naloxone. None of the peptides had any effect on resting, unstimulated spleen cells or on the response to a mixture of lipopolysaccharide and dextran sulfate, which is specifically mitogenic for B lymphocytes. The pharmacological properties of the β-endorphin potentiation indicate that the effect may be mediated by a nonopiate but β-endorphin-specific mechanism. These results suggest a possible role for peripheral β-endorphin and may provide a link between stress and disease susceptibility.**

Both β-endorphin and corticotropin (adrenocorticotropic hormone, ACTH) are neuropeptides released into the blood stream from the pituitary gland (1). In rats, this secretion is increased severalfold in response to acute stress, producing plasma β-endorphin concentrations up to 10 ng/ml (3.3 nM). The target for blood-borne ACTH is assumed to be the adrenal cortex, but the cellular target for circulating β-endorphin has not been defined. The possible relationship between stress, immune function, and disease (2–4), as well as the recent demonstration of opioid-like receptors on lymphoid cells (5), prompted us to investigate the effects of β-endorphin on lymphocyte function. We present here studies designed to evaluate the ability of α-endorphin, β-endorphin, and [D-Ala², Met⁵]enkephalin to modulate the proliferative responses of splenic lymphocytes to mitogenic stimulation. The results indicate that β-endorphin, but not α-endorphin or [D-Ala², Met⁵]enkephalin, potentiates the lymphocyte proliferative response to the T cell mitogens concanavalin A (Con A) and phytohemmagglutinin (PHA) but has no effect on the response to the B cell mitogen lipopolysaccharide/dextran sulfate (LPS/DS). These observations suggest that β-endorphin has a specific effect on one cellular component of the immune system and may indicate one possible link between the neuroendocrine and immune systems.

## MATERIALS AND METHODS

**Cells and Cell Cultures.** Spleen cells were obtained from young adult (3- to 4-month-old) male and female Lewis and Brown Norway rats from the breeding colony of the Research Institute of Scripps Clinic. Spleens were removed aseptically and single-cell suspensions were prepared by teasing apart the organs into medium [RPMI 1640 medium supplemented with 2 mM glutamine, penicillin at 100 μg/ml, streptomycin at 100 μg/ml, 20 mM Hepes, and 10% heat-inactivated (30 min, 56°C) normal Lewis rat serum] and filtering through nylon mesh. Erythrocytes were lysed by treatment of the cells for 10 min on ice with Tris-buffered ammonium chloride (17 mM Tris/136 mM $NH_4Cl$). After two washes, the cells were resuspended in medium at a concentration of $4 \times 10^6$ per ml. Viability, determined by trypan blue exclusion, was 85–95%. Spleen cells were cultured in 96-well flat-bottomed microtiter plates (Falcon). Each well contained $2 \times 10^5$ spleen cells and appropriate concentrations of mitogens and neuropeptides in a final volume of 200 μl. The plates were incubated at 37°C in a 5% $CO_2$ atmosphere. On day 3, the cultures were pulsed with 1 μCi ($3.7 \times 10^4$ becquerels) of [*methyl*-³H]thymidine ([³H]dThd; specific activity 2 Ci/mmol; Amersham) and 24 hr later the cells were harvested on glass filter papers (MASH II multisample harvester, Microbiological Associates, Walkersville, MD). Results are expressed as the mean cpm ± SD of triplicate cultures. Student's *t* test was used for determining significant differences between experimental and control groups; $P \leq 0.05$ was considered to be statistically significant.

**Reagents.** Con A (Miles–Yeda, Rehovot, Israel) was used at 1–50 μg/ml; PHA (GIBCO) was used at 10–50 μl/ml. For stimulation of rat B lymphocytes, LPS derived from *Salmonella minnesota* (Difco) was used in conjunction with DS (Sigma) at 10–50 μg/ml. Synthetic α-endorphin, β-endorphin, and [D-Ala², Met⁵]enkephalin were a generous gift from N. Ling of the Salk Institute and were synthesized by using methods described elsewhere (6, 7). Naloxone was purchased from Endo Laboratories (Garden City, NY). All reagents were diluted in RPMI 1640 medium.

## RESULTS

**β-Endorphin Potentiates Proliferative Responses to Con A.** When rat spleen cells are cultured in the presence of Con A for 3–4 days, a subpopulation of these cells is induced to proliferate, as shown by an increased uptake of [³H]dThd. To determine if β-endorphin had any effect on these proliferating lymphocytes, spleen cells from Lewis rats were cultured with Con A at 1–50 μg/ml in the presence or absence of various concentrations of β-endorphin for the entire 4-day culture period. Cells cultured in the presence of β-endorphin and Con A

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Abbreviations: Con A, concanavalin A; PHA, phytohemagglutinin; LPS/DS, mixture of lipopolysaccharide and dextran sulfate; [³H]dThd, [*methyl*-³H]thymidine; ACTH, corticotropin (adrenocorticotropic hormone).

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showed a significant increase in [$^3$H]dThd uptake as compared to cells cultured with Con A alone (Fig. 1). The degree of enhancement was dependent on the dose of β-endorphin and could be observed at β-endorphin concentrations as low as 0.33 nM (1 ng/ml, Fig. 1, Exp. 1). In the absence of Con A there was no appreciable enhancement of [$^3$H]dThd uptake by β-endorphin (data not shown), indicating that β-endorphin alone is not mitogenic.

There was considerable variation between experiments in the degree of enhancement of [$^3$H]dThd uptake obtained in the presence of β-endorphin. For example, Fig. 1 shows data from two separate experiments with spleen cells from two individual Lewis rats. In experiment 1, a significant enhancement was obtained at 0.33 nM β-endorphin, whereas in experiment 2 the same peptide concentration produced no significant increase in [$^3$H]dThd uptake over control values, although significant increases in [$^3$H]dThd uptake were observed at higher β-endorphin concentrations. In 18 separate experiments with cells from 18 Lewis rats, a significant enhancement of [$^3$H]dThd uptake was observed in the presence of β-endorphin in 10 experiments. A similar enhancement was observed with spleen cells from two of five Brown Norway rats. In positive experiments the [$^3$H]dThd uptake in Con A-stimulated cultures containing β-endorphin was generally 30–60% greater than the [$^3$H]dThd uptake of control (no β-endorphin) cultures, but as much as a 300% enhancement has been observed.

**Effect of α-Endorphin and [D-Ala$^2$, Met$^5$]Enkephalin on [$^3$H]dThd Uptake of Con A-Stimulated Rat Spleen Cells.** To determine if the ability of β-endorphin to enhance [$^3$H]dThd uptake in Con A-stimulated cultures was specific for this peptide or was a property of neuropeptides in general, α-endorphin was tested for its ability to modulate the Con A response of rat spleen cells (α-endorphin consists of the $NH_2$-terminal 16 amino acid sequence of β-endorphin). Spleen cells from Lewis rats were cultured with Con A at 1–25 μg/ml and either no neuropeptide or various concentrations of α- or β-endorphin. The results demonstrated that α-endorphin did not enhance [$^3$H]dThd uptake in Con A-stimulated cultures, whereas β-endorphin produced a significant increase in [$^3$H]dThd uptake at the same peptide concentrations (Table 1). α-Endorphin failed to enhance [$^3$H]dThd uptake in Con A-stimulated cultures from 18 Lewis rats.

Both α- and β-endorphin contain the $NH_2$-terminal enkephalin pentapeptide sequence, Tyr-Gly-Gly-Phe-Met. It was of interest, therefore, to test the effect of enkephalin on the spleen cell response to Con A. The enkephalin analogue [D-Ala$^2$, Met$^5$]enkephalin had no significant effect on [$^3$H]dThd uptake in the presence of Con A at concentrations similar to those employed for β-endorphin (Table 1). The synthetic enkephalin analogue [D-Ala$^2$, Met$^5$]enkephalin rather than [Met$^5$]enkephalin was employed in this case to avoid problems due to peptide degradation.

**Effect of β-Endorphin Is Not Naloxone Reversible.** Because only β-endorphin and not other opiate peptides causes an enhancement in the Con A response it was critical to test whether the β-endorphin was acting via an opiate-like receptor that could be blocked by the opiate antagonist naloxone. To determine if the effect of β-endorphin was naloxone sensitive, Lewis spleen cells were cultured with Con A at 10 μg/ml and triplicate cultures were given medium only (control) or 3.3 nM β-endorphin, 10 μM naloxone, or β-endorphin plus naloxone. In two separate experiments, the enhancement of [$^3$H]dThd uptake by β-endorphin was not reversed by a 3,000-fold molar excess of naloxone (Table 2). At a concentration of 10 μM, naloxone alone had no effect on the Con A-stimulated [$^3$H]dThd uptake. Naloxone at higher concentrations (>10 μM) resulted in decreased

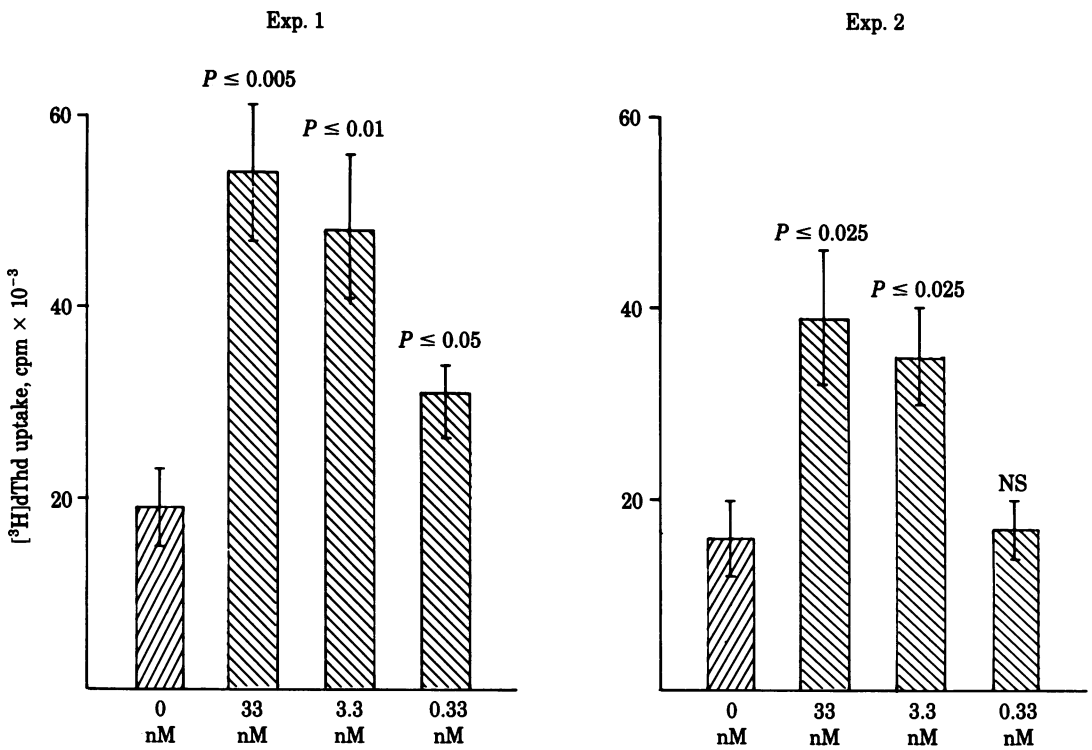


FIG. 1. β-Endorphin potentiates the proliferative response of rat spleen cells to Con A stimulation. Spleen cells from a Lewis rat were cultured for 4 days with Con A at 10 μg/ml in the absence (control) or presence of 33, 3.3, or 0.33 nM β-endorphin. Cultures were pulsed with 1 μCi of [$^3$H]dThd on day 3 and harvested 24 hr later. The mean [$^3$H]dThd uptake (cpm) for triplicate cultures and standard deviations are shown. *P* values were calculated by using Student's *t* test and compare [$^3$H]dThd uptake in cultures with β-endorphin to uptake in cultures without β-endorphin. NS, not significant.

Table 1. Effect of α-endorphin, β-endorphin, and [D-Ala$^2$, Met$^5$]enkephalin on Con A responses of rat spleen cells

| Peptide | Conc., nM | Exp. 1 Uptake, cpm × $10^{-3}$ | Exp. 1 % change | Exp. 1 P | Exp. 2 Uptake, cpm × $10^{-3}$ | Exp. 2 % change | Exp. 2 P |
|---|---|---|---|---|---|---|---|
| Control | — | 150 ± 18 | — | — | 210 ± 9 | — | — |
| β-Endorphin | 33 | 225 ± 5 | +50 | <0.01 | 282 ± 14 | +34 | <0.01 |
| | 3.3 | 179 ± 7 | +19 | <0.01 | 304 ± 10 | +45 | <0.001 |
| | 0.33 | 155 ± 2 | +3 | NS | 186 ± 10 | −11 | NS |
| α-Endorphin | 33 | 173 ± 9 | +15 | NS | 184 ± 12 | −12 | NS |
| | 3.3 | 171 ± 5 | +14 | NS | 186 ± 13 | −11 | NS |
| [D-Ala$^2$, Met$^5$]Enkephalin | 33 | 157 ± 3 | +5 | NS | 215 ± 4 | +2 | NS |
| | 3.3 | 167 ± 3 | +11 | NS | ND | — | — |

Spleen cells were cultured for 4 days with Con A at 10 μg/ml plus either no peptide (control) or various concentrations of α-endorphin, β-endorphin, or [D-Ala$^2$, Met$^5$]enkephalin. Cultures were pulsed with 1 μCi of [$^3$H]dThd on day 3 and harvested 24 hr later. Uptake values are mean ± SD of triplicate cultures. *P* values were calculated by Student's *t* test for the comparison of [$^3$H]dThd uptake in cultures without peptide to [$^3$H]dThd uptake in cultures containing α-endorphin, β-endorphin, or [D-Ala$^2$, Met$^5$]enkephalin. NS, not significantly different from control; ND, not done.

[$^3$H]dThd uptake, probably due to a nonspecific toxic effect, and therefore could not be used to compete with β-endorphin.

**Effect of β-Endorphin on the Responses to PHA and LPS/DS.** The lectin Con A specifically stimulates a subpopulation of splenic T cells. To determine if the effect of β-endorphin was restricted to Con A responses, spleen cells from a Lewis rat were cultured with Con A, PHA (another T cell mitogen), or LPS/DS (a mitogen specific for β cells) in the presence or absence of various concentrations of β-endorphin or α-endorphin. β-Endorphin potentiated the proliferative responses to both T cell mitogens, Con A and PHA, but did not affect the proliferative response to the B cell mitogen LPS/DS (Table 3). In eight experiments, no effect of β-endorphin was seen over a large range of LPS/DS (LPS at 10–100 μg/ml plus DS at 10–50 μg/ml) and β-endorphin concentrations (3.3–330 nM). There was no effect of α-endorphin on the spleen cell response to any of the three mitogens tested. These results suggest that β-endorphin selectively affects T lymphocyte proliferation.

## DISCUSSION

This study demonstrates that β-endorphin potentiates the proliferative response of rat splenic lymphocytes to the T cell mitogens Con A and PHA but has no effect on response to the B cell mitogen LPS/DS. The data suggest that one function for peripherally circulating β-endorphin may be to regulate some aspects of lymphoid cell function. The pharmacological properties of the potentiation indicate that this effect may be mediated by a mechanism distinct from that associated with the analgesic effects of the neuropeptide.

Table 2. Effect of β-endorphin is not naloxone reversible

| Peptide, antagonist | Exp. 1 Uptake, cpm × $10^{-3}$ | Exp. 1 % change | Exp. 1 P | Exp. 2 Uptake, cpm × $10^{-3}$ | Exp. 2 % change | Exp. 2 P |
|---|---|---|---|---|---|---|
| Control | 191 ± 8 | — | — | 180 ± 6 | — | — |
| β-Endorphin | 266 ± 15 | +39 | <0.01 | 201 ± 8 | +12 | <0.01 |
| Naloxone | 176 ± 18 | −8 | NS | 178 ± 17 | −1 | NS |
| β-Endorphin + naloxone | 245 ± 14 | +28 | <0.01 | 212 ± 12 | +18 | <0.01 |

Spleen cells from a Lewis rat were cultured for 4 days with Con A alone at 10 μg/ml (control) or with Con A at 10 μg/ml plus 3.3 nM β-endorphin, 10 μM naloxone, or 3.3 nM β-endorphin plus 10 μM naloxone. Cultures were pulsed with 1 μCi of [$^3$H]dThd for the final 24 hr of culture. Uptake values are mean ± SD of triplicate cultures. *P* values were calculated by Student's *t* test for the comparison of [$^3$H]dThd uptake in control cultures to the [$^3$H]dThd uptake in cultures containing β-endorphin, naloxone, or both. NS, not signficantly different from control value.

The inability to inhibit the β-endorphin potentiation with the opiate antagonist naloxone and the inactivity of the opioid peptides α-endorphin and [Met]enkephalin suggest that the effect of β-endorphin is not mediated by an opiate receptor of the classical type but rather by a receptor that interacts with the COOH-terminal portion of β-endorphin. A receptor with similar properties has been defined by binding studies using cultured human lymphoblastoid cell lines (5). This receptor has an apparent $K_d$ of 3 nM for β-endorphin and would have a sufficiently high affinity to mediate the effects of β-endorphin at the peptide concentrations we have used in this study. Furthermore, the binding of 0.5 nM β-endorphin to human lymphocytes was not inhibited by 10 μM naloxone (5). Opiate receptors have also been demonstrated on human phagocytic leukocytes (8). Our studies and those of Hazum *et al.* (5) suggest that lymphocytes may possess a physiologically functional receptor for β-endorphin that is distinct from the μ, δ, or κ opiate receptors defined in other systems (9–11).

Although cellular proliferation as determined by [$^3$H]dThd uptake is increased in cultures containing β-endorphin, the mechanism by which β-endorphin exerts a positive second signal during cellular activation is unknown. The proliferative response of splenic lymphocytes to T cell mitogens is a complex process involving the participation of macrophages and at least two subsets of T lymphocytes as well as soluble factors such as lymphokines that are produced by these cells. Our results, however, do not delineate the cellular target for β-endorphin. The peptide may act directly upon the proliferating T cell blast. Alternatively, β-endorphin could exert its effects indirectly by acting primarily on accessory cells, for example, the interleukin-1-producing macrophage (12) or the nonproliferating subset of T lymphocytes responsible for interleukin-2 production (13). In this respect it is intriguing that preparations of human leukocyte interferon contain material that is reactive with antibodies against β-endorphin and ACTH (14). Cell fractionation and reconstitution experiments should allow us to determine the cellular target(s) for β-endorphin.

In all the experiments described here β-endorphin was pres-

Table 3. Effect of α- and β-endorphin on lymphocyte proliferative responses to Con A, PHA, and LPS/DS

| Peptide | Conc., nM | Con A Uptake, cpm × $10^{-3}$ | Con A % change | Con A P | PHA Uptake, cpm × $10^{-3}$ | PHA % change | PHA P | LPS/DS Uptake, cpm × $10^{-3}$ | LPS/DS % change | LPS/DS P |
|---|---|---|---|---|---|---|---|---|---|---|
| Control | — | 99 ± 14 | — | — | 77 ± 6 | — | — | 73 ± 13 | — | — |
| β-Endorphin | 33 | 203 ± 12 | +105 | <0.001 | 105 ± 10 | +36 | <0.01 | 67 ± 7 | −8 | NS |
| | 3.3 | 192 ± 5 | +94 | <0.001 | 99 ± 5 | +29 | <0.05 | 81 ± 10 | +11 | NS |
| | 0.33 | 164 ± 4 | +66 | <0.001 | ND | — | — | ND | — | — |
| α-Endorphin | 33 | 107 ± 10 | +8 | NS | 82 ± 6 | +6 | NS | 84 ± 11 | +15 | NS |
| | 3.3 | 102 ± 10 | +3 | NS | ND | — | — | ND | — | — |

Spleen cells from a Lewis rat were cultured for 4 days with Con A at 10 μg/ml, PHA at 50 μl/ml, or a mixture of LPS at 100 μg/ml and DS at 50 μg/ml. In addition, the cultures contained no (control) or the indicated concentrations of α- or β-endorphin. Cultures were pulsed with 1 μCi of [$^3$H]dThd on day 3 and harvested 24 hr later. Uptake values are mean ± SD of triplicate cultures. *P* values were calculated by Student's *t* test for the comparison of the [$^3$H]dThd uptake in cultures without endorphin (control) to the [$^3$H]dThd uptake in cultures with α- or β-endorphin. NS, not significantly different from control; ND, not done.

ent throughout the entire 4-day culture period and we do not know, as yet, when β-endorphin is exerting its effect. It may act during the initial 24 hr of culture when cells are recruited into the mitogenic process. If we assume that the level of [$^3$H]dThd incorporation is related to the number of cells that have been induced to proliferate (15), then the increased [$^3$H]dThd uptake seen with β-endorphin may reflect an increase in the number of cells that were induced to proliferate in the presence of the peptide. Alternatively, β-endorphin may act throughout the culture period as a growth or maintenance factor. Interestingly, the high-affinity binding site for β-endorphin on human lymphocytes has been detected only on transformed or proliferating cells (5), suggesting that β-endorphin may act in the later stages of the culture period. Receptors for insulin are also induced in lymphocyte blasts (16, 17), and insulin has a potentiating effect on the action of some mitogens in other systems (18–20). In addition, murine splenic lymphocytes have been reported to have β-adrenergic binding sites (21) and the Con A response of these cells was modulated by β-adrenergic agents (22) in a fashion similar to that seen here with β-endorphin.

Although the enhancement of the Con A response by β-endorphin was seen in many experiments, it was not observed in all experiments. The level of enhancement by different doses of β-endorphin varied considerably between individual experiments. Part of this variation is simply due to differences in the responsiveness of different spleen cell preparations to Con A. The effect of β-endorphin is probably seen only when the cells are suboptimally stimulated by mitogen: if a particular dose of Con A is maximally stimulating the cells, no additional effect of β-endorphin will be detected. Part of the experimental variation in the effect of β-endorphin may also be due to differences in the response of the cells to β-endorphin itself.

We have little knowledge of any physiological functions of β-endorphin in the periphery. At high doses the peptide stimulates adrenal steroidogenesis, skin darkening, and lipolysis (23) and at doses of 50 nM β-endorphin influences the secretion of hormones from the endocrine pancreas (24). Systemic administration of β-endorphin has also been reported to induce hypotension (25) and has been observed to increase the activity of ornithine decarboxylase in the rat kidney (26).

The effects of β-endorphin on lymphocyte proliferation were observed at peptide concentrations that exist *in vivo*. The normal circulating levels of β-endorphin in the rat (1) are very low, approximately 0.33 nM (i.e., 1 ng/ml). Pituitary secretion of both ACTH and β-endorphin is increased dramatically during stress (1). Acute stress induces a rapid increase in the plasma levels of both peptides to as much as 10 ng/ml. Clinically, stress is associated with increased susceptibility to disease, possibly through an alteration in normal immune function. Present evidence suggests that the stress-induced release of pituitary ACTH results in immunosuppression due to the deleterious effects of corticosteroids, released from the adrenal cortex, on lymphocytes (3, 4, 27, 28). In this light, the enhancement of immune function by β-endorphin, which is released from the pituitary simultaneously with ACTH, appears paradoxical. The overall effect of stress on immune function and consequent disease susceptibility, however, may thus depend upon a balance between positive and negative signals originating from the pituitary.

We thank Dr. N. Ling for providing synthetic peptides, Ms. Cindi Biazak for excellent technical assistance, and Ms. Nancy Callahan for secretarial assistance. This work was supported by U.S. Public Health Service Grants AA 17007 and AI/CA 16205 to J.D.F. and National Research Service Award Individual Fellowship MH 07899 to J.M.S.

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