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Research Article

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Immunoreactive β-Endorphin in a Subpopulation of Mouse Spleen Macrophages

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Abstract. Using radioimmunoassay and immunofluorescence with antibodies to β-endorphin (βEP) and ACTH, we have shown that a subpopulation of mouse spleen cells, expressing Mac-1, a marker of macrophage differentiation, contains immunoreactive (ir)-βEP, ir-ACTH, and smaller amounts of presumptive higher molecular weight forms of both. Neither nonadherent spleen cells, nor adherent or nonadherent cells from peripheral blood, contained detectable levels of these peptides. These findings suggest that βEP and ACTH may be synthesized in a subpopulation of spleen macrophages, and are consistent with the possibility that these or related peptides may modulate lymphocyte function in the specific microenvironment of the spleen.

Introduction

A number of recent observations suggest a role for derivatives of pro-opiomelanocortin in the regulation of the immune response. β-Endorphin (βEP)[1] has been shown to enhance lymphocyte proliferative responses to the T cell mitogens concanavalin A and phytohemagglutinin (1). ACTH and α-endorphin likewise have been claimed to inhibit antibody response to T

cell-dependent (sheep erythrocyte) and T cell-independent (dinitrophenyl-Ficoll) antigens (2). Virus-infected lymphocytes have also been reported to produce ACTH- and γ-endorphin-related substances (3, 4). In this study, we examined spleen cells to determine if immunoreactive (ir)-βEP or ir-ACTH were confined to a single cell type.

Methods

Cell culture. Spleens from five mice were minced with a sterile razor blade and incubated at 37°C for 20 min in 10 ml Dulbecco's phosphate-buffered saline (DPBS; Commonwealth Serum Laboratories, Parkville, Victoria, Australia), containing 1% bovine serum albumin (BSA), 7.5 mM glucose, 1.5 mg/ml trypsin (Sigma Chemical Co., St. Louis, MO), and 40 μg/ml DNase (Worthington Biochemical Co., Freehold, NJ). The minced tissues were washed twice with 10 ml Dulbecco's modified Eagles medium (DME) with 5% horse serum and 2.5% fetal calf serum, incubated for 3 min in 10 ml of DPBS/1% BSA (Ca^{++}- and Mg^{++}-free) with 2 mM EDTA, washed three times with Ca^{++}- and Mg^{++}-free DPBS, and dispersed by repeated passage through 12- and 18-gauge sterile stainless steel needles and a siliconized pipette. Cells were washed, resuspended in DME (5 ml/spleen), loaded on 5 ml-chilled Ficoll-Paque (Pharmacia Fine Chemicals, Uppsala, Sweden), and centrifuged at 350 g for 30 min at 4°C. Leukocytes were removed, washed, and resuspended in enriched culture medium (DME with 5% horse serum, 5% fetal calf serum, 2 mM glutamine, 1% nonessential amino acids, 0.1% sodium bicarbonate, and 40 U/ml penicillin). Leukocyte yield was 6 to 8 × 10^7 cells/spleen; viability was >90% on trypan blue exclusion.

Immunofluorescence. Immunofluorescence staining was performed as described previously (5). Briefly, Bouin's fixed (4 h, room temperature [RT]), paraffin-embedded mouse spleen (4-μm sections), or Bouin's fixed 24-h cultures of adherent spleen cells were washed in phosphate-buffered saline (PBS), pH 7.4, and reacted with rabbit antisera against βEP (R56; 1/40-1/80) or ACTH (R1-3; 1/20-1/40) for 24 h at 4°C (sections) or 2 h at RT (adherent cells). Following PBS washes (3 × 10 min), fluoresceinated (fluorescein isothiocyanate [FITC]; Wellcome, Beckenham, U.K.), or tetramethylrhodamine isothiocyanate- (TRITC; Nordic, Tilburg, Holland) labeled, anti-rabbit immunoglobulin (1/20) was added for 30 min at RT. For double labeling studies, fixed cultures were reacted with monoclonal antibody to Mac-1 ([6]; gift of Dr. A. Gomez, Walter

and Eliza Hall Institute, Melbourne; neat supernatant, 20 min, RT), washed, and FITC-labeled anti-rat immunoglobulin (1/20, Wellcome) was added for 20 min at RT. Cells were washed and reacted sequentially with βEP or ACTH antiserum, and TRITC anti-rabbit immunoglobulin, as described above. Cells were mounted and viewed with a Leitz-Dialux 20 microscope equipped with narrow band blue (for FITC-labeled) and narrow band green (for TRITC-labeled) illumination.

Radioimmunoassay. Spleens were collected in 2 ml of 0.1 N HCl at 4°C, and processed for radioimmunoassay as previously published (5, 7). The antiserum (R56) raised against ovine βEP (Peninsula Laboratories, Inc., Belmont, CA) crossreacted 100% with human and ovine βEP, and with βEP1-27, but <0.1% with βEP1-17. The antiserum (R1-3) raised against Synacthen crossreacted 100% with human ACTH1-39 (MRC 74/555), but <0.1% with αMSH (ACTH1-13amide) and with CLIP (ACTH18-39).

Results

Ir-βEP and ir-ACTH were consistently found in spleen extracts from adult male and female Balb/c mice (14.8±1.2 [nanograms per gram wet weight; mean±SEM, n = 6] and 15.2±1.4 for male; 13.5±1.1 and 14.6±1.3 for female). These levels are one-third those in hypothalamus, and about three orders of magnitude lower than those in the pituitary, on a whole tissue basis (7). To ascertain the cell population(s) of origin of the immunoreactive material, mouse spleen cells were dissociated. The dissociated cells comprised 6–9% monocytes, 51–63% T cells, and 37–43% B cells (as shown by reactivity with Mac-1, Thy-1, and anti-mouse immunoglobulin).

Adherent cells were isolated by incubating spleen leukocytes on glass coverslips for 2 h at 37°C. Values of ir-βEP and ir-ACTH were 0.21±0.02 and 0.19±0.02 ng/well at 1.25×10^5 cells/well, and 0.41±0.03 and 0.43±0.04 at 2.5×10^{-5} cells/well. After 24 h, 79–86% of the adherent cells possessed esterase-positive granules, while 68–77% expressed the macrophage differentiation antigen Mac-1.

βEP- and ACTH-like immunoreactivity were demonstrated in 24-, 48-, and 96-h cultures of adherent spleen cells (Fig. 1). Serial dilutions of cell extracts were parallel with standard curves of synthetic βEP and ACTH. In contrast, levels were below detection (ir-ACTH <2 pg/tube; ir-βEP <5 pg/tube) in non-adherent spleen cells; levels were similarly below detection limits in adherent and nonadherent peripheral blood leukocytes, and in conditioned medium from all cell types examined. In adherent spleen cells, levels of ir-βEP closely paralleled those of ir-ACTH, and appeared to increase slowly with time in culture. Levels of ir-ACTH and ir-βEP in fresh medium (1 ml/well) were below detection limit (<20 pg/ml, <50 pg/ml), evidence that the increases shown in Fig. 1 ($\sim$150 pg/well) represent net synthesis rather than merely uptake from the medium.

Extracts of adherent spleen cells were characterized further by Sephadex G50 gel chromatography (Fig. 2). The chromatographic profile of ir-βEP showed three distinct peaks: one in the void volume, a second eluting in the same position as 11,500-mol-wt β-lipotropin (βLPH), and a major peak eluting with synthetic 3,500-mol-wt βEP. For ir-ACTH, two major peaks
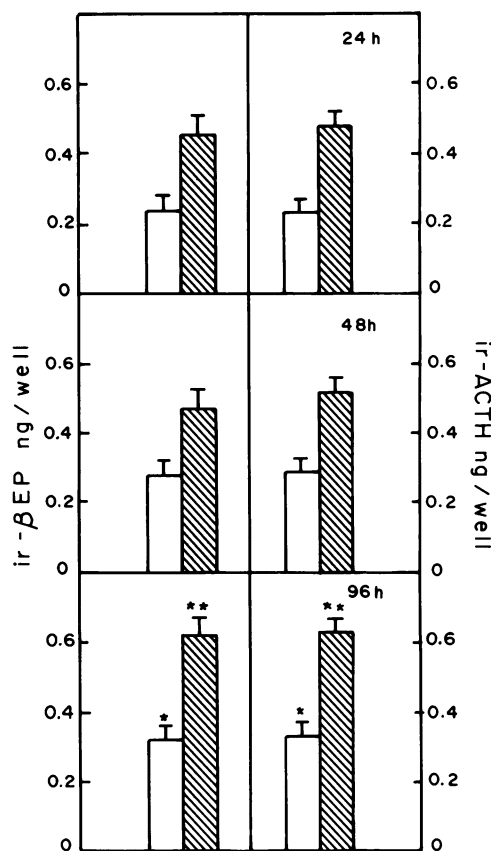

Figure 1. Levels of ir-βEP and ir-ACTH (nanograms per well) in adherent spleen leukocytes cultured for 24, 48, and 96 h. Open bars show values for 1.25×10^5 cells/well, and hatched bars 2.5×10^5 cells/well. Recovery of added synthetic human-βEP (Peninsula Laboratories, Inc., 500 pg in 1 ml of medium) after 24-h coincubation with the culture in newly replaced medium was 91±8% (mean±SEM, n = 4), while recovery for human-ACTH1-39 (MRC 74/555, 500 pg) was 86±5%. Values shown are mean±SEM, nanograms per milligram, n = 6. $*P < 0.05$; $**P < 0.01$ compared with 24-h culture.

were found, one eluting in the void volume and the other with human ACTH1-39, with a less prominent intermediate peak at $\cong$22,000 mol wt.

To determine whether all adherent spleen cells contained ir-βEP and ir-ACTH, or whether immunoreactivity was restricted to a subpopulation, 24-h cultures of adherent spleen cells were reacted by indirect immunofluorescence with antisera to βEP and ACTH. βEP- and ACTH-immunofluorescence-positive cells were found in 12–19% of the adherent cells. Double labeling with antisera to βEP and Mac-1 showed that all βEP-positive cells were also Mac-1 positive. High-power magnification of the positive cells showed that the immunoreactive material was located in cytoplasmic granules (Fig. 3).

Immunofluorescence localization of ir-βEP and ir-ACTH in mouse spleen sections revealed positive cells sparsely dis-
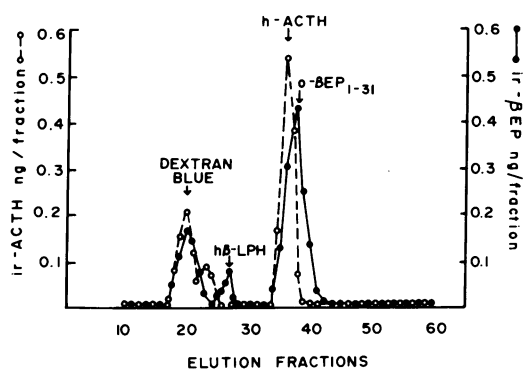
Figure 2. Chromatographic profiles of ir-βEP (●) and ir-ACTH (○) from adherent spleen cell extracts. The extract was lyophilized and reconstituted in 5 ml of 1% formic acid and a 3-ml sample applied to a Sephadex G-50 fine column (1.9 × 54 cm), equilibrated and eluted at RT with 1% formic acid containing BSA (0.2 mg/ml). Fractions (2-ml) were collected, lyophilized, reconstituted in 1-ml assay buffer, and assayed with βEP antiserum (R56) and ACTH antiserum (R1-3). The column was calibrated with blue dextran (void volume), purified human βLPH (gift of Dr. P. Lowry, St Bartholemew's Hospital, London, United Kingdom), synthetic ovine βEP (Peninsula Laboratories, Inc.), and purified human ACTH(1-39) (MRC 74/555).

tributed in the subcapsular region of the tissue (Fig. 4). Companion hematoxylin and eosin staining indicated that these cells were located in the venous sinusoidal region of the red pulp, but not in spleen germinal centers, periarteriolar sheaths, or marginal zones. Only a small number of positive cells were present, but in many cases the staining was so intense that the nuclear image was masked.

Discussion

Although the chemical identity of the peaks of ir-βEP and ir-ACTH was not established, the profile of βEP-like immunoreactivity on gel chromatography strongly suggests the presence of 3,500-mol-wt βEP, 11,500-mol-wt βLPH, and a higher molecular weight species. The profile of ACTH-like immunoreactivity is similarly consistent with the presence of 4,500-mol-wt ACTH, and a higher molecular weight form (or forms). This higher molecular weight species may include a common ACTH and βEP precursor. Taken with the absence of an intermediate molecular weight peak of ir-ACTH, corresponding to a glycosylated pituitary form, this higher molecular weight form argues strongly against the immunoreactivity being a consequence of uptake from plasma.
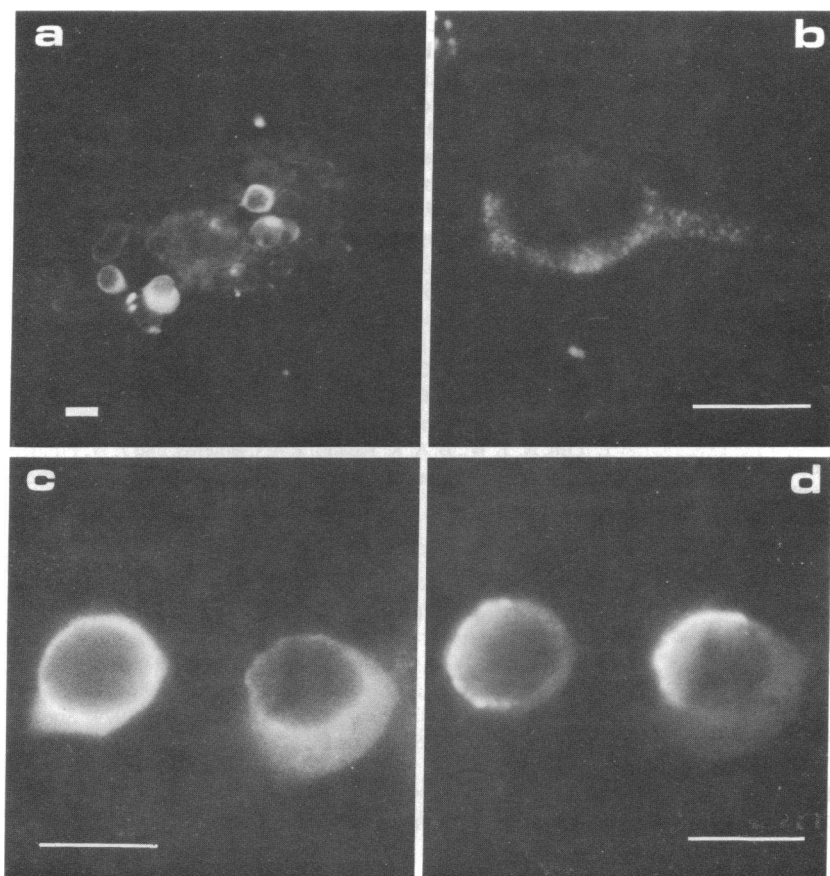

Figure 3. Indirect immunofluorescence microscopy of adherent mouse spleen cells. Specificity of the staining reaction was demonstrated by the absence of specific staining with PBS, with normal rabbit serum, or with βEP antiserum at 1:20 dilution preabsorbed with an equal volume of 5 μg/ml synthetic human-βEP. Staining was unaffected by prior absorption with γ- or α-endorphin, ACTH, met-enk, vasopressin, or substance P at concentrations of 50–100 μg/ml. (*a*) Immunofluorescence-positive cells stained with βEP antiserum (1:40); (*b*) βEP-positive cells, showing localization of immunoreactive material in granules; (*c*) βEP-positive cells; (*d*) same field as in *c* with cells showing reactivity to Mac-1 (8). Scale bars equal 10 μm.
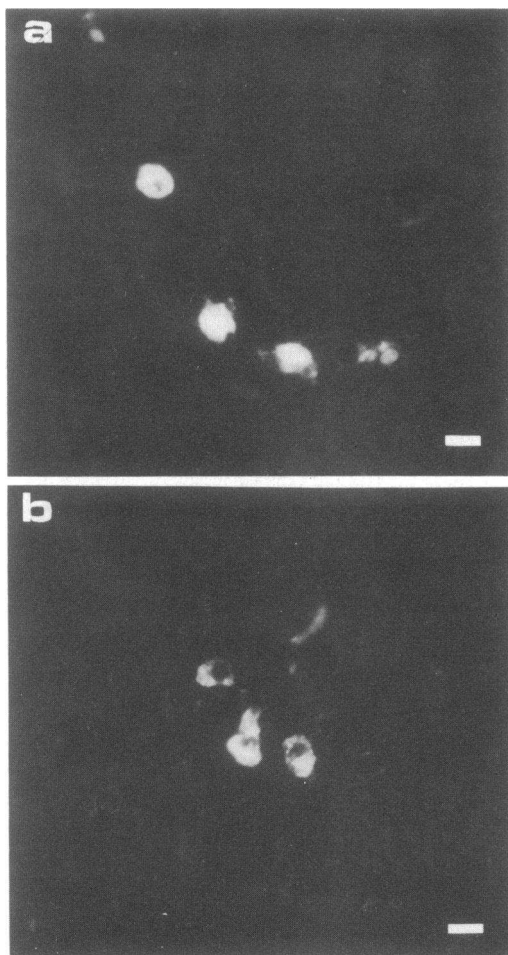
Figure 4. 4-μm section of mouse spleen, fixed in Bouin's solution and reacted with (*a*) βEP antiserum (1:80) and (*b*) ACTH antiserum (1:40). Equivalent immunofluorescence staining was obtained with an anti-βEP antiserum raised by Dr. S. Yen (University of California, San Diego), provided by Dr. John Oliver (Flinders Medical Centre, Bedford Park, South Australia), an antiserum that recognizes different determinants in the βEP sequence (9) from our in-house R56 antiserum. Both our in-house R56 and Dr. Yen's anti-βEP also showed equivalent immunofluorescence staining of sections of rat anterior pituitary, and of cultured rat ovarian follicular cells (5). In addition, indistinguishable immunofluorescence staining was also obtained with an antiserum to ACTH from Immuno Nuclear Corp., Stillwater, MN.

βEP-like material has been detected in the anterior and intermediate lobes of the pituitary (8), hypothalamus (8), pancreas (9), and reproductive tract (5). The extent of ir-βEP distribution outside the central nervous system suggests that βEP, or other products from a common precursor, may have both central and peripheral actions. Studies reporting opiate receptors on lymphoid cells (10), and the regulatory effects of opioid peptides on lymphoid cell function (1, 2), have been interpreted as suggesting a role for pro-opiomelanocortin-derived peptides as modulators of immune responsiveness. In this context, opioid peptides released into the bloodstream from the pituitary gland have been suggested to be responsible for the effects on immune function (1). In our studies, the localization of ir-ACTH and ir-βEP to a subpopulation of spleen cells, and its absence from other spleen cells, is consistent with a paracrine, rather than endocrine, role.

Our results demonstrate that spleen macrophages contain ir-βEP and ir-ACTH, and strongly suggest that such cells synthesize the peptides. While they do not appear to secrete immunoreactive material in vitro under the conditions of culture, such peptides may be released by macrophages in vivo to perform a local immune regulatory role in the spleen microenvironment. Since macrophages are well known to release immune regulatory substances in response to antigen stimulation (11), we are currently investigating the relationship between antigen challenge and the production and release of opioid-like peptides by spleen macrophages.

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