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DIFFERENTIAL EFFECT OF OPIOIDS ON IMMUNOGLOBULIN PRODUCTION BY LYMPHOCYTES ISOLATED FROM PEYER'S PATCHES AND SPLEEN

D.J.J. Carr°, R.T. Radulescu+, B.R. deCosta∞, K.C. Rice∞, and J.E. Blalock°

°Department of Physiology and Biophysics, University of Alabama at Birmingham,
UAB Station, Birmingham, AL 35294
+University of Muenster Medical School, 4400 Muenster, FRG
∞Laboratory of Medicinal Chemistry, NIH, NIDDK, Bethesda, MD 20892

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Summary

The mucosal immune system plays an important role in blocking the penetration of invasive organisms into various mucosal surfaces. Evidence now suggests neuroendocrine peptide hormones have immunomodulatory properties, including the ability to alter mucosal immunity. The potential for opioid compounds and corticotropic hormone (ACTH) to modulate mucosal immune function was investigated. We have found β-endorphin, ACTH, and naltrindole (delta-class opioid receptor antagonist) to significantly suppress concanavalin A-stimulated Peyer's patch lymphocyte immunoglobulin production of IgA, IgG, and IgM isotypes. Oxymorphindole, a delta class opioid receptor agonist, significantly decreased IgM but not IgA or IgG production by the mitogen-stimulated Peyer's patch lymphocytes. Both oxymorphindole and naltrindole modestly reduced interleukin-2 receptor expression of concanavalin A- (Con A)-stimulated splenic and Peyer's patch lymphocytes. Neither compound appreciably affected immunoglobulin production by lipopolysaccharide-stimulated Peyer's patch lymphocytes. Collectively, these results indicate stress-related peptides such as ACTH and opioids may be involved in the regulation of immunoglobulin synthesis by Peyer's patch lymphocytes.

The mucosal immune system constitutes a portion of the immune system which has the distinction of being one of the first lines of defense against invasive microorganisms. The gut-associated lymphoid tissue (GALT) which includes the Peyer's patches, is similar to other lymphoid tissue in that there is compartmentalization into B- and T-cell zones, antigen-presenting dendritic cells, and germinal centers which develop upon antigenic challenge. Interestingly, the GALT possesses more lymphoid tissue than the spleen (1), is continuously exposed to various ingested and endogenous bacterial antigens, and is innervated with peptidergic nerve fibers (2).

Previous work has shown somatostatin, vasoactive intestinal peptide (VIP), and substance P (SP) affect GALT homeostasis. For example, SP has been shown to enhance the proliferative responses and augment IgA, IgG, and IgM synthesis by Peyer's patch lymphocytes (3). Unlike SP, somatostatin appears to dampen cell proliferation and IgA secretion (4). Interestingly, mucosal immune cell trafficking has been shown to be significantly affected by vasoactive intestinal peptide (VIP) (5). All of these events are mediated by specific receptors for these peptides on immune cells (6,7).

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ACTH and opioids have been shown to be potent immunomodulatory agents within the systemic immune system (i.e., peripheral blood and spleen). For example, ACTH has been shown to suppress immunoglobulin production to both T-dependent and T-independent antigens (8). Likewise, the endogenous opioids including endorphins and enkephalins have been shown to dampen immunoglobulin production in the primary immune response (8-10), enhance natural killer cell activity (11), and the generation of cytotoxic T lymphocytes (12), and augment IgG_{2a}-mediated antibody-dependent cytotoxicity by macrophages to sheep red blood cells (13).

The potential for these molecules to influence GALT function seems evident since they have been observed to be associated with lymphoid cells of the gut (14). Likewise, lymphocytes possess both ACTH (15,16) and opioid (17) receptors which appear to be structurally and functionally similar to their neuroendocrine counterparts (18).

With the continuous environmental stimuli in the intestinal milieu as well as the occurrence of the proopiomelanocortin-derived peptides (i.e., ACTH, endorphins) associated with immune cells in the GALT, we investigated possible effects these peptides have on the production of immunoglobulin by Peyer's patch lymphocytes. The results of these findings implicate both ACTH and opioids as immunoregulatory molecules within the GALT.

Materials and Methods

Mice. Male and female C57BL/6J mice were obtained from Charles Rivers Breeding Laboratory, Madison, Wisconsin.

Splenic leukocyte preparation and culture conditions. Murine spleens were aseptically removed from C57BL/6J (6-8 weeks old) and used to obtain splenocytes by mechanical dispersion. Red blood cells were osmotically lysed using 0.84% NH_4Cl solution. Spleen cells (2 X 10^6 cells/2ml) were cultured in 10% fetal bovine serum (FBS, GIBCO, Grand Island, NY) in RPMI media containing 100 U/ml penicillin, 100 μg/ml streptomycin, and 40 U/ml nystatin for 7 days at 37°C in 5% CO_2 atmosphere in the presence of concanavalin A (Con A, 5-8 μg/2 ml). Neuropeptides or opioid compounds were added daily to the cultures until the harvest of the supernates at day 7.

Peyer's patch lymphocyte preparation and culture conditions. Peyer's patches (on average 6-8/intestine) were aseptically removed from the small intestine and mechanically sheared from the surrounding tissue. The nodules containing lymphocytes were incubated in a 37°C shaker in RPMI containing collagenase (1 mg/ml) for 45 minutes. After the incubation period, undigested Peyer's patch tissue was allowed to settle and the supernate containing cells was removed and washed twice with 5% FBS in RPMI. The process was repeated again with the undigested tissue. Cells recovered in the supernates were then placed on a 30% discontinuous Percoll gradient and centrifuged for 10 min to separate lymphocytes from columnar epithelial and glandular tissue (19). The resultant lymphoid population contained less than 10% contamination by epithelial and glandular cells and greater than 95 viability as determined by trypan blue and microscopic evaluation. Peyer's patch lymphocytes (2 X 10^6 cells/2 ml) were cultured in 10% FBS in RPMI media containing 100 U/ml penicillin, 100 μg/ml streptomycin, and 40 U/ml nystatin for 7 days at 37°C in a 5% CO_2 atmosphere in the presence of Con A (5 μg/2 ml) or lipopolysaccharide (6 μg/2 ml). Neuropeptides or opioid compounds were added daily up to the time of harvest.

Enzyme-linked immunosorbent assay (ELISA) detection of immunoglobulin production. Goat anti-mouse polyvalent immunoglobulin in carbonate buffer

(35 mM $NaHCO_3$, 15 mM Na_2CO_3, pH 9.6) was plated (86 μg/well) into wells of 96-well microtiter plates and incubated overnight at 4°C. Unbound antibody was removed by washing wells (3X) with phosphate buffered saline (PBS)-Tween 20 (contains 8.0 g NaCl, 0.2 g KH_2PO_4, 2.9 g Na_2HPO_4, 0.2 g KCL, and 0.5 mls Tween-20/liter, pH 7.4). Supernates from the Con A-stimulated cultures were added (200 μl/well) to each well followed by a 2h incubation at room temperature. After incubation, unbound material was removed by excess washing (3X) with PBS-tween 20. Goat anti-mouse isotype specific, alkaline phosphatase-conjugated antibodies (diluted 1:500 in PBS-tween 20) were added to each well (200 μl) followed by an additional 2h incubation. Following the incubation, excess unbound conjugated antibody was removed by washing wells (3X) with PBS-tween 20. The amount of immunoglobulin bound to the coated wells was determined following the addition of the substrate, p-nitrophenol phosphate and using the values obtained from immunoglobulin (IgA, IgG, IgM) standards and linear regression analysis (20).

Lymphocyte culture for interleukin-2 receptor expression. Lymphocytes were obtained from the spleen of C57BL/6J mice as previously described and cultured (2 X 10^6 cells/2 ml) in 10% FBS in RPMI in the presence or absence of Con A (8 μg/well) in 24 well culture dishes for 72h at 37°C. Wells received either media alone (control), naltrindole (5 X 10^{-8} M), oxymorphindole (5 X 10^{-8} M), or both once every 24h period for 72h. After the incubation period, cells were collected and passed over nylon wool columns to eliminate B cells (21). Cells were enumerated and used for fluorescent activator cell sorter (FACS) analysis. T cell purification was consistently >70% as assessed by FACS analysis using anti-Ig antibody.

FACS analysis for IL-2 receptor expression. Enriched T cell populations (5 X 10^5 - 1 X 10^6 cells/condition) were washed in 1.0 ml PBS containing 0.1% bovine serum albumin (BSA) and 0.05 M NaN_3 and resuspended 0.1 ml PBS-BSA with azide containing an anti-mouse interleukin-2 receptor monoclonal antibody (1:30 final dilution). After a 30 min incubation at 4°C, cells were washed twice with cold (4°C) PBS-BSA and azide. Cells were resuspended with 0.1 ml of rabbit anti-rat immunoglobulin (fluorescein isothiocyanate conjugated) diluted in PBS-BSA plus azide and incubated for 45 min at 4°C. Cells were subsequently washed three times with PBS-BSA plus azide and fixed in a 1% paraformaldehyde solution. Single parameter FACS analysis was performed with a single argon ion laser (Becton Dickinson, Mountain View, CA) operating at 400 mW. Fluorescein staining (5000 cells/FACS exposure) was excited at 488 nm and the emitted light was passed through a long pass filter and analyzed at 515 nm.

FACS analysis for Ig^+ cells. Con A-stimulated lymphocytes, which had been cultured for 7 days, were collected and washed in 1.0 ml PBS-BSA plus azide. Cells (1 X 10^6) were then resuspended in 0.1 ml PBS-BSA plus azide containing goat $(Fab')_2$ anti-mouse immunoglobulin conjugated with phycoerythrin (diluted 1:40) and allowed to incubate for 30 min at 4°C. Cells were subsequently washed three times with PBS-BSA plus azide, fixed in a 1% paraformaldehyde solution, and analyzed by FACS for the percentage of Ig^+ cells. Phycoerythrin staining (5000 cells/FACS exposure) was excited at 488 nm and the emitted light was passed through a long pass filter and analyzed at 580 nm.

Reagents. Concanavalin A, β-endorphin, naloxone, collagenase type IV, goat anti-mouse IgA, IgG, and IgM specific alkaline phosphatase conjugated antibodies, goat antimouse polyvalent immunoglobulin, and p-nitrophenol phosphate were purchased from Sigma Chemical Company (St. Louis, MO). Mouse IgA, IgG, and IgM used as standards were obtained from Zymed Laboratories (San Francisco, CA). The phycoerythrin-conjugated goat $(Fab')_2$ antimouse

immunoglobulin was obtained from TAGO, Inc. (Burlingame, CA). The rat anti-IL-2 receptor antibody was purchased from Boehringer Mannheim (Indianapolis, IN). Naltrindole and oxymorphindole were synthesized as previously described (22).

Statistics. Statistical analysis (student t-test; unpaired, one-tail) was performed using the Statview II program (Abacus Concepts, Inc., Calabascas, CA).

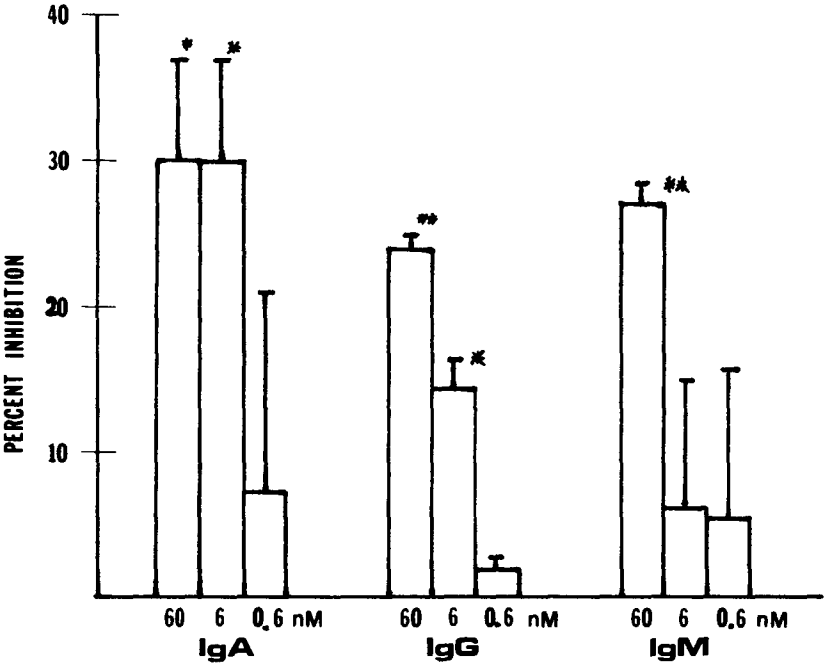

FIG. 1

ACTH suppresses immunoglobulin production by Con A-stimulated Peyer's patch lymphocytes. Peyer's patch lymphocytes (2 X 10^6 cells/culture) were cultured with Concanavalin A (5 μg/2 ml culture) and treated with ACTH (0.6-60 nM) daily for 7 days. Supernates were collected and assayed for immunoglobulin content using an ELISA procedure. Immunoglobulin concentrations were determined using plotted curves generated with known standards. Error bars indicate standard deviation, n = 3. Con A-stimulated lymphocyte supernates contained 200 ng/ml IgA, 100 ng/ml IgG, and 45 ng/ml IgM. *p<.05, **p<.01 student t-test comparing treated cultures versus untreated cultures.

Results

ACTH and β-endorphin suppress Peyer's patch immunoglobulin production. The ability of ACTH to suppress antibody production to T-dependent and T-independent antigen (8) led us to investigate its effects on concanavalin A (ConA)- stimulated Peyer's patch lymphocytes. Previous studies employed Con A-stimulated Peyer's patch lymphocytes to determine the effects substance P and vasoactive intestinal peptide have on immunoglobulin production (4). The present results (Fig. 1) show that ACTH in a dose-dependent fashion significantly suppressed immunoglobulin production of all isotypes.

Likewise, β-endorphin was also investigated for its ability to modulate mitogen-stimulated immunoglobulin production. Similar to ACTH, β-endorphin inhibited concanavalin A-stimulated immunoglobulin production with the mu isotype most dramatically effected (Fig. 2). β-endorphin levels of 10^{-7}-10^{-9} M were consistently immunosuppressive whereas lower concentrations were not effective (data not shown).

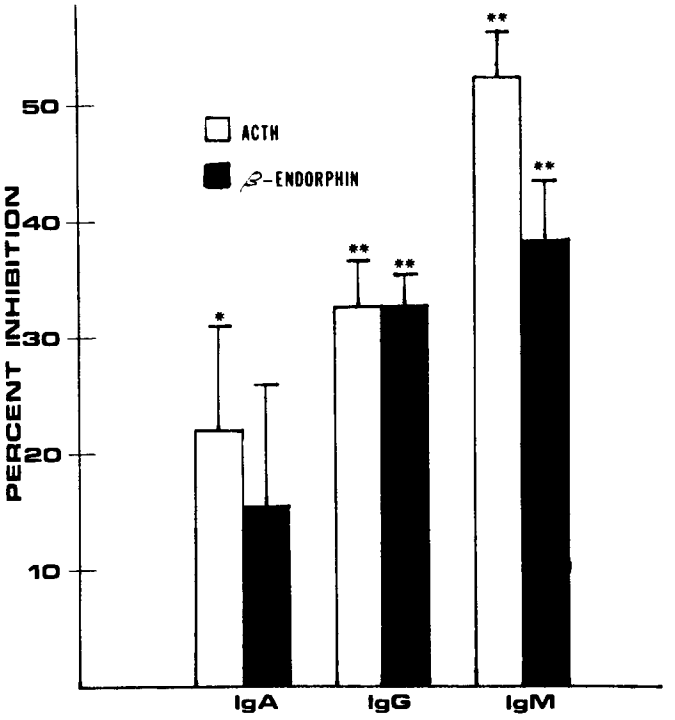

FIG. 2

β-endorphin suppresses immunoglobulin production by Con A-stimulated Peyer's patch lymphocytes. Peyer's patch lymphocytes (2 X 10^6 cells/culture) were cultured with Con A (8 μg/2 ml culture) and treated with β-endorphin (20 nM) or ACTH (60 nM) daily for 7 days. Supernates were collected and assayed for immunoglobulin content using an ELISA procedure. Immunoglobulin concentrations were determined using plotted curves generated with known standards. Error bars indicate standard deviation, n = 3. Con A-stimulated lymphocyte supernates contained 651 ng/ml IgA, 613 ng/ml IgG, and 171 ng/ml IgM. This is a representative experiment of four independent experiments.
*p <.05 (student t-test comparing treated versus untreated lymphocyte cutures; n=3).
**p <.001 (student t-test comparing treated versus untreated lymphocyte cutures; n=3).

Delta-class opioid receptor antagonist, naltrinidole, suppresses Peyer's patch immunoglobulin production. The previous results showed the endogenous opioid peptide, β-endorphin, suppressed antibody secretion. To determine if the effect elicited by β-endorphin was mediated through a classical ligand-receptor interaction, naloxone (opiate receptor antagonist) was added to cultures which received β-endorphin. Whereas naloxone alone had no immunoregulatory charac-

TABLE I
Opioids Modulate Immunoglobulin Production by Concanavalin A (Con A)-stimulated Peyer's Patch Lymphocytes[a]

Treatment	IgA	IgG	IgM
β-endorphin	35.7 ± 2.2[b]	32.3 ± 1.4	38.0 ± 4.5
β-endorphin + naloxone	50.4 ± 5.1	48.3 ± 1.7	56.7 ± 2.1
naloxone alone	10.4 ± 3.7	2.5 ± 0.9	0 ± 3.5
β-endorphin + naltrindole	53.4 ± 2.0	57.6 ± 1.4	63.0 ± 3.5
naltrindole alone	55.1 ± 1.4	58.1 ± 0.9	65.4 ± 2.4
oxymorphindole alone	9.1 ± 3.8	12.3 ± 2.1	32.0 ± 5.3

[a] β-endorphin (20 nM), naloxone (2 μM), naltrindole (200 nM), or oxymorphindole (200 nM) were added daily to cultures of Con A (5 μg/2 ml culture) stimulated Peyer's patch lymphocytes (2 X 10^6 cells/culture) for 7 days. Supernates were collected and assayed for immunoglobulin content using an ELISA procedure. Immunoglobulin concentrations were determined using plotted curves generated with known standards.
[b] Percent inhibition ± standard deviation, n = 3. Con A-stimulated lymphocyte supernates contained 557 ng/ml IgA, 509 ng/ml IgG, and 205 ng/ml IgM.

teristics, the antagonist was unable to block the immunosuppression mediated by β-endorphin (Table I). In fact, naloxone significantly augmented the β-endorphin effect (Table I). Another opioid receptor antagonist, naltrindole, was added to β-endorphin-treated cultures to determine if a potent delta-class opioid receptor antagonist (22) might block the action of β-endorphin. Contrary to naloxone, naltrindole alone dramatically suppressed immunoglobulin production (Table I). Similar to naloxone, naltrindole was unable to block the immunosuppressive effect of β-endorphin. The maximum effect of naltrindole was observed between 10^{-6}-10^{-8} M. Lesser concentrations were ineffective (data not shown). Oxymorphindole, a selective delta-class opioid receptor agonist was also studied to determine if the inhibitory qualities mediated by other opioids was a general trend. Unlike the other opioid compounds tested, oxymorphindole was not suppressive except for the production of IgM which was inhibited by 32% (Table I).

Investigations using these compounds were also conducted using lymphocytes isolated from the spleen. None of the opioid compounds (β-endorphin, oxymorphindole, and naltrindole) significantly enhanced or reduced immunoglobulin production (data not shown).

Naltrindole reduces the expression of cultured Ig^+ cells. The ability of naltrindole to substantially reduce immunoglobulin production by Peyer's patch lymphocytes but not splenic lymphocytes suggested either (i) a reduction in the number of B cells following culture and/or (ii) a decreased synthesis rate of antibody as reflected by the ELISA. In order to assess the former prediction, the percentage of B cells as determined by immunoglobulin (Ig) expression was evaluated by fluorescence-activated cell sorter (FACS) analysis. Naltrindole-treated, Con A-stimulated Peyer's patch lymphocyte cultures expressed far fewer Ig^+ cells (22±6%) versus the untreated, Con A-stimulated cells alone (45±4%) (Fig. 3). There was no difference in Ig^+ lymphocytes taken from spleen cultures treated with (54±4%) or without (56±4%) naltrindole (Fig. 3). Interestingly, oxymorphindole had a similar effect on Ig^+ cells compared to naltrindole (Table II). Moreover, oxymorphindole could partially block the suppressive effect of naltrindole on Peyer's patch lymphoid cultures (Table II).

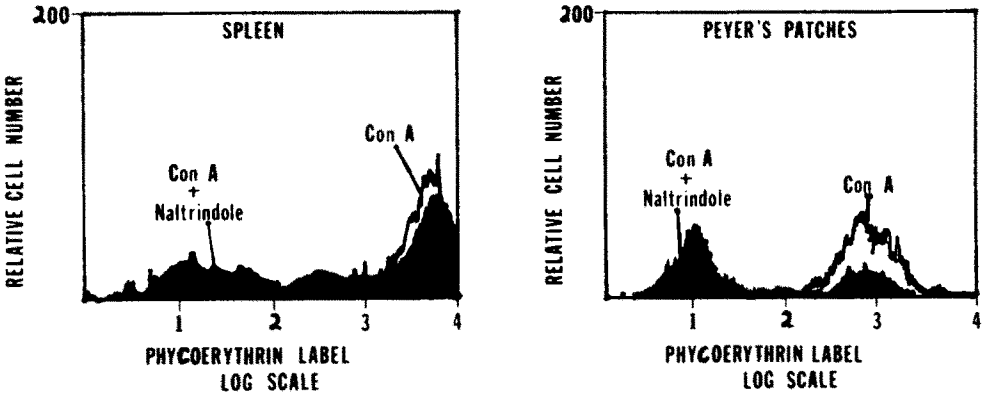

FIG. 3

Naltrindole reduces Ig$^+$ cells taken from Peyer's patches. Naltrindole (50 nM) was added daily to cultures of Con A-stimulated splenic or Peyer's patch lymphocytes (2 x 10^6 cells/culture) for 7 days. Cells were collected, washed, labeled with goat $(Fab')_2$ antimouse Ig conjugated with phycoerythrin, and subsequently analyzed by FACS analysis. This is a representative experiment of three independent experiments.

TABLE II

Naltrindole and Oxymorphindole Treatment Decreases Ig$^+$ Lymphocytes Taken from Peyer's Patches but not Spleen[a]

Treatment	Spleen	Peyer's Patches
Con A alone	56.9 ± 4[b]	52.7 ± 5
Con A + naltrindole	55.4 ± 4	36.7 ± 3
Con A + oxymorphindole	50.9 ± 4	34.7 ± 4
Con A + naltrindole + oxymorphindole	60.5 ± 3	44.5 ± 1

[a]Naltrindole (50 nM) and oxymorphindole (50 nM) were added daily to cultures of Con A (8 μg/2 ml culture)-stimulated splenic or Peyer's patch lymphocytes (2 X 10^6 cells/culture) for 7 days. Cells were collected, washed, labeled with goat $(Fab')_2$ antimouse Ig conjugated with phycoerythrin, and analyzed by FACS analysis.

[b]Percent Ig$^+$ cells ± standard deviation. Numbers represent the mean of three different experiments.

Naltrindole modulates immunocompetence of T lymphocytes. The results obtained in the previous experiments used the T cell mitogen, Con A. To determine if the effect by the opioids was a general phenomena, the B cell mitogen, lipopolysaccharide (LPS), was employed. Unlike Con A-stimulated cultures, LPS-stimulated Peyer's patch lymphoid cultures were not affected in the ability to produce immunoglobulin (predominately IgM) by the various opioid compounds tested including oxymorphindole, and naltrindole (Table

III). The fact that the opioids tested had no regulatory capacity on LPS-stimulated immunoglobulin production by lymphocytes suggested the opioid compounds might mediate their immunosuppressive effects through T cells. Since the expression of the interleukin-2 (IL-2) receptor correlates closely with T cell activation (23), we measured IL-2 receptor expression of Con A-stimulated T lymphocytes treated with naltrindole, oxymorphindole, or both compounds. Both spleen and Peyer's patch lymphocytes' IL-2 receptor expression was altered by these compounds (Fig. 4). IL-2 receptor expression decreased 15% following naltrindole treatment (Fig. 4B), decreased 20% following oxymorphindole treatment (Fig. 4C), but only decreased 7% in the presence of oxymorphindole and naltrindole (Fig. 4D). In Con A-stimulated lymphocytes taken from Peyer's patches, naltrindole decreased IL-2 receptor expression by 11% and oxymorphindole decreased expression by 17% (data not shown).

Table III
Opioid Compounds Do Not Affect Immunoglobulin Production by Lipopolysaccharide (LPS)-stimulated Peyer's Patch Lymphocytes[a]

Treatment	IgA	IgG	IgM
Naltrindole	0 ± 14.0[b]	0 ± 2.5	0 ± 5.2
Oxymorphindole	2 ± 1.7	0 ± 15.4	0 ± 7.9
Oxymorphindole + naltrindole	0 ± 5.0	9.5 ± 6.3	9.7 ± 5.6

[a]Naltrindole (200 nM) and oxymoprhindole (200 nM) were added daily to cultures of LPS (6 μg/2 ml culture)-stimulated Peyer's patch lymphocytes (2X10⁶ cells/ culture) for 7 days. Supernates were collected and assayed for immunogloblin content using an ELISA procedure. Immunoglobulin concentrations were determined using plotted curves generated with known standards.

[b]Percent inhibition ± standard deviation, n = 3. LPS-stimulated lymphocyte supernates contained 267 ng/ml IgA and 11.63 μg/ml IgM. IgG concentrations were not determined.

Discussion

The ability of opioids to suppress Peyer's patch lymphocyte immunoglobulin production but not splenic lymphocyte immunoglobulin production following Con A-stimulation indicates the possibility of a unique population of immune cells which take up residence in the GALT. This selective and separate lymphoid population may possess structurally different opioid-like receptors on their surface and, thus, respond differently to the compounds. Precedence for unique populations of lymphocytes in the GALT has previously been described (24).

Opioid compounds and ACTH were added daily to cultures to maximize the effect since the half-life of these molecules is short. Moreover, if the substances were added as one bolus on day 0 of culture, only those which were given in high concentrations (10^{-6} M) effected immunoglobulin production 7 days later (unpublished observation). Using B cell and T cell mitogens, the opioid compounds were shown to mediate the immunosuppression indirectly through T cells. Consequently, IL-2 receptor expression which mirrors T cell proliferation (23) was investigated using the naltrindole- and oxymorphindole-treated cells. Since both compounds reduced IL-2 receptor

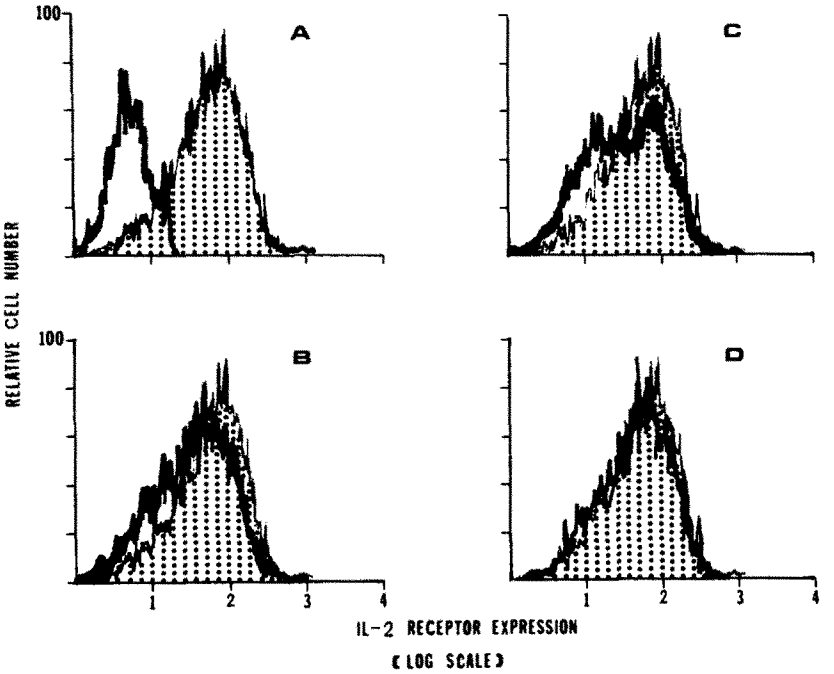

FIG. 4

Oxymorphindole and naltrindole modestly decrease IL-2 receptor expression on splenocytes. Spleen cells (2 X 10^4 cells/culture) were cultured with Con A (8 μg/culture) and treated with oxymorphindole (50 nM), naltrindole (50 nM), or both compounds daily for 3 days. Following the treatment regimen, cells were collected, washed, and assessed for IL-2 receptor expression using an anti-IL-2 receptor antibody. A. Shaded: Con A-stimulated cells. Unshaded (thick line) Secondary antibody only (rabbit anti-rat). B. Shaded: Con A-stimulated cells. Unshaded (thick line): naltrindole + Con A-stimulation. C. Shaded Con A-stimulated cells. Unshaded (thick line): oxymorphindole + Con A-stimulation. D. Shaded: Con A-stimulated cells. Unshaded (thick line) oxymorphindole, naltrindole + Con A-stimulation. Control, untreated lymphocytes expressed 36%. IL-2 receptor expression above cells stained with secondary antibody only. This is a representative experiment of three independent experiments.

expression by spleen or Peyer's patch lymphocytes, no definitive conclusions can be drawn with regard to the differences observed in immunoglobulin production between the two anatomically distinct lymphoid organs. Although the expression of the IL-2 receptor on these cells which parallels T cell activation may have a role in the immunosuppressive effects of the opioid compounds, other cytokines (e.g., IL-4 and IL-5) may also be regulated by the opioids.

The observations that the delta-selective antagonist, naltrindole, possessed agonist activity is not unique in the immune system since naloxone has previously been reported to have agonist activity (25). However,

naltrindole has previously been shown to block opioid-mediated enhancement of splenic natural killer cells without any residual effect alone (26). Since the effects elicited by oxymorphindole on the suppression of immunoglobulin production were less than those obtained by naltrindole, these results are consistent with oxymorphindole being a partial agonist (27). Interestingly, oxymorphindole could partially antagonize the effects of naltrindole indicating that both compounds are directed toward at least one common recognition site. Alternately, naltrindole may have a greater affinity to the binding site of the opioid-like receptor compared to oxymorphindole. Still another possibility may reside with the fact that lymphocytes isolated from Peyer's patches or spleen are heterogenous with heterogeneity of the opioid receptors present. Therefore, lymphocyte subpopulations which synthesize a specific cytokine may or may not express the appropriate opioid receptors and, thus, may or may not be responsive to the ligands.

Collectively, the data point to the immunoregulatory characteristics of opioid compounds on the mucosal immune system. Evidence indicating the existence of opioids in the GALT as well as peptidergic innervation of the GALT (2) suggests the endogenous opioid peptides (i.e., β-endorphin and Met-enkephalin) influence mucosal immune homeostasis. This relationship could clearly play a role in intestinal maladies especially under stressful conditions. Finally, these results illustrate the interaction of the immune and neuroendocrine systems through what appears to be a selective localization of specialized immune cells (spleen versus Peyer's patch) which respond differently to the same neuroendocrine hormones.

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