NEUROPEPTIDES (SP AND CGRP) AUGMENT IL-1 BETA PRODUCTION IN HSV-INFECTED MACROPHAGES

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ABSTRACT

Neuropeptides, possessing specific and functional receptors on various cells of the immune system, have the potential to regulate immune responses; and the macrophages as important components of defense against various agents, are at their influence. In this study the effect of neuropeptides substance P (SP) and calcitonin gene-related peptide (CGRP) on IL-1beta production by herpes simplex type-1 (HSV-1)-infected and also uninfected mouse peritoneal macrophages were considered. Each neuropeptide separately has upregulated IL-1beta production by HSV-1 infected macrophages with the greatest effect at the concentrations of 10^{-9}M for both SP and CGRP, but no synergistic effect on IL-1 production has been observed in the presence of both neuropeptides at optimal concentrations. IL-1β production by uninfected macrophages was also moderately enhanced in the presence of each neuropeptide, but not in the presence of both neuropeptides simultaneously. It can be concluded that IL-1β production, which is part of macrophage mediated inflammatory response to HSV-1, is enhanced by specific doses of neuropeptides.

Keywords: Substance P, Calcitonin Gene-Related Peptide, Macrophage, Herpes Simplex Virus Type -1, Interleukin-1beta.

INTRODUCTION

Substance P (SP) and Calcitonin Gene-Related Peptide (CGRP) are neuropeptides found in sensory neurons with 11- and 37-amino acids respectively.[1,2] These neuropeptides are mediators of neurogenic inflammation.[3] There is increasing evidence that neuropeptides have regulatory effects on the immune system.[4,5] Various immunologically important cells, including lymphocytes and macrophages, have neuropeptide receptors,[6-10] which render their functions to be under the influence of these mediators. Various macrophage functions including production of cytokines, nitrogen and oxygen metabolites are affected in the presence of neuropeptides.[11-13] At almost all of these studies, the macrophages are stimulated by the neuropeptide itself or together with lipopolysaccharide (LPS) as an additional stimulator. However, the effects of neuropeptides on the macrophage response to a viable infectious agent such as herpes simplex virus type-one (HSV-1), remains unknown. HSV-1, a very common virus in human beings, is able to remain latent in sensory ganglions (14). Macrophages are important components of defense against HSV-1 as shown by macrophage depletion[15,16] or other treatments.[17-20] They are able to degrade viral

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particle[21] and to produce different cytokines and various metabolites[22-24] in response to them. In this study the effects of neuropeptides SP and CGRP (individually and concurrently) on production of pro-inflammatory cytokine Interleukin-1 beta in both HSV-1-infected and uninfected mouse peritoneal macrophages were considered.

MATERIALS AND METHODS

Virus

KOS strain of HSV-1 was propagated on Vero cell line (NCBI C101) and stored at -70°C. The virus titer was determined by plaque forming assay on Vero cells.[25] Briefly, 3×10^5 Vero cells were cultured on 24 well microplates to form a monolayer. Then the supernatants were removed and various dilutions of virus[10-2-10-9] in 200 βl RPMI medium (Sigma, USA) containing various dilutions of virus (10^2PFU-10^9PFU) were added in duplicate. After one hour adsorption, the remaining media were removed and medium containing 0.5% agarose and 5% FCS (Seromed, Germany) was added. After 72 hours the plaques were fixed with formaldehyde (10% in PBS) and counted after staining with Crystal violet.

Neuropeptides

The lyophilized neuropeptides SP and CGRP (Sigma, USA) were dissolved in injectable-grade deionized water (at stock concentrations of 2×10^{-4}M and 2×10^{-5}M respectively) and after aliquotion were stored at -70°C.

Macrophage culture and infection

Peritoneal macrophages obtained from 8-10 weeks old, male BALB/c mice using cold normal saline (injectable grade), were washed twice and resuspended in DMEM medium (Sigma, USA) supplemented with 10% FCS (Seromed, Germany). The cells were added to 96-well tissue culture treated microplates (Falcon, USA) 4×10^5/well or 6×10^5/well (in different experiments) and after 2 hours incubation at 37°C and 5% CO2 were washed with warm normal saline (injectable grade, 37°C) to remove non-adherent cells. About half of the cells (54% +/- 4) were removed as determined by counting the washed cells from six wells and more than 95% of adherent cells were macrophages as shown by Giemsa staining. After washing the non-adherent cells, the remaining macrophages were infected with the virus at a multiplicity of infection (MOI) of three. After one-hour absorption of the virus, the media (supplemented with 10% FCS) containing different concentrations of neuropeptides were added to final volume of 250βl and incubated at 37°C, 5% CO2. All media and solutions used were endotoxin free. All tests with macrophages were repeated twice or three times.

Cytokine assay

The amount of cytokine IL-1beta was determined 12 hours after infection in the supernatants of each culture by sandwich ELISA using avidin-biotin system (Biosource, Belgium), and TMB (3, 3', 5, 5'-tetramethyl benzidine) as substrate. The absorbance was read at 450nm.

Statistical Analysis

Data were analyzed using one-way analysis of variance (ANOVA) followed by Student T test and presented as mean +/- SEM.

RESULTS

Determining the optimal concentration of SP and CGRP for induction of cytokine release:

Peritoneal macrophages ($2x10^5$/well) were exposed to HSV-1 virus (KOS strain) at virus to cell ratio (multiplicity of infection or MOI) of three. After one-hour adsorption of the virus, the HSV-infected macrophages were incubated with SP at concentrations of 10^{-10}M-10^{-5}M, CGRP at concentrations of 10^{-11}M-10^{-6}M or medium alone (control). The supernatants were removed after 12 hours for IL-1beta assay. As can be observed in Fig. 1, IL-1 beta production is upregulated significantly in the presence of SP at concentrations of 10^{-10}M-10^{-5}M (with p values of <0.0006, <0.0015, <0.00002, < 0.00006, <0.00007, <0.002 respectively). Maximal response was obtained at concentration of 10^{-9}M. Therefore, this concentration of SP was chosen for further studies. Increasing effect of CGRP on cytokine production was significant only at concentrations of 10^{-8}M and 10^{-9}M ($p<0.02$). Maximal response was obtained at 10^{-9}M CGRP (Fig. 1), therefore this concentration was chosen for further studies.

Effect of neuropeptides SP and CGRP on IL-1beta secretion by infected and uninfected macrophages

Peritoneal macrophages ($3x10^5$/cell) were infected with HSV-1 virus (KOS strain) at virus to cell ratio or multiplicity of infection (MOI) of three. Neuropeptides at selected concentration (10^{-9}M) or medium alone (control groups) were added to HSV-infected and uninfected macrophages and cytokine assay of supernatants was performed 12 hours after infection.

IL-1 secretion was significantly upregulated in the presence of 10^{-9}M SP or 10^{-9}M CGRP both in infected (Fig. 2A) and uninfected groups (Fig. 2B). Substance P at selected concentration has induced more than three times increase ($p<3\times10^{-12}$) in IL-1 production by infected macrophages (comparing to infected control), and about 70% increase in IL-1 production ($p<0.00003$)

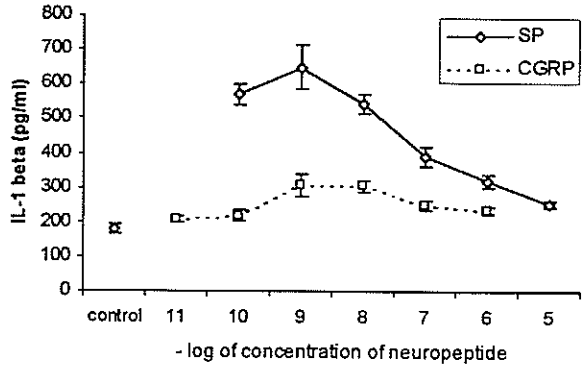

Fig. 1. IL-1 beta production was assayed by ELISA method in supernatants of $2x10^5$ macrophages 12 hours after infection with KOS strain of HSV-1 (MOI=3). The FCS supplemented medium containing different concentrations of neuropeptide substance P (10^{-10}M-10^{-5}M) or CGRP (10^{-11}M-10^{-6}M) was added to culture after one-hour absorption of the virus (controls were medium without neuropeptide). The results are shown as mean +/- SEM (each point is the mean of four separate wells).

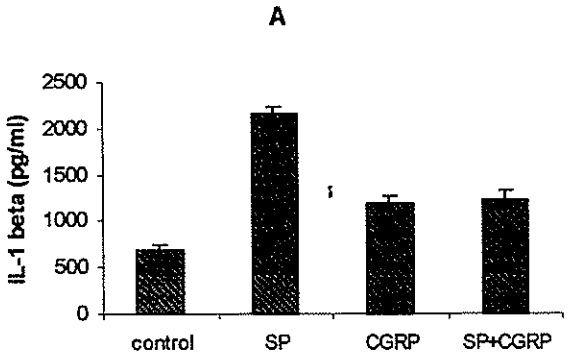

Fig. 2A.

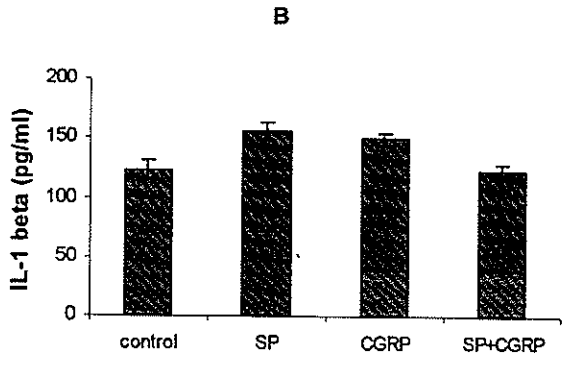

Fig. 2B. IL-1β production of HSV-infected (A) and unifected (B) mouse peritoneal macrophages at the presence of 10^{-9}M substance P, 10^{-9}M CGRP, SP plus CGRP (each at final concentration of 10^{-9}M), or none (controls). The number of macrophages was 3×10^5\well and virus to macrophage ratio was three (MOI= 3). ELISA assay was performed 12 hours after infection. The results are shown as mean +\-SEM (each point is the mean of eight separate wells for infected and five for uninfected macrophages). This experiment has been repeated three times.

was observed at the presence of selected concentration of CGRP. In the presence of both neuropeptides at 10^{-9}M, increasing effect was approximately similar to CGRP alone and about 70% more than control ($p<0.0004$). SP and CGRP up-regulated IL-1beta production in uninfected macrophages as well (about 11% with $p<0.03$ and 9% with $p<0.02$ respectively) but no enhancing effect on IL-1 beta production of uninfected macrophages was observed at the presence of both cytokines simultaneously (Fig. 2B).

Cytokine production by HSV-infected macrophages in comparison to uninfected ones in the presence or absence of the same neuropeptide has also shown significant differences. Infection of macrophages has induced several times more IL-1beta production comparing to uninfected groups. IL-1 beta release by HSV-1-infected macrophages was about 15,8, and six times more than uninfected macrophages in SP, CGRP and control groups respectively (with p values smaller than 1×10^{-8}).

DISCUSSION

Macrophages respond to infectious agents in various ways including cytokine production. Amongst them pro-inflammatory cytokines are very important, with both beneficial and potentially harmful effects. Macrophages are among the essential components of defense against HSV-1 as shown in various studies.[15,17,19] They can produce pro-inflammatory cytokines including IL-1beta and TNF in response to the virus, which are measurable within the first hours of infection by HSV-1 and decline within 24 or 48 hours.[22-24,26] In this study, supernatants of HSV-1-infected macrophages were removed for cytokine assay 12 hours after infection and the results indicated that IL-1 beta had produced in response to HSV-1, had been several times more than uninfected macrophages (last paragraph of results). After 48 hours, however, IL-1beta was not detectable in the supernatants (data not shown).

As mentioned before, macrophages have functional receptors for various neuropeptides including SP and CGRP.[7,9,27] These neuropeptides are present in sensory nerves all over the body,[28,29] they are mediators of neurogenic inflammation[3] and their release may be trig-

gered or increased by diverse stimulators.[30,31] Thus the assumption that these neuropeptides influence immune responses could be realizable in various conditions. Increasing reports about the regulatory role of sensory neuropeptides (including SP and CGRP) on immune system and also studies in which HSV-1-induced mortality in mice is reduced in capsaicin pretreated mice (32, 33), encourage the guest in understanding the regulatory effect of neuropeptides on responses of immune system particularly macrophage response to HSV-1.

There are numerous studies, which indicate that substance P has regulatory effects on macrophage cytokine production. Substance P-mediated induction of IL-1beta release from both LPS-stimulated macrophages[13,34] and unstimulated macrophages[13] has been reported. In several studies, however, the ability of SP to enhance IL-1beta production had been observed only in the presence of another stimulator, which was (LPS in almost all cases), and it had no effect on unstimulated macrophages.[36,40] It should be noted that the conditions of the experiments and the sources of macrophages used in these studies were dissimilar, which might explain diversity of results.

We have observed an enhancing effect for SP on cytokine production. In our study, IL-1beta production in both HSV-infected and uninfected macrophages were enhanced significantly in the presence of various concentrations of SP (Figs. 1). The effect of SP on uninfected macrophages is minimal when compared with HSV-infected macrophages (Fig. 2). IL-1beta release by HSV-infected macrophages were augmented three times in the presence of SP (from 692 pg/ml to 2156 pg/ml), but in uninfected groups only 11% increase (from 123 pg/ml to 157 pg/ml) was observed. It may be stated that activated macrophages have a higher potential to augment cytokine release in response to neuropeptides, which may be attributed to various explanations including the enhanced expression of neuropeptide receptors on activated macrophages.[39] According to more recent studies, substance P is able to activate p38 mitogen-activated protein kinase within 5-10 min after treatment.[40] Substance P induction of nuclear factor-kappaB via cytoplasmic protein kinase activation is an independent and important mechanism[41] and both pathways are able to mediate cytokine production. Therefore, substance P may have a synergistic effect in stimulating macrophage cytokine production along with other infectious agents like HSV-1. Since the necessary precautions regarding LPS or endotoxins in the culture media have been taken, on the basis of the reagents and media employed, we can conclude that our cultures did not contain endotoxin. Therefore the results obtained with necessary controls were reproducible and indicated the effect of neuropeptides under the laboratory and test circumstances described.

There are not many reports regarding the effect of CGRP, on IL-1beta production by macrophages. Recently, Cuesta and coworkers have reported an increasing effect of CGRP on basal secretion of IL-1beta (and also TNF and IL-6) from human peripheral blood mononuclear cells at concentrations of $0.3\text{-}1\times10^{-6}$ M of CGRP (42). We have observed that CGRP significantly enhances IL-1beta secretion in response to HSV-1 (from 692 pg/ml to 1185 pg/ml), its effect on IL-1beta production by uninfected macrophages has been minimal (about 9%) but significant ($p<0.02$). According to reports concerning the mechanism of action of CGRP in cells other than macrophages, including CGRP-induced AP-1 binding activity in the nucleus of B cells;[43] and cAMP-dependent activation of JNK in neuroblastoma cells,[44] and also reports declaring an enhancing effect of CGRP on nitric oxide production[45] or IL-6 release from mouse peritoneal and bone marrow macrophages,[27,46] it can be concluded that this neuropeptide is able to regulate expression of specific genes via mechanisms different than SP. In the presence of optimal concentrations of both neuropeptides, an enhancing effect similar to CGRP alone has been observed in infected macrophages (from 692 pg/ml to 1231 pg/ml). Since there are reports indicatng that NF-kappa B activation, the important mechanism of SP to induce cytokine production, is inhibited by CGRP (47) it can be postulated that CGRP is able to neutralize out the effect of SP in this condition. In uninfected macrophages, no significant effect has been observed in the presence of both neuropeptides, although each neuropeptide alone has been able to mildly enhance IL-1beta release by them.

In this study, both neuropeptides enhanced pro-inflammatory cytokine (IL-1 beta) production in response to HSV-1. This localized effect may, in certain organs such as the eye, aggravate the injuries caused by the virus itself. These studies shed some light on the complex interactions between mediators of the nervous and immune systems. Further research on the signaling and biochemical pathways involved as well as on the effect of other neuropeptides on various parameters of macrophage function is necessary to elucidate the role of these networks in regulating the immune response.

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