Is the pretreatment effect of low dose Freund's adjuvant on adjuvant arthritis due to an activation of the nervous system?

I. Bileviciute¹, E. TheodorssoW, T. Lundeberg¹,²

¹Department of Physiology and Pharmacology, Karolinska Institute
²Department of Clinical Chemistry, University Hospital, Linköping
³Department of Rehabilitation and Physical Medicine, Karolinska Hospital, Stockholm, Sweden

Summary It has been recently shown that pretreatment with a low dose of Freund's adjuvant decreases the severity of adjuvant arthritis in rats. To study the involvement of the central and peripheral nervous systems in the pretreatment effect, concentrations of substance P (SP)-, neurokinin A (NKA)-, calcitonin gene-related peptide (CGRP)- and neuropeptide Y (NPY)-like immunoreactivities (LI) were measured in the cerebrospinal fluid, plasma and synovial fluid 2 and 24 h after a single s.c. injection of 0.05 mg Freund's adjuvant. Increased concentrations of CGRP-LI were found in the cerebrospinal fluid, plasma and synovial fluid. NPY-LI was decreased in the cerebrospinal fluid while NKA-LI was decreased in plasma. In the synovial fluid, SP-LI was increased at 24 h and NKA-LI was increased at 2 h following treatment.

Our results indicate that part of pretreatment effect of low dose of subcutaneous Freund's adjuvant in the rat may be attributed to neurogenic mechanisms.

INTRODUCTION

It is well known that subcutaneous (s.c.) inoculation of concentrated complete Freund's adjuvant (FA), containing more than 0.5 mg Mycobacterium antigen, induces adjuvant arthritis (AA) in rats. The AA is characterized by a systemic inflammation with disseminated joint inflammation. This inflammation has been shown to be mediated through immunological mechanisms, and is commonly used as a model of human rheumatoid arthritis. Also, both the central and peripheral nervous systems contribute to the severity of AA in rats.

We have reported that injection of 0.05 mg complete FA into the right knee joint of the rat induced significant changes in the neuropeptide concentrations in the cerebrospinal fluid (CSF), plasma and synovial fluid (SF) after 2, 6 and 24 h indicating an early activation of the central and peripheral nervous systems in experimentally induced monoarthritis.

Recently it has been reported that pretreatment with a low dose of complete (0.1 mg) or incomplete FA (0.1 mg) before the induction of AA reduced the severity of polyarthritic lesions and the changes seen in the nervous system. The pretreatment effect has been attributed to the activation of the immune system and/or to the activation of the hypothalamo-pituitary-adrenal axis.

The aim of the present study was to assess the effect on the central and peripheral nervous systems of 0.05 mg complete FA inoculated subcutaneously by measuring concentrations of substance P (SP), neurokinin A (NKA), calcitonin gene-related peptide (CGRP)- and neuropeptide Y (NPY)-like immunoreactivity (LI) in the CSF, plasma and SF 2 and 24 h after injection.
MATERIALS AND METHODS

The study was carried out on 33 male albino Sprague-Dawley rats, weighing 250–300 g, allowed to become habituated to the laboratory for at least 7 days before experimentation. All rats were maintained under identical conditions which included alternate cycles of 12 h light and 12 h darkness, temperature of 24°C, 60% relative humidity, and food and water and libitum. On the day of the experiment the rats were anaesthetized with chloralhydrate (0.4 g/kg) i.p. The rats were grouped as follows: 16 rats were inoculated with 0.05 ml complete FA (1 mg/ml, Sigma) s.c. at the base of the tail and 16 rats were given 0.05 ml saline s.c. at the base of the tail.

At 2 or 24 h following the FA/saline injection the rats were again anaesthetized with chloralhydrate i.p. The skin overlying the knees was shaved and a 2 cm longitudinal skin incision exposing the knees was made bilaterally. A 27 gauge needle was inserted into each knee joint from the medial aspect and a 22 gauge needle from the lateral side. Both knee joints were simultaneously perfused with 0.9% saline through the 27 gauge needle using a syringe pump set at 0.2 ml/min and the SF was collected through the 22 gauge needles. Perfusion was carried out for 10–15 min, resulting in a 1.5–2 ml perfusate. For collection of CSF, rats were placed in a stereotactic frame. The atlanto-occipital membrane was exposed by retracting the overlying muscles and samples of 80–150 μl of CSF were obtained through a 27 gauge needle connected to a 1 ml syringe via a polyethylene tube. Blood (1.5–4.5 ml) was collected by a puncture of the heart with a vacutainer tube containing heparin 143 iu, and thereafter Trasylol 500 iu ml⁻¹ was added. The samples were centrifuged and plasma was removed and frozen.

All samples were rapidly cooled and stored at −45°C until analysis. Samples from the SF were extracted using a reverse-phase C18 cartridge (Sep Pak, Waters) and analysed using competitive radioimmunoassays. Radioimmunoassay of SP (SP-LI) was analysed using antiserum SP2 which reacts with SP and SP sulfoxide, but not with other tachykinins. Intra- and interassay coefficients of variation were 7% and 11%, respectively. Neurokinin A (NKA-LI) was analysed using antiserum K12 which reacts with NKA (100%), NKA (3–10) (48%), NKA (4–10) (45%), neurokinin B (26%), neuropeptide K (61%) and eledoisin (50%), but not with SP. Intra- and interassay coefficients of variation were 7% and 12%, respectively. Calcitonin gene-related peptide (CGRP-LI) was analysed using antiserum CGPR8 raised against conjugated rat CGRP. HPLC-purified 125 I-Histidyl rat CGRP was used as radioligand, and rat CGRP as standard. The cross-reactivity of the assay to SP, neurokinin A, neurokinin B, neuropeptide K, gastrin, neurotensin, bombesin, neuropeptide Y and calcitonin was less than 0.01%. Cross-reactivity toward rat CGRP α and β was 100% and 120%, respectively. Intra- and interassay coefficients of variation were 8% and 14%, respectively. Neuropeptide Y (NPY-LI) was analysed using antiserum N1 which cross-reacts with avian pancreatic polypeptide 0.1%, but not with other peptides. Intra- and interassay coefficients of variation were 7% and 12%, respectively. The lower detection limit in all extracted samples was 0.1 fmol/ml for all peptides.

Statistical analyses were carried out using the SPSS software (release 6). Neuropeptide concentrations in the CSF, plasma and SF of FA- and saline-treated rats were examined and compared using analysis of variance followed by multiple comparison procedures (Bonferroni test). P values less than 0.05 were considered significant. Correlation analysis was carried out by using Pearson correlation test.

RESULTS

Cerebrospinal fluid

In CSF a significant increase in CGRP-LI concentrations was detected at 2 and 24 h as compared to the controls (Fig. 1). Concentrations of CGRP-LI were significantly increased from median concentration <0.1 fmol/ml to 664.0 fmol/ml at 2 h and from <0.1 fmol/ml to 1076.8 fmol/ml at 24 h. Concentrations of NPY-LI were significantly decreased from median concentration 3063.5 fmol/ml to 41.9 fmol/ml at 24 h. Concentrations of SP-LI had a strong tendency to be decreased at 2 h and 24 h as compared to the saline-treated animals, however, not reaching significant differences (Fig. 1).

Plasma

In plasma, CGRP-LI was found to be increased from median concentration 2.9 fmol/ml to 11.1 fmol/ml at 2 h and from 2.6 fmol/ml to 8.1 fmol/ml at 24 h as compared to saline-treated animals (Fig. 2). Concentrations of NKA-LI were measured to be decreased from median concentration 8.1 fmol/ml to 3.3 fmol/ml at 2 h and from 75 fmol/ml to 1.2 fmol/ml at 24 h. There were no changes in the concentrations of SP- and NPY-LI at 2 or 24 h as compared to the rats treated with saline (Fig. 2).

Synovial fluid

In the right and left joint SF CGRP-LI was increased bilaterally at 2 h (from median concentration <0.1 fmol/ml to 2.5 fmol/ml in the right joint and from 0.2 fmol/ml to
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2.5 fmol/ml in the left) (Fig. 3). A significant increase in CGRP-LI was also measured at 24 h (from median concentration <0.1 fmol/ml to 2.6 fmol/ml in the right joint and from <0.1 fmol/ml to 3.1 fmol/ml in the left) (Fig. 4). NKA-LI was increased from median concentration <0.1 fmol/ml to 3.3 fmol/ml at 2 h in the left joint as compared to the controls, the right joint and to the rats examined at 24 h, Fig. 3, Fig. 4. No significant changes in NKA-LI of SF were found at 24 h when comparing to the control group. SP-LI was increased at 24 h in the right knee joint SF from median concentration <0.1 fmol/ml to 0.9 fmol/ml in the right knee joint and from <0.1 fmol/ml to 1.6 fmol/ml in the left (Fig. 4). There were no significant changes in the concentrations of NPY-LI in the SF either at 2 or 24 h.

Correlation analysis was performed considering neuropeptide-LI in plasma vs CSF, CSF vs SF of the right and left knee joints and plasma vs SF of the right and left knee joints. No correlation was found between neuropeptide-LI examined in different tissues.

DISCUSSION

The results of the present study show that administration of a single dose of complete FA results in a significant release of neuropeptide-LI in the CSF, plasma and SF. This would indicate that part of the arthritis reducing effect of pretreatment with a low dose of FA is mediated through sensory nerves. It is possible that the reduced severity is due to an interaction between the immune and nervous system. Lymphoid structures such as thymus, liver, lymph nodes and femoral lymphatic vessels have been reported to be innervated by...
peptidergic nerves.\textsuperscript{18-20} Recently, Fink & Weihe suggested that neuropeptides released in lymphoid structures might modulate the activity of immune system.\textsuperscript{21} In the present study there was marked increase of CGRP in CSF, plasma and SF at 2 and 24 h. This is interesting to note as anti-inflammatory effects of CGRP have been demonstrated recently.\textsuperscript{22-24}

It can be also speculated that an activation of the nervous system after pretreatment with a low dose of FA may be important in the immunization against neurogenic inflammation taking place in AA-induced rats.\textsuperscript{5-8} Neuropeptides such as SP, NKA and CGRP have been demonstrated to play a significant role in neurogenic inflammation.\textsuperscript{25-28} It is interesting to note that immunization with antibodies against SP and CGRP reduced the neurogenic inflammation in rats,\textsuperscript{29} and antibodies against CGRP reduced the severity of FA in rats.\textsuperscript{30} This indicates that immunization against neurogenic inflammation may contribute to the reduced severity.

It is possible that pretreatment with a low dose of FA through activation of the nervous system and release of neuropeptides has an effect on the hypothalamo-pituitary-adrenal axis. Recently, we have shown that i.p. administration of IL-1\alpha induces significant changes in neuropeptide-LI in CSF, plasma and SF indicating that an early activation of central and peripheral nervous systems might be part of a host defense reaction.\textsuperscript{31} Part of this host defense reaction in AA-induced rats may include the activation of the hypothalamo-pituitary-adrenal axis as shown by Neidhart & Larson.\textsuperscript{22} The part of the arthritis reducing effect with a low dose of FA due to an activation of hypothalamo-pituitary-adrenal axis is

\textbf{Fig. 2} Concentrations of SP-, NKA-, CGRP- and NPY-LI in plasma of control (CNT) and FA (FA) innoculated rat at 2 (2) and 24 (24) h are presented as raw data. Median concentration in fmol/ml for each group is indicated at the top of the frame. * Denotes significant difference from the control group: * P<0.05.
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**Fig. 3** Concentrations of SP-, NKA-, CGRP- and NPY-LI in synovial fluid in the right (R) and left (L) knee joint of control (CNT) and FA (FA) inoculated rat at 2 h are presented as raw data. Median concentration in fmol/ml for each group is indicated at the top of the frame. n indicates number of animals in the group. * Denotes significant difference from the control group: * P<0.05; + denotes significant difference from the contralateral knee joint; § denotes significant difference from the same knee joint at 24 h.

strongly supported by Knight et al who have reported that activation of hypothalamo-pituitary-adrenal axis plays a significant role in the pretreatment effect of low dose FA.14 Interestingly, Kustova et al reported on improved learning ability and decreased nociception in rats given s.c. injection of complete FA at doses not enough to induce AA.15 Moreover, pretreatment with a low dose of FA i.p. has been shown to have a protective effect against infection induced by *Fasciola hepatica* in rats.16 Also, immunization with incomplete FA modulates lung infection induced by *Pseudomonas aeruginosa* in rats.17 This indicates that pretreatment with complete or incomplete FA might be useful not only against the same mycobacterial antigen. It also emphasises a common generalized protective effect of pretreatment with low dose FA.

Taken together, a dose of complete FA not sufficient to induce arthritis causes significant changes in neuropeptide concentrations in CSF, plasma and SF which may play an important role in the pretreatment effect against AA in rats.

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Fig. 4 Concentrations of SP-, NKA-, CGRP- and NPY-LI in synovial fluid in the right (R) and left (L) knee joint of control (CNT) and FA (FA) inoculated rat at 24 h are presented as raw data. Median concentration in fmol/ml for each group is indicated at the top of the frame. * Indicates number of animals in the group. * Denotes significant difference from the control group: * P<0.05; § Denotes significant difference from the same knee joint at 2 h.

REFERENCES


