Acute pain induces an instant increase in natural killer cell cytotoxicity in humans and this response is abolished by local anaesthesia

J. Greisen¹*, M. Hokland², T. Grøfte¹, P. O. Hansen³, T. S. Jensen³, H. Vilstrup¹ and E. Tønnesen⁴

¹Department of Medicine V, ³Department of Neurology and ⁴Department of Anaesthesiology, Aarhus University Hospital, DK-8000 Aarhus C, Denmark. ²Department of Medical Microbiology and Immunology, University of Aarhus, Denmark
*Corresponding author

We have investigated the effect of pain without tissue injury on natural killer (NK) cell activity in peripheral blood in humans and the effect of local anaesthesia on the response. Ten subjects were investigated during two sessions. First, self-controlled painful electric stimulation was applied to abdominal skin for 30 min to an intensity of 8 on a visual analogue scale (0–10). Next, the electric intensity profile was reproduced during local anaesthesia (mepivacaine 10 mg ml⁻¹ s.c. to a total dose of 2.5 mg kg⁻¹). NK cell cytotoxicity was measured using a 4-h ⁵¹Cr-release assay against K562 target cells. NK cell activity increased from mean 22 (SEM 4)% (baseline) to 35 (6)% and 36 (5)% after 15 and 30 min of painful stimulation, respectively (P<0.02). A simultaneous increase in the number of CD56⁺ cells in peripheral blood during pain was found. Stimulation after local anaesthesia did not change either NK cell activity or number. Parallel and significant increases in concentrations of plasma epinephrine and serum cortisol were observed. These changes were abolished by local anaesthesia. We conclude that acute severe pain without tissue injury markedly increased NK cell cytotoxicity. Local anaesthesia completely abolished this immunological and hormonal response.

Br J Anaesth 1999; 83: 235–40

Keywords: blood, natural killer cells; pain, experimental; model, pain; pain, mechanism; immune response; sympathetic nervous system, catecholamines; hormones, cortisol; anaesthetics local

Accepted for publication: January 12, 1999

Many stressful situations are accompanied by an increase in afferent stimuli to the central nervous system, often as a perception of pain. Pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage.¹ Furthermore, pain is an integrated part of trauma, exercise, infections and surgery, and these conditions have been investigated with respect to alterations in immune function.² ³ However, little is known of the consequences of pain per se on immune function as the above mentioned conditions represent both pain and tissue lesions. It has been shown that epidural block of afferent nervous activity from the surgical field modifies the cellular immune response normally seen after surgery performed during general anaesthesia.² ⁴ These findings suggest that the natural killer (NK) cell system is activated via neuroendocrine stimuli and that pain per se may be a sufficient stimulus to increase NK cell activity.

NK cells are a subpopulation of large granular lymphocytes exerting spontaneous cytotoxicity against a variety of tumour cells, virus-infected cells and certain normal cells, and they probably play an important role as a primitive first line of defence against virus infections and certain malignancies.⁵ ⁷ NK cell activity increases instantaneously during physical stress such as surgery⁸ and exercise.⁹ Similarly, acute mental stress has been shown to increase NK cell activity.¹⁰ The endocrine response to stress with increased concentrations of epinephrine and cortisol influences the NK cell response. Several studies have shown that epinephrine enhances NK cell activity in vitro and in vivo¹¹ ¹² whereas cortisol may be responsible for the decrease in NK cell activity found hours and days after surgery.¹³ ¹⁴

We have investigated whether acute pain without tissue lesion increased NK cell activity, and if this effect was modified by local anaesthesia.
Subjects and methods

We studied 10 healthy male subjects of mean age 23 (range 20–27 yr, body weight 77 (69–89) kg and height 182 (177–188) cm. Before participation, the nature, purpose and potential risks of the study were explained to the subjects, and their written informed consent was obtained. The study was approved by the Regional Ethics Committee.

Study design

The 10 subjects were studied in two experimental sessions. In the first session, electric stimulation (square wave, 0.3 ms in duration, 2 Hz, intensity of 0–100 mA) was applied via a wet felt electrode on the abdominal skin for 30 min. Four stimulation marks on a horizontal line through the umbilicus were stimulated successively, 1 min in turn. The volunteers controlled current intensity via a hand-held potentiometer, and were instructed to reach and maintain a pain intensity corresponding to 8 on a visual analogue scale (VAS) from 0 (no pain) to 10 (unbearable pain). The electric intensity profiles were recorded and stored on computer.

In the second session, the abdominal skin was blocked by local anaesthesia consisting of subcutaneous injections of mepivacaine 10 mg ml
−1 to a total of 2.5 mg kg
−1. The volunteers controlled current intensity via a hand-held potentiometer, and were instructed to reach and maintain a pain intensity corresponding to 8 on a visual analogue scale (VAS) from 0 (no pain) to 10 (unbearable pain). The electric intensity profiles were recorded and stored on computer.

At each session, subjects were brought by car to the laboratory in the morning after a 10-h overnight fast and placed in bed. The sessions started at 07:00 (t
=240 min) and ended 8 h later at 15:00 (t
=240 min). The painful stimulation was applied from t
=0 min to t
=30 min. Four to five weeks elapsed between the two sessions.

Blood sampling and handling

Blood samples were obtained from a cubital vein. Samples for NK cell analysis (cytotoxicity and number of CD56+ cells in peripheral blood) were obtained using sterile 10-ml CPD (citrate, phosphate, dextrose) tubes (Venoject, code VT-100SCPD17, Terumo Europe, Leuven, Belgium). Samples were obtained at t
=−180, −60, 15, 30, 60 and 120 min. Mononuclear cells were isolated within the next 16 h in the following way. Blood was mixed with equal volumes of medium RPMI 1640 (Gibco BRL, Life Technologies, Paisley, Scotland) and the mixture was carefully layered on Ficoll–Hypaque (Lymfoprep, density 1.077 g ml
−1 ; Nyegaard and Co., Oslo, Norway) and centrifuged at 450 g for 20 min. Mononuclear cells were harvested from the interphase, washed once with RPMI 1640 and then resuspended in 0.5 ml of fetal calf serum before addition of 0.5 ml of freeze medium (1 part DMSO (Fluka Chemie A.G., Buchs, Schweiz) and 4 parts RPMI 1640) and the cells were frozen at −135°C until assayed.

Blood samples for leucocyte and differential counts were obtained at t
=−180, 15 and 120 min and analysed immediately. Samples for concentrations of serum cortisol and plasma epinephrine and norepinephrine were obtained at t
=−240 and every 60 min for the rest of the experimental period. Additional blood samples for hormone analysis were obtained at t
=10, 20, 30 and 40 min and serum was frozen immediately after centrifugation at −20°C (−80°C for catecholamines) until assayed.

4-h ⁵¹Cr release assay

The NK sensitive erythroleukaemic cell line K562 was grown in continuous culture and harvested in exponential growth phase. Five to ten million K562 cells were incubated with ⁵¹NaCrO₄ 200 μCi (Dupont-NEN Research Products, Boston, MA, USA) for 1 h at 37°C, washed twice and resuspended to 4×10⁵, 2×10⁵, 1×10⁵ and 0.5×10⁵ cells ml
−1. Assays were performed in round-bottomed microplates (Nunc, Roskilde, Denmark) with 100 μl per well of mononuclear cell suspension (2×10⁵ ml
−1) as effector cells and 100 μl per well of the varying target cell concentrations. Cultures were set up in triplicate, and after 4 h of incubation at 37°C the supernatant was harvested and counted for isotope release. The percentage of specific lysis for each effector/target combination was calculated as follows:

\[
\frac{(\text{experimental release–spontaneous release})}{(0.8\times\text{maximum release–spontaneous release})} \times 100
\]

where spontaneous release was determined from the supernatant of cultures where target cells were incubated without effector cells, whereas maximum release was determined after resuspending the target cells before harvesting. Results were discarded when spontaneous ⁵¹Cr release exceeded 10% (seen only on one occasion). Inter-day variation of the assay was evaluated continuously by including peripheral blood from the same healthy adult volunteer at each day of testing of a given donor.

Immunofluorescence staining and flow cytometry

Saturating amounts of PE (Phycyo-Erythrin)-conjugated IgG₁ anti-CD56 monoclonal antibody (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) were added to mononuclear cell suspensions (100 μl) obtained from each subject and incubated for 15 min at room temperature. The isotype-matched irrelevant antibody IgG₁-PE (Becton Dickinson Immunocytometry Systems) was used as a negative control. After incubation, cells were washed twice with phosphate-buffered saline (PBS), 0.1% sodium azide (NaN₃) and finally fixed in PBS, 1% formaldehyde.

Fluorescence-labelled cells were analysed on a Counter Elite flow cytometer (EPICS Division of Counter Corp., Hialeah, FL, USA), equipped with a 15 mW air-cooled argon ion laser (488 nm). Red–orange fluorescence (PE) was collected via a 575-nm bandpass filter. A minimum of 10 000 mononuclear cells were analysed per labelled cell sample. The negative isotype control was analysed for determination of the limit between positive and negative cells, and the limit was set at 1% nonspecific binding. The
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The absolute number of NK cells in peripheral blood was calculated from the percentage of CD56+ cells among mononuclear cells and the absolute number of mononuclear cells in peripheral blood.

Measurement of total peripheral blood leucocyte and differential counts were made by Coulter Counter STKS (Coulter Electronics, UK).

**Hormone assays**

Serum concentrations of cortisol were measured by an immunofluorimetric assay (AutoDELFIA, Wallac Oy, Finland). Plasma concentrations of catecholamines were measured by electrochemical detection after high-pressure liquid chromatography. Intra- and inter-assay coefficients of variation were, respectively, 3.6% and 1.9% for cortisol, 7.7% and 6.0% for epinephrine and 6.5% and 6.1% for norepinephrine. Limits of detection of the assays were 6 µg litre\(^{-1}\) for cortisol, 20 ng litre\(^{-1}\) for epinephrine and 10 ng litre\(^{-1}\) for norepinephrine.

**Statistical analyses**

Statistical analysis was performed by two-way analysis of variance for repeated measurements, using Bonferroni’s test for multiple comparisons. Values are given as mean (SEM). \(P<0.05\) was considered significant.

**Results**

In the first session, skin hyperaemia developed around the stimulation area which lasted for approximately 1 h after stimulation. Surrounding the hyperaemic zone, an area of hyperaesthesia to pinprick and brush was present which persisted for a few hours after cessation of stimulation. In the second session, hyperaemia was either absent or reduced markedly. There were no other signs or symptoms of tissue damage. During painful stimulation, all subjects experienced a pain intensity of 8 (VAS 0–10) and during stimulation under block, pain intensity was scored as 0.5 (0.3).

**NK cell cytotoxicity and absolute number in peripheral blood**

A significant and transient increase in NK cell activity was seen during painful stimulation. Activity increased from 22 (4)% \((t=-60\text{ min})\) to 35 (6)% and 36 (5)% after 15 and 30 min of painful stimulation, respectively \((P<0.02)\) (Fig. 1). Cytotoxicity returned to pre-stimulation levels within 1 h after stimulation.

The number of NK cells identified as CD56+ cells in peripheral blood increased slightly, but not significantly, during pain \((P=0.21)\) (Fig. 2).

After local anaesthesia, NK cell activity and the number of CD56+ cells did not change. The difference in NK cell activity between the sessions was significant during stimulation at \(t=30\text{ min}\) \((P=0.02)\) but not at \(t=15\text{ min}\) \((P=0.40)\). Baseline \((t=-180)\) values of CD56+ cells were different \((518 (50)\times10^6\text{ litre}^{-1}\) in the first session vs \(387 (28)\times10^6\text{ litre}^{-1}\) in the second session; \(P=0.01\), paired \(t\) test). Thus the relative change during stimulation from an index of 1 at baseline was significantly higher in the first session \((1.32 (0.15)\text{ vs }1.01 (0.09); P=0.03\), paired \(t\) test).

**Leucocyte distribution**

Leucocytosis, consisting of neutrophils, was demonstrated in both sessions (Fig. 3). The magnitude of the leucocytosis was significantly higher in the first session \((P=0.03)\).

In the first session, the number of lymphocytes increased significantly during stimulation followed by a decrease after stimulation. However, during block, these lymphocyte changes were not seen. At \(t=120\text{ min}\), the difference between sessions was significant \((P<0.001)\).
Fig 3 Number of leucocytes, neutrophils and lymphocytes in peripheral blood (mean (SEM), n=10) under the same conditions as in Figure 1.

**Plasma epinephrine**

In the first session, mean plasma epinephrine concentration was 40 (2) ng litre$^{-1}$ at baseline. Immediately before stimulation ($t=0$), epinephrine concentration increased to 70 (16) ng litre$^{-1}$ and during pain it continued to increase to a peak concentration of 104 (16) ng litre$^{-1}$ ($t=30$) ($P<0.001$ for $t=10, 20$ and 30 min compared with baseline) (Fig. 4). After painful stimulation, concentrations returned to pre-stimulation values within 30 min. After block, no changes were seen during or after stimulation. Baseline values were comparable in the two sessions.

**Plasma norepinephrine**

In the first session, there was no change in plasma concentrations of norepinephrine during or after stimulation. In the block session, decreased concentrations were demonstrated during stimulation. Baseline plasma norepinephrine concentrations ($t=-240$ to $-60$ min) were significantly higher in the first session (363 (20) ng litre$^{-1}$) than in the block session (274 (9) ng litre$^{-1}$) ($P=0.001$).

**Serum cortisol**

In both sessions, serum cortisol concentrations decreased at baseline to approximately 100 µg litre$^{-1}$. Pain increased cortisol concentrations to a maximum of 164 (6) µg litre$^{-1}$ ($t=30$ min) ($P<0.001$), whereas cortisol concentration decreased to 80 (7) µg litre$^{-1}$ ($t=30$ min) after block.

**Discussion**

In this study, the immunological consequences of severe, controlled and standardized pain without concomitant tissue injury were investigated. The main results were that acute, severe experimental pain without tissue injury elicited a marked, instantaneous increase in NK cell cytotoxicity, plasma concentrations of epinephrine and serum cortisol concentrations. However, these responses were blocked by local anaesthesia.

Little tissue damage was produced in our pain model as hyperaemia was transient. Furthermore, we measured plasma cytokines as markers of tissue injury in four pilot subjects not participating in the study but undergoing the same pain stimulus. No pro-inflammatory cytokines (IL-1β, IL-6 and TNFα) were detectable in plasma while the anti-inflammatory cytokine IL-10 was detectable at constant
levels during the study (J. Greisen, K. Bendtzen, unpublished data). These findings indicate that tissue injury from the electric current did not play a role as cytokines, especially IL-6, are markers of tissue injury.16

As lymphocytes express a variety of hormone receptors, it has been suggested that hormones mediate stress-induced changes in immune function, and catecholamines seem to play an important role in the endocrine–immune network. Our findings of simultaneous increases in plasma epinephrine and NK cell cytotoxicity in addition to the number of CD56+ cells during pain are in accordance with other studies that demonstrated an increase in NK cell number and activity after in vivo administration of epinephrine.11 12

After block, plasma norepinephrine concentrations decreased markedly during stimulation. The reason for this is uncertain but venous blood sampling may have affected the result as blood flow and re-uptake of norepinephrine in the arm probably changes during pain. The pattern of decrease was seen previously in the isometric exercising arm and during mental stress.19

Cortisol inhibits NK cell cytotoxicity in vitro13 and in vivo.20 However, in another study, administration of cortisol did not affect NK cell activity in healthy volunteers12 but this may be explained by the short observation time of only 5 h. In our study, serum concentrations of cortisol increased markedly during pain and the cortisol response was probably responsible for granulocytosis and lymphopenia which are well known effects of increased cortisol concentrations.21 22 After painful stimulation, there was no rebound phenomenon in NK cell activity consisting of decreased activity. This may be because of the limited observation time of 90 min after stimulation. The observed time course of cortisol with high values in the morning and gradually decreasing values thereafter probably reflects the diurnal variation in the production of this hormone.23

Local anaesthesia completely abolished the pain-induced increase in NK cell cytotoxicity and number of CD56+ cells in peripheral blood. The electric profile was identical for each subject in the two experimental sessions. The effect of mepivacaine may be a result of block of afferent neuronal activity to the spinal cord or a systemic effect on NK cell activity. Local anaesthetics are known to inhibit NK cell cytotoxicity in vitro in a dose-dependent manner.24–26 Thus lidocaine instilled into the airways inhibits NK cell cytotoxicity in rat lung lavage fluid.27 In a study comparing the in vitro effect of various local anaesthetics on NK cell cytotoxicity, an inhibitory effect of lidocaine but not of mepivacaine was found.28 We used mepivacaine 2.5 mg kg⁻¹, which resulted in an estimated maximal blood concentration of 0.5 mg litre⁻¹. This concentration is very low compared with concentrations used in the above mentioned in vitro studies, and no inhibition of NK cell activity was probably caused by a systemic effect of mepivacaine. However, the best method of controlling the systemic effect of mepivacaine would have been to inject it remote from the stimulation area in the control situation.

Subjects were investigated in the same sequence: stimulation in the first session followed by the same stimulation plus local anaesthesia in the second session. Randomization was not possible because the electric intensity profile recorded in the first session was used in the second session to obtain comparable trauma to the tissues. This systematic sequence may have influenced the results because anxiety and stress levels may have been higher in the first session. The findings of increased baseline plasma norepinephrine concentrations and NK cell number in the first session support this notion. However, epinephrine and cortisol concentrations were comparable during baseline in the two sessions and it seems obvious that the sensation of pain overruled this possible preconditioning.

Our results raise the question of whether infiltrating the surgical field with local anaesthetic for intraoperative and postoperative pain relief has a beneficial effect. The endocrine stress response to surgery may be blocked to such an extent that the increase in NK cell cytotoxicity is also prevented. Hence infiltration block may impair the NK cell response against certain microbial infections and dissemination of tumour cells.

In summary, acute severe pain without concomitant tissue injury markedly increased NK cell cytotoxicity, probably by increasing the number of these cells in peripheral blood, concentrations of plasma epinephrine and serum cortisol. Furthermore, local anaesthesia completely abolished this hormonal and immunological response. These results support the hypothesis that the NK cell system is activated via neuroendocrine pathways and that pain per se is a sufficient stimulus to increase NK cell activity.

Acknowledgements
We thank Elin Jacobsen for technical assistance. This study was supported by grants from the Danish Medical Association Research Fund, the Danish Foundation for the Advancement of Medical Science, the Danish Cancer Society, the Aarhus University Research Foundation, the Institute of Experimental Clinical Research, University of Aarhus and the Novo Nordisk Foundation.

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