Int J Legal Med (1996) 109:130–133

ORIGINAL ARTICLE

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Interleukin-1α (IL-1α) and N-formyl-methionyl-leucyl-phenylalanine (FMLP) as potential inducers of supravital chemotaxis

Received: 29 January 1996 / Received in revised form: 28 August 1996

Abstract The phenomenon of artificially induced local leucocyte reactions during the supravital period could be of practical importance, but has not yet been comprehensively investigated. For a more detailed evaluation, experiments with the chemotactic agents interleukin-1α (IL-1α) and N-formyl-methionyl-leucyl-phenylalanine (FMLP) were performed by subcutaneous injection into various anatomical regions (back, abdomen, limbs) of NMRI-mice (National Medical Research Institute) and pigs 0–5 min after circulatory arrest. Phosphate buffered saline (PBS) without effective components was administered to equivalent areas of the animals as a control. Tissue specimens were collected at 6 h postmortem (mice) and 12–14 h postmortem (pigs), cut into serial sections, stained with H&E and examined under the microscope. A leucocyte reaction did not develop in pigs (n = 10, 30 tissue samples) following injection of FMLP, however, dermal, subcutaneous and perivascular infiltration of leucocytes (in particular mononuclear cells and a few granulocytes) was found in 3 out of 30 tissue specimens in murine experiments. In addition intravascular cell accumulations were detected in 2 out of 30 samples. The injection of IL-1α to mice gave similar results, i.e. aggregations of leucocytes and intravascular cell accumulations in 4 out of 30 and 3 out of 30 tissue samples, respectively. In negative controls no leucocyte reaction was detectable. This shows that potent chemotactic factors such as IL-1α and FMLP administered in the early supravital period can induce moderate local leucocyte reactions in animal models in at least some cases. A clear morphological differentiation between vital and supravital chemotaxis does not seem to be possible. The supravitally stimulated accumulations of leucocytes are interpreted as an aggregation of resident macrophages in combination with a slight migration of blood leucocytes. Presumably, these alterations are restricted to the very early supravital period as long as sufficient energy reserves are available. It must be stated that the observed changes are reactions, not spontaneous actions, so that the general validity of the phenomenon of leucocyte infiltration as a vital parameter is not affected.

Key words Supravital chemotaxis · Interleukin-1α (IL-1α) · N-formyl-methionyl-leucyl-phenylalanine (FMLP) · Animal tests · Histology

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Introduction

The term "chemotaxis" can be defined as locomotion oriented along a chemical gradient [4]. Granulocytes, monocytes and, to a lesser extent, lymphocytes respond to various exogenous and endogenous stimuli such as bacterial products (e.g. N-formyl-methionyl-peptides), components of the complement system (e.g. C5a), products of the lipoxygenase pathway (e.g. LTB_4) and cytokines (e.g. IL-1, IL-8) [1, 2, 4, 5, 16]. The result of extravasation, migration and directed cell movement toward the site of injury (e.g. wound) is a local leucocytic infiltration. This is regarded as one of the most important histological signs of vitality in forensic medicine and can be used for wound age estimation. However, it seems to be possible that in the supravital period – a phase of intermediary life following irreversible circulatory and respiratory arrest with the survival of some tissues [10, 11] – the capacity of cell migration is still intact. This could have practical consequences if stimuli in the (early) supravital period were able to induce chemotaxis and simulate local vital phenomena. To our knowledge, there has only been one detailed investigation by Ali [1] dealing with this problem. This author observed the development of a marked and partially progressive migration of white blood cells in 50% of cases after postmortem injection of a chemotactic agent (N-formyl-methionyl-leucyl-phenylalanine, FMLP) into the abdominal wall and skin of rats (Fig. 1). How-

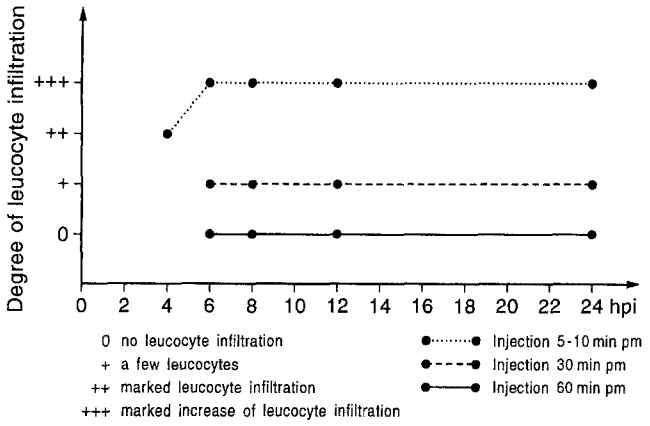

Fig. 1 White blood cell infiltration following postmortem injection of FMLP into the skin of rats: degree of leucocyte reaction versus hours after injection (hpi) (according to Ali [1])

ever, only very few animals were tested in each subgroup, the histological findings were not described in detail (no illustrations) and some data in the methodology appear to be questionable (e.g. taking of multiple samples from the same site of injection). The aim of this study was to verify and extend the observations of Ali by conducting animal experiments with mice and pigs with two different chemotactic substances, FMLP and interleukin-1α [5, 16].

Materials and methods

Experiments with FMLP

FMLP (Sigma) was administered to NMRI-mice (National Medical Research Institute) (n = 10, age: 2–8 months, weight: 25–35 g) and pigs (n = 10, weight: 15–25 kg) in endotoxic shock. The mice were sacrificed by cervical distorsion, the thorax and abdomen were opened and the great vessels at the heart base were clamped to produce sudden and total circulatory arrest. The narcotised pigs in endotoxic shock were sacrificed by i.v. injection of T 61 (Hoechst; mixture of embutramide, mebezoniumiodide and tetracain hydrochloride resulting in narcosis, paralysis of respiratory centre and skeletal/respiratory musculature and rapid circulatory collapse) and circulatory arrest was verified by blood pressure monitoring. After circulatory arrest FMLP and controls were injected within 5 min into the skin and subcutaneous tissue of the back, abdomen and hind legs (mice) or chest, abdomen and hind legs (pigs). FMLP was dissolved in 0.1 M phosphate buffered saline (PBS, pH 7.4) to a final concentration of 1 μg/ml (mice) and 0.5 mg/ml (pigs) and 1 mg/ml Evan's blue was added as a colour indicator. Aliquots of 50 μl (mice) or 1 ml (pigs) of the working solution were injected by a tuberculin syringe into the right side of the body. Equal amounts of 0.1 M PBS containing Evan's blue served as controls and were applied to the left side. The animals were stored in the supine position at room temperature (mice in humid chambers to avoid drying). Tissue samples were taken at 6 h (mice) and 12–14 h (pigs) postmortem, fixed in 4% buffered formalin, embedded in paraffin, cut into serial sections 4 μm thick and stained with H&E. The leucocyte infiltration was graduated semiquantitatively (i.e. negative/slight/moderate/strong reaction).

Experiments with IL-1α

IL-1α (mouse, recombinant; Sigma) was applied to NMRI-mice (n = 10, age: 2 months, weight: 25–35 g) at activities of 27500 U and 2750 U (dissolved in 0.1 M PBS pH 7.4, and admixture of 1 mg/ml Evan's blue for tissue marking). The working solution or PBS as control (50 μl) were injected subcutaneously into the same sites. The further procedure was identical to the FMLP experiments with mice.

The concentrations of IL-1α and FMLP were derived from the literature [1, 5] with respect to the weight of the test animals. In addition to postmortem experiments, the efficacy of FMLP and IL-1α concentrations was tested by vital injection into the skin and subcutaneous tissue of four mice which were sacrificed 6–8 h after injection. All animal experiments were performed within the scope of licensed clinical investigations. The principles of laboratory animal care were followed. Most of the tests were done postmortem.

Immunohistochemical investigations

For an evaluation of mononuclear cells in murine skin, immunohistochemical investigations were carried out with the monoclonal rat anti-mouse antibody BM 8 (Dianova) which detects a major subpopulation of resident tissue macrophages. The APAAP procedure [3] was used which briefly comprised the following main steps:

1. Enzymatic predigestion of paraffin sections with 0.01% pronase (15 min, room temperature).
2. Incubation with primary BM 8 antibody (dilution 1:50, 1 h, 37° C).
3. Incubation with secondary rabbit anti-rat antibody (Dako, dilution 1:50, 30 min, room temperature).
4. Incubation with APAAP complex (rat) (Dako, dilution 1:50, 30 min, room temperature).
5. Detection with neofuchsin, embedding in glycerol gelatin.

Results

Normal skin histology

Control samples from murine and porcine skin showed only a few and widely scattered mononuclear cells in the dermal and subcutaneous layers. Immunohistochemically, most of these cells in murine tissue samples stained positively with the antibody BM 8 proving them to be (resident) tissue macrophages. Granulocytes were absent from normal specimens.

Skin histology after supravital application of PBS (controls)

The same pattern was observed as found in specimens from normal skin. Infiltrations by macrophages or granulocytes were not present.

Skin histology after supravital application of FMLP and IL-1α

Following injection of FMLP into pigs, leucocytic reactions did not develop in a single case. Histologically, only morphologically avital subcutaneous hemorrhages were detected at the site of injection. By contrast, in the murine animal model slight to moderate infiltration by leucocytes (mainly macrophages reacting positively with BM 8, several lymphocytes and a few neutrophil granulocytes) was observed in the subcutaneous and muscular layers as well as in the perivascular tissue in 3 out of 30 specimens (Figs. 2 and 3). Intravascular cell accumulations were demonstrable in 2 out of 30 samples. However, in the ma-

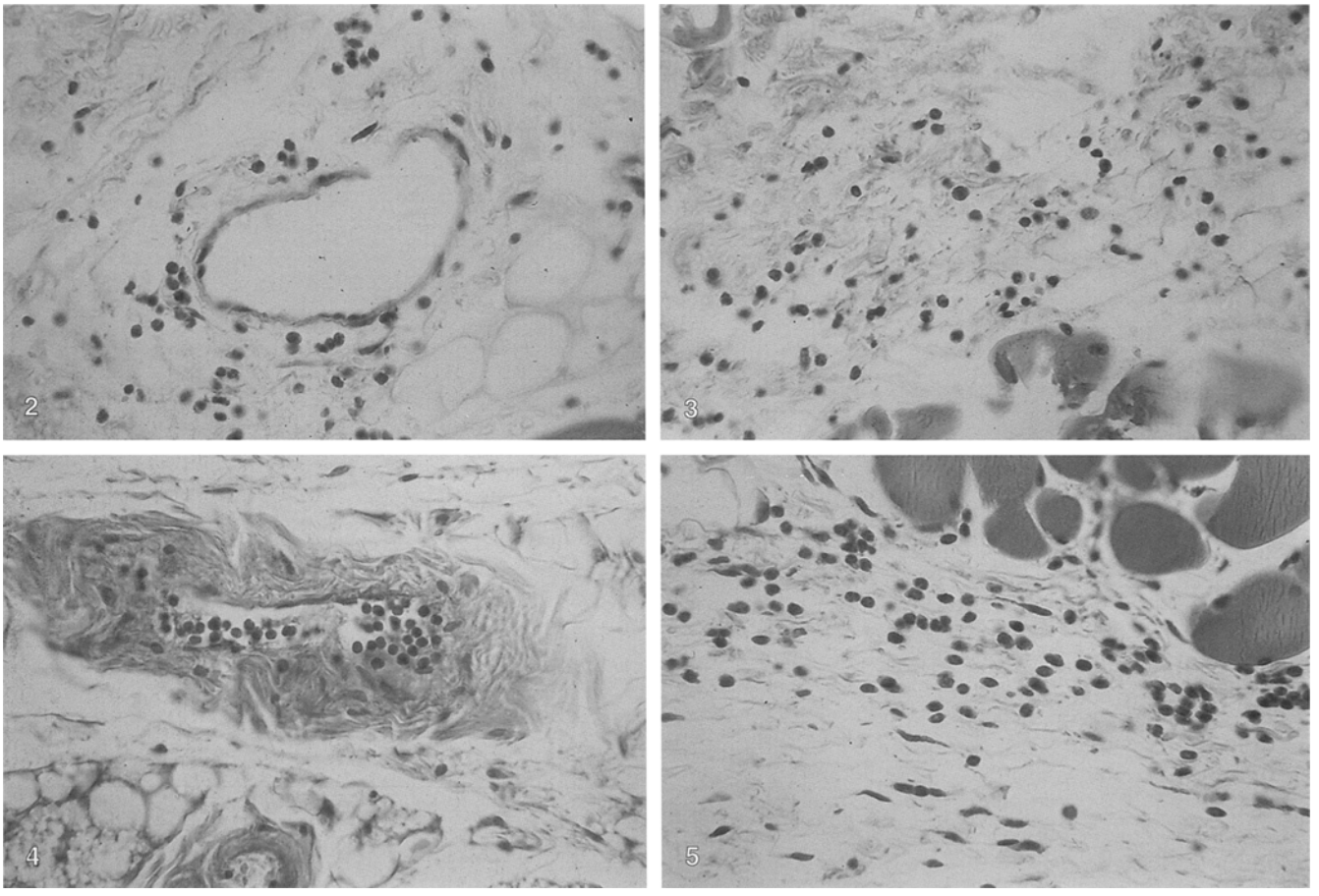

Fig. 2 Perivascular accumulation of leucocytes following supravital injection of FMLP into murine skin. H & E, × 500

Fig. 3 Leucocyte infiltration of murine connective tissue after supravital injection of FMLP. H & E, × 500

Fig. 4 Intravascular cell accumulation after supravital injection of IL-1α into murine skin. H & E, × 500

Fig. 5 Marked leucocyte reaction after vital injection of IL-1α into murine skin. H & E, × 500

jority of cases no cell reaction was observed. In positive cases the reactivity was independent of the anatomical site and younger animals tended to develop stronger leucocyte reactions.

The administration of IL-1α to mice gave similar results i.e. slight/moderate infiltration of leucocytes and intravascular cell accumulations in 4 out of 30 and 3 out of 30 tissue samples, respectively (Fig. 4). The reaction pattern was independent of the IL-1α activity.

Skin histology after vital application of FMLP and IL-1α

Vital injection of both substances into the subcutaneous tissue of mice resulted in a marked/strong infiltration by macrophages and granulocytes (survival time 6–8 h) (Fig. 5), which exceeded supravital reactions. Both chemotactic agents proved their efficacy but IL-1α was slightly more potent at an activity of 27500 U compared to 2750 U.

Discussion

To investigate the potential phenomena of supravital chemotaxis we used FMLP and IL-1α as stimulating agents. FMLP belongs to a group of synthetic formylated peptides which resemble bacterial products and is a strong chemo-attractant for neutrophils and macrophages at very low concentrations of 10^{-9}–10^{-11} M as assays on the cellular level demonstrated [13, 16–18]. IL-1 with its subtypes IL-1α and IL-1β is a well-known representative of the cytokine family [2, 5, 6, 8, 9, 15, 20] which plays a central role in the mediation and coordination of inflammatory processes and immune/defense mechanisms as well as in the regulation of wound reaction and wound repair [6, 9, 15]. IL-1 proved to be an extremely potent chemo-attractant for neutrophils [5]. In this respect it is superior to FMLP and induced detectable neutrophil migration in rabbits at doses of approximately 2×10^{-15} to 2×10^{-16} mol/intradermal site [5]. Leucocyte migration in these experiments (intradermal injection of 3.2 U/site in living rabbits) was rapid and occurred within the first 30 min with maximal rates between 30 and 90 min. Equivalent concentrations for humans are difficult to designate. With respect to different body weights they may be approximately 2,000–3,000-fold higher than for mice.

Using these two chemotactic factors in postmortem supravital investigations, we observed no comparable leucocyte reaction in the majority of cases. Obviously either leucocytes have reduced migratory capability very early

after death or supravital chemotaxis is insufficient to be diagnosed microscopically. The negative results in porcine experiments may be due to the clinical study design (i.e. endotoxic shock, narcosis).

Nevertheless, it is in our opinion noteworthy, that a more or less distinct leucocyte infiltration was detectable in 10–15% of the mice. These histological alterations underline the fundamental existence of supravital chemotaxis as a phenomenon. Ali [1] described similar changes after postmortem injection of FMLP into the skin of rats, but did not use the term „supravital chemotaxis". Ali observed a clear localized infiltration by white blood cells in 50% of animals injected with FMLP 5–10 min after sacrifice (Fig. 1). He found a dependence on the position of animals (prone versus supine position) and the concentration of FMLP without providing further details. In addition, he did not specify his results as to the cell type and gave no histological illustrations, so that it remains unclear what was regarded as a significant infiltration. Therefore, a comparison with our results seems to be of limited value. In another very short report by Uher [21] a proliferation and mobilization of histiocytes, macrophages and round cells was described 10–20 h after injection (1 h postmortem) of turpentine oil into the skin of rats. Further details were not given, so that these results cannot be clearly evaluated.

Supravital chemotaxis can be assessed as one part within the broad spectrum of supravital reaction [see survey in 10, 11]. The supravital migration of leucocytes seems possible as long as energy reserves are available providing ATP for cell movement and membrane stability (anaerobic glycolysis in the first 10 h postmortem [10]). Energy rich phosphates and stable cell membranes are prerequisites for the complex mechanisms of chemotaxis and leucocyte migration which are regulated by further factors such as adhesion molecules [4, 7, 12–14, 18, 19, 22]. FMLP, for instance, is known to induce a two- to threefold increase of intracellular cAMP levels in human neutrophils 5–15 s after administration [18].

Presumably, supravital chemotaxis with simulation of local vital reactions is restricted to the early supravital period. Firstly, a sufficient energy supply is necessary and secondly, it must be kept in mind that it takes some time to produce a marked tissue infiltration which can be diagnosed microscopically. Granulocytes, for instance, migrate at rates of approximately 30 μm/min [22].

The leucocyte infiltrations observed are interpreted as an aggregation of resident tissue macrophages combined with a moderate migration of blood leucocytes. Due to the limited possibilities of postmortem migratory activity, a movement of local cells is more likely than a locomotion of intravascular cells in the state of circulatory arrest. This hypothesis would also explain the predominance of macrophages in supravital specimens, whereas more granulocytes appeared in vital samples despite comparable temporal conditions (time intervals of 6–8 h between injection and sampling). However, a clear morphological differentiation between the results of vital and supravital chemotaxis does not seem to be possible, even if supravitally induced cell aggregates were not as marked as vital infiltrates. Finally, it must be stated that the observed changes are reactions (to artificial injection of chemo-attractants), not spontaneous actions, so that the general validity of the phenomenon of leucocyte infiltration as a vital parameter primarily is not affected.

Acknowledgements The authors would like to thank Mrs. A. Schoofs, Mrs. E. Schmitt-Nau and Mrs. K. Meis for excellent technical assistance.

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