STATE OF NEW MEXICO
IN THE FIRST JUDICIAL DISTRICT COURT
SANTA FE COUNTY

Arthur Firstenberg, Plaintiff,

-against- No. D101CV201000029

Raphaela Monribot, Defendant.

MOTION FOR RECONSIDERATION AND ISSUANCE OF PRELIMINARY INJUNCTION

Comes now the Plaintiff, Arthur Firstenberg, and moves the Court to reconsider its decision, dated April 5, 2005, denying Plaintiff's Motion for Preliminary Injunction. Arguments supporting this Motion are presented in the Memorandum filed herewith.

Wherefore, the Plaintiff requests that the Court enter its order granting a preliminary injunction as prayed in that motion.

Respectfully submitted,

Lindsay A. Lovejoy, Jr.
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3600 Cerrillos Road, Unit 1001A
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April 20, 2010

CERTIFICATE OF SERVICE

The undersigned hereby certifies that on April 20, 2010, he caused a copy of the following to be served by first class mail upon counsel for the Defendant:

1. The foregoing Motion,

2. The Affidavit of Olle Johansson, executed on April 20, 2010, with attached curriculum vitae,

3. Johansson, O., Hilliges, M., Björnhagen, V., Hall, K. (1994). "Skin changes in patients claiming to suffer from "screen dermatitis": a two-case open-field provocation study", *Exp. Dermatol.* 3:234–238,

4. Johansson, O., Hilliges, M., Han, S. W. (1996). "A screening of skin changes, with special emphasis on neurochemical marker antibody evaluation, in patients claiming to suffer from screen dermatitis as compared to normal healthy controls." Exp. Dermatol. 5:279–285,

5. Rajkovic, V., Matavulj, M., Johansson, O. (2005b). The effect of extremely low-frequency electromagnetic fields on skin and thyroid amine- and peptide-containing cells in rats: an immunohisto-chemical and morphometrical study. Environ. Res. 99:369–377.

To:

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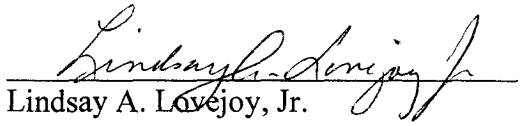

Lindsay A. Lovejoy, Jr.

STATE OF NEW MEXICO
IN THE FIRST JUDICIAL DISTRICT COURT
SANTA FE COUNTY

Arthur Firstenberg, Plaintiff,

-against- No. D101CV201000029

Raphaela Monribot, Defendant.

AFFIDAVIT OF OLLE JOHANSSON

Kingdom of Sweden)
ss:
City of Stockholm)

Olle Johansson deposes and says:

1. I am an Associate Professor, Head of the Experimental Dermatology Unit in the Department of Neuroscience at the Karolinska Institute in Stockholm, Sweden. I received my Ph.D. in neuroscience from the Karolinska Institute in 1983. I also am a Professor in basic and clinical neuroscience at the Royal Institute of Technology, Stockholm. An excerpt of my curriculum vitae is attached.
2. I have published more than 500 original articles, reviews, book chapters and conference reports within the field of basic and applied neuroscience, and participated in more than 300 congresses and symposia as an invited speaker. I have been employed as a peer reviewer by a large number of scientific journals, including Acta Dermato-Venereologica, Acta Obstetrica & Gynecologica, Acta Physiologica Scandinavica, Acta Stereologica, Archives of Dermatological Research, Brain Research, Canadian Journal of Zoology, Diabetologia, European Journal of Dermatology, Experimental Brain Research, Experimental Dermatology, Histochemical Journal, Journal of Chemical Neuroanatomy, Journal

of Comparative Neurology, Journal of Investigative Dermatology, Journal of Microscopy, Neurobiology, Neuroscience, Regulatory Peptides, and Skin Pharmacology.

3. I am a member of the European Neuroscience Association (ENA), IBAS Users of Scandinavia (IBUS), The International Brain Research Organization (IBRO), The International Society for Stereology (ISS), The Royal Microscopical Society (RMS), Scandinavian Society for Electron Microscopy (SCANDEM), Society for Neuro-science, Svenska Fysiologföreningen (Swedish Association of Physiologists), Svenska Intressegruppen för Grafisk Databehandling (SIGRAD) (Swedish Computer Graphics Association), Svenska Läkaresällskapet (Swedish Society of Medicine), and the Svenska Sällskapet för Automatiserad Bildanalys (SSAB) (Swedish Society for Automated Image Analysis).
4. I have been acquainted with the Plaintiff, Arthur Firstenberg, for over twelve years, and have conversed with him numerous times over the phone or by letter or email concerning the functional impairment electrohypersensitivity.
5. My group, the Experimental Dermatology Unit, investigates health effects of modern, man-made electromagnetic fields as well as the functional impairment electrohyper-sensitivity. I introduced the clinical term "screen dermatitis" to explain the cutaneous damages that developed in the late 1970's when office workers, first mostly women, began to be placed in front of computer monitors. I called for action along lines of occupational medicine, biophysics and biochemistry, as well as neuroscience and experimental dermatology. The working hypothesis early became that persons with the impairment electrohypersensitivity react in a cellularly correct way to the electro-magnetic radiation, maybe in concert with chemical emissions such as plastic components, flame retardants, etc., in a

highly specific way and with a completely correct avoidance reaction -- just as one would do if exposed to, *e.g.*, sun rays, X-rays, radioactivity or chemical odors.

6. I and my collaborators have, in addition, worked in great depth in other areas of health science such as skin diseases, cancer, child delivery, female urine incontinence, oral mucosa diseases, dental care for elderly and medically compromised persons, brain and spinal cord morphology, synaptology and chemical transmission, peripheral nervous system-related issues, cardiac function, skeletal muscle function and disease, and connective tissue ripening phenomena.
7. In our experience in the Experimental Dermatology Unit, symptoms of the skin, the nervous system and the heart dominate the picture in electrohypersensitive persons.
8. In one study that we published in 2005, the functional impairment electrohypersensitivity was investigated with the aim to characterize the complex set of symptoms and to order them according to the WHO's ICQ10 register of diagnoses. Twenty-two individuals with this disability participated, aged 25 to 79. Among the most common symptoms were skin redness, eczema and sweating, loss of memory, concentration difficulties, sleep disturbances, dizziness as well as muscular and joint-related pain, and muscular and joint-related weakness. Headache, faintness, nose blockade, and fatigue were also common. In addition, 19 of the people had symptoms from the gastrointestinal tract. All the individuals had tinnitus. (Holmboe, G., Johansson, O. (2005). "Symptombeskrivning samt förekomst av IgE och positiv Phadiatop Combi hos personer med funktionsnedsättningen elöverkänslighet", ("Description of symptoms as well as occurrence of IgE and positive Phadiatop Combi in persons with the physical impairment electrohypersensitivity", in Swedish), Medicinsk Access 1:58–63.)

9. In one of the early papers we made an important discovery when we exposed two electrically sensitive individuals to a TV monitor. When we looked at their skin under a microscope, we found something that surprised us. In this article, we used an open-field provocation, in front of an ordinary TV set, of persons regarding themselves as suffering from skin problems due to work at video display terminals. Employing immunohistochemistry, in combination with a wide range of antisera directed towards cellular and neurochemical markers, we were able to show a high-to-very high number of somatostatin-immunoreactive dendritic cells as well as histamine-positive mast cells in skin biopsies from the anterior neck taken before the start of the provocation. At the end of the provocation the number of mast cells was unchanged, however, the somatostatin-positive cells had seemingly disappeared. The reason for this latter finding is discussed in terms of loss of immunoreactivity, increase of breakdown, etc. The high number of mast cells present may explain the clinical symptoms of itch, pain, edema, and erythema. Some electrically sensitive people have symptoms similar to heart attacks after exposure to electromagnetic fields. The cardiac mast cells may be responsible for these changes due to degranulation after exposure to electromagnetic fields. (Johansson, O., Hilliges, M., Björnhagen, V., Hall, K. (1994). "Skin changes in patients claiming to suffer from "screen dermatitis": a two-case open-field provocation study", *Exp. Dermatol.* 3:234–238)
10. We have compared facial skin from electrohypersensitive persons with correspond-ing material from normal healthy volunteers. The aim of the study was to evaluate possible markers to be used for future doubleblind or blind provocation investigations. Differences between electrohypersensitive persons and normal volunteers were found for the biological markers calcitonin gene-related peptide

(CGRP), somatostatin (SOM), vasoactive intestinal polypeptide (VIP), peptide histidine isoleucine amide (PHI), neuropeptide tyrosine (NPY), protein S-100 (S-100), neuron-specific enolase (NSE), protein gene product (PGP) 9.5, and phenylethanolamine N-methyltransferase (PNMT). The samples were blind-coded so that the investigators evaluated the samples without knowing which individuals they came from. The overall impression was such that it turned out easy to blindly separate the two groups from each other. (Johansson, O., Hilliges, M., Han, S. W. (1996). "A screening of skin changes, with special emphasis on neurochemical marker antibody evaluation, in patients claiming to suffer from screen dermatitis as compared to normal healthy controls." Exp. Dermatol. 5:279–285)

11. One very fierce criticism from certain skeptics has been that all symptoms, including mast cell and other skin alterations in people with electrohypersensitivity cannot be due to the action of electromagnetic fields (EMFs) but must be due to psychological or psychiatric personality disturbances, cognitive malfunction, or likewise. In my opinion, based on over two decades of research and acquaintance with thousands of electrosensitive individuals, this is not the case. Furthermore, we have also done a series of animal experiments, whose results cannot be attributed to psychological factors. These have been a collaborative effort between the Department of Biology, Faculty of Sciences, Novi Sad, Serbia and Montenegro, and my own research group at the Karolinska Institute, Stockholm, Sweden. We investigated the influence of extremely low-frequency electromagnetic fields (ELF-EMFs) on mast cells, para-follicular cells, and nerve fibers in rat skin and thyroid gland, as seen using light and transmission electron microscopy. Significant changes were seen in both the skin and thyroid gland, very similar to what we have also seen in the tissues of patients with electrohypersensitivity. The

animal results cannot be understood by psychological or psychiatric theories, but can only be due to the EMF exposure. (Rajkovic, V., Matavulj, M., Johansson, O. (2005a). "Histological characteristics of cutaneous and thyroid mast cell populations in male rats exposed to power-frequency electro-magnetic fields". Int. J. Radiat. Biol. 81:491–499; Rajkovic, V., Matavulj, M., Johansson, O. (2005b). The effect of extremely low-frequency electromagnetic fields on skin and thyroid amine- and peptide-containing cells in rats: an immunohisto-chemical and morphometrical study. Environ. Res. 99:369–377; Rajkovic, V., Matavulj, M., Johansson, O. (2006). Light and electron microscopic study of the thyroid gland in rats exposed to power-frequency electromagnetic fields. J. Exp. Biol. 209:3322–3328; Rajkovi,c V., Matavulj, M., Johansson, O. (2010) Combined exposure of peripubertal male rats to the endocrine-disrupting compund atrazine and power-frequency electromagnetic fields causes degranulation of cutaneous mast cells: A new toxic environmental hazard? Arch. Environ. Contam. Toxicol. Feb 11 [Epub ahead of print])

12. In Sweden electrohypersensitivity (EHS) is an officially fully recognized functional impairment. Survey studies published by the Swedish National Board of Health and Welfare show that somewhere between 230,000–290,000 Swedish men and women report a variety of symptoms when being in contact with electro-magnetic field (EMF) sources. They have the legal right to work in spite of their impairment. For example, they can get low-emission computers, high-frequency fluorescent lamps can be changed to ordinary light bulbs, and wireless telephones can be removed from their work spaces. Their governmental subsidized handicap organization, The Swedish Association for the Electrohypersensitive, F.E.B., was founded in 1987, and some of its members were once electrical engineers who

worked in the telecommunications or electronics industries. In fact, the Institute for Working Life, in Sweden, has reported that 12.5% of the engineers in the electronics industry are electrohypersensitive. (Eriksson N, Höög J, Hansson Mild K, Sandström M, Stenberg B, "Förekomst av symtom liknande "sjuka hus-sjuka", bildskärmsrelaterade hudbesvär och 'elöverkänslighet' i den vuxna svenska befolkningen" ["Prevalence of symptoms similar to 'sick building syndrome', computer screen-related skin conditions and 'electrohypersensitivity' in the adult Swedish population", in Swedish], Arbetslivsrapport nr 2000:5, Arbetslivsinstitutet, Umeå)

13. I have been informed that Arthur Firstenberg has been diagnosed with electrohypersensitivity by nine doctors and that he receives disability benefits from the U.S. Social Security Administration on that basis. I have been informed that upon exposure he suffers from subjective symptoms such as dizziness, nausea, concentra-tion and memory difficulties, and insomnia, as well as objective neurological signs such as tremors and hyperreflexia. I have also been informed that in October 2009 he was diagnosed with a heart arrhythmia that he never had before, and which resolved several weeks after he stopped sleeping at his house. I have also been informed that about one week before he was diagnosed with a heart arrhythmia, his neighbor, Raphaela Monribot, moved into a neighboring house that is joined to his house by common wiring, that that neighboring house had previously been vacant, and that Mrs. Monribot has an iPhone which is on 24 hours a day, wireless Internet, and dimmer switches. It is thus likely that his heart arrhythmia was due to electromagnetic exposure from next door, especially taking into account recent papers in the scientific literature. In my opinion, for Arthur Firstenberg it is of paramount importance to immediately achieve a proper

accessibility measure through elimination of the sources of electromagnetic radiation causing his health problems, so that he can enjoy an equal and healthy life in accordance with the UN 22 "Standard Rules on the Equalisation of Opportunities for People with Disabilities", since 2007 upgraded into the UN "Convention on Human Rights for Persons with Functional Impairments".

Further affiant saith not.

Signed and affirmed under penalty of perjury under the laws of the State of New Mexico to be true and correct:

Olle Johansson — April 20, 2010

Olle Johansson — Date

C.V. for Olle Johansson

Olle Johansson, associate professor, head of the Experimental Dermatology Unit, Department of Neuroscience, at the Karolinska Institute (famous for its Nobel Prize in Physiology or Medicine) in Stockholm, Sweden, is a world-leading authority in the field of EMF radiation and health effects. He is also a professor in basic and clinical neuroscience at the Royal Institute of Technology, Stockholm. He has published more than 500 original articles, reviews, book chapters and conference reports within the field of basic and applied neuroscience. He has worked with a number of international colleagues that, later on, became Nobel Laureates, including professors Andrew V. Schally and Roger Guillemin among others.

His doctoral thesis at the Karolinska Institute had the title "Peptide Neurons in the Central and Peripheral Nervous System. Light and Electron Microscopic Studies". He has participated in more than 300 congresses and symposia as an invited speaker, and with free contributions and as an invited 'observer' at an additional 100.

He is a member of, inter alia, The European Neuroscience Association (ENA), IBAS Users of Scandinavia (IBUS), The International Brain Research Organization (IBRO), The International Society for Stereology (ISS), The Royal Microscopical Society (RMS), Scandinavian Society for Electron Microscopy (SCANDEM), Society for Neuroscience, Svenska Fysiologföreningen (Swedish Association of Physiologists), Svenska Intressegruppen för Grafisk Databehandling (SIGRAD) (Swedish Computer Graphics Association), Svenska Läkaresällskapet (Swedish Society of Medicine), and the Svenska Sällskapet för Automatiserad Bildanalys (SSAB) (Swedish Society for Automated Image Analysis).

He is often used as referee for a large number of scientific journals, including the Acta Dermato-Venereologica, Acta Obstetrica & Gynecologica, Acta Physiologica Scandinavica, Acta Stereologica, Archives of Dermatological Research, Brain Research, Canadian Journal of Zoology, Diabetologia, European Journal of Dermatology, Experimental Brain Research, Experimental Dermatology, Histochemical Journal, Journal of Chemical Neuroanatomy, Journal of Comparative Neurology, Journal of Investigative Dermatology, Journal of Microscopy, Neurobiology, Neuroscience, Regulatory Peptides, and Skin Pharmacology.

He has on-going international scientific collaborations with, inter alia, Japan, Brazil, India, Serbia, Germany and the US. His studies have been widely recognized in the public media, including newspapers, radio and TV as well as on the Internet, both nationally as well as internationally.

Selection of papers:

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Skin changes in patients claiming to suffer from "screen dermatitis": a two-case open-field provocation study

Johansson O, Hilliges M, Björnhagen V, Hall K. Skin changes in patients claiming to suffer from "screen dermatitis": a two-case open-field provocation study.
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Abstract: An open-field provocation, in front of an ordinary TV set, of 2 patients regarding themselves as suffering from skin problems due to work at video display terminals (VDTs) is presented. Using immunohistochemistry, in combination with a wide range of antisera directed towards cellular and neurochemical markers, we were able to show a high-to-very high number of somatostatin-immunoreactive dendritic cells as well as histamine-positive mast cells in skin biopsies from the anterior neck taken before the start of the provocation. At the end of the provocation the number of mast cells was unchanged; however, the somatostatin-positive cells had seemingly disappeared. The reason for this latter findings is discussed in terms of loss of immunoreactivity, increase of breakdown, etc. The high number of mast cells present may explain the clinical symptoms of itch, pain, edema and erythema. Naturally, in view of the present public debate, the observed results are highly provocative and, we believe, have to be taken seriously.

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Key words: human skin – immunohistochemistry – somatostatin – histamine – "screen dermatitis"

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Introduction

Reports of skin complaints in people exposed to video display terminals (VDTs) are becoming an increasing phenomenon in several countries (for refs., see 1). Very little is known about the cause of these health complaints. The symptoms may be grouped into objective ones, including erythema, papules and pustules, as well as subjcetive ones including sensations of heat, itch, pain, smarting, etc. (2). Clinical dermatologists have regarded the symptoms to be mostly of rosacea or rosacea-like dermatitis nature (cf. 2). A large scale epidemiological study has shown that the subjective facial skin symptoms were more common among VDT-exposed persons, but there were no significant difference between exposed and non-exposed groups in objective skin signs or skin disease (3). The early notion that employees with VDT work might have specific facial histological changes could not be confirmed by Berg et al. in their histopathological study (4). In the present investigation, the aim was to study possible morphological as well as histochemical changes in the skin before and after an open-field provocation, in front of an ordinary TV set, of 2 patients believing themselves to suffer from skin problems due to work at VDTs, i.e. "screen dermatitis".

Material and methods

Subjects

Two patients (females; 40 and 54 years of age) claiming to have suffered for several years from "screen dermatitis" were investigated in the study. The patients did not have any other on-going medication or any systemic or dermatological diseases, including acute infections. Routine and special (including peptide radioimmunoassay) laboratory blood tests were performed before and after provocation (see below). Regularly (each 15 min) during the provocation the blood pressure was also monitored.

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Provocation situation

The patients arrived in the morning, one at a time, at the clinic (Department of Endocrinology), in an objectively and subjectively unaffected state. They were immediately subjected to the first pair of biopsies and blood tests. Directly following this, they were placed in front of an ordinary household television set (distance 40–50 cm) and the blood pressure analysis was commenced. Care was taken to ensure that the patients were not able to inspect their own mirror-images, thus, they were not in a situation of visual self-suggestion. An interviewer continuously examined the subjective and objective reactions during the provocation. The patients were told to interrupt the on-going provocation at any time, and, finally, when they could not stand further time in front of the TV screen. At this point, the second pair of biopsies and blood tests were taken. Finally, the patients were interviewed after an additional 24–48 hours.

Preparation of tissue

Double punch biopsies (3 mm; 1 cm apart) were taken under local anesthesia with lidocaine (0.5%) without epinephrine from the anterior neck skin (20 mm below angulus mandibulus) before the start of and after the cessation of the provocation (see above). One of the two biopsies was immersed for 2 h at 4°C in a solution of 14% saturated picric acid and 10% formalin. The other biopsy in each pair (to be incubated with the histamine antiserum; cf. Ref. 5) was immersed in 4% carbodiimide (1-ethyl-3-3-dimethylaminopropyl-carbodiimide; Sigma Chem. Comp., USA) diluted in phosphate buffer (pH 7.4)) for 2 h at 4°C. All the tissue was then rinsed for at least 24 h in 0.1 M Sörensen's buffer containing 10% sucrose, 0.01% NaN_3 and 0.02% Bacitracin, and 14 µm sections were cut using a cryostat (Microm, Heidelberg), thawed on to gelatine-coated slides and processed for indirect immunohistochemistry (see below).

Antibodies

Rabbit or mouse antibodies to substance P (SP; 1:400; Amersham), calcitonin gene-related peptide (CGRP; 1:400; Peninsula), neurokinin A (NKA; 1:100; E. Theodorsson-Norheim, Stockholm), galanin (GAL; 1:400; Peninsula), vasoactive intestinal polypeptide (VIP; 1:400; Peninsula), peptide histidine isoleucine amide (PHI; 1:3,200; J. Fahrenkrug, Copenhagen), neuropeptide tyrosine (NPY; 1:400; L. Terenius, Stockholm), enkephalin (ENK; 1:25; Kemila (Sera-Lab)), dynorphin (DYN; 1:400; L. Terenius, Stockholm), somatostatin (SOM; 1:800; R.P. Elde, Minneapolis), protein S-100 (S-100; 1:400; K. Haglid, Göteborg, and L. Olson, Stockholm), neuron-specific enolase (NSE; 1:800; UC), protein gene product 9.5 (PGP 9.5; 1:2,000; UC) and histamine (HIST; 1:2,000; Milab) were used. All antibodies were checked in parallel in positive controls from normal human skin, to avoid any false-negative interpretations.

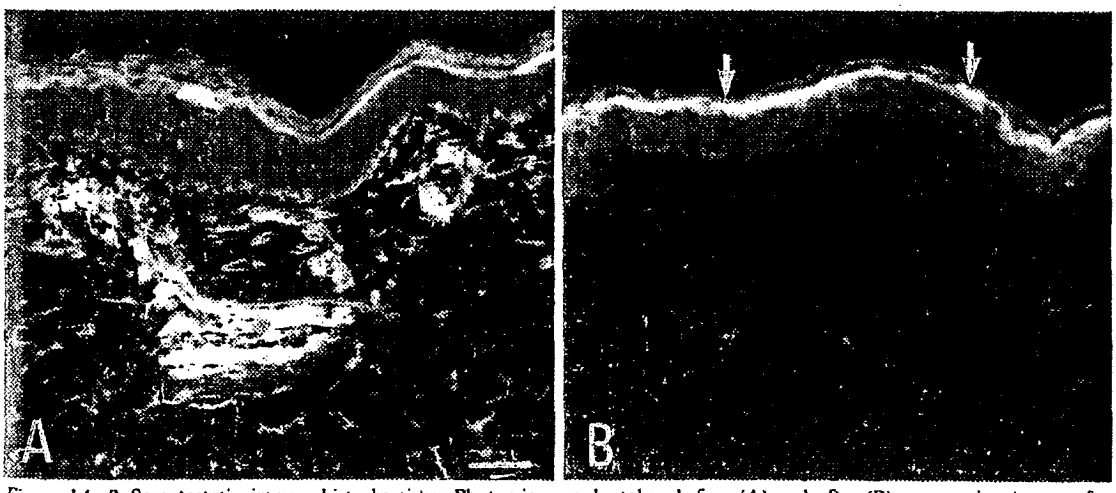

Figure 1A, B. Somatostatin immunohistochemistry. Photomicrographs taken before (A) and after (B) provocation (see text for further details) from patient A. In A, a high number of somatostatin-immunoreactive dendritic cells is seen in the epidermis and dermis. In B, all these cells are seemingly gone, i.e. most probably they have lost their capacity to react with the somatostatin antiserum used. Arrows in B point to unspecific background fluorescence. Bar in A indicates 50 µm.

Immunohistochemistry

The indirect immunofluorescence technique was used. The sections were kept in a humid atmosphere, incubated with the above-mentioned antibodies overnight at 4°C, rinsed in PBS, incubated for 30 min at 37°C in rhodamine (TRITC)-conjucated goat anti-rabbit or anti-mouse IgG (1:80 or 1:40; Boehringer Mannheim), rinsed and mounted. All antibodies were diluted in 0.3% Triton X-100. For observation and photography a Nikon Microphot-FXA or Optiphot fluorescence microscope was used. The material was blind-coded and evaluated by 2 independent observers.

Results

Clinical assessment

Objectively, patient A responded with skin redness already after 10-15 min. This redness was further aggravated until the patient stopped the provocation (after 60 min). The skin was at that moment swollen and gave an impression of general edema. The patient was also sweating somewhat. Furthermore, the patient reported sensations of tingling in the body parts facing the TV screen. At the end of the provocation, the patient complained of dizziness and gave incomplete and inadequate answers to the interviewer's questions. Her speech was also slurred. Patient A was, after a couple of weeks, provoked once more, at which she withstood the situation for 30 min, showing the same objective and subjective symptoms as above.

Patient B, on the other hand, did not reveal any objective or subjective signs at all, apart from some temporal faint reddening of the skin of the neck which only lasted for approx. 10 min. She stopped the provocation after 3.5 hours without any feelings of illness. The patient was disappointed at not having reacted at all.

Both patients reported profound feelings of subjective illness 24 hours (and onwards) after the provocation. At inspection of patient A 24 h after the end of the provocation, a large number of papules and pustules was seen in the skin of the face.

Immunohistochemistry

In the biopsies taken before provocation a remarkably high number of SOM-immunoreactive dendritic cells was found in the dermis, preferentially around the blood vessels and hair follicles as well as in the basal layer of the epidermis (Figs. 1A and 2A). Furthermore, a profound amount of histamine-positive mast cells could be detected in the carbodiimide-fixed tissue before the start of the provocation (Fig. 3A). The cells were granulated and observed preferentially around the blood vessels.

After provocation, no somatostatin-immunoreactive cells at all could be revealed in either patient A or patient B using the presently employed immunohistochemical method (Figs. 1B and 2B). This observation was the basis for the repetition of the provocation for patient A, i.e. to further establish this finding. Regarding the histamine cells, no changes in morphology, number or fluorescence intensity were observed after the provocation (Fig. 3B), as compared to the pre-provocation state.

There were no difference in the SP, CGRP, NKA, GAL, VIP, PHI, NPY, ENK, DYN, S-100, NSE or PGP 9.5 immunoreactivities before and after the provocation, and the patterns generally looked normal. Furthermore, no changes could be

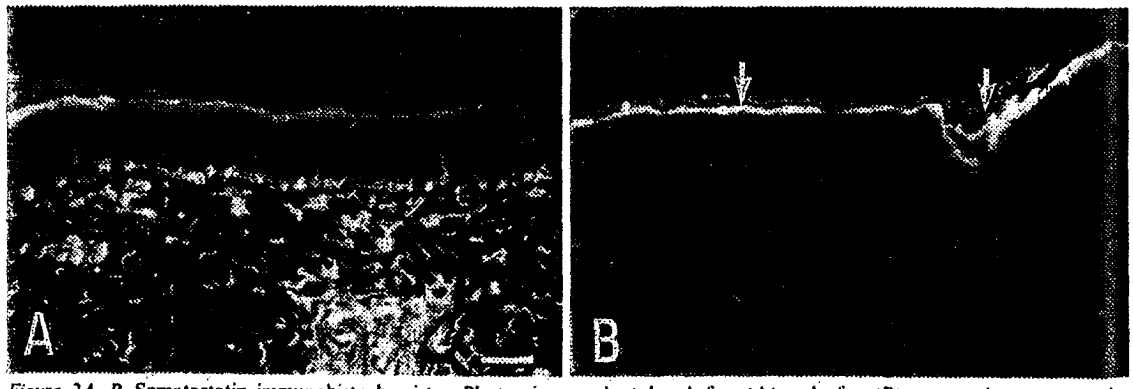

Figure 2A, B. Somatostatin immunohistochemistry. Photomicrographs taken before (A) and after (B) provocation (see text for further details) from patient B. In A, a very high number of somatostatin-immunoreactive dendritic cells is seen in the epidermis and dermis. In B, all these cells are seemingly gone, i.e. most probably they have lost their capacity to react with the somatostatin antiserum used. Arrows in B point to unspecific background fluorescence. Bar in A indicates 50 μm.

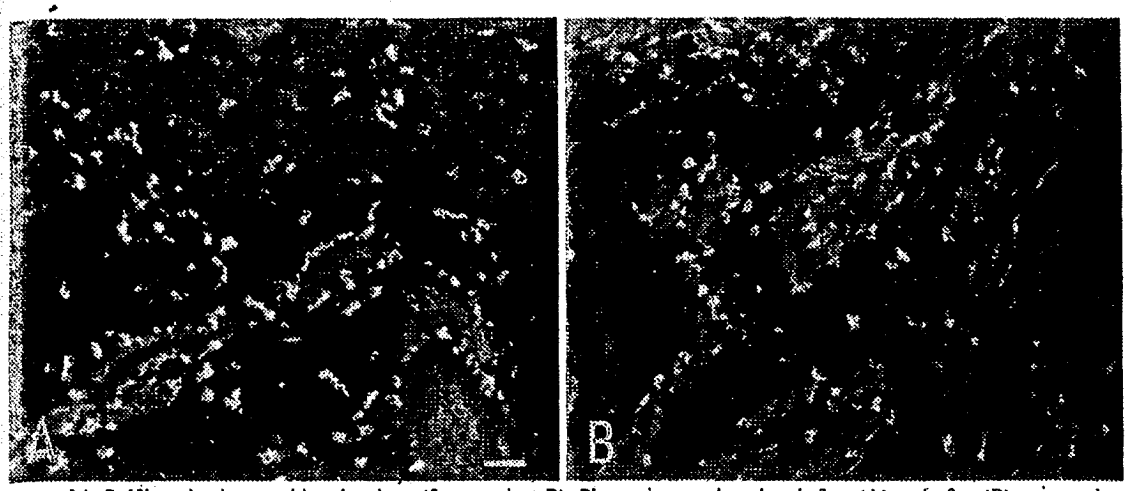

Figure 3A, B. Histamine immunohistochemistry (from patient B). Photomicrographs taken before (A) and after (B) provocation (see text for further details). A high-to-very high number of mast cells is observed both in A and B. There is no clear-cut difference between the two occasions. Bar in A indicates 50 μm.

seen in the routine blood tests or in the blood pressure monitoring, however, both patients had significant changes in the blood level of pancreatic polypeptide.

Discussion

In the following, our results will be discussed. However, it has to be pointed out that we cannot, based upon the present results, draw any conclusions about the cause of the changes observed. Whether this is due to electric or magnetic fields, a surrounding airborne chemical, stress factors, or something else, still remains an open question. As the basis for an explanation of our present observations, it is tempting to speculate about an effect of the electric and/or magnetic fields emitted by the TV set, but such a correlation can only be obtained in true blind or double-blind experiments.

In the present study, a high number of somatostatin-positive dendritic cells was encountered in the dermis and epidermis of 2 patients claiming to suffer from "screen dermatitis". Compared to our ongoing studies regarding such somatostatin-immunoreactive dendritic cells in normal healthy controls (6; Johansson et al., in preparation), we were immediately struck by the very dense population of these cells, both within the basal layer of the epidermis and around the dermal blood vessels and within the connective tissue.

After the open-field provocation, to our great surprise, the somatostatin-immunoreactive cells were no longer detectable using the presently employed immunohistochemical method. It is our belief that the cells still remained in the tissue, but, for some unknown reason they were no longer immunoreactive towards the somatostatin antibodies used. The cells may have released their content of somatostatin-like immunoreactivity, or the degradation of the molecule(s) responsible for the immunoreactivity may have been enhanced. However, also direct cytotoxic effects have to be taken into consideration as well as migration of the dendritic cells from the skin to other organs, such as the lymphoid system.

We also investigated the presence of mast cells in the skin using histamine-based immmunohistochemistry (cf. Ref. 5). There was no change in number before compared to after the provocation; however, the number of mast cells in their affected areas was remarkably high already from the beginning. Again, it has to be pointed out that the material is too small to allow for any general statement, but, a mastocytosis could very well, due to histamine effects, explain the subjective sensations of itch and pain as well as changes in the blood vessel system leading to edema and erythema reported in this patient category (cf. Refs. 1 and 2). In this context, it must be mentioned that Berg et al. (4) were unable to observe any difference, as compared to normal human skin, in their material.

It is of great importance to note that the 2 patients, subjectively and objectively, from a clinical point of view did not respond in an equal manner during the provocation. In spite of this, our method was sensitive enough to detect the same changes in both patients. With these 2 patients at hand, we cannot fully explain the observed effects as only a Pavlovian-type conditioning reflex or a general stress reaction. It should also be pointed

out that both patients reported profound feelings of subjective illness 24 h (and onwards) after the provocation. It may, therefore, be argued that the time spans generally used for inspection of these patients in earlier studies may very well have been simply too short.

It is evident from our preliminary data that biological changes are present in the patients claiming to suffer from "screen dermatitis". In view of the recent epidemiological studies pointing to a correlation between long-term exposures from magnetic fields and cancer (7, 8), our data ought to be further analyzed. One question that immediately arises is how ordinary healthy normal humans will react in this kind of open-field provocation situation. Blind or double-blind provocations in a controlled environment are also necessary to elucidate possible underlying causes for the changes reported in this investigation.

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A screening of skin changes, with special emphasis on neurochemical marker antibody evaluation, in patients claiming to suffer from "screen dermatitis" as compared to normal healthy controls

Johansson O, Hilliges M, Han S-W. A screening of skin changes, with special emphasis on neurochemical marker antibody evaluation, in patients claiming to suffer from "screen dermatitis" as compared to normal healthy controls. Exp Dermatol 1996: 5: 279–285. © Munksgaard, 1996

Abstract: In the present study, facial skin from so-called "screen dermatitis" patients were compared with corresponding material from normal healthy volunteers. The aim of the study was to evaluate possible markers to be used for future double-blind or blind provocation investigations. Differences were found for the biological markers calcitonin gene-related peptide (CGRP), somatostatin (SOM), vasoactive intestinal polypeptide (VIP), peptide histidine isoleucine amide (PHI), neuropeptide tyrosine (NPY), protein S-100 (S–100), neuron-specific enolase (NSE), protein gene product (PGP) 9.5 and phenylethanolamine N-methyltransferase (PNMT). The overall impression in the blind-coded material was such that it turned out easy to blindly separate the two groups from each other. However, no single marker was 100% able to pin-point the difference, although some were quite powerful in doing so (CGRP, SOM, S-100). However, it has to be pointed out that we cannot, based upon the present results, draw any definitive conclusions about the cause of the changes observed. Whether this is due to electric or magnetic fields, a surrounding airborne chemical, humidity, heating, stress factors, or something else, still remains an open question. Blind or double-blind provocations in a controlled environment are necessary to elucidate possible underlying causes for the changes reported in this investigation.

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Key words: human – skin – immunohistochemistry – "screen dermatitis" – neurochemical markers – neuropeptides

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Reports of skin complaints in people exposed to video display terminals (VDTs) are becoming an increasing phenomenon in several countries (1–4). Very little is known about the cause of these health complaints. The symptoms may be grouped into objective ones, including erythema, papules and pustules, as well as subjective ones including sensations of heat, itch, pain, smarting, etc. (3–8). Clinical dermatologists have regarded the symptoms to be mostly of rosacea or rosacea-like dermatitis nature (9). A large-scale epidemiological study has shown that the subjective facial skin symptoms where more common among VDT-exposed persons, but no significant differences between exposed and non-exposed groups in objective skin signs or skin disease were reported (10). The early notion that employees with VDT-work might have specific facial histological changes could not be confirmed by Berg et al. (11) in their histopathological study. In the present investigation, the highly sensitive indirect immunofluorescence methodology (12) has been utilized. The aim of the study was to evaluate possible markers to be used for future double-blind or blind provocation investigations.

Material and methods

Subjects

3 groups were investigated. The material was sampled and taken by 2 professional dermatologists (see

Acknowledgements) at the Department of Dermatology, Karolinska Hospital. The groups consisted of normal healthy controls (no VDU work; no skin symptoms; n=3; males; 33, 34 and 44 years of age), "screen dermatitis" patients (VDU work; subjective skin symptoms; n=4; 1 male, 3 females; 42, 43, 44 and 52 years of age) and "screen dermatitis" patients (VDU work; subjective and objective (erythema, telangiektasia) skin symptoms; n=8; 2 males and 6 females; 26, 38, 42, 49, 49, 52, 59 and 60 years of age). Of the latter patients, 3 (females; 38, 52 and 60 years of age) were omitted from the study because of improper technical handling, such as inferior fixation. The "screen dermatitis" patients had been suffering for several years from facial skin symptoms. The patients did not have any on-going medication or any systemic or dermatological diseases, including acute infections.

Biopsies

The subjects arrived, one at a time, to the clinic (Department of Dermatology, Karolinska Hospital). Punch biopsies (2 mm) were taken under local anaesthesia with lidocaine (0.5%) without epinephrine from the lateral part of the face, over the arcus zygomaticus. The biopsies were taken at exactly the same place regardless of the actual symptoms. The idea behind this strategy was to enable for the dermatologists to have an anatomically defined spot and, thus, to avoid variation in the material due to different localities. Finally, the biopsies were blind-coded by the dermatologists.

Preparation of tissue

The biopsies were immersed for 2 h at 4°C in a solution of 14% saturated picric acid and 10% formalin. All the tissue samples were then rinsed for at least 24 h in 0.1 M Sörensen's buffer containing 10% sucrose, 0.01% NaN_3 and 0.02% Bacitracin, and 14 µm sections were cut using a cryostat (Microm, Heidelberg). The sections were thawed on to gelatine-coated slides and processed for indirect immunohistochemistry (see below).

Antibodies

Rabbit or mouse antibodies to calcitonin gene-related peptide (CGRP), neuropeptide KG_2($NPKG_2$), galanin (GAL), somatostatin (SOM), γ-melanocyte stimulating hormone (γ-MSH), vasoactive intestinal polypeptide (VIP), peptide histidine isoleucine amide (PHI), neuropeptide tyrosine (NPY), protein S-100 (S–100), neurofilament (NF), neuron-specific enolase (NSE), protein gene product 9.5 (PGP 9.5) and phenylethanolamine N-methyltransferase (PNMT) were used. The characteristics of the antibodies are summarized in Table 1. Two (SOM polyclonal; PGP 9.5) of the antibodies were added at a later stage of the investigation, therefore, the first biopsies were not incubated with these antibodies.

Immunohistochemistry

The indirect immunofluorescence technique (12) was used for demonstrating the neuropeptides and neuroactive substances. The sections were kept in a humid atmosphere, incubated with the above-mentioned antibodies overnight at 4°C, rinsed in phosphate buffered saline (PBS), incubated for 30 min at 37°C in rhodamine (TRITC)-conjugated goat anti-rabbit or anti-mouse IgG (1:80 or 1:40; Boehringer Mannheim), rinsed and mounted. All antibodies were diluted in 0.3% Triton X–100. For observation and photography a Nikon Microphot-FXA or Optiphot fluorescence microscope was used. The material was evaluated by 2 independent observers using

Table 1. Description of the primary antibodies

Antibody	Abbreviation	Dilution	Species	Source
calcitonin gene-related peptide	CGRP	1:400	rabbit	Peninsula
neuropeptide KG_2	$NPKG_2$	1:400	rabbit	E. Theodorsson-Norheim, Stockholm
galanin	GAL	1:400	rabbit	Peninsula
somatostatin monoclonal	SOM	1:200	mouse	R.P. Elde, Minneapolis
somatostatin, polyclonal	SOM	1:800	rabbit	R.P. Elde, Minneapolis
γ-melanocyte stimulating hormone	γ-MSH	1:200	rabbit	L. Terenius, Stockholm
vasoactive intestinal polypeptide	VIP	1:400	rabbit	Peninsula
peptide histidine isoleucine amide	PHI	1:400	rabbit	J. Fahrenkrug, Copenhagen
neuropeptide tyrosene	NPY	1:400	rabbit	L. Terenius, Stockholm
protein S-100	S-100	1:400	rabbit	K. Haglid, Göteborg & L. Olson, Stockholm
neurofilament	NF	1:500	rabbit	K. Haglid, Göteborg & L. Olson, Stockholm
Neuron-specific enolase	NSE	1:200	rabbit	UC
protein gene product 9.5	PGP 9.5	1:2,000	rabbit	UC
phenylethanolamine N-methyltransferase	PNMT	1:800	rabbit	M. Goldstein, New York

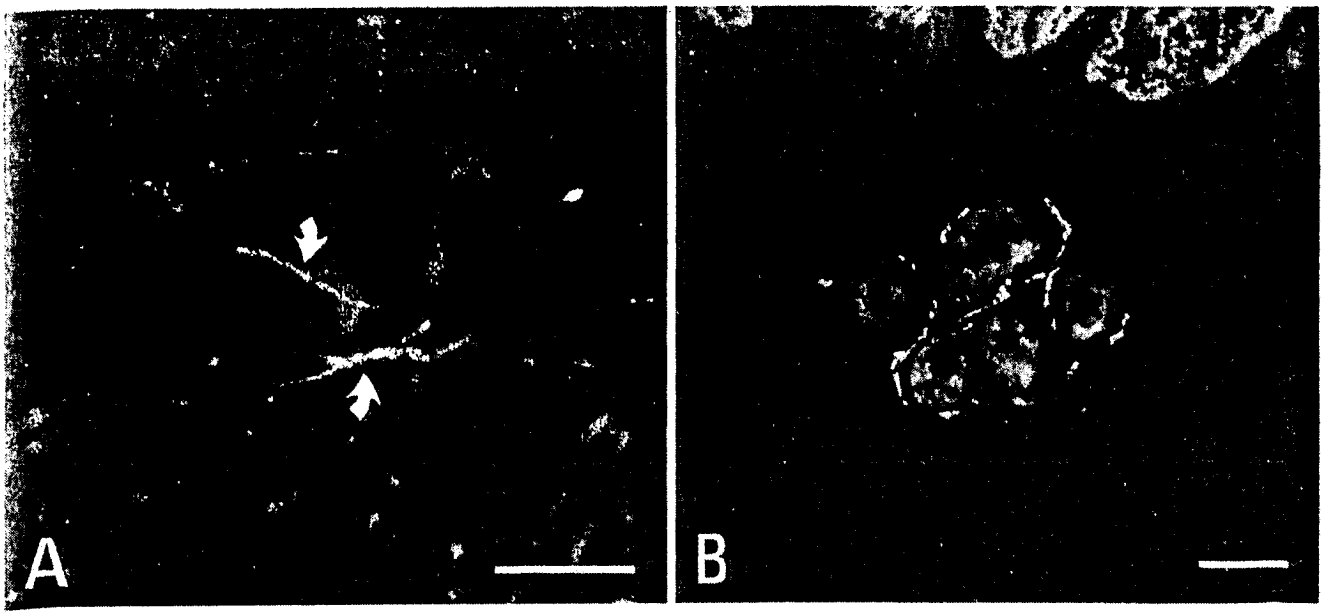

Figure 1. Immunofluorescence micrographs from "screen dermatitis" skin. (A) CGRP immunoreactive nerve fibers (arrows) in the dermis. (B) PHI immunoreactive nerve fibers surrounding eccrine sweat glands. Bars indicate 100 µm (A) and 50 µm (B), respectively.

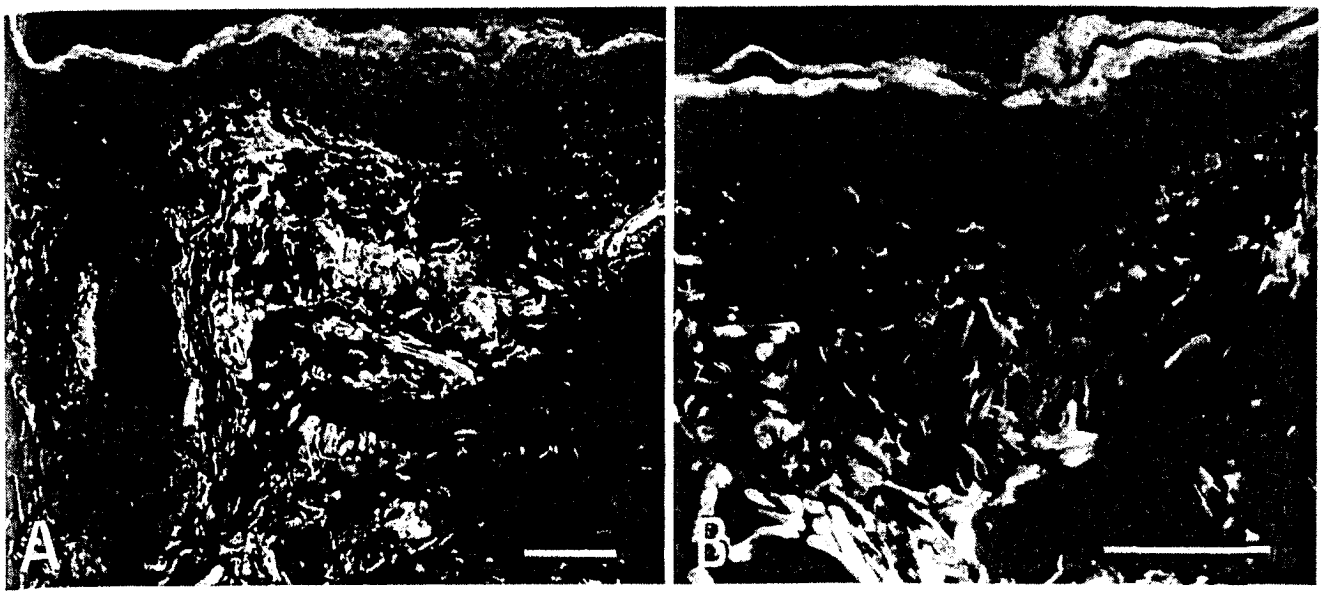

Figure 2. Immunofluorescence micrographs from "screen dermatitis" skin after incubation with a polyclonal antiserum to somatostatin. Immunoreactive cells in large numbers are seen all over the dermis. Single cells are also found in the basal part of the epidermis. Note, that the chain of cells in the upper granular layer is also seen in normal skin. Bars indicate 50 µm.

a 5-graded semiquantitative scale. For further technical details, see Ljungberg & Johansson (13).

Results

In the following, no description of the normal material is given, since this has been published in extenso in the literature. Only differences observed in the "screen dermatitis" material compared with the normal control tissue are given below. This is against the background given in our aim, thus, to evaluate possible markers to be used for future double-blind or blind provocation investigations.

CGRP: 4 out of the 9 (4/9) "screen dermatitis" patients had few-to-very few nerve fibers in the dermis (Fig. 1A). The other patients revealed a normal-looking image concerning the nerve fiber number and distribution. However, 3 others had a very high

background in the papillary dermis. Thus, in summary, 7/9 showed an abnormal pattern.

$NPKG_2$: no conclusive differences were observed between the normal healthy control material and the "screen dermatitis" patients.

GAL: no conclusive differences were observed between the normal healthy control material and the "screen dermatitis" patients. It may be noted, that in 2/9 patients single GAL immunoreactive, round-to-oval dermal cells with a central, oval nucleus were observed. Such cells have not previously been observed in normal material.

SOM, monoclonal: 5/9 patients had single, very weak and very thin processes in the dermis. 6/9 had a very weak staining of the myelin.

SOM, polyclonal: 7 patients were investigated. All (7/7) showed a higher number of SOM positive dendritic cells. 1 patient had a remarkably high number as well as cells entering the epidermis (Fig. 2A). Another patient also had a remarkably high number of SOM immunoreactive dendritic cells in the dermis, but, no cells were seen in the epidermis (Fig. 2B). Four patients had cells located perivascularly. One patient instead showed very few cells.

γ-MSH: polymorphonuclear cells immunoreactive to γ-MSH were found in 3/9 "screen dermatitis" patients. 8/9 had a higher background fluorescence in the stratum papillare.

VIP: 2/9 did not reveal any nerve fibers at all around blood vessels, sweat glands or other dermal appendages. Furthermore, yet another 2/9 had a decreased number of nerve fibers as compared to normal healthy skin.

PHI: 2/9 did not reveal any nerve fibers at all around blood vessels, sweat glands or other dermal appendages. Furthermore, yet another 2/9 had a decreased number of nerve fibers as compared to normal healthy skin (Fig. 1B). PHI immunoreactive cells were seen in 8/9 patients. Of these, 5/9 revealed small cells with paranuclear staining. 2/9 had somewhat larger, round cells in groups. Their nuclei were eccentrically located. 3/9 had polymorphonuclear-like cells.

NPY: 1/9 did not reveal any NPY positive nerve fibers at all. 3/9 had a decreased number as compared to normal, healthy material. Single, small dermal cells were observed in 4/9 of the patients. Such cells have not been described in normals.

S-100: in the dermis, generally the pattern looked normal. However, the S-100 immunoreactive dendritic cells of the epidermis could be grouped into several different patterns of change. First, 3/9 pa-

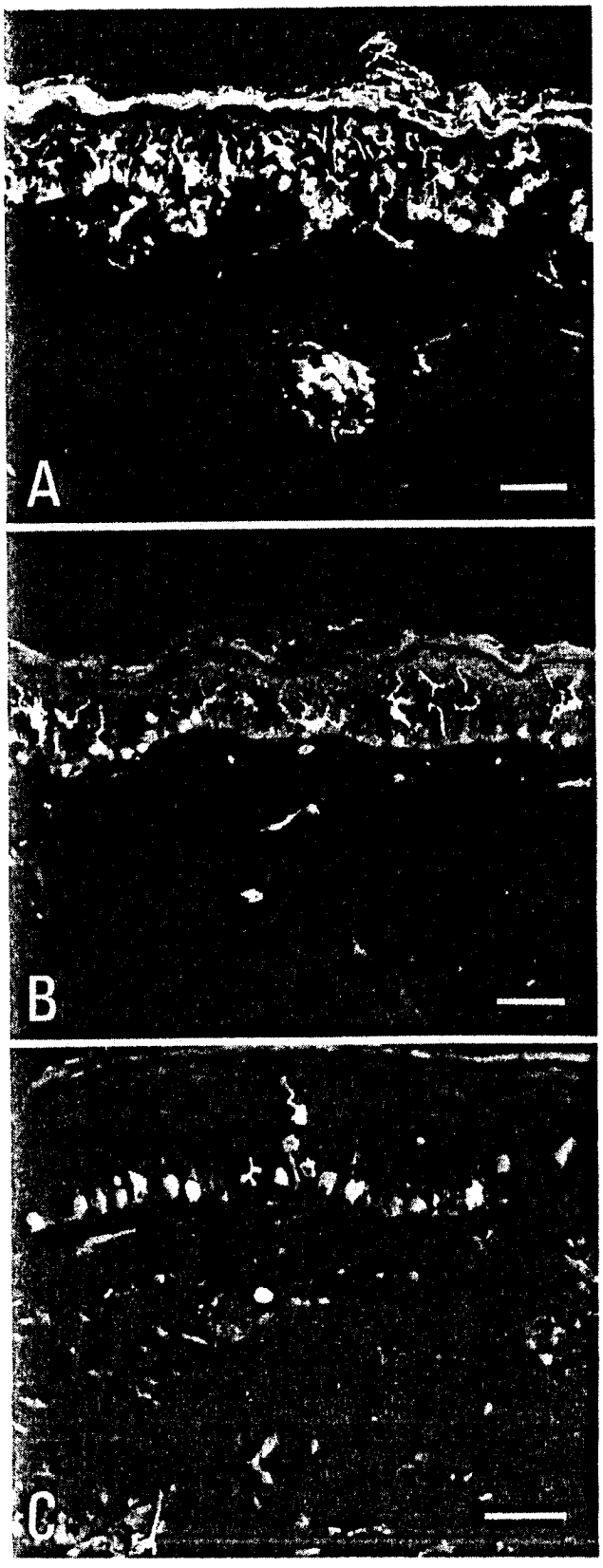

Figure 3. Immunofluorescence micrographs from normal (A) and "screen dermatitis" (B, C) skin after incubation with S-100 antibodies. A partial loss of epidermal dendritic cells could sometimes be seen (cf. A and B). In addition, certain cells revealed fewer and weaker dendritic processes, and even showing a complete loss of them (C). Bars indicate 50 μm.

tients showed partial loss of epidermal dendritic cells, i.e., along the epidermis areas of complete cellular loss were seen (cf. Fig. 3A, B). In addition, the remaining cells were located to the stratum basale and revealed fewer and weaker dendritic processes, even sometimes showing complete loss of processes (cf. Fig. 3A, C). Secondly, one group (5/9) had cells located at their normal position, but their processes were fewer, weaker and sometimes even absent. A certain overlap could be observed between these two groups. Finally, only one patient had a normal-looking pattern regarding frequency, location as well as morphology.

NF: no conclusive differences were observed between the normal healthy control material and the "screen dermatitis" patients.

NSE: in the dermis, no clear-cut differences were observed between the normal healthy control material and the "screen dermatitis" patients. In the epidermis, a complete loss of nerve fibers were found in 2/9, in 2/9 nerve fibers were only seen in the stratum basale, and in yet 2/9 fewer nerve fibers were revealed, however, with a normal location within the epidermis.

PGP 9.5: 5 patients were investigated. All (5/5) showed epidermal fibers running all the way up to, and including, the stratum granulosum, however, one patient revealed a highly decreased number. This patient did not reveal nerve fibers equally high up in the epidermis. 3/5 had an increased number. In addition, it may be noted that one of these latter patients had nerve fibers running in a more straight fashion.

PNMT: 1/9 had a massive number of PNMT positive cells (with granular fluorescence) in the entire dermis (cf. Fig. 4A, B). 4/9 had a somewhat lower number of equally-looking cells (cf. Fig. 4A, C). The other "screen dermatitis" patients had a more normal appearance, i.e., single PNMT immunoreactive cells were revealed.

In summary, the two independent observers could easily, in the blind-coded fashion, distinguish, based on the above-given description, the "screen dermatitis" patients from the normal healthy volunteers. It may be noted, that two rosacea patients being processed in parallel did not differ from the normal material apart from earlier reported findings, but, were clearly not similar to the "screen dermatitis" tissue.

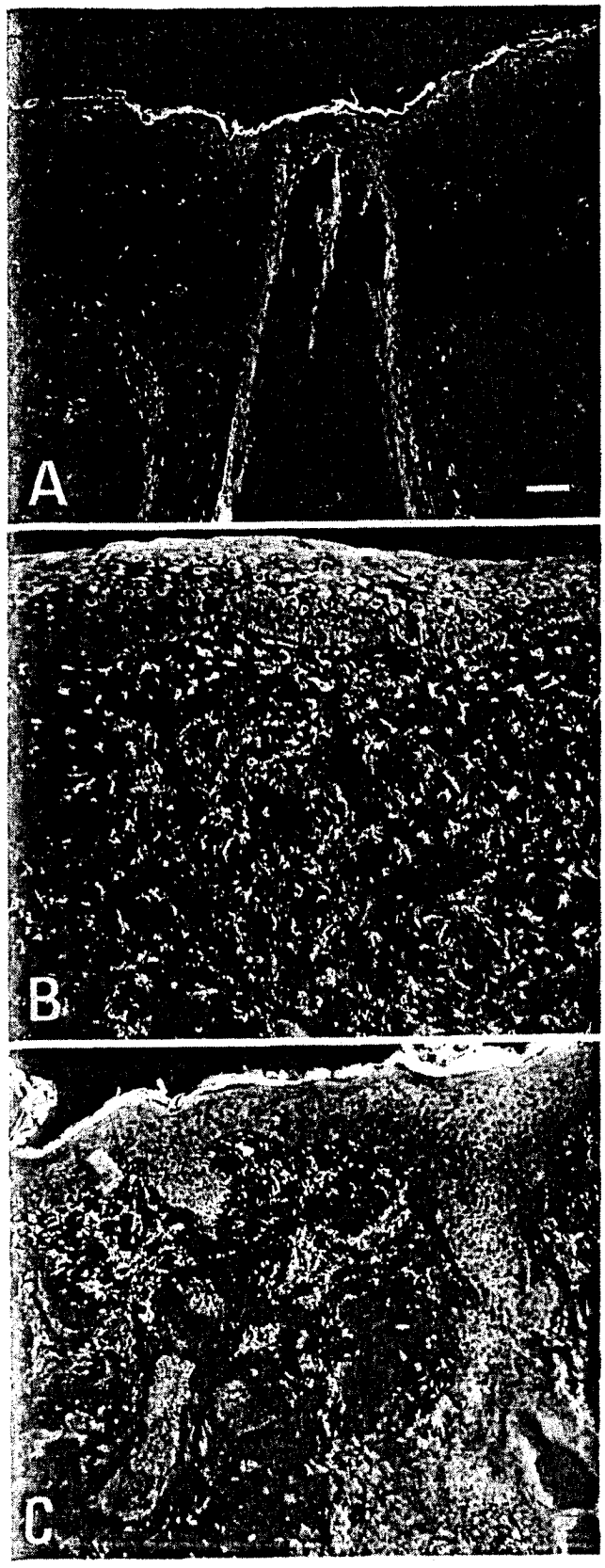

Figure 4. Immunofluorescence micrographs from normal (A) and "screen dermatitis" (B, C) skin using PNMT immunohistochemistry. One patient had a massive number of PNMT positive cells (with granular fluorescence) in the entire dermis (cf. A and B). 4 other patients had a somewhat lower number of equally-looking cells (C). In addition, the epidermis often revealed a general increase in fluorescence. Bar in A indicates 50 μm.

Discussion

In the following, our results will be discussed. However, it has to be pointed out that we cannot, based upon the present results, draw any definitive conclusions about the cause of the changes observed. Whether this is due to electric or magnetic fields, a surrounding airborne chemical, humidity, heating, stress factors, or something else, still remains an open question. Blind or double-blind provocations in a controlled environment are necessary to elucidate possible underlying causes for the changes reported in this investigation.

In the present study, clear differences between normal healthy skin and corresponding tissue from "screen dermatitis" patients were found for the biological markers CGRP, SOM (polyclonal), VIP, PHI, NPY, S-100, NSE, PGP 9.5 and PNMT. The overall impression in the blind-coded material was such that it turned out easy to blindly separate the two groups from each other. However, no single marker was 100% able to pin-point the difference, although some were quite powerful in doing so (CGRP, SOM (polyclonal), S-100). From a statistical point of view, it is not likely at all, that the observed differences can be explained by mass significance phenomena, but, naturally further and larger studies have to be initiated to rule out any such influences.

Certain markers could very well explain some of the claimed clinical, subjective and/or objective, symptoms. For instance, changes in the CGRP immunoreactive nerve fibers could be the basis for sensory symptoms, such as itch, pricking pain, and smarting. The autonomic markers VIP, PHI and NPY (but perhaps also (through axon-reflex actions) CGRP) may explain redness and oedema. SOM and S-100 within epidermal and dermal dendritic cells could account for the general subjective sensation of an on-going inflammation and susceptibility to skin infections as well as sensitivity to ordinary light. The morphologic markers NSE and PGP 9.5 most likely reflect structural differences between the two groups, however, it is not easily understood if they are primary or secondary in sequence. Furthermore, they are also seemingly contradictory to each other, but it should be pointed out that only 5/9 patients were incubated with PGP 9.5. It may also reflect the possibility that NSE and PGP 9.5 actually show 2 different nerve fiber populations, something never clearly investigated. Finally, PNMT is more difficult to understand, since this is regarded as a more or less negative control marker in normal healthy skin, only showing single immunoreactive cells. But, in one patient a massive number of PNMT positive cells (with granular fluorescence) was seen in the entire dermis, and 4/9 patients had a somewhat lower number of equally-looking cells. It should be remembered that PNMT is the norepinephrine-converting enzyme, leading to the production of epinephrine. Maybe this is the chemical basis for a local stress-like reaction, not dependent on stress-mediated increased levels of adrenal medullary catecholamines, but, instead dependent on true physical factors, such as electric and/or magnetic fields, humidity, heating, etc., influencing the skin? If electric and/or magnetic fields are involved, with the on-going public debate in mind, they most probably are of high frequency nature, including both MHz and GHz ones. Of course, our data cannot exclude psychological stress as an important confounder.

The initial aim of the present study was to evaluate possible markers to be used for future double-blind or blind provocation investigations. This goal has been fulfilled. At the same time, new and highly remarkable observations were made. The fact that the two independent observers easily could, in the blind-coded fashion, distinguish the "screen dermatitis" patients from the normal healthy volunteers came as a big surprise to us. Naturally, this will lead us into further strong efforts to throw more light onto the very difficult health issue of "screen dermatitis".

It is evident from our preliminary data that biological differences are present in the patients claiming to suffer from "screen dermatitis". In view of the recent epidemiological studies pointing to a correlation between long-term exposures from magnetic fields and cancer (14, 15; Flodérus B. et al., personal communication), our data definitely ought to be further analyzed.

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The effect of extremely low-frequency electromagnetic fields on skin and thyroid amine- and peptide-containing cells in rats: An immunohistochemical and morphometrical study ☆

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Abstract

The aim of this study was to investigate the influence of extremely low-frequency electromagnetic fields (ELF-EMFs) on mast cells (MCs), parafollicular cells, and nerve fibers in rat skin and thyroid gland. The experiment was performed on 24 2-month-old Wistar male rats exposed for 4 h a day, 7 days a week for 1 month to EMFs (50 Hz, 100–300 μT, 54–160 V/m). After sacrifice, samples of skin and thyroid were processed for indirect immunohistochemistry or toluidine blue staining and then were analyzed using the methods of stereology. The antibody markers to serotonin, substance P, calcitonin gene-related peptide (CGRP), and protein gene product 9.5 (PGP) were applied to skin sections and PGP, CGRP, and neuropeptide Y (NPY) markers to the thyroid. A significantly increased number of serotonin-positive MCs in the skin and NPY-containing nerve fibers in the thyroid of rats exposed to ELF-EMF was found compared to controls, indicating a possible EMF effect on skin and thyroid vasculature.

Keywords: Electromagnetic field; Skin; Thyroid gland; Rat; Stereology

1. Introduction

Among a broad range of biological effects of extremely low-frequency electromagnetic fields (ELF-EMFs) on almost all organ systems in various mammalian organisms, alterations in the neuroimmune system have also been proposed. EMFs have been found to alter the level of neurotransmitters or their metabolites (Seegal et al., 1989; Prato et al., 1995; Lai and Carino, 1999) and the content of biogenic amines in the spinal ganglion (Merkulova, 1990). The contents of calcitonin gene-related peptide (CGRP), somatostatin, and protein S-100 were found altered in the cutaneous nerve fibers in subjects expressing sensitivity to EMF exposure (Johansson et al., 1996). Based on criteria of morphological appearance, number, and the distribution of mast cells (MCs) in the skin of healthy subjects exposed to EMFs, it has been shown that these cells are susceptible to the EMF influence (Johansson et al., 2001), as are MCs in the intestine, lymph nodes, thyroid, and brain (Iurina et al., 1997; Matavulj et al., 1999; Cook et al., 2000).

Nerve fibers in skin and thyroid gland contain a number of mediators targeting adjacent cells via synaptic as well as paracrine actions that maintain the homeostasis of these organs under physiological conditions. The microanatomical relations of nerve fibers and MCs as well as their functional relationship are

☆ The study was performed on white laboratory rats of the Wistar strain and was conducted with the permission of the Ethical Committee on Animal Experiments of the University of Novi Sad.

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recognized in skin (Bauer and Razin, 2000), but these are not confirmed for the thyroid.

The nociceptive nerve fibers of the skin contain, among other peptides, substance P (SP) and CGRP. It is known that CGRP exerts vasodilatation in skin, while SP is responsible for vasodilatation and protein extravasation (Holzer, 1998) and is considered to induce degranulation of MCs (Suzuki et al., 1995). The population of MCs in the skin contains a number of preformed mediators in its granules (histamine, serotonin, etc.), but can de novo synthesize additional biologically active molecules (leukotrienes, prostaglandins, etc.) upon cell activation. Apart from CGRP and SP, MC-derived serotonin has a vasoactive role in the skin as well (Gershon et al., 1975; Askenase, 1979).

The thyroid gland harbors three populations of cells from which bioactive molecules originate: sympathetic–adrenergic nerves, MCs, and parafollicular (PF) cells (Melander, 1977). In the intrathyroidal adrenergic nerve fibers that terminate near blood vessels and thyroid follicles, noradrenalin is colocalized with neuropeptide Y (NPY), which is known to increase glandular blood flow (Michalkiewicz et al., 1993). The influence on thyroid microcirculation, and also on thyroid hormone synthesis and secretion, is attributed to amine-containing MCs, which store histamine and serotonin in their granules (Melander and Sundler, 1972; Melander et al., 1975). PF cells contain several peptides, including CGRP, which is also found in nerve fibers around blood vessels, follicles, and PF cells. The coexistence of CGRP with SP has been shown for many nerve fibers, while for PF cells indications of colocalization with calcitonin within the same granules have been found (Grunditz et al., 1986). Under certain experimental conditions, the CGRP from PF cells has been shown to display an inhibitory role in thyroid hormone secretion (Ahren, 1991).

In the present study, we set out to investigate whether power-frequency EMFs can affect skin, which is permanently exposed to a variety of external environmental stimuli, and the thyroid gland, a well-vascularized organ with a superficial anatomical position in mammals. We used selected antibody markers to cutaneous and thyroid gland nerve fiber peptides and for the granular content of MCs and PF cells to demonstrate whether ELF-EMFs could affect them in a manner visible by morphological screening and stereological quantification. The immunohistochemical methods we used enabled us to demonstrate serotonin in cutaneous MCs, SP and CGRP in nerve fibers of the skin, as well as NPY, and protein gene product (PGP) and CGRP in thyroid nerve fibers; the latter two were also used for the demonstration of PF cells in the thyroid gland. For the visualization of intrathyroid MCs, the classical histological staining of toluidine blue was performed.

2. Materials and methods

2.1. Animals

The experiment was performed on 24 white male rats of the Wistar strain. Animals were 1.5 months old when they arrived in the laboratory, and they were kept for 2 weeks, until the beginning of the experiment. Animals were housed under laboratory conditions at $20\pm2\,^{\circ}C$ and subjected to a controlled photoperiod (14 h light, 10 h dark). Access to tap water and pelleted food was omitted during exposure hours. Twelve animals were exposed to the influence of ELF-EMF for 4 h a day, 7 days a week for 1 month. Twelve animals served as controls; they were handled in the same manner as the exposed group and maintained in a similar environment, but without the presence of artificially produced ELF-EMF. The investigation was made with the permission of the Ethical Committee on Animal Experiments of the University of Novi Sad.

2.2. Exposure system and the EMF

The exposure system, by which ELF-EMF was produced, was composed of a single coil of solenoid equipped with a cooling system and energized from 50 Hz, 220 V, and 10 A via an autotransformer, which provided a 100-V output. Cages with animals were placed on both sides of the coil, perpendicular to the coil axis, at a 12-cm distance, and were covered with a plastic lid. The coil axis was parallel to the lines of force of the geomagnetic field (north–south direction). The EMF produced by the coil was in the horizontal direction regarding the geomagnetic field; it was inhomogeneous and of decaying intensity along the animal cages, with values of 300 μT and 160 V/m on the side of the cage near the coil and 100 μT and 54 V/m on the opposite side, while the value of the electric field at any other point in the room was less than 10 V/m. The residential values of the magnetic (AC Milligaussmeter, Model 42B-1, Monitor Industries, USA) and electric fields (HI-3607 E.L.F. Sensor, Holaday Industries, USA) were measured to be 0.2 μT and 2.9 V/m, while the value of the geomagnetic field (Gauss/Tesla Meter, Model 4048, F.W. Bell, USA) was 40 μT. The estimated value of the magnetic field inclination was 61.2°.

2.3. Specimen preparation

Immediately after the last hour on the last day of exposure, animals were sacrificed via diethyl ether narcosis. Samples of skin from the interscapular region were taken as well as samples of thyroid gland with adjacent parts of the trachea and surrounding connective tissue. All specimens were fixed at 4 °C in a mixture of paraformaldehyde (4%) and saturated picric

acid (14%). Thereafter, the tissue samples were rinsed in 0.1 M Sörensen's buffer containing 10% sucrose, 0.01% NaN_3, and 0.02% bacitracin and cut into 14-μm-thick sections using a cryostat (Microm, Heidelberg, Germany). Sections were further processed for indirect immunohistochemistry or toluidine blue staining.

2.4. Immunohistochemistry

In order to demonstrate MCs or nerve fibers in skin and nerve fibers and/or PF cells in thyroid samples, the indirect immunofluorescence technique was used. Sections were kept at 4 °C overnight in a humid atmosphere during incubation with the following primary antibodies: rabbit antibodies to serotonin (5-HT; 1:500; Verhofstad et al., 1983), mouse or rabbit antibodies to protein gene product 9.5 (PGP 9.5; 1:2000; UltraClone), rabbit antibodies to substance P (1:400; a gift from professor A.C. Cuello, Department of Pharmacology & Therapeutics, McGill University, Montreal, Canada), rabbit antibodies to CGRP (1:600; Peninsula Laboratories), and rabbit antibodies to NPY (1:400; Amersham International). Sections were further rinsed in phosphate-buffered saline (PBS), incubated for 30 min at 37 °C in rhodamine (TRITC)-conjugated donkey anti-rabbit IgG (1:160; Jackson), rinsed, and mounted. For PGP and serotonin double-staining, fluorescein (FITC)-conjugated donkey anti-mouse IgG (1:160; Jackson) was also used. All antibodies were diluted in 0.3% Triton X-100. To test for any possible nonspecific binding of the primary antisera, PBS was applied to certain sections instead of each primary antibody. For observation, a Nikon Microphot-FXA fluorescence microscope was used. All sections were blind-coded and analyzed by the same observer.

2.5. Stereological analysis

Sections stained according to the immunohistochemistry protocol were used for quantitative estimation of MCs, nerve fibers, and PF cells, as were toluidine blue-stained thyroid sections. Skin sections were analyzed starting from the epidermal–dermal junction and thyroid sections from the middle of the lobe (facing the trachea) to the periphery.

Serotonin-containing MCs, PGP-, SP-, CGRP-, and NPY-positive nerve fibers, and CGRP- or PGP-labeled PF cells were estimated according to the principles of design-based stereology. The number of immunoreactive cells or nerve fibers per projected square millimeter was counted using a special microscopic frame under the 20 × objective on two skin sections per sample and two test fields per section or two thyroid sections per sample and four test fields per section. Immunoreactive nerve fibers innervating hair follicles and/or blood vessels were not counted.

Analysis on toluidine blue sections was performed using multipurpose stereological grid M42 placed in the ocular of a Reichert light microscope on four thyroid sections per sample and 100 test fields per animal using ocular 10 × and objective 40 × magnification. The numerical and volume density of degranulated (partially and fully) perifollicular and stromal MCs was determined. Further, the ratio of these two cell groups for both stereological parameters was also calculated.

Estimations were made by the same observer on blind-coded sections. A nonparametric Mann–Whitney test was used for statistical analysis.

3. Results

3.1. Analysis of skin samples

3.1.1. Serotonin (MCs)

Serotonin-positive MCs in skin of the control and the exposed animals were mainly situated in the upper and deep dermis (Figs. 1a and b). An increased number of serotonin-positive MCs with a weak fluorescence and a decreased cell volume was observed in samples of the exposed group (Fig. 1b). MCs under the epidermis were characterized by a variable morphology accompanied by

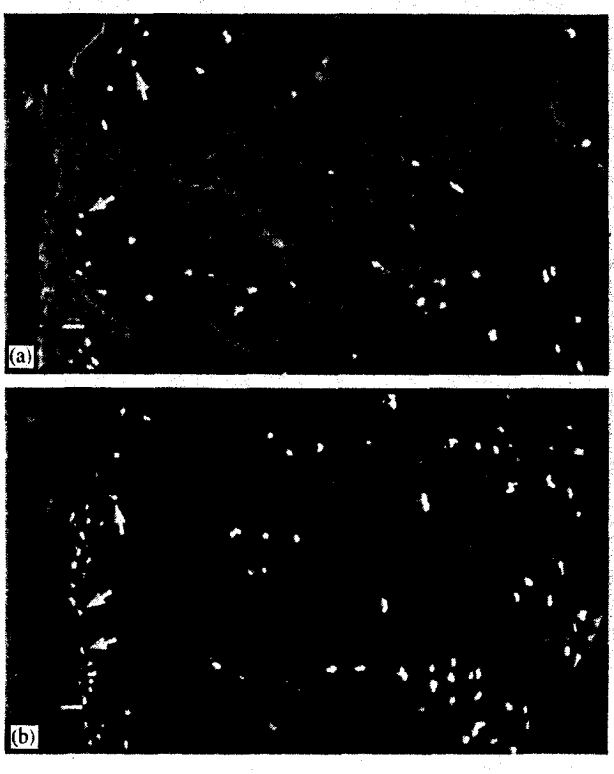

Fig. 1. Serotonin-positive mast cells in the skin of a control animal (a) and an animal exposed to ELF-EMF (b). Brightly fluorescent cells of mainly uniform size and shape (arrows) are seen in (a). The papillary dermis in (b) was found populated with a higher number of cells with diverse shape, size, and fluorescence intensity (arrows). Bars, 50 μm.

a reduced cell size, which suggests degranulation of these cells (Fig. 2b). This was noted in both groups, but most prominently in the exposed group (Figs. 2a and b). In double-stained sections, degranulated serotonin-positive MCs were seen in frequent contact with PGP-positive nerve fibers in the upper dermis of exposed animals. According to the stereological analysis, the increased number of MCs containing serotonin in the exposed group was statistically significant ($P<0.05$) compared to the number in the control group (Fig. 3).

3.1.2. SP (nerve fibers)

Immunoreactivity to SP was found in thin nerve fibers of the skin epidermis and upper dermis in both investigated groups (Figs. 4a and b). Fibers were rarely observed in other parts of the dermis. In the control, SP-containing fibers were mostly brightly fluorescent (Fig. 4a), while weakly stained in the exposed group, and, therefore, occasionally hardly visible (Fig. 4b). Statistical analysis showed a lowered number of SP fibers in exposed animals compared to controls, but it was not significant ($P = 0.95$).

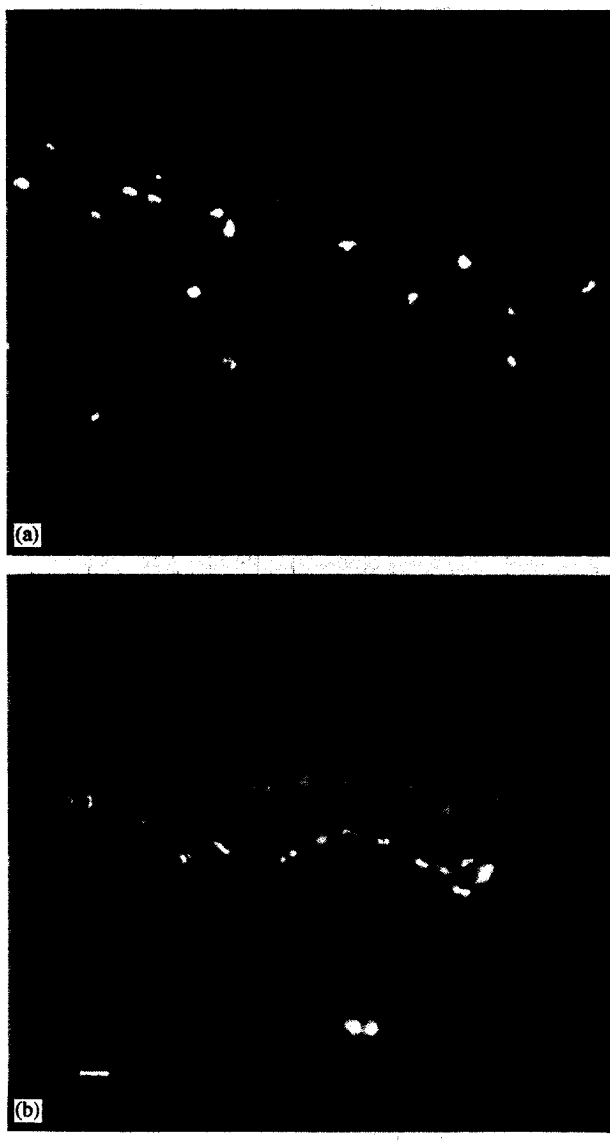

Fig. 2. Serotonin-positive mast cells in the skin of a control animal (a) and an animal exposed to ELF-EMF (b). Degranulated cells apposed to the epidermis and serotonin-containing granules released into the cutaneous connective tissue are observed in (b) and predominantly intact mast cells in (a). Both photomicrographs are of the same magnification. (b) Bar, 50 μm.

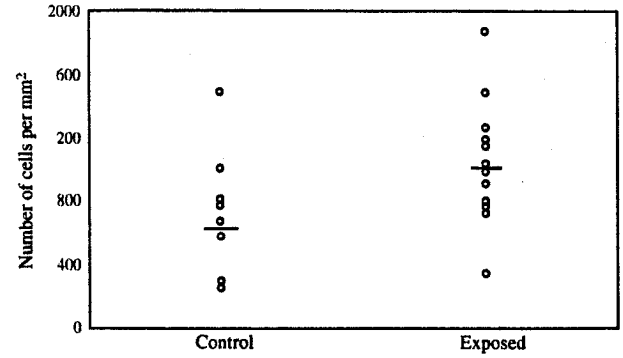

Fig. 3. Number of serotonin-positive mast cells per projected square millimeter of skin in control animals and animals exposed to ELF-EMF. According to the Mann–Whitney U test the difference between the groups was significant at $P<0.05$.

3.1.3. CGRP (nerve fibers)

Nerve fibers containing CGRP were found primarily in the epidermis and upper dermis of the skin, but they were also present in other parts of the dermis (Figs. 4c–f). The area of upper dermis was usually populated with thicker fibers (Figs. 4c–e) and the epidermis with thinner ones (Fig. 4e). In the exposed group, thin fibers were found in a noticeably higher number, especially in the epidermal region. These fibers were often characterized by weaker fluorescence. The calculated value of the CGRP-positive nerve fiber number in the skin of the exposed group was higher than in the control group, but it was not statistically significant ($P = 0.64$).

3.2. Analysis of thyroid gland samples

3.2.1. PGP 9.5 (nerve fibers)

The neuronal marker PGP 9.5 enabled a general overview of the thyroid gland nerve fibers found around thyroid follicles and blood vessels (Figs. 5a and b). There were no apparent differences between the exposed and the control groups of animals except in the counted number of fibers. However, the lowered number of fibers in the exposed group was not significant compared to the controls ($P = 0.50$).

3.2.2. CGRP (nerve fibers)

Nerve fibers containing CGRP were visible around thyroid follicles and blood vessels in both groups, but they were abundantly observed in the gland connective

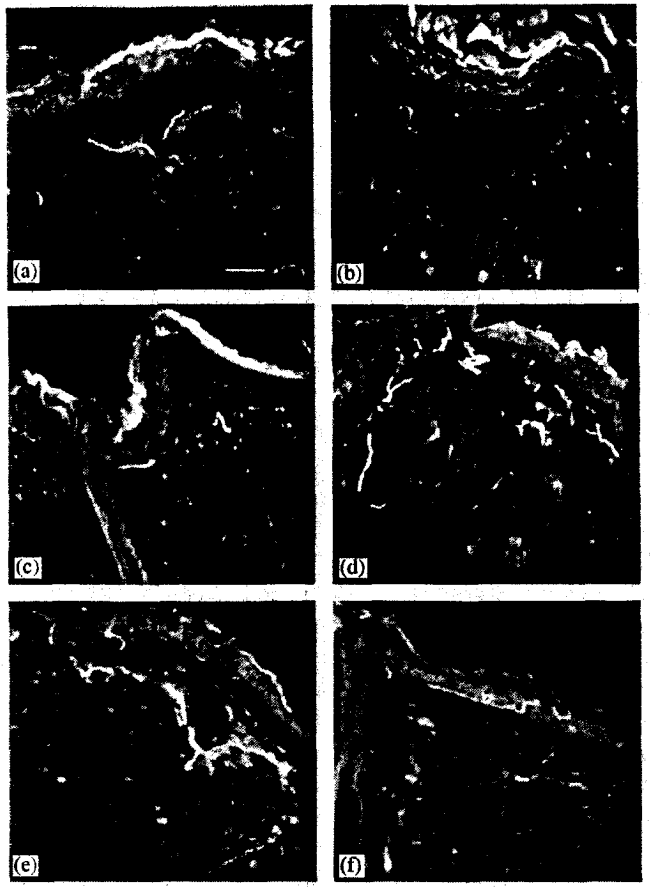

Fig. 4. SP- and CGRP-positive nerve fibers in the skin of control animals (a and c) and animals exposed to ELF-EMF (b, d, e, and f). Brightly fluorescent SP-containing nerve fibers are found in (a) while very thin and weakly fluorescent fibers are seen in (b). A few CGRP-positive nerve fibers are observed in (c) and numerous in (d) and (e). Note the CGRP-containing nerve fibers of different thickness and fluorescence intensity in the epidermis in (e) and (f). All photomicrographs are of the same magnification. (a) Bar, 100 μm.

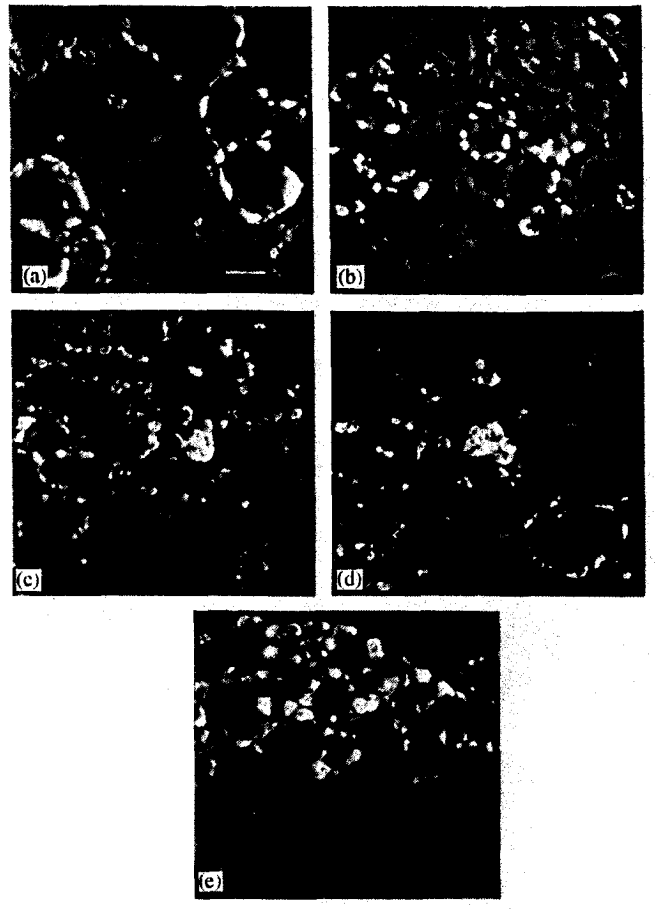

Fig. 5. PGP- and CGRP-positive nerve fibers and parafollicular cells in the thyroid gland of control animals (a and c) and animals exposed to ELF-EMF (b, d, and e). A pattern of distribution of nerve fibers and parafollicular cells labeled with antibodies to PGP 9.5 is observed (a and b). A higher population density of CGRP-containing parafollicular cells is found in (c) compared to (d). Large groups of cells in the stroma of the gland are seen in (c) and (d). Parafollicular cells populate certain areas of the thyroid lobe, with the adjacent parenchyma lacking such positive cells (e). All photomicrographs are of the same magnification. (a) Bar, 100 μm.

tissue of the exposed group. In these animals, nerve fibers showed a decreased fluorescence compared to controls, especially those in contact with PF cells. The prominent increase in the number of CGRP-positive nerve fibers in exposed animals was, however, not significant in comparison to the controls ($P = 0.07$).

3.2.3. NPY (nerve fibers)

Thyroid sections stained using the NPY antiserum revealed nerve fibers situated in close proximity to follicles and blood vessels. The NPY-positive nerve fibers related to thyroid follicles appeared as thin fibers with pale fluorescence in both the control and the exposed groups (Figs. 6a and b). The increased number of these fibers in the exposed animals was statistically significant compared to controls ($P<0.01$) (Fig. 7).

3.2.4. Toluidine blue (MCs)

Partially and fully degranulated MCs in the thyroid gland were divided into cells situated perifollicularly and

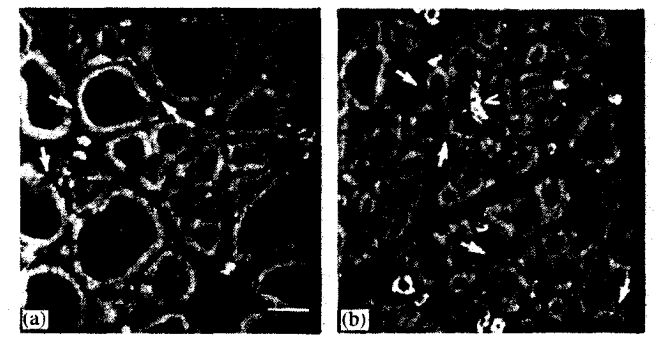

Fig. 6. NPY-positive nerve fibers in the thyroid gland of a control animal (a) and an animal exposed to ELF-EMF (b). Fibers terminating in the vicinity of thyroid follicles are thin and express a pale fluorescence (arrows) in (a) and (b). Note the higher number of fibers and the richly innervated blood vessels with bright fluorescent fibers (arrowhead) in (b). Both photomicrographs are of the same magnification. (a) Bar, 100 μm.

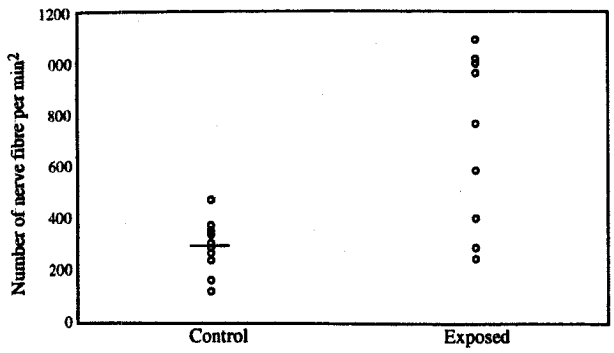

Fig. 7. Number of NPY-immunoreactive nerve fibers per projected square millimeter in the thyroid gland of control animals and animals exposed to ELF-EMF. According to the Mann–Whitney U test, the difference between the groups was significant at $P<0.01$.

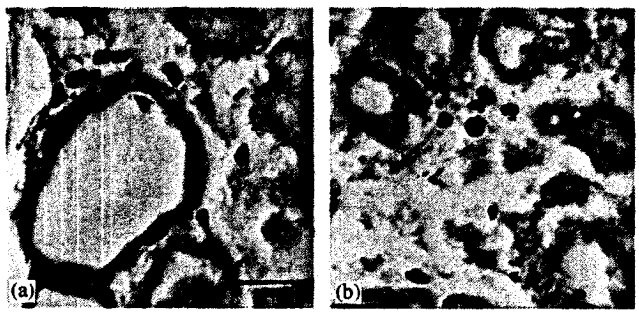

Fig. 8. Mast cells stained with toluidine blue in the thyroid gland of a control animal (a) and an animal exposed to ELF-EMF (b). Degranulated perifollicular mast cells are found in (a) and stromal cells in (b). Both photomicrographs are of the same magnification. (a) Bar, 100 μm.

in the connective tissue stroma of the gland (Figs. 8a and b). Stereological analysis of their numerical and volume density in control and exposed animals showed differences, but they were not statistically significant. The calculated ratio between the two MC groups for both investigated stereological parameters indicated that the stromal MCs exceeded the perifollicular MCs in both groups. However, this was much more prominent in the exposed group than in the control group.

3.2.5. PGP 9.5 (PF cells)

The PGP-positive PF cells were, as is common, found in the connective tissue stroma and in thyroid follicles. PF cells in both control and exposed animals showed a similar pattern of distribution in the thyroid lobes and in their contacts with PGP-positive nerve fibers (Figs. 5a and b). The difference between the groups was the observation of larger groups of stromal PF cells in the exposed specimens and some PF cells with weak fluorescence compared to the controls. The increased number of PF cells in exposed animals showed no statistical significance when compared to controls ($P = 0.54$).

3.2.6. CGRP (PF cells)

The PF cells expressed a strong fluorescence after staining with CGRP in both investigated groups, and a number of cells with weaker fluorescence were also noticed in both groups (Figs. 5c–e). The CGRP-positive cells were found in larger groups in the connective tissue of each group (Figs. 5c and d) and solitary cells in contact with CGRP-containing nerve fibers. The number of cells stained with CGRP was lower in the exposed group compared to the number in the control group (Figs. 5c–e), but it was not significant ($P = 0.45$).

4. Discussion

The results presented above show significantly increased numbers of serotonin-containing MCs and NPY-immunoreactive nerve fibers in skin and thyroid, respectively, of rats exposed to ELF-EMF with the characteristics defined by our exposure protocol.

In rodents, serotonin (or 5-hydroxytryptamine, 5-HT) is synthesized by MCs and released into the surrounding connective tissue after cellular degranulation. The physiological role of this biogenic amine in the skin is known to be vasoactive. Gershon et al. (1975) found that serotonin released from MCs caused the development of gaps between neighboring endothelial cells of postcapillary venules in the skin, leading to increased vascular permeability. Intradermal injections of serotonin were shown to have the same effect (Keahey et al., 1991). Serotonin-positive MCs in samples of skin taken from animals exposed to ELF-EMF in our study were predominantly situated in the upper dermis of the skin. Many of these MCs were characterized by a decreased cell volume and a weak fluorescence, both indications of cell degranulation. Therefore, it could be presumed that the 5-HT released from MCs, which richly populated the papillary dermis, diffused through the connective tissue in the skin and affected cutaneous microvasculature.

MCs containing serotonin were found apposed to nerve fibers positive to SP and CGRP, positioned closely to or being in direct contact with MCs, in the rat mesentery (Crivellato et al., 1991). Substance P has an ability to provoke the release of granules from rat peritoneal and human cutaneous MCs when challenged with this vasoactive transmitter (Ebertz et al., 1987; Amano et al., 1997). CGRP showed the same effect, but was several-fold less potent in augmenting this effect than SP (Piotrowski and Foreman, 1986). Acting upon MCs, SP elicited vasodilatation and vascular permeability via histamine and serotonin (Lam and Ferrell, 1990). However, the microanatomical relations and functional interactions of MCs and nerve fibers are bidirectional: histamine and serotonin released from MCs can affect afferent nerve endings, causing SP and CGRP release from peripheral varicosities, leading to

hyperemia and protein leakage (reviewed in Holzer, 1998).

Considering our results of small alterations in the number of nerve fibers containing SP and CGRP, but also the decreased intensity of SP and CGRP immunoreactivity within the fibers, the interaction of these two neuropeptides with MCs under our experimental conditions may be questionable. This particularly refers to SP because of the very low distribution density of peptidergic fibers harboring this peptide in the skin samples taken from our experimental animals. On the other hand, the number of CGRP-containing fibers was much higher per analyzed field of vision of the skin compared to the number of SP-containing fibers. This could indicate a possible role of CGRP in the skin physiology of rats exposed to EMFs. Under these conditions, MCs would, probably, express their overall effect on the cutaneous vascular bed directly, via serotonin release into the connective tissue of the skin dermis. In our study, serotonin-positive MCs were observed in frequent contact with PGP-positive nerve fibers, indicating that the interaction between them probably occurred under EMF exposure, but whether this is true only for MCs with nerve fibers storing CGRP or for other mediator(s) remains to be elucidated. In addition, one of our previous findings showed a small increase in the number of eosinophil cationic protein-immunoreactive cells in rats exposed to ELF-EMF (data not published). This observation favors a proposed mediator effect on skin vasculature accompanied by moderate eosinophil recruitment, pointing to the involvement of certain nerve fiber-derived mediators.

A number of studies point to neuroimmune responses to the influence of various types of electromagnetic fields. MCs in rats and humans were found to be susceptible to constant magnetic fields, microwaves, and static fields by means of alteration in their number, morphology, and/or mediator content (Doeva et al., 1990; Kalabekov et al., 1995; Donnellan et al., 1997; Johansson et al., 2001). Estimation of the number of CGRP-positive nerve fibers in humans expressing subjective and/or objective skin symptoms during exposure to EMFs showed an increase of these fibers in the papillary dermis (Johansson et al., 1996). Both MCs and sensory nerve fibers in the skin are known to be involved in the response to ultraviolet (UV) B irradiation, playing an important role in the development of UVB-induced immunosuppression (reviewed in Aubin, 2003). Upon UVB irradiation, CGRP is released from nerve endings in the skin, triggering MCs to deplete tumor necrosis factor-alpha (Niizeki et al., 1997). Although it was revealed that UVB exposure increases CGRP, but also SP content, in cutaneous nerve fibers, CGRP was proven to be a dominant neuropeptide in mediating UV effects on skin (Legat et al., 2002; Seiffert and Granstein, 2002). Our results of an increased number of CGRP nerve fibers and degranulated MCs in the skin of exposed animals resemble the cutaneous effects of UVB irradiation, except that the released MC mediator is different. Considering the data in the literature and our findings, we hypothesize that ELF-EMF could indirectly activate MCs to release serotonin by inducing cutaneous nerve terminals to free CGRP.

NPY, another known vasoactive peptide, is considered to induce vascular effects through mediator secretion from MCs (Shen et al., 1991; Mousli and Landry, 1994). The spatial association between MCs and nerve fibers containing neuropeptides has been demonstrated in a number of organs and tissues (Williams et al., 1995), but was not substantiated for the thyroid gland. However, both NPY and MC-derived histamine independently influence thyroid microcirculation. NPY is involved in the regulation of thyroid function by acting as a vasoconstrictor agent and, thereby, increasing thyroid blood flow after binding to NPY Y1 receptors on capillaries (Michalkiewicz et al., 1993; Matsuda et al., 2002). Histamine released from MCs is also known to increase thyroid blood flow and capillary permeability (Melander et al., 1975). Our current results show an increased number of intrathyroid NPY-immunoreactive nerve fibers and a predominance of degranulated stromal MCs over perifollicular ones in rats exposed to ELF-EMF. Additionally, we have previously found a considerable increase of histamine-containing MCs in the thyroid of exposed rats (data not published). Altogether, these observations indicate that thyroid vascular tissue in rats is probably affected during EMF exposure. As a consequence, the microenvironment of increased blood flow and capillary permeability in the gland may enhance the uptake of substrate by thyroid follicular cells used in different physiological processes, including thyroid hormone synthesis.

CGRP localized within thyroid nerve fibers is shown to have no effect on basal or thyroid-stimulating hormone (TSH)-stimulated thyroid hormone secretion, but it enhances VIP-stimulated iodothyronine secretion (Grunditz et al., 1986). CGRP from PF cells is also without an effect on thyroid hormone secretion, but when administrated together with two other PF cell-derived peptides, calcitonin and katacalcin, the TSH-induced increase in thyroid hormone release in mice is inhibited (Ahren, 1989). Since CGRP is found around blood vessels in the thyroid and its role as a potent vasodilator is well known, it is possible that CGRP is involved in the regulation of local microcirculation, as noted earlier (Grunditz et al., 1986). According to our results, the number of CGRP-positive nerve fibers was increased in rats exposed to ELF-EMF, suggesting CGRP as a possible neuronal modulator of NPY

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