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Constitutive Production of Lymphocyte Activating Factors by Normal Tissues in the Adult Rat

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Abstract Lymphocyte activating factors (LAFs), e.g., interleukin-1 (IL-1) and IL-1-like factors, have previously been demonstrated outside the immune system in the skin, thymus epithelium, and the human and rat testis. We have studied the presence of LAFs in normal tissues of the adult rat, utilizing a highly IL-1 sensitive murine thymocyte proliferation assay. We have demonstrated high amounts of LAF activity in the tongue, esophagus, proventricular part of the stomach, and the liver. Some activity was also demonstrated in the duodenum, placenta, spleen, Peyer's patches, glandular stomach, and jejunum, but no bioactivity was present in other gastrointestinal, endocrine, lymphoid, or haematopoietic tissues. We were also unable to detect any LAF activity in the reproductive organs (except for the testis), urinary tract, skeletal and muscular tissues, brain, eyes, salivary glands, or lung. In the esophagus the activity was mainly localized to the mucosa. The LAF activity in the skin was partly inhibited by treatment with a mixture of antibodies against human IL-1α and IL-1β. Dose response curves and gel filtration on a Sephacryl S-200 column suggested the presence of a high molecular weight (90,000–100,000 Da) LAF inhibitory factor in the liver. In all positive tissues, the demonstrated LAFs had a molecular weight of 15,000–25,000 Da, as determined by Sephacryl S-200 gel filtration. Of the positive tissues, the skin, tongue, esophagus, and the proventricular part of the stomach all contain stratified squamous epithelium. It is tempting to suggest that the detected LAFs have a similar function in these barrier tissues, e.g., to serve as host defence factors, or, alternatively or additionally, as tissue growth factors.

Key words: interleukin-1, growth factor, cytokine, proliferation, host defence

Cytokines are a group of potent immunoinflammatory mediators, originally described as products of immune cells and mononuclear phagocytes, but they have recently been shown to be produced by almost any cell type [1]. Interleukin-1 (IL-1) is a family of polypeptides with at least two distinct members, IL-1α and IL-1β [2]. They were initially described as lymphocyte activating factors (LAFs) produced by activated macrophages. They are important in the inflammatory host defence, e.g., by inducing the hepatic acute phase protein synthesis, increasing the production of prostaglandins (particularly PGE_2), inducing the proliferation and activation of T- and B-lymphocytes, and inducing fever by a direct effect on the brain [3].

IL-1 or related factors, have also been detected in the skin, where they are primarily produced by keratinocytes. The main IL-1 protein found in the skin seems to be IL-1α [4–7]. It has been speculated that IL-1 in the skin exerts an immunomodulatory effect, since the skin serves as an important host defence barrier [8,9]. We have previously demonstrated the presence of an IL-1α-like protein with an isoelectric point (pI) of 5.7 and a relative molecular mass (Mr) of approximately 17,000 (17 K) in the rat testis, where it is produced by the Sertoli cells [10–12]. Other authors have demonstrated the constitutive production of IL-1 and IL-1-like factors in vivo in murine corneal epithelial cells [13], the cortex of rat brain [14], human amniotic fluid [15], and human ovarian follicular fluid [16]. By using antibodies against synthetic peptides corresponding to murine pro-IL-1α sequences, IL-1-like immunoreactivity has been localized to rat adrenal chromaffin cells [17], and vas deferens, gastrointestinal tract, urinary bladder, sympathetic ganglia, thymus, spleen, and lymph nodes in rats [18]. IL-1-like factors have also been demonstrated in normal tissues and cells cultured in vitro, such as murine and human thymus epithelium [19,20], mesangial cells of the

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rat kidney [21], the microglia of the human and rat central nervous system [22,23], mouse calvarial cultures [24], human lung tissue [25], pregnant rabbit cervix [26], and human decidua [27]. IL-1 has been shown to exert a variety of effects on several non-lymphoid tissues such as the gonads [28–30], brain [31,32], islets of Langerhan's [33], and the CNS regulation of gastric secretion [34,35]. Synergism between IL-1 and parathyroid hormone in stimulating bone resorption has been demonstrated [36]. This reported production of IL-1 (-like factors) by a variety of tissues and the diverse effects of IL-1 on different target organs have prompted us to systematically investigate the constitutive production of LAFs, including IL-1 (-like factors), in normal tissues in the adult rat. For this purpose we have utilized a thymocyte proliferation assay with responder cells from a highly IL-1 responsive mouse strain.

MATERIALS AND METHODS

Experimental Animals

Male and female Sprague-Dawley rats, aged 60–90 days, were obtained from ALAB, Sollentuna, Sweden, and were kept under standard laboratory conditions with a standard diet and water ad libitum. Thymocytes were obtained from a NMRI mouse substrain (NMRI/KI), aged 4–8 weeks, kindly donated by Professor Tore Midtvedt, Department of Medical Microbial Ecology, Karolinska Institute, Stockholm, Sweden. The animals were killed by CO_2 inhalation.

Tissue Extraction

The animals were dissected immediately after sacrificing, and all tissues and organs to be investigated were removed and frozen at −20°C. Tissues were pooled from 10 animals before freezing. After thawing, the tissues were homogenized in 0.15 M sodium chloride (saline; 1:4 w/v) by means of an Ultraturrax T25 tissue homogenizer (IKA-Labortechnik), or, for volumes smaller than 1.5 ml, a glass piston homogenizer, worked manually. After centrifugation at 750g for 15 minutes to remove non-dispersed material, the supernatants were frozen, thawed, and again centrifuged at 10,000g for 15 minutes in order to sediment the obtained cryoprecipitates. The supernatants were then collected and passed through a Sephadex G-25 column (PD10, Pharmacia) equilibrated in saline, to remove low Mr (<5 K) compounds (e.g., prostaglandins and thymidine) that might interfere with the murine thymocyte bioassay. After filtration (0.22 μm), the samples were frozen at −20°C until further assayed. The esophageal mucosa was prepared by stripping the mucosa from the underlying muscular layer and was then extracted using the same procedure as above. Colon tissues were kept in 0.05 M hydrochloric acid prior to freezing in order to prevent bacterial growth. Ovarian follicular fluid was prepared by puncturing each follicle with a sharp needle and centrifuging the whole ovary on top of a 25 μm filter paper in a test tube at 120g for 5 minutes in order to collect the released fluid. The fluid was analyzed separately in the LAF assay, and the remaining part of the ovary was extracted as above. Placenta was collected from two animals at a gestational age of 14–16 days and was homogenized in saline 1:10 w/v, but otherwise was extracted as above. Rat islets of Langerhan's, prepared by collagenase digestion of pancreas [37], and conditioned medium from 24-hour cultures of islets of Langerhan's, were obtained from Dr. C.G. Östensson, Department of Endocrinology, Karolinska Hospital, Stockholm. Kidney tissues from adult rats (renal corpuscles, outer cortex, inner cortex, and medulla—all isolated by microdissection) were supplied by Dr. B. Sahlgren, Department of Pediatrics, St Göran's Children's Hospital, Stockholm. The protein concentration of the samples was determined by using the method of Bradford [38], with bovine serum albumin (BSA) as a standard.

Lymphocyte-Activating Factor (LAF) Assay

LAF activity was determined by a murine thymocyte proliferation assay as previously described [39,40]. Thymocytes from NMRI/KI mice were suspended in 100 μl cultures of α-modified Eagle's Minimum Essential Medium (α-MEM) and were incubated for 48 hours with phytohemagglutinin-P (5 μg/ml). The sample to be tested was added in a volume of 10 μl from the start. At 46 hours, tritiated thymidine (0.5 μCi/well) was added, and the cells were harvested at 48 hours. The DNA synthesis, as assessed by the incorporated radioactivity, was used as an estimation of the LAF activity and was determined by liquid scintillation counting and expressed as mean counts per minute (cpm) of duplicate cultures. Dose response curves were established for all samples, and the magnitude of the LAF activity was compared at a dose interval where all dose

response curves were parallel. Several concentrations of each sample were tested in order to establish the concentration that produced the maximal LAF activity and to dilute, if possible, any factors that might inhibit thymocyte proliferation in the bioassay. Recombinant human IL-1α and IL-1β (Genzyme) show high activity in this assay, with a detection level for IL-1α of 2 pg/ml and for IL-1β of 1 pg/ml, whereas recombinant and tissue-derived human IL-6 shows no bioactivity alone or in synergy with IL-1 (Fig. 1). The thymocytes also respond to IL-2, IL-4, and IL-7 during these conditions, but the presence of those cytokines was excluded by an identical bioassay, utilizing thymocytes from a different NMRI mouse substrain (NMRI/H; 41). This substrain is resistant to IL-1α and IL-1β, but responds equally to IL-2, IL-4, and IL-7 (Söder et al., to be published). Negative control cultures received culture medium only.

Inhibition of LAF Activity With Antibodies Against IL-1

Aqueous extract of rat skin was incubated for 4 hours at 20°C with a mixture of polyclonal rabbit antibodies against recombinant human IL-1α and IL-1β covalently bound to agarose gel (Endogen, Boston, MA). After incubation, the sample was centrifuged at 2500g for 5 minutes, and the supernatant was tested directly in the LAF assay. Samples incubated either without gel, or with an agarose gel coupled to normal rabbit serum immunoglobulin G, were used as controls.

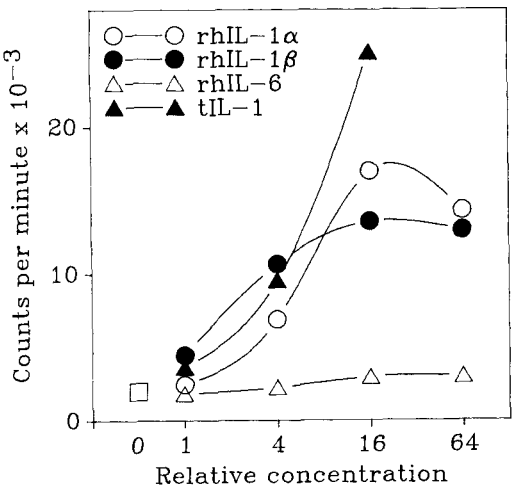

Fig. 1. Proliferation of NMRI/KI mouse thymocytes in response to recombinant human IL-1α (rhIL-1α), rhIL-1β, rhIL-6, and rat testicular IL-1-like factor [tIL-1; 10]. The highest relative concentration (= 64) of rhIL-1α and rhIL-1β was 57 pg/ml, and for rhIL-6, 45 ng/ml. TIL-1 was tested as a crude tissue extract with a highest protein concentration of 190 μg/ml. Data points are mean cpm of duplicate cultures.

Sephacryl S-200 Chromatography

In order to remove high Mr compounds that might interfere with the LAF bioassay, 100 μl of the crude tissue extracts were passed through a Sephacryl S-200 (Pharmacia) column (resin size 185 × 7 mm) and eluted with saline. The flow rate was 20 cm/h. 130 μl fractions were collected and individually tested in the LAF bioassay. Fractions corresponding to Mr 10–40 K from tissues that were negative as crude extracts were pooled before being tested in the bioassay. Human transferrin (Mr 95 K), BSA (67 K), soybean trypsin inhibitor (20.1 K), and cytochrome c (12.4 K) were used as molecular weight markers.

RESULTS

LAFs in Crude Tissue Extracts

The LAF assay, utilizing the NMRI/KI mouse substrain as a source of thymocytes, was shown to be highly sensitive for IL-1α and IL-1β, whereas IL-6 did not induce any thymocyte proliferation during these conditions. Testicular IL-1-like protein (tIL-1) was also bioactive in this assay (Fig. 1).

High amounts of LAF activity were detected in the esophagus, proventricular part of the stomach, and the tongue. The liver contained variable activity. We could also confirm previous findings of high LAF (or IL-1) bioactivity in the skin and in the testis (Fig. 2). No bioactivity was detected in aqueous extracts of lymphoid and haematopoietic organs (spleen, lymph nodes, Peyer's patches, whole thymus, bone marrow), endocrine organs other than the testis (pineal gland, pituitary, thyroid, adrenal gland, whole pancreas, isolated islets of Langerhan's, conditioned medium from islets of Langerhan's, ovary, follicular fluid), reproductive organs (Fallopian tube, uterus, epididymis, vas deferens, prostate gland, seminal vesicles), urinary tract (whole kidney, isolated renal corpuscles, outer and inner renal cortex, renal medulla, ureter, urinary bladder), other gastrointestinal tissues (glandular part of the stomach, pylorus, jejunum, ileum, colon), skeletal and muscular tissues (bone, car-

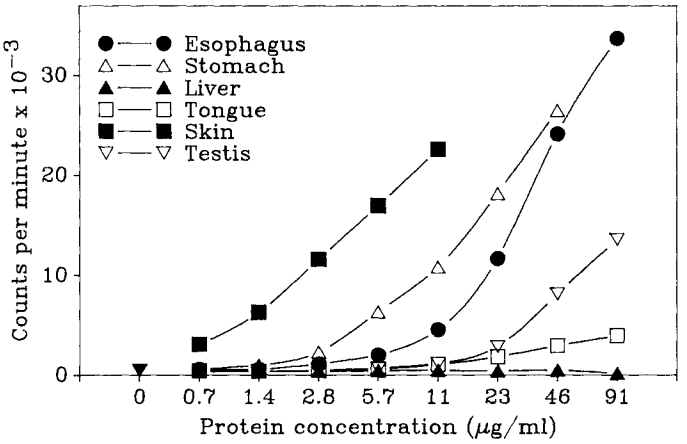

Fig. 2. Dose response curves of LAF bioactivity produced by crude tissue extracts of rat esophagus, proventricular part of the stomach, liver, tongue, skin, and testis. The tissue extracts were adjusted to the same protein concentration before assay. Data points are mean cpm of duplicate estimations. The x-axis expresses final concentration of tissue protein in the test cultures. The highest LAF bioactivity was detected in the skin, and the lowest in the liver. Ten animals were used for all tissue extractions. Identical results were obtained in repeated LAF assays.

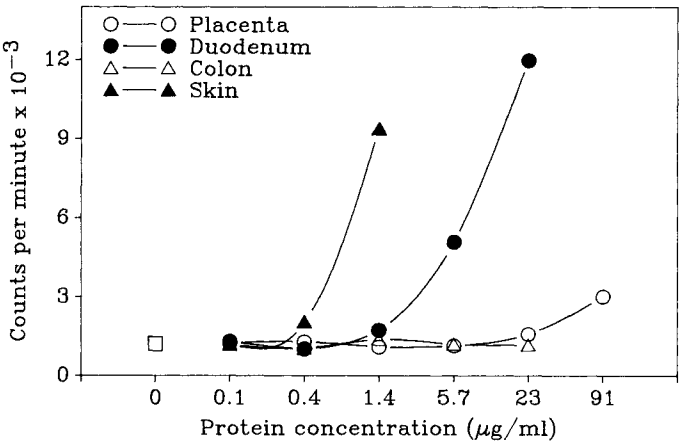

Fig. 3. Dose response curves showing LAF bioactivity in aqueous extracts of the duodenum and placenta. Skin was used as a positive control, and colon as a negative control. The x-axis expresses the final dilution of tissue extracts in the test cultures.

tilage, skeletal muscle, smooth muscle from uterus), whole brain, whole eyes, salivary glands, or lung. The duodenum and the placenta (gestational age 14–16 days) contained slight activity (15% or less of maximal activity; Fig. 3). The esophageal mucosa contained much more bioactivity than the submucosal and muscular layers (Fig. 4). This was also observed with the proventricular part of the stomach (data not shown), though the separation of the mucosa from the underlying layers was technically more difficult in this tissue. Comparing the LAF bioactivity per amount of extracted protein in different tissues, the skin contained higher amounts than the proventricular stomach, and the esophagus, testis, tongue, and liver contained decreasing amounts in comparison (Fig. 2).

All tissue extracts that were positive in the NMRI/KI mouse thymocyte bioassay were also tested in a bioassay using thymocytes from the IL-1 resistant NMRI/H mouse substrain to exclude the presence of IL-2, IL-4, and IL-7, which are bioactive in both assays. The tested tissues were all found to be negative (Fig. 5).

The LAF activity in aqueous extracts of rat skin was partly inhibited by incubation with an immunoaffinity agarose gel containing a mixture of antibodies against human IL-1α and IL-1β, as demonstrated by a shift to the right of the dose response curve (Fig. 6).

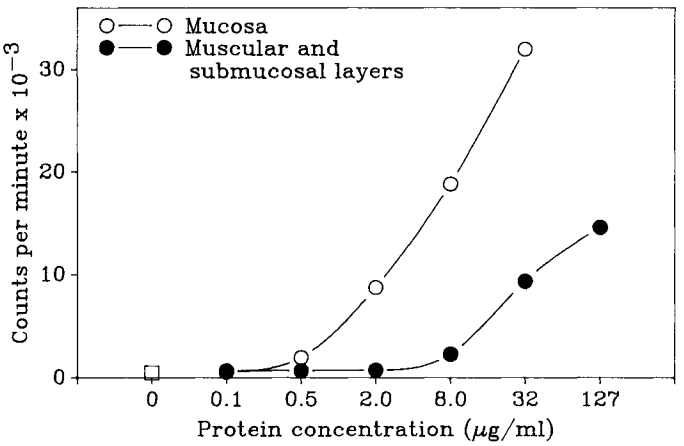

Fig. 4. LAF bioactivity in tissue extracts of rat esophagus. The mucosa was mechanically stripped from the underlying muscular layers and submucosa and was extracted separately. The highest bioactivity was detected in the esophageal mucosa.

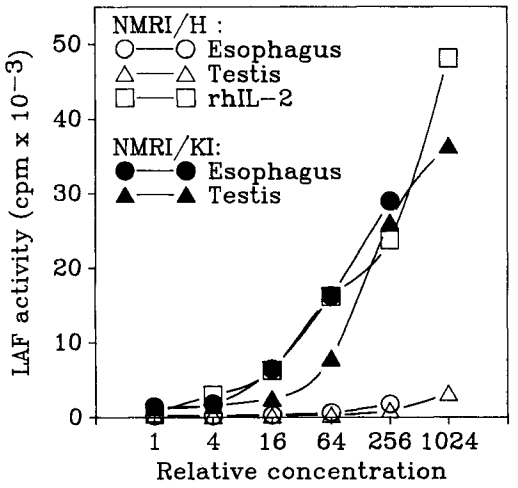

Fig. 5. LAF bioassay utilizing NMRI/KI (IL-1 responsive; filled symbols) and NMRI/H (IL-1 unresponsive; open symbols) mice. No LAF activity was detected with responder thymocytes from the NMRI/H mice with crude tissue extracts of testis and esophagus, whereas high activity was detected in the same tissues at the same protein concentrations using responder thymocytes from the NMRI/KI substrain. Human recombinant IL-2 (rhIL-2; Boehringer Mannheim) was used as a positive performance control in the IL-1 resistant assay. The highest protein concentration of testicular extract was 240 μg/ml (relative concentration 1024), esophageal extract was tested at a maximal protein concentration of 25 μg/ml (256), and the maximal relative concentration of IL-2 (1024) was 900 units/ml. Identical results with no responsiveness in cultures of NMRI/H thymocytes were obtained with crude tissue extracts of all other positive tissues, i.e., the proventricular part of the stomach, the liver, tongue, skin, duodenum, and placenta (data not shown).

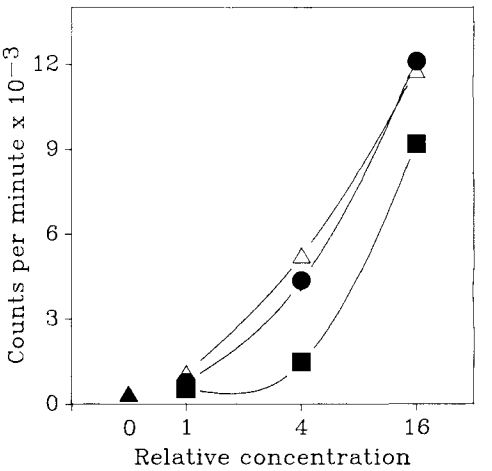

Fig. 6. Partial neutralization of LAF activity of rat skin by antiserum against human IL-1α and IL-1β. The material was tested either as untreated crude tissue extract (△), or after incubation with immunoaffinity agarose gel coupled to antibodies against human IL-1α and IL-1β (■) or normal rabbit serum IgG (●).

LAF Activity After Sephacryl S-200 gel Filtration

In order to remove high Mr compounds, all crude tissue extracts that were negative in the LAF assay were passed through a Sephacryl S-200 column. The Mr 10–40 K fractions were collected and tested in the LAF bioassay. The glandular part of the stomach, the jejunum, Peyer's patches, and spleen exhibited slight bioactivity (less than 10% of maximal activity). All positive tissue extracts were also passed through the Sephacryl S-200 column, and the fractions corresponding to Mr 10–90 K tested individually. In all tissues, including the skin and testis, the LAF activity was eluted as a single peak with an apparent Mr of 15–25 K (Fig. 7). The liver was shown to contain a Mr 90–100 K factor that was inhibitory in the bioassay. After removal of this factor, the liver showed high amounts of LAF activity (Fig. 8).

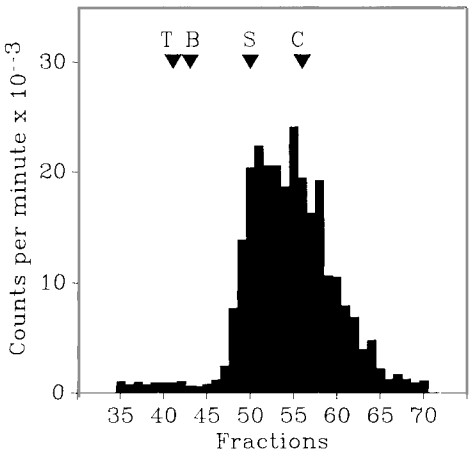

Fig. 7. Sephacryl S-200 elution profile of LAF activity of esophageal tissue extract. The maximal bioactivity eluted with an apparent Mr of 15–25 K. Transferrin (T, Mr 95 K), bovine serum albumin (B, 67 K), soybean trypsin inhibitor (S, 20.1 K), and cytochrome c (C, 12.4 K) were used as Mr markers.

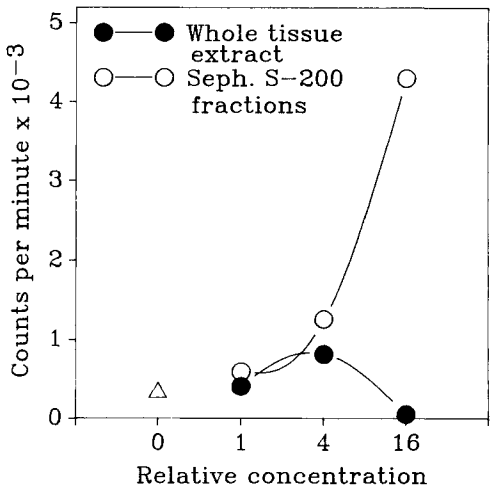

Fig. 8. LAF bioactivity in the liver in the presence or absence of high Mr inhibitory material. Bioactive Sephacryl S-200 fractions were pooled after separation of liver extract, and the crude liver extract was diluted accordingly. The x-axis expresses the final sample dilution in the test cultures. The inhibitory activity was maximal at the highest concentration tested, but as can be seen from the dose response curve, the inhibition was less obvious when the sample was diluted. No inhibition was present in the material pooled from the Sephacryl S-200 column.

DISCUSSION

In this study, we have systematically explored most tissues and organs of intact adult rats for the production of LAFs. We here demonstrate that LAF bioactivity is present in the tongue, esophagus, proventricular part of the stomach, and the liver, and confirm previous findings of such activity in the testis and the skin of adult rats. Some activity was also demonstrated in the duodenum and placenta. After removal of high Mr compounds, that seemed to interfere with the bioassay, slight activity was found in the glandular part of the stomach, the jejunum, Peyer's patches, and the spleen. There was no bioactivity present in other lymphoid and haematopoietic, endocrine, or gastrointestinal tissues. The reproductive organs (except the testis), urinary tract, skeletal and muscular tissues, brain, eyes, salivary glands, and lung were also negative. Further, we have demonstrated the presence of a Mr 90–100 K factor in the liver that seems to inhibit the LAF activity in the bioassay. The LAF activity in the positive tissues was not due to the presence of IL-2, IL-4, or IL-7, as demonstrated by using NMRI/H mice which are highly responsive to those factors, but with no responsiveness to IL-1α and IL-1β. IL-3, IL-5, IL-6, IL-8, γ-IFN, and TNFα were also excluded, since these factors show no LAF activity during the present conditions.

It has been unequivocally demonstrated that LAF activity in the skin, which has been shown to be due to IL-1 or related factors [9,42], originates in the epidermis, and mainly in keratinocytes [5,9,42]. The skin, tongue, esophagus, and proventricular stomach, which were all positive in the present study, all contain stratified squamous epithelium. In the esophagus (Fig. 4) and the stomach, the LAF activity was mainly localized to the mucosa. Therefore, it is reasonable to suggest that the detected LAFs are constitutively produced by the squamous epithelial cells in the above tissues, although a minor contribution from dendritic cells cannot be excluded. However, the exact location of LAF production cannot be determined until immunohistochemical methods or in situ hybridization are applied. LAF in the skin, also designated epidermal thymocyte activating factor [ETAF; 4], has been suggested to serve as an important host defence factor [8,9]. Stratified squamous epithelium constitutes a protective defence barrier against injury and invading microorganisms. The present findings of high amounts of LAF activity in epithelial tissues are compatible with this function. A similar defence function explaining the production of LAFs, although less obvious, might also be attributed to the liver, placenta, and testis. However, an additional or alternative

function of the detected LAFs is also possible. Cytokines including IL-1 have mitogenic activity with wide target cell specificity [21,43–47], and such a role has been proposed for IL-1 (ETAF) in the epidermis [42] and other tissues, including certain tumors [48], as well as in the testis [49]. Stratified squamous epithelium has a high proliferation rate, and in the testis, testicular IL-1-like factor (tIL-1) is produced by Sertoli cells in close contact with the rapidly proliferating spermatogonia [50]. This might suggest an additional role for LAFs in these tissues as growth factors. In the liver, the presence of LAF activity might in part be due to the fact that the liver functions as a haematopoietic organ during fetal life, and IL-1 has been shown to act as a haemopoietic growth factor [2]. We have recently shown that the amount of LAF bioactivity is the same in conventional and germ-free rats in all positive tissues, thus indicating that the production of LAFs is not induced by the exposure to microbial antigens [51].

At present, the exact identity of the demonstrated LAF(s) is not known. The presently used bioassay is highly sensitive to IL-1α and IL-1β, but not at all to IL-6 alone or in synergy with IL-1 (Fig. 1). IL-2 has also been excluded. The neutralization experiment with antibodies against human IL-1 (Fig. 6) suggests that IL-1 or a related protein might be responsible for at least part of the activity. To our knowledge there are no specific immunoassays against rat IL-1α and IL-1β available. The recently reported cloning of rat IL-1α [52] seems to indicate that rat IL-1α might not be fully identical with murine [53] and human IL-1α [54]. The reported rat IL-1α cDNA [52] has not been available in the present study. To our knowledge there are no reports on the cloning of rat IL-1β.

Using the rat IL-1α probe, Nishida and coworkers were unable to detect IL-1α mRNA in the brain, lung, and liver of normal or *E. coli* lipopolysaccharide (LPS)-treated rats. In the spleen IL-1α mRNA was only present after LPS-treatment. Others have studied the expression of IL-1α and IL-1β mRNA in mice using in situ hybridization with human IL-1 cDNA probes [55]. IL-1α and β transcripts were found in all lymphoid tissues, bone marrow, lung, digestive tract, uterus, liver, kidney, and immature bone in the mouse. These authors, however, were unable to find any IL-1 mRNA in the mouse testis and brain, in contrast to our demonstration of IL-1 bioactivity in the rat testis and the previous findings of IL-1 bioactivity in the rat brain [14]. In the mouse only the IL-1α probe hybridized to the skin [55]. The findings of IL-1 mRNA in all lymphoid tissues in the mouse seem to disagree with our results in the rat. However, we have measured bioactivity demonstrating the presence of an active protein, whereas the presence of mRNA does not always correspond to an active protein synthesis. Another explanation for the discrepancies could be a species difference. IL-1α has also been demonstrated in several human tumors and tumor cell lines derived from stratified squamous epithelium, e.g., esophageal carcinoma and nasopharyngeal carcinoma [56–59], where it has been primarily associated with hypercalcemia and osteolytic properties. However, these authors did not investigate the corresponding normal tissues for the presence of IL-1.

In our present study we could confirm previous findings of LAF activity in the skin, but not in the rat brain, corneal epithelial cells (whole eyes), or ovarian follicular fluid. Also, there was no LAF or IL-1 activity in most rat tissues previously found to display IL-1 immunoreactivity in tissue sections [17,18]. The reason for these discrepancies could be that IL-1 (-like factors) are present in these tissues in bioinactive forms or that the antibodies used for tissue staining detect some cross-reacting factor(s). Another possibility is that the amounts of IL-1 produced are too low to be detected by the bioassay, despite its high sensitivity with a detection limit of 1 pg/ml. We were also unable to confirm most in vitro findings of IL-1-like factors. However, some of these previous results might be due to cellular activation caused by the in vitro conditions which might not reflect the situation in vivo, whereas our studies were performed on normal intact rat tissues without any apparent previous cellular activation. Another explanation for the conflicting data could be that detection of mRNA does not always reflect the production of the corresponding protein. Some results, including the findings of IL-1-like factors in human ovarian follicular fluid, might be species specific, since we were unable to confirm these results in the normal rat. It must also be pointed out that we, in the present study, have only demonstrated the presence, and not necessarily the production, of LAFs in the studied tissues. The LAFs, thus, could be produced elsewhere and transported to the positive tissues, though

this seems unlikely in view of the high activity in comparison to other tissues in close proximity.

It remains to be studied whether or not the high Mr inhibitor detected in the liver is related to previously described IL-1 inhibitors, e.g., uromodulin [60–64]. However, one can speculate that the action of IL-1 and IL-1-like factors is controlled locally in the tissues by specific inhibitors.

The demonstrated IL-1-like factor(s) in all positive tissues, including the skin and the testis, has an apparent Mr of 15–25 K as determined by separation on a Sephacryl S-200 gel filtration column. This is in agreement with the reported Mr of 17 K of macrophage IL-1 [54]. Further studies are presently being conducted in order to determine the exact biochemical nature of the detected factor(s).

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