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Responses of human skin to intradermal injection of leukotrienes C_4, D_4 and B_4

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1 The ability of intradermally injected leukotrienes C_4 (LTC_4), LTD_4 and LTB_4 to produce inflammatory changes in human skin alone and in combination with prostaglandin E_2 (PGE_2) has been investigated.

2 LTC_4 and D_4 (0.012–0.38 nmol) caused dose-related erythema and wealing. No evidence of synergism between PGE_2 and LTC_4 or LTD_4 was detected, although only single dose combinations were studied.

3 LTB_4 (0.15–1.5 nmol) caused areas of induration which persisted for more than 4 h and which showed perivascular neutrophil infiltrates on histological examination. Only slight synergism between PGE_2 and LTB_4 was found.

4 It was concluded that these pro-inflammatory properties of LTC_4, LTD_4 and LTB_4 are consistent with their proposed roles as mediators of inflammation in the skin and other tissues.

Introduction

Oxidative metabolism of arachidonic acid leads to the formation of a wide range of putative mediators of inflammation. Products of the cyclo-oxygenase pathway, including prostaglandin E_2 (PGE_2), PGD_2 and prostacyclin (PGI_2) may play a role as mediators of the vascular events of acute inflammation in human skin (see Camp, 1982, for review). Recently, other oxidative pathways of arachidonic acid metabolism have been described, which are catalysed by lipoxygenases. Of the several compounds formed by these pathways, the leukotrienes (Samuelsson & Hammarström, 1980) have generated interest and speculation as potential mediators of inflammation.

Leukotriene C_4 (LTC_4, 5**S**-hydroxy-6**R**-**S**-glutathionyl-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid) and LTD_4 (5**S**-hydroxy-6**R**-**S**-cysteinylglycinyl-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid) are components of the pharmacological substance, slow reacting substance of anaphylaxis (SRS-A) (see Sirois & Borgeat, 1980, for review) and both induce vasoconstriction in guinea-pig skin and cause increased vascular permeability in the presence of the vasodilator, PGE_2. LTC_4 was the more potent vasoconstrictor, and its permeability-increasing effect was partly masked by this property (Peck, Piper & Williams, 1981). LTC_4 and LTD_4 (1 nmol each) were injected intradermally in human skin (Soter, Lewis, Corey & Austen, 1983) and persistent weals with central pallor and surrounding erythema were seen, but in this study little information about dose-related effects is given.

LTB_4 (5**S**, 12**R**-dihydroxy-6,14-*cis*-8, 10-*trans*-eicosatetraenoic acid) (Sirois, Roy, Borgeat, Picard & Corey, 1981) is one of the most potent known chemotactic agents for polymorphonuclear leucocytes (Ford-Hutchinson, Bray, Doig, Shipley & Smith, 1980; Palmer, Stepney, Higgs & Eakins, 1980; Nagy, Lee, Goetzl, Pickett & Kay, 1982). It has been shown to cause increased vascular permeability (Wedmore & Williams, 1981) and to induce leucocytic infiltration on intradermal injection in rabbits (Carr, Higgs, Salmon & Spayne, 1981). Soter *et al.* (1983) have also reported that intradermal injection of 1.6 nmol LTB_4 in human skin caused an immediate weal and flare reaction followed by persistent induration in one subject, but no visible effect was seen in two other subjects. However, histological examination of biopsies of LTB_4 injection sites in each of the three subjects at 6 h showed perivenular neutrophil infiltrates. Both LTB_4 and LTC_4 were reported to enhance the expression of complement receptors on human neutrophils and eosinophils (Nagy *et al.*, 1982). We have therefore investigated the pro-inflammatory activity of LTC_4, LTD_4 and LTB_4 in human skin, and we now describe the effects of intradermal injection of a range of doses of these

compounds. Some of this work was communicated to the British Pharmacological Society as a poster (Camp, Coutts, Greaves, Kay & Walport, 1982).

Methods

Subjects

The study was carried out on the skin of the backs of six healthy adult male subjects aged 33–47 years, all of whom were members of the medical or scientific staff of the Institute of Dermatology, and who were fully aware of the nature and objectives of the study. The protocol had previously been approved by the Institute of Dermatology Ethical Committee. None of the subjects had taken any systemic drugs within 24 h preceding the study, and none had any history of atopic disorders.

Materials

LTC_4 and LTD_4 were kindly supplied by Dr J. Rokach, Merck Frosst Canada Inc., Quebec, Canada. LTB_4 was biosynthesized as described by Camp, Woollard, Mallet, Fincham, Ford-Hutchinson & Bray (1982) using methods based on those of Borgeat & Samuelsson (1979). The LTB_4 fraction was finally purified by straight phase high performance liquid chromatography (h.p.l.c.) in order to separate the 5S,12S-*trans-cis-trans* isomer of LTB_4 (Borgeat, Picard, Vallerand and Sirois, 1981) which is a much less active stimulator of leucocyte motility (R.D.R. Camp and S.D. Brain, unpublished observations). The identity of LTB_4 was confirmed by ultraviolet spectrophotometry and gas chromatography – mass spectrometry. PGE_2 (Prostin E_2) was obtained from Upjohn, Crawley. Histamine acid phosphate injection B.P. was obtained from McCarthy's, Romford. Phosphate buffered saline, pH 7.3, contained 0.01 M sodium phosphate and 0.154 M sodium chloride and was sterilized by Millipore filtration.

Dose-response curves

Skin was injected intradermally with 50 μl phosphate buffered saline alone or containing LTC_4, LTD_4, LTB_4, PGE_2, histamine or combinations of these agents. Injections were carried out between 11 h 00 min and 13 h 00 min in a temperature-controlled room (22–25 °C). The area of erythema induced by each injection was delineated at 1 min and again at 5 min. These two areas were often quite different in outline and intensity. The outlines were traced on to paper and the areas of erythema measured planimetrically. Two perpendicular diameters of each weal, including the longest axis and ignoring small pseudopodia, were measured at intervals after injection. The size of the weal was given as the mean of the two diameters. Subjective sensations observed by the subjects in response to each injection were also recorded.

Statistics

The significance of differences between the mean area of erythema induced by injection of vehicle and that due to injection of each test substance was determined by the Mann-Whitney U-test. The mean maximum diameter of weals due to injection of vehicle was similarly compared with that due to test substances. Because sample numbers were low, tables of U for small numbers were consulted (Siegel, 1956).

Histological studies

Punch biopsies of injection sites (4 mm in diameter) were removed after intradermal infiltration of 1% lignocaine as a local anaesthetic. These were fixed in formalin, subjected to routine paraffin section and stained with haematoxylin and eosin before examination by light microscopy.

Results

Leukotrienes C_4 and D_4

Intradermal injections of solutions containing LTC_4 or LTD_4 caused similar responses (Figure 1 and 2). The first was an erythematous reaction in the surrounding skin. This appeared within 60 s of injection, reached a maximum between 1 to 3 min, was diminished by 5 min (as indicated in Figures 1 and 2) and faded completely over the following 60 min. As little as 0.012 nmol of each agent caused significant erythema. The extent of erythema was dose-related up to 0.19–0.38 nmol with the exception of a relative depression of the responses to 0.09 nmol LTD_4. In other experiments (data not shown) it was found that increasing the dose of LTC_4 or LTD_4 as high as 7.5 nmol in four subjects caused no further significant increase in erythema. Figures 1 and 2 also show the erythemal response to histamine for comparison. The maximum erythema response to either leukotriene was less than that due to 1.63 nmol histamine.

LTC_4 and LTD_4 also produced weal formation (Figures 1 and 2). The mean diameters of the weals were maximal at 15 min and were dose-related up to 0.19–0.38 nmol, with the exception of a relative depression of the response to 0.09 nmol LTD_4. The lowest dose of LTC_4 (0.012 nmol) and 0.047 nmol LTD_4 caused weals that were greater than the

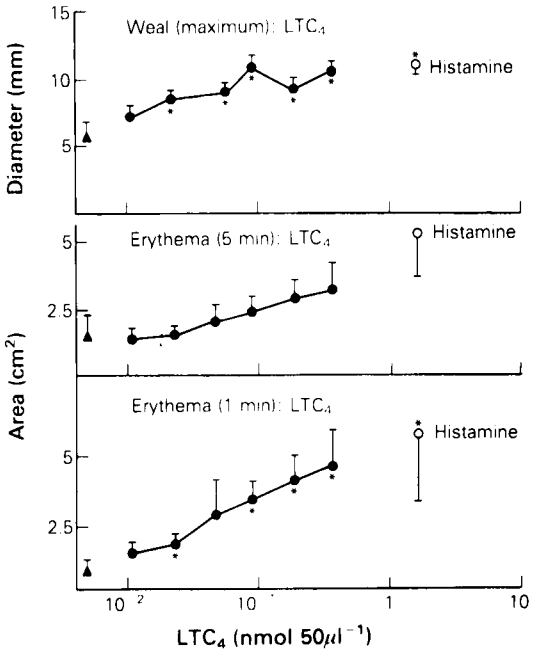

Figure 1 Dose-response curves (mean values with s.e. shown by vertical lines) for area of 1 min and 5 min erythema and for maximum diameter of weal evoked by intradermal injection of leukotriene C_4 (LTC_4), compared with responses to a single dose of histamine. (▲) = response to an equal volume of vehicle. Number of subjects = 4. $^*P<0.05$, on comparison with (▲) (Mann-Whitney U-test).

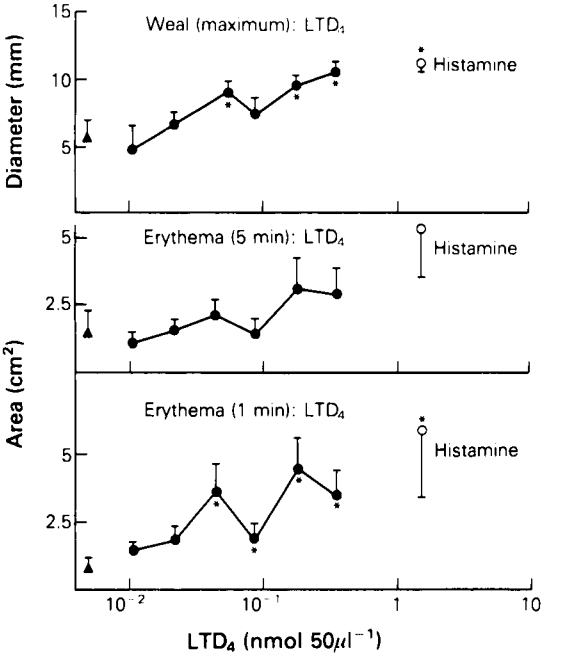

Figure 2 Dose-response curves (mean values with s.e. shown by vertical lines) for area of 1 min and 5 min erythema, and for maximum diameter of weal evoked by intradermal injection of leukotriene D_4 (LTD_4), compared with responses to a single dose of histamine. (▲) = response to an equal volume of vehicle. Number of subjects = 4. $^*P<0.05$, on comparison with (▲) (Mann-Whitney U-test).

vehicle-induced weals. In other experiments (data not shown) further increase of the dosage of LTC_4 or LTD_4 up to 7.5 nmol caused no greater diameter of wealing than that due to 0.38 nmol of either agent. Maximum wealing due to these two leukotrienes was approximately equal in magnitude to that due to 1.63 nmol histamine. Wealing subsided 45–60 min after injection and no subsequent visible or palpable reactions developed at the injection sites. Central pallor was seen in the weals induced by LTC_4 and LTD_4, but this was no more prominent than that seen in the histamine-induced weals. The sensations caused by intradermal injection of LTC_4 and LTD_4 did not differ significantly in either quality or intensity from those due to injection of the vehicle.

Leukotriene B_4

LTB_4 was injected at dosages of 0.15, 0.30 and 1.5 nmol in four subjects. In all subjects areas of induration developed at about 30 min after injection. The induration was ill-defined, erythematous and slightly raised, and achieved a maximum diameter of about 8 mm at 60–120 min after injection. The diameters of these areas were not dose-related. These lesions persisted for at least 4 h, were tender and, in one subject, were associated with persistent itching. Neither the vehicle nor histamine (1.63 nmol) caused any delayed induration.

Prostaglandin E_2

The effect of injection with PGE_2 alone and in combination with LTC_4, LTD_4 and LTB_4 was investigated in two subjects. PGE_2 alone (0.18 nmol) caused erythema and wealing which were approximately equal in extent to the maximal response to LTC_4 and LTD_4. A solution containing 0.18 nmol PGE_2 and 0.38 nmol LTC_4 or 0.75 nmol LTD_4 caused no greater erythema or wealing than would be expected from the sum of the responses individually. However, combined injections using lower doses of leukotrienes were not performed. Injection of a combination of LTB_4 (0.30 nmol) and PGE_2 (0.14 nmol) caused slight enhancement of the visible responses; at 4 h the diameters of the ill-defined swellings were

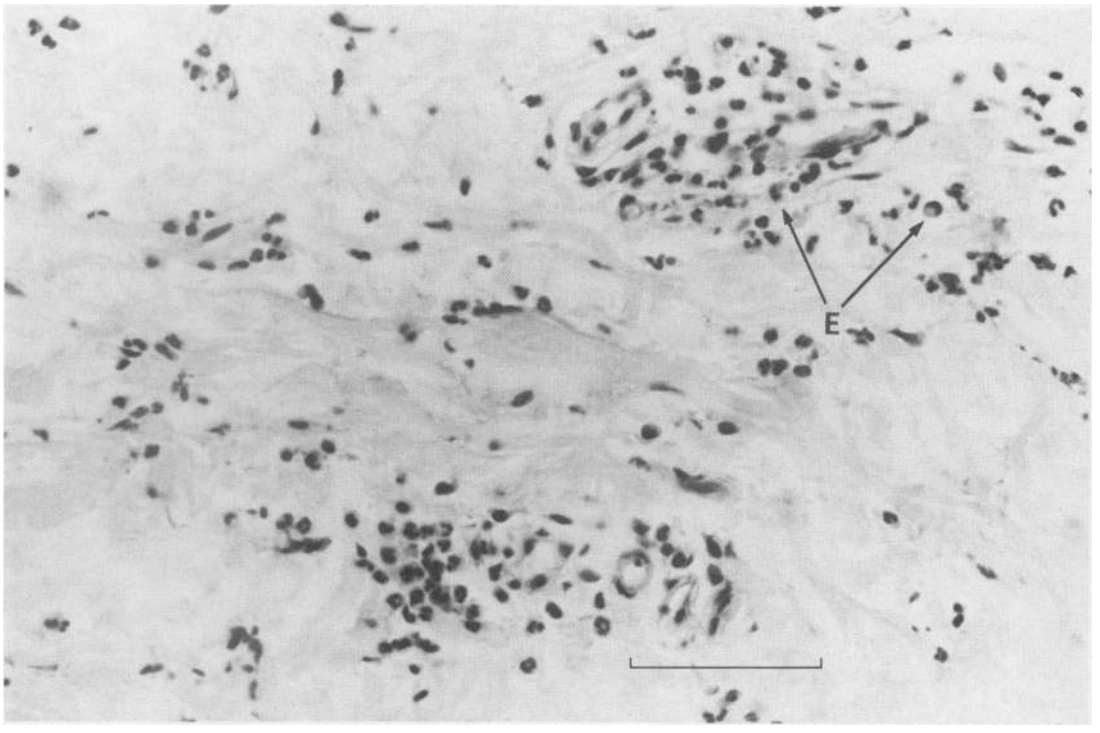

Figure 3 Histological appearance of human skin 4 h after intradermal injection of 0.3 nmol leukotriene B_4 (LTB_4), demonstrating a dermal perivascular neutrophil infiltrate with a few eosinophils. E = eosinophil. Bar = 100 μm.

approximately 3 mm larger at the sites of the combined injections compared with the sites of injection of 0.30 nmol LTB_4 alone. PGE_2 (0.14 nmol) alone caused no detectable response at 4 h.

Histological appearances

In two subjects 4 mm punch biopsies were removed 4 h after injection of 0.30 nmol LTB_4, and were processed for light microscopy as described. Biopsies from both subjects showed a pronounced dermal neutrophil infiltrate (Figure 3), with perivascular accentuation, and containing a few eosinophils. In contrast, similar biopsies removed 2 h after injection of 7.5 nmol LTC_4 or LTD_4 in the same two subjects showed little or no evidence of polymorphonuclear infiltration.

Discussion

This study demonstrates that both LTC_4 and LTD_4 produce dose-related erythema and wealing in human skin at very low concentrations, although dose-response curves are shallow and control values for weals and erythema are high. Although no attempt was made to make a full direct comparison of dose-response curves, consideration of our present and earlier results (Marks & Greaves, 1977) suggests that LTC_4 and LTD_4 are at least as potent as histamine in production of erythema and wealing at the lower dose range, especially in respect of weal production.

Much existing information on the pro-inflammatory actions of LTC_4 and LTD_4 derives from studies on the microvasculature of guinea-pig skin (Peck *et al.*, 1981). In this species, both LTC_4 and LTD_4 cause vasoconstriction, the former being the more potent. LTD_4, but not LTC_4, caused local increase in vascular permeability which was greatly potentiated by addition of the vasodilator PGE_2. Evidence was obtained that LTC_4 had some permeability increasing activity, but this was masked by its potent vasoconstrictor properties. Soter *et al.* (1983) report the presence of pallor in the centre of weals due to intradermal injection of LTC_4 and LTD_4 (1 nmol each) in human subjects and interpret this as being due to intrinsic arteriolar constriction. While we have also observed pallor in the centre of weals

induced by LTC_4 and LTD_4, equivalent pallor was seen in histamine-induced weals. We believe that pallor in weals of human skin may simply be a function of the degree of dermal oedema, which, by extrinsic compression of the microvasculature, transiently interferes with dermal blood flow. We commonly observe pallor in wealing reactions induced experimentally and seen in clinical disease. Our interpretation is supported by the experiments of Bisgaard, Kristensen & Sondergaard (1982), in which the effects on cutaneous blood flow of intradermal injection of LTC_4 and LTD_4 (1 μg each) in humans were determined by a laser-Doppler technique. They found that wealing interfered with blood flow values, and therefore measured blood flow at a distance of 5 mm from injection sites. Increased blood flow was seen and there was no evidence of vasoconstriction.

In the study of Soter *et al.* (1983) erythema due to LTC_4 and LTD_4 was found to be maximal at 1 h and to persist for more than 4 h, whereas in the present study erythema was maximal between 1 and 3 min and had faded completely by 1 h. In addition, the study of Soter *et al.* indicated that wealing was maximal at 1 h and had dissipated by 4 h, whereas in the present study wealing was maximal at 15 min and had dissipated by 1 h. These differences may be explained by the higher doses of LTC_4 and LTD_4 used by Soter *et al.* (1983). It may also be relevant that their injections were carried out on the flexor aspect of the forearm, whereas ours were done on the back.

We were unable to demonstrate any synergism between PGE_2 and LTC_4 or LTD_4, but similar combination studies ought to be carried out using a wider leukotriene dose range and with other vasodilator mediators including PGI_2 and PGD_2. Direct evidence of increased generation of LTC_4 and LTD_4 in inflamed tissues has yet to be obtained. Nevertheless, our findings are consistent with the possibility that these compounds may behave as mediators of inflammation.

The results obtained with LTB_4 show that subnanomolar amounts of this agent are capable of inducing neutrophil infiltrates in human tissues *in vivo*. Although there is evidence that LTB_4 is chemokinetic (Bray, Ford-Hutchinson & Smith, 1981) and chemotactic (Nagy *et al.*, 1982) towards eosinophils, only a few eosinophils were seen in the biopsies of LTB_4 injection sites in our experiments. The number of eosinophils infiltrating in response to LTB_4 injection may depend on the concentration of blood eosinophils, and may possibly be increased when the blood eosinophil count is high. Soter *et al.* (1983) have reported that PGD_2 enhanced the response to intradermal LTB_4. We have demonstrated only slight enhancement of the visible LTB_4 response in human skin by PGE_2.

There have been few conclusive reports of elevated LTB_4 levels in inflamed tissues. The report of Klickstein, Shapleigh & Goetzl (1980) that LTB_4 levels were raised in joint fluid of patients with inflammatory arthropathies appears to be based on analytical techniques of limited specificity, and is thus open to challenge. Their report has also been questioned by Davidson, Rae & Smith (1982), who analysed synovial fluid samples from patients with rheumatoid arthritis by h.p.l.c. and bioassay. They reported bioassayable concentrations of LTB_4 of about 0.34 ng ml^{-1} joint fluid, levels approximately 400 fold lower than those reported by Klickstein *et al.* (1980). Brain, Camp, Dowd, Kobza Black, Woollard, Mallet & Greaves (1982) have demonstrated the release of LTB_4 from the involved skin of patients with psoriasis, as determined by assay for chemokinetic activity in h.p.l.c. fractions of skin extracts.

The properties of the leukotrienes we have studied are entirely consistent with their proposed role as mediators of inflammation in skin and other tissues. Whilst specific integrated sequential measurements of the levels of these compounds in inflamed tissues need to be carried out, final establishment of their importance will have to await the development of specific antagonists or inhibitors.

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