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THE INFLUENCE OF LIMONENE ON INDUCED DELAYED HYPERSENSITIVITY TO CITRAL IN GUINEA PIGS
I. HISTOLOGICAL STUDY

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Abstract. The effect of limonene on citral sensitization and elicitation was studied by histological methods. Limonene decreases the intensity of citral sensitization and of the citral test. Possible interpretations of the influence of limonene are discussed; in particular, an effect of limonene at the macrophage level could explain the results.

Key words: Contact dermatitis; Quenching of contact dermatitis; Citral; Limonene; Histological study

Delayed hypersensitivity (DH) induced by a skin contact with a sensitizer schematically involves two phases: an induction phase and an elicitation phase. The induction phase leads to the formation of memory and effector cells; the purpose of the elicitation phase is to eliminate the hapten from the skin surface. During this latter phase, at the cutaneous contact point and via the release of soluble substances, lymphokines, activated effector cells accumulate a non-specific cellular population, consisting essentially of lymphocytes and macrophages, thus constituting the basis of the superficial inflammatory dermal infiltrate of eczema.

In order for the two phases to take place, the conjugation of the hapten with one or several carriers is necessary and so is the presence of macrophages and T-lymphocytes. The hapten, conjugated to a carrier (most probably a skin protein) or/and to the antigens of the major histocompatibility complex (MHC) is presented by the macrophages to the T-lymphocytes (6).

Many natural substances in cosmetology, in perfumes, and in household products can induce DH; one of those products is an aldehyde, citral (Fig. 1).

In 1976, Opdyke (5) showed in a study of human volunteers that the presence of some compounds in perfumes could cause quenching of sensitization to known sensitizers. In particular, although experimental induction of sensitization to citral was successful, no such induction could be achieved with for instance, *lemongrass*, a natural 4: 1 mixture of citral and *d*-limonene (Fig. 1). Other substances, such as eugenol and phenylethanol, could also play the role of "quencher".

These facts prompted us to reproduce in the guinea pig a hypersensitivity to citral and to a citral and limonene mixture and to check, essentially with qualitative and quantitative histological criteria, the existence of such quenching in animals.

MATERIALS AND METHODS

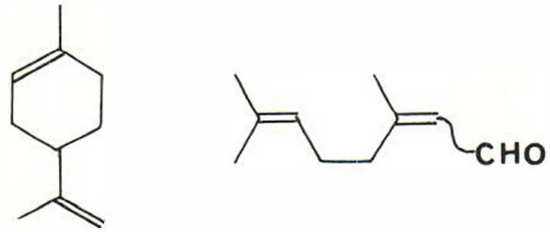
Citral and *d*-limonene were provided by Haarmann und Reimer GmbH, Holzminden, Germany. The animals were albino Hartley females (from R. Versault, 77250 Luisetaines, France) weight range 300–350 g.

Two series of guinea pigs of 5 and 4 animals respectively and noted C1 to C5 and C6 to C9 were sensitized to citral alone (C); two other series of animals, noted CL1 to CL5 and CL6 to CL10 respectively, were sensitized to a mixture of citral and limonene (CL).

d-Limonene was shown in previous experiments, in the same guinea pig strain, to be non-sensitizing. The sensitization method used was FCAT (4) (Freund Complete Adjuvant Test): the sensitizer (0.5 ml of citral, or a mixture of 0.5 ml of citral and 0.5 ml of limonene) was dissolved in 4.75 ml of FCA and then emulsioned with 4.75 ml of saline, using a syringe. Each animal received intradermally, in the shaved nuchal region, 5 injections (of 0.1 ml each) of the emulsion, on alternate days. Controls, noted T1 to T3 also received 5 injections of the same emulsion of FCA and saline, but devoid of sensitizer.

After a 2-week rest, elicitation was performed. For

Abbreviations used throughout the article: C: citral-sensitized animals; CL: (citral and limonene)-sensitized animals; c: citral-tested animals; cl: (citral and limonene)-tested animals; l: limonene-tested animals; FCA: Freund Complete Adjuvant; DH: delayed hypersensitivity; MHC: major histocompatibility complex.



d-Limonene Citral

Fig. 1. Chemical structures of citral and limonene.

clinical evaluation, 25 μl of a solution of 1 g citral in 100 ml ethanol (c-test) or of 1 g citral with an equimolar amount of l in 100 ml ethanol (cl-test) was deposited with a micropipette at the same time but at a distance from each other on the shaved flank of the animal on a 2 cm^2 circular area. For the histological study, the same solutions were used but on a 0.5 cm^2 area; only 6.5 μl of citral, citral and limonene, or limonene (0.0066 mole/l) solutions were deposited and the test area was then covered with a "Finn chamber" kept in place by means of hypoallergic adhesive plaster (Fig. 2).

Reading was effected at the 24th hour for the open epicutaneous test, using the following scale: 0: no reaction; 0.5: slight erythema covering *part* of the test area; 1: erythema covering the entire test area; 2: erythema + swelling on the test area; 3: erythema + swelling extending well beyond the test area.

For the histological study, the skin was excised at the 14th hour (3) using a 6-mm punch after anesthetizing the animal by means of ether. The biopsy was then fixed for 24 h in Bouin's solution, then dehydrated classically and dried with hematoxylin-eosin safran. The *qualitative* reading for which subjective and comparative criteria were used, was expressed by either the absence 0 or the presence of a weak (+) moderate + or strong ++ mononuclear

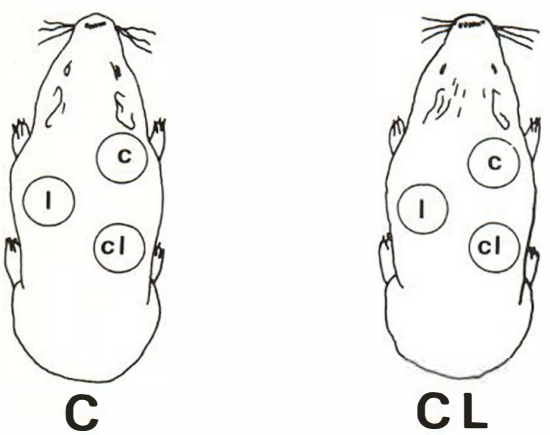


Fig. 2. C- and CL-sensitized animals; test sites on the guinea pigs' flanks (c-, cl- and l-) are shown.

Table I. *Results of open epicutaneous tests in C- and CL-sensitized guinea pigs*

Test intensity shown: 0=no reaction, 0.5=test area partly erythematous, 1 = test area erythematous, 2 = erythema + swelling on test area, 3 = erythema + swelling extending well beyond test area

Animal number	Test to citral (c)	Test to a citral +limonene mixture (cl)
Sensitization to citral C		
C1	1	1
C2	1	1
C3	0.5	0.5
C4	1	1
C5	1	2
Citral + limonene CL		
CL1	1	1
CL2	1	1
CL3	2	1
CL4	1	1
CL5	1	1

exocytosis, *spongiosis* or *superficial dermal infiltrate*. Quantitative readings denoted the nature (lymphocytes, macrophages, eosinophils and neutrophils, unknown cells) and the number of inflammatory cells present in the superficial dermis just beneath the epidermis and this, in 20 successive high-power fields using an oil-immersion objective (1). This reading was a double-blind one.

Statistical analysis

To compare c, cl and l epicutaneous tests effected on the same animals, we used a Friedman two-way analysis of variance by ranks (7) (factor test and factor animal). When this analysis was statistically significant, it was completed by a Student's *t*-test for two related samples. The two-way analysis of variance for unequal numbers was used to compare groups of animals sensitized (C or CL), taking into account the fact that we have studied two successive experimental series for each of these groups (7).

RESULTS

Reading of the open epicutaneous tests (Table I) does not reveal significant differences between the c-test and the cl-test in C- and CL-sensitized animals.

Qualitative histological studies effected on one series of C- and one series of CL- sensitized animals showed that C-animals reacted more strongly to citral than did CL-animals (Table II) (Figs. 3, 4).

This difference in reactivity—though weaker—was also oberved in the cl-test; moreover, the reactivity of both groups of animals to that test was

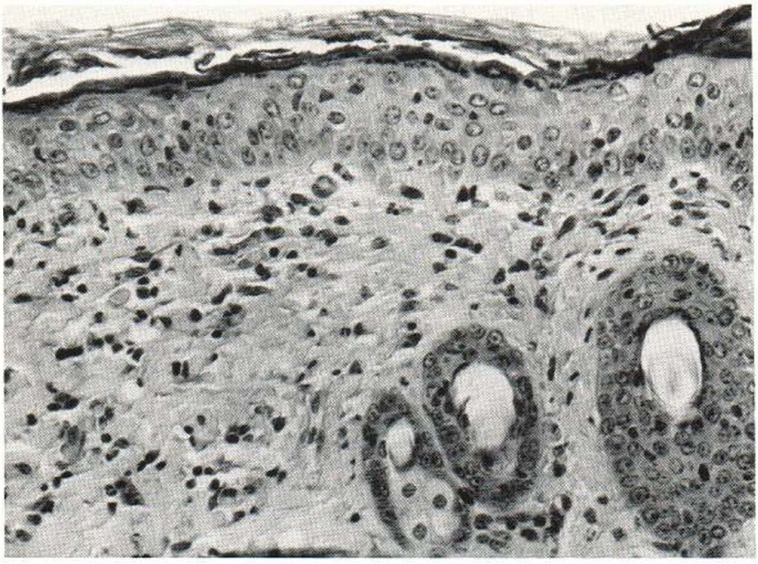


Fig. 3. Patch test with 1% citral in an animal sensitized to a citral + limonene mixture. Faint spongiosis and exocytosis, dispersed lymphocytic infiltration of the upper dermis (×310).

weaker than the one observed in the c-test (Table III) (Figs. 5, 6).

Results of *quantitative* histological reading are reported in Table IV. They showed for both the lymphocyte reaction and the total infiltrate, a stronger reactivity to the c-test, as compared with the cl-test or the l-test. For the three controls, the reactivity was almost the same to citral, citral and limonene, or limonene, and generally weaker than in sensitized animals.

The Friedman two-way analysis of variance by ranks (7) showed that the factor "test" is significant to 1% in both C- and CL-sensitized animals, tested with citral, citral and limonene, or limonene.

We have therefore completed these analyses with a Student's *t*-test for two related samples and studied the differences of the results between the c- and the cl-test, the c- and l-test and, lastly, the cl- and l-tests, in both C- and CL-sensitized animals. Results are recorded in Table V. They show that

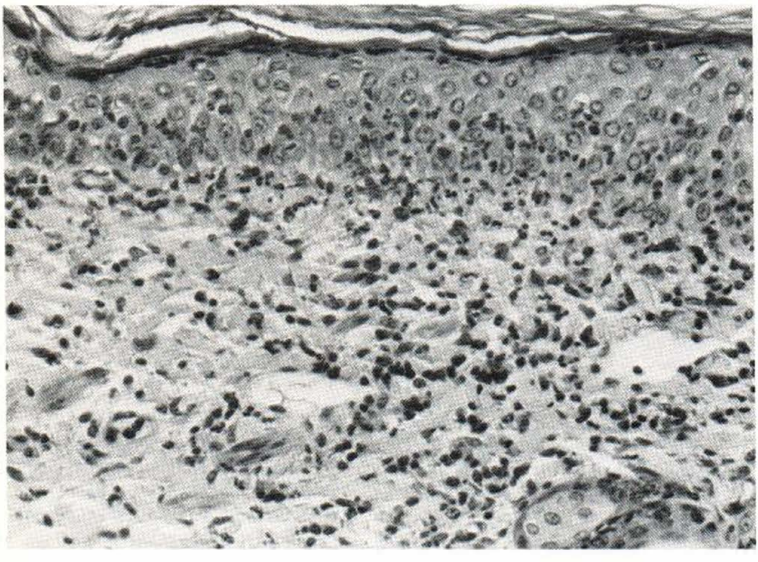


Fig. 4. Patch test with 1% citral in an animal sensitized to citral. Spongiosis, exocytosis and dense dermal infiltrate rich in lymphocytes (×310).

Table II. *Qualitative study of cutaneous biopsies of 1% citral tests*

Arbitrary scale = 0 absence, (+) weak, + moderate, ++ significant

	Exocytosis	Spongiosis	Superficial dermal infiltrate
Animals sensitized to citral			
C1	+	+	+
C2	+	++	+
C3	+	+	+
C4	+	++	(+)
C5	+	+	+
Animals sensitized to citral + limonene			
CL1	0	0	+
CL2	(+)	(+)	++
CL3	(+)	(+)	++
CL4	(+)	0	(+)
CL5	0	0	(+)
Controls			
T1	0	0	+
T2	(+)	0	(+)
T3	0	0	(+)

Table III. *Qualitative study of cutaneous biopsies of citral + limonene tests*

Arbitrary scale = 0 absence, (+) weak, + moderate, ++ significant

	Exocytosis	Spongiosis	Superficial dermal infiltrate
Animals sensitized to citral			
C1	0	0	+
C2	0	0	+
C3	+	(+)	+
C4	0	0	+
C5	0	0	(+)
Animals sensitized to citral + limonene			
CL1	0	0	+
CL2	(+)	(+)	+
CL3	0	0	+
CL4	(+)	(+)	(+)
CL5	0	0	(+)
Controls			
T1	0	0	(+)
T2	0	0	(+)
T3	0	0	(+)

within a group (C- or CL-sensitized animals) within a 5% error, there is a significant difference between c- and cl-tests and also between c- and l-tests. There is however no significant difference between the cl- and l-tests.

The two-way analysis of variance for unequal numbers did not reveal significant differences between the C- and CL-sensitized groups of animals.

DISCUSSION

Several authors, particularly Polak (6), have discussed the mechanism of the modulation of DH and shown that it can occur at either the induction or the elicitation phase in the nodes and in the skin.

This modulation can be effected by a specific tolerance mechanism involving either suppressor cells or clonal deletion of lymphocytes.

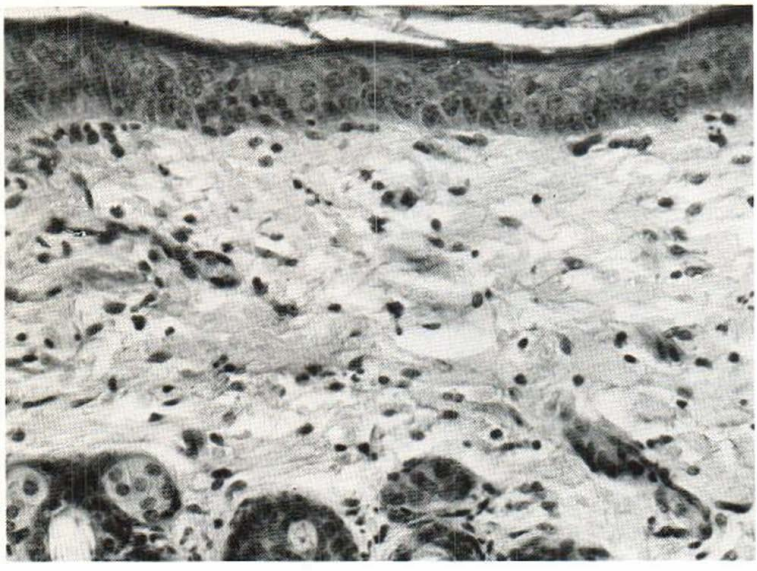


Fig. 5. Patch test with 1% citral + limonene (1 : 1 molar) in the same animal as in Fig. 3. Dispersed lymphocytic infiltration of the dermis, few lesions of the overlying epidermis (×310).

Table IV. *Quantitative study of cutaneous biopsies (cell counts (/) in citral (C), citral + limonene (CL) sensitized[a] animals and in controls[b]*

Animals	Test[c]	Lymphocytes	Macrophages	Neutrophils	Eosinophils	Unidentified cells	Total infiltrate
C1	c	269	105	11	3	13	401
	cl	220	102	6	10	1	339
	l	209	100	2	1	7	319
C2	c	291	83	36	71	68	549
	cl	222	92	51	32	70	467
	l	145	48	10	7	7	217
C3	c	333	215	109	45	67	769
	cl	188	189	22	57	22	478
	l	155	73	6	0	3	237
C4	c	263	76	52	21	9	421
	cl	154	50	23	11	2	240
	l	194	77	7	10	2	290
C5	c	534	172	60	18	32	816
	cl	269	124	16	13	3	425
	l	146	77	0	0	1	224
C6	c	129	76	11	7	3	226
	cl	57	47	5	11	8	128
C7	c	389	125	89	6	8	617
	cl	308	112	75	0	1	496
C8	c	295	115	50	21	10	491
	cl	182	81	5	10	2	280
C9	c	320	146	43	20	6	535
	cl	221	105	15	18	0	359
CL1	c	269	156	80	18	35	558
	cl	179	134	77	37	12	439
CL2	c	231	111	121	4	2	469
	cl	180	105	154	2	4	445
	l	184	127	6	54	5	376
CL3	c	345	146	115	61	0	667
	cl	282	109	164	1	0	556
	l	241	125	256	0	12	634
CL4	c	231	90	6	42	18	387
	cl	119	82	68	1	3	273
	l	95	71	18	3	10	197
CL5	c	202	100	30	9	6	347
	cl	177	111	35	1	16	340
	l	115	50	8	0	6	179
CL6	c	339	135	54	0	6	534
	cl	318	152	64	17	11	562
CL7	c	417	122	121	8	6	674
	cl	372	105	82	10	7	576
CL8	c	254	99	19	11	6	389
	cl	202	85	25	5	7	295
CL9	c	334	129	40	8	7	518
	cl	206	78	17	17	6	324
CL10	c	151	38	4	2	7	202
	cl	80	44	0	0	0	124
T1	c	231	197	98	18	22	566
	cl	166	75	20	13	2	276
	l	159	72	4	2	4	241
T2	c	111	53	19	16	22	221
	cl	125	128	9	42	10	314
	l	133	67	1	1	0	202
T3	c	125	66	45	7	13	256
	cl	117	61	26	12	11	227
	l	137	81	17	20	3	258

[a] The animals were sensitized according to the FCA technique using citral (C) or citral + limonene (CL) equimolar mixture and FCA.

[b] Controls were animals which received injections of FCA *alone*.

[c] Tests were performed with 1% citral (c) 1% citral + limonene (cl) equimolar mixture and limonene at a concentration identical with the cl test.

Table V. *Student's t-values for two related samples*

C = citral-, CL = citral + limonene-sensitized animals; c, cl, l = animals tested with citral (c), citral + limonene (cl), limonene (l); for instance, Ccl 1 = animal no. 1 sensitized with Citral and tested with a citral + limonene mixture

Comparison between	Lymphocytes			Total infiltrate		
	Student's *t*-values	Degrees of freedom	Significance	Student's *t*-values	Degrees of freedom	Significance (%)
Cc(1–5)–Ccl(1–5)	3.33	4	5%	3.22	4	5
Cc(6–9)–Ccl(6–9)	9.96	3	1%	5.89	3	1
Cc(1–5)–Cl(1–5)	2.83	4	5%	3.25	4	5
Ccl(1–5)	1.46	4	Non-significant	2.10	4	Non-significant
CLc(1–5)–CLcl(1–5)	4.50	4	2%	3.06	4	5
CLc(6–9)–CLcl(6–9)	3.79	4	2%	2.47	4	Non-significant
CLc(1–5)–CLl(1–5)	5.04	3	2%	3.36	3	5
CLcl(1–5)–CLl(1–5)	2.2	3	Non-significant	1.33	3	Non-significant

It can also be caused by blocking the lymphokines released by the effector lymphocytes on the site of the cutaneous reaction, this latter mechanism being invoked by Hasegawa et al. (2) who showed that α-L-fucose was able to reduce significantly the patch test to DNCB in sensitized animals if this carbohydrate was injected intravenously within 6 hours after application of the patch test.

To interpret our study, if on the one hand we consider the qualitative results to c-tests—in C- and CL-sensitized animals (Table II)—we could invoke a tolerance mechanism caused by limonene and exerting its effects on citral. CL-sensitized animals did in fact react less strongly to the c-test than did C-sensitized animals.

If, on the other hand, we consider both the qualitative and quantitative results of cl-tests—while comparing them with c-tests—in C- and CL-sensitized animals, we could invoke a mechanism identical with the one described by Hasegawa et al. (2) for α-L-fucose: indeed the addition of limonene to citral (cl-test) brings about a weakening of the reactivity to citral (c-test).

Yet the weakening of the reactivity to citral when limonene is used, both in the induction phase and in the elicitation phase, leads us to consider other types of mechanisms. It could be a *mechanical phenomenon*. Limonene (or its metabolites) could facilitate elimination of citral through the epidermis, thus reducing the amount of citral bound to Langer-

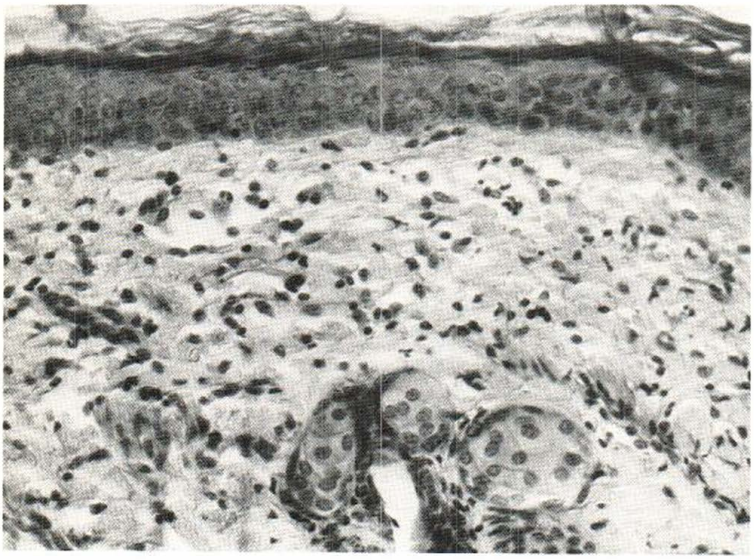


Fig. 6. Patch test with 1% citral + limonene (1 : 1 molar) in the same animal as in Fig. 4. No epidermal lesions, faint mononuclear infiltration of the upper dermis (×310).

hans cells and consequently the intensity of the c-test. Such a mechanism would result in "shunting" the Langerhans cells and in inducing tolerance to citral. We might also invoke another possibility: the impact point of the mechanism might be the macrophage, in which case limonene (or its metabolites) could "block" the node macrophages at the induction phase (interference with the MHC, modification of the membrane properties, etc.) and the Langerhans cells in the elicitation phase. Thus the presence of limonene would account for both the difference in reactivity between the two groups of animals and the weakening of the reactivity to citral within the same group. Work is in progress in our laboratory to try to decide between these different possibilities.

CONCLUSION

The above results show that *d*-limonene is able to quench delayed hypersensitivity to citral. This quenching effect, as demonstrated by histological studies, operates at two levels: induction and elicitation. Both results can be tentatively explained by a blocking of the node macrophages (induction phase) and of the Langerhans cells (elicitation phase) or by another mechanism.

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