

Lymphocyte Transformation Test for Diagnosis of Isothiazolinone Allergy in Man

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The lymphocyte transformation test (LTT) has been used for evaluation of *in vitro* lymphocyte responses in 18 patients with dermatitis and positive patch tests to 200 ppm of a combination of 5-chloro-2-methylisothiazolinone and 2-methylisothiazolinone (MCI) in nine patients with dermatitis unrelated to MCI and in seven subjects without skin diseases. Two workers sensitized by occupational exposure to a formulation containing 1,2-benzisothiazolin-3-one (BIT) were also studied. Lymphocytes from nine patch-test-positive patients proliferated vigorously to MCI *in vitro*. Lymphocytes from the remaining nine patients were not stimulated. Lymphocytes from two BIT-sensitized workers responded to BIT *in vitro*. The lymphocyte proliferation to isothiazolinones indicates the presence of memory cells in the patients' blood and confirms immunologic reaction to the inducing agent. To establish clinical relevance of LTT results, 12 MCI patch-test-positive patients underwent "use test" with lotion containing 15 ppm MCI. Four of five LTT-

positive patients were use-test-positive, whereas seven of seven LTT-negative patients were use-test-negative. LTT-positive and lotion-positive patients responded to 100 ppm or lower concentrations of MCI on patch testing, whereas seven of eight LTT-negative and lotion-negative patients responded to 200 ppm only. In the case of MCI, proliferation was due to the chlorinated component, indicating that this part contains an allergenic epitope. Finally, MCI-specific lymphocyte proliferation was observed only in patients with MCI-positive skin test, but not in nine patients with dermatitis induced by other agents, or in seven subjects without skin diseases.

Thus, the lymphocyte transformation test is able to distinguish between irritant and allergic skin responses. It may also be valuable in establishing the clinically relevant patch-test concentration of allergens with irritative properties. *J Invest Dermatol* 94:798-802, 1990

Because of its antimicrobial efficacy at low concentrations, the combination of 5-chloro-2-methylisothiazolinone plus 2-methylisothiazolinone (MCI) is widely used in household products, industrial products, and toiletries. Intrinsically, MCI is an irritant and may also induce sensitization at certain concentrations [1,2], much like other antimicrobial agents with similar end-use applications. As is the case for any allergen, it is important to establish both its prevalence rate (patch-positive findings) and its relevance in allergic contact dermatitis (ACD). Clinical data in this respect have been conflicting due to variability in patch-test protocols and criteria for the selection of patients. For example, the prevalence rates for MCI have been reported to range from 0 to 4.5% [3]. The relevance of these findings has not been clearly established.

At present it is difficult to distinguish between irritant responses and true allergic responses by routine patch testing, because both

responses are T-cell mediated [4]. Histopathology is also inconclusive [5,6]. We have recently demonstrated that the lymphocyte transformation test (LTT) may be a valuable tool in the differential diagnosis of ACD due to occupational drug exposure [7-9]. Thus, subjects suffering from drug-induced ACD have drug-specific memory cells in their blood capable of reacting with the drug *in vitro*. On the other hand, such specific lymphocytes are absent in exposed individuals showing skin irritation but not ACD. In the current study we used LTT to diagnose immunologic responses to MCI observed in subjects positive to MCI at routine patch testing.

SUBJECTS AND METHODS

Twenty-nine patients, of both sexes, ranging from 19 to 71 years, had attended Södersjukhuset Dermatology Clinic for dermatitis located in face and extremities. Eighteen patients reacted to MCI in the patch test and the majority were also positive to one or more of the following substances: nickel, cobalt, fragrance mix, parabens, and colophony. Two women had a history of atopy. Two workers in industrial paint production were accidentally exposed to a formulation containing 20% 1,2-benzisothiazolin-3-one (BIT) as active ingredient on the hands and legs. The exposure was followed by burning and vesiculation. The acute reaction subsided in a few weeks but they still responded with a dermatitis following a repeat with the same formulation. The blood samples for *in vitro* studies were drawn in these workers before patch testing.

Blood from nine patients with dermatitis unrelated to MCI and from seven persons without dermatitis was also tested for MCI-specific lymphocyte responses.

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Abbreviations:

ACD: allergic contact dermatitis

BIT: 1,2-benzisothiazolin-3-one

LTT: lymphocyte transformation test

MCI: 5-chloro-2-methylisothiazolinone and 2-methylisothiazolinone

PPD: purified protein derivative

T cell: thymus-derived lymphocyte

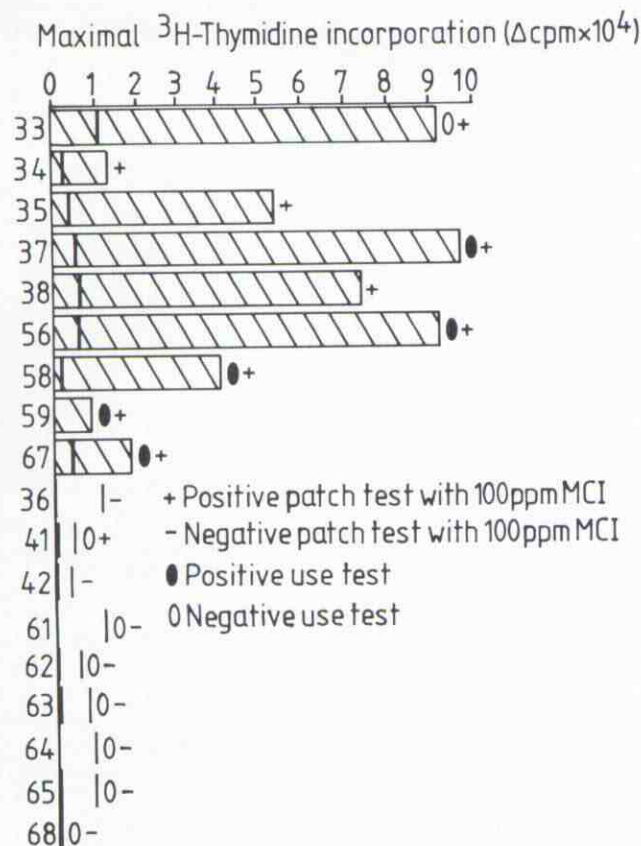


Figure 1. Summarized results of LTT, patch tests, and use tests in patients responding to 200 ppm of MCI in patch test. Vertical line, borderline for positive responses to MCI.

Patch Tests Patients were routinely patch tested with an ICDRG standard series of 26 substances including MCI (0.02% and 0.01% active ingredient, Chemotechnique Diagnostics AB), employing Finn chamber (Epitest Oy, Helsinki, Finland), and Semipore (Norges Plastics A/S, Oslo Norway). Four patients with positive reactions to 200 and 100 ppm of MCI were further tested with 50 and 25 ppm. Two workers sensitized to formulation containing BIT were tested with 1% and 2% of formulation in alcohol. Ten control patients were randomly selected from outpatients being treated for dermatitis at the Clinic. Upon testing with formulation, no positive reactions were recorded.

Patch tests were read after 72 h and judged to be positive if erythema, infiltration, and papules (+) or erythema, infiltration, papules, and vesicles (++) were present. The patients were told to report any reactions after the test have been read but none were reported.

Use Test Use tests were performed on subjects who were patch positive to MCI by twice-daily applications to the elbow flexure of a skin-care lotion containing 15 ppm of MCI. The same lotion without MCI was applied on the other. The patients were asked to continue application for at least 7 d or until a skin reaction occurred, and report any skin reactions within the tested area.

The test was conducted in a double-blind fashion. Where they were positive, reactions generally appeared within 2-3 d of the initial application.

Lymphocyte Transformation Test Defibrinated blood for in vitro lymphocyte studies was obtained at various intervals following patch testing. The isolation and cultivation of lymphocytes was performed as previously described with minor modifications [7].

The number of monocytes was reduced by incubation of cells on plastic surfaces. Lymphocytes were cultivated with various concentrations of isothiazolinones for 5 d at 37°C. MCI dilutions for LTT were obtained from Rohm and Haas Company (Philadelphia) or from Chemotechnique Diagnostics, Malmö. N-methyl malonic acid, malonic acid, and malonic acid were obtained from the Rohm and Haas Company. 1,2-benzisothiazolin-3-one [BIT], was obtained from Dr. M. Bruze, Occupational Dermatology Clinic, Lund Hospital. PPD (purified protein derivative) was used as a positive control of lymphocyte proliferation [7]. Lymphocyte transformation in control and antigen-treated cultures was measured by ³H-thymidine incorporation into lymphocyte DNA after 5 d cultivation. The increase of ³H-thymidine incorporation in antigen-treated cultures is expressed as mean cpm ± SD of triplicates or as Δcpm, where Δcpm is the mean cpm in antigen-treated cultures minus the mean cpm in control cultures.

Cells from the 5-d cultures were also screened morphologically for the presence of lymphoblasts using May-Grünwald-Giemsa stained smears [7]. Morphologic evaluation was performed by one researcher only and always preceded the radioactively labeled isotope evaluation of lymphocyte proliferation. The results of LTT were considered positive only if the mean cpm of antigen-treated cultures was more than twice that of control cultures, and if such cultures contained at least twice as many lymphoblasts as the control cultures.

RESULTS

MCI induced lymphocyte proliferation in nine of 18 patients who exhibited patch tests positive to 200 ppm of MCI (Fig 1). MCI-specific response was dose-dependent, approaching maximum at 0.12-0.25 μg of MCI in culture and decreasing at higher concentrations (Fig 2). Interestingly, LTT-positive patients also responded to 100 ppm of MCI or less (50 μg and 25 μg) upon patch testing. Patient 41 was LTT negative, but showed positive skin reactions to 100 ppm of MCI. He also reacted to chromium and fragrance mix. The remaining LTT-negative patients exhibited negative patch tests with 100

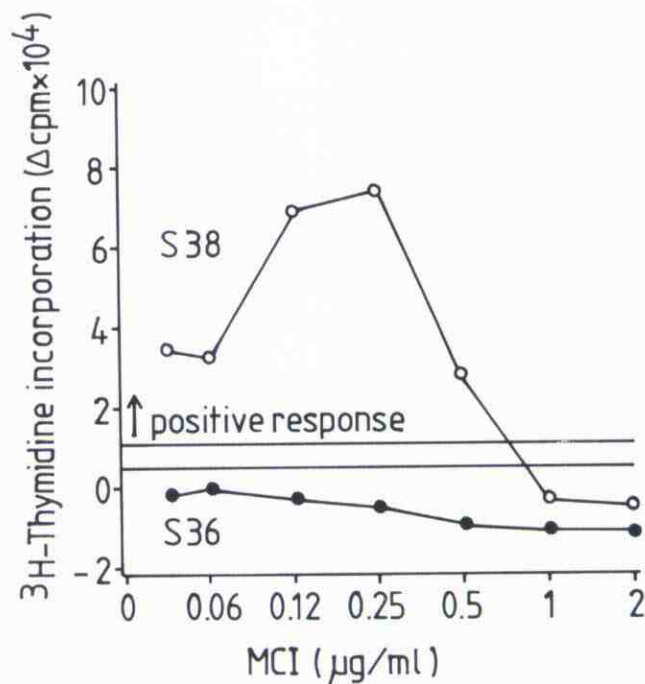


Figure 2. Lymphocyte proliferation to MCI in patients 36 and 38, who responded to 200 ppm of MCI upon skin testing. Lymphocyte tests were done simultaneously.

Table I. Lymphocyte Proliferation to Various Isothiazolinones and to Isothiazolinone Metabolites in MCI Patch-Test-Positive Patient 37

Antigen in Culture	Optimal Concentration and Range of Concentrations Tested ($\mu\text{g/ml}$) ^a	Maximal Lymphocyte Proliferation (cpm \pm SD)
Control		244 \pm 40
MCI	0.062 (1.6×10^{-5} –1)	15,783 \pm 82 ^b
Chlorinated isothiazolinone	0.02 (6.4×10^{-7} – 2×10^{-2})	16,177 \pm 678 ^b
Nonchlorinated isothiazolinone	0.1 (13.2×10^{-6} –0.5)	282 \pm 28
N-methyl-malonamic acid	2 (1–8)	261 \pm 13
Malanomic acid	4 (1–8)	230 \pm 25
1,2-benzisothiazolin-3-one (BIT)	0.04 (4×10^{-3} –5)	263 \pm 52

^a Concentration giving maximum lymphocyte proliferation. Two- to fourfold dilution of antigen in a given range.

^b Positive response.

ppm of MCI. In order to assess the clinical relevance of results obtained through LTT and patch testing, "use test" with lotion containing MCI was performed (Fig 1). Five out of six LTT-positive patients reacted to MCI-containing lotion, whereas seven of seven LTT-negative patients showed no response.

To investigate the allergenic epitopes of isothiazolinones, lymphocytes from several patients were tested with chlorinated and nonchlorinated isothiazolinones, their metabolites, and BIT. Four experiments were performed with similar results. The results of one such experiment are shown in Table I. Lymphocytes from MCI-allergic patients proliferated to chlorinated isothiazolinone, whereas no proliferation was observed with the nonchlorinated isothiazolinone, isothiazolinone metabolites, or BIT. The importance of chlorine as a part of the allergenic epitope is further demonstrated by stimulation of MCI-specific lymphocytes with dichlorinated isothiazolinone (Fig 3).

The absence of lymphocyte proliferation to MCI in some patch-test-positive patients might be due to the occurrence of lymphocytes sensitized to metabolites of isothiazolinones formed in vivo, but not under experimental conditions in vitro. Therefore, similar experiments with lymphocytes from patch-test-positive but LTT-negative patients were conducted (Table II). No isothiazolinone-specific responses were observed, suggesting the absence of MCI-specific memory cells of these patients.

Because all previously described patients were patch tested prior to the LTT, the possibility of sensitization through patch testing was considered. Therefore, we tested lymphocytes from two industrial workers accidentally exposed to high concentrations of BIT during their work (Table III). LTT revealed strong sensitization to BIT and this was confirmed by a subsequent patch test. Lymphocyte and patch-test responses to MCI were also positive. The implications of this finding are discussed below.

To study the specificity of MCI-specific lymphoproliferative responses, lymphocytes from seven volunteers with no dermatitis and from nine patients with dermatitis unrelated to MCI were studied. Mean cpm incorporated by control lymphocytes from former group were 1993 ± 1636 , whereas mean cpm incorporated by MCI-stimulated lymphocytes were 2391 ± 2504 . Mean cpm incorporated by individual MCI-stimulated cultures were less than twice that of control cultures (data not shown). The similar results were obtained in the latter group (Table IV). Thus, lymphocytes from these groups did not respond to MCI in vitro.

DISCUSSION

The results of LTT show that nine of 18 patients with patch tests positive to MCI had isothiazolinone-specific lymphocytes in the blood. Such lymphocytes were absent in the remaining patients. In the latter group the inability of LTT to demonstrate MCI-sensitization could be due to the fact that these patients were sensitized by isothiazolinone metabolites generated in vivo. The metabolites may have been absent under experimental conditions in vitro. This explanation is unlikely because isothiazolinone metabolites did not stimulate lymphocytes from isothiazolinone-allergic patients. The absence of isothiazolinone-specific lymphocytes in MCI patch-test-positive patients suggests that their skin reactions were irritant rather than allergic in nature. Use test with MCI-containing lotion was performed to establish clinical relevance of positive patch-test responses. The results indicate that patients with lymphocytes reacting to MCI in vitro respond to MCI-containing lotion,

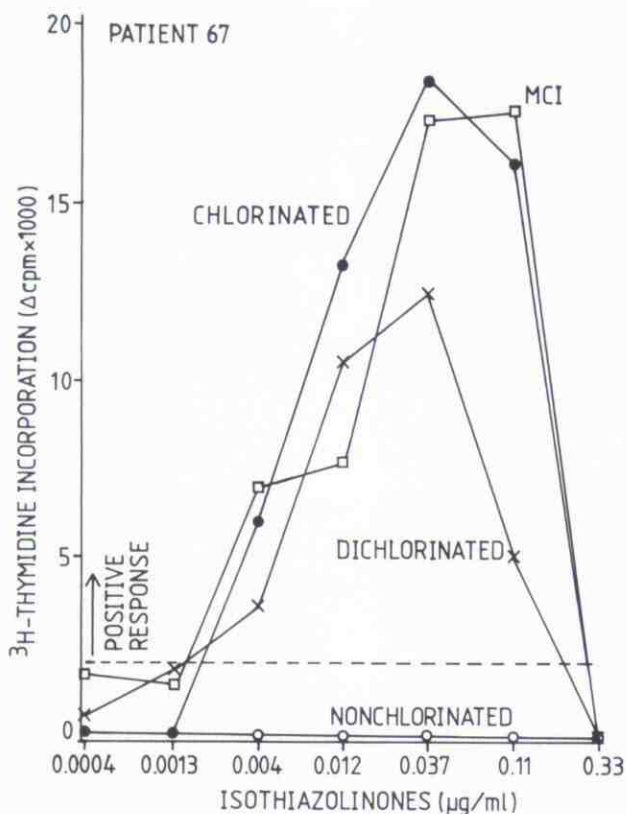


Figure 3. Lymphocyte proliferation to various isothiazolinones in patient 67.

Table II. Lymphocyte Transformation Test Performed with Tuberculin, Isothiazolinone, and Isothiazolinone Metabolites in Two Patients (41 and 42) Exhibiting MCI-Positive Patch Tests

Antigen in Culture	Range of Concentrations Tested ($\mu\text{g/ml}$) ^a	Maximal Lymphocyte Response (cpm \pm SD)	
		Patient 41	Patient 42
Control		2,376 \pm 803	2,389 \pm 218
PPD	0.1, 1	26,383 \pm 1051 ^b	5,338 \pm 360 ^b
MCI	2.4×10^{-4} –1	3,715 \pm 348	2,974 \pm 436
Chlorinated isothiazolinone	9.8×10^{-6} – 4×10^{-2}	4,758 \pm 481	4,617 \pm 240
Nonchlorinated isothiazolinone	2.9×10^{-5} –0.12	4,105 \pm 202	2,241 \pm 67
N-methyl-malonamic acid	1.6×10^{-2} –4	2,242 \pm 67	2,241 \pm 224
Malanamic acid	1.6×10^{-2} –4	2,097 \pm 189	2,462 \pm 291
Malonic acid	6.3×10^{-2} –4	3,126 \pm 129	1,978 \pm 294

^a Fourfold dilution of antigen concentrations in a given range.

^b Positive response.

Table III. Results of Patch Tests and LTT in Two Male Workers Accidentally Sensitized to Formulation Containing BIT at Work

Patient Code	Age	Patch Tests						
		Maximal Proliferative Response (cpm \pm SD)			BIT ^b		MCI	
		Control	BIT	MCI	1%	2%	100 ppm	200 ppm
48	30	854 \pm 224	17,830 \pm 465 ^a	7,305 \pm 339 ^a	++	++	neg	neg
49	21	1,251 \pm 306	9,543 \pm 214 ^a	2,923 \pm 234 ^a	neg	+	neg	++

^a Positive responses.

^b Formulation containing BIT.

Table IV. Lymphocyte Responses to MCI and to Tuberculin (PPD) in Patients with Dermatitis Unrelated to MCI

Patient Code	Age/Sex	Patch Test and/or LTT Positivity	Maximal Proliferative Response		
			Control (cpm)	MCI (cpm)	PPD (cpm)
30	40/F	Cobalt, chromium, nickel	883	328	298,590 ^a
39	26/F	Nickel	23,292	15,627	56,989 ^a
45	27/F	Formalin	520	196	32,328 ^a
236	36/F	0	240	490 ^b	41,201 ^a
237	45/F	Zimelidine, bacampicillin	3,579	3,714 ^b	169,368 ^a
238	25/F	Nickel, cobalt	8,318	1,753	102,776 ^a
239	19/M	Quinidine	1,552	590	74,950 ^a
241	46/F	Bacampicillin	3,191	-692	148,671 ^a
242	38/F	Bacampicillin	117	96	19,746 ^a

^a Positive responses.

^b No MCI-specific lymphoblasts in culture.

whereas patients lacking MCI lymphocytes are use-test negative. Interestingly, use-test and LTT-negative patients often reacted only upon patch testing with 200 ppm active ingredient but not with 100 ppm active ingredient of MCI. The reverse was true for the group of LTT- and use-test-positive patients; they all reacted positively upon skin testing with 100 ppm or less. Consequently, the prevalence of MCI allergy may be overestimated in some clinical populations. This is also suggested by other investigations [15–18].

The chlorinated isothiazolinone was responsible for the induction of proliferative responses in lymphocytes from MCI-allergic patients. The importance of the chlorine atom as a part of an allergenic epitope of isothiazolinones was further supported by findings of cross-reactions with dichlorinated isothiazolinones. As reported

by others [11–19], the dichlorinated isothiazolinones produced positive skin reactions in MCI-sensitized humans and guinea pigs. No cross-reaction was observed with non-chlorinated isothiazolinone or with various isothiazolinone metabolites. Further, MCI-sensitized lymphocytes did not cross-react with industrial MCI and with BIT. These results are also confirmed in human patch-test studies.

LTT testing was also conducted prior to patch testing in two workers who had been accidentally exposed to high concentrations of BIT. Lymphocytes from these workers reacted to BIT and also to MCI. This suggests either cross-reaction between the two isothiazolinones or specific sensitization to both. The latter seems more plausible because MCI-specific lymphocytes did not respond to BIT

in vitro. Later, it was found that workers 48 and 49 had also been exposed to industrial products containing MCI.

At present it is not clear if some of the MCI-LTT-positive patients were sensitized by patch testing with 200 ppm of MCI. A possible sensitization by patch testing has been reported by others [1,19]. Formaline and metoprolol epoxides are two examples of substances which may produce flare up and sensitization of non-allergic patients if patch tested at high concentrations.

In regard to the specificity of LTT, immunologic responses to MCI were found only in patients with dermatitis and who were also patch-test-positive to MCI. Isothiazolinone-specific responses were never found in patients with dermatitis unrelated to MCI or in donors without dermatitis.

In conclusion, 50% of patients positive in patch testing with 200 ppm of MCI had isothiazolinone-specific lymphocytes in the blood. In these patients, positive patch test could also be demonstrated with 100 ppm of MCI and as positive use tests demonstrated, they were truly allergic to MCI. The sensitizing properties were not restricted to chlorinated isothiazolinones because accidental exposure to BIT resulted also in allergic sensitization. LTT may be used to establish relevant patch-test concentrations of irritative chemicals and for the more precise diagnosis of allergic contact dermatitis induced by irritative substances.

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