

T lymphocyte dynamics in methylisothiazolinone-allergic patients

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Summary

Background. Methylisothiazolinone (MI), a preservative that is commonly used in personal care products, is now recognized as an important contact allergen in both cosmetic and occupational settings.

Objectives. To analyse T lymphocyte responses to MI, in order to provide important information regarding the relationship between the nature of such responses and skin sensitization potency.

Methods. Proliferative responses to free MI and to an MI–human serum albumin (HSA) conjugate were measured according to [³H]thymidine incorporation (n = 56 donors; patch test scores of + in 20, ++ in 29, and +++ in 7). Peripheral blood mononuclear cells were cultured in the presence of MI (0.001–1 µg/ml) or MI–HSA (0.001–100 µg/ml). Proliferating CD4⁺ and CD8⁺ T lymphocytes were identified by flow cytometry with the intracellular marker Ki-67.

Results. For free MI, modest positive responses were recorded for 7 of 31 donors. In contrast, MI–HSA stimulated more marked responses in 17 of 31 donors. Characterization of positive proliferative responses showed variable patterns of proliferating CD4⁺ and CD8⁺ T lymphocytes from donors with the same patch test scores and similar maximal values.

Conclusions. MI–HSA is able to induce secondary responses in lymphocytes drawn from sensitized subjects, and provides a more effective source of antigen than free MI. Furthermore, individual donors show differential activity profiles with respect to T lymphocyte subsets.

Key words: allergic contact dermatitis; methylisothiazolinone; patch testing and skin sensitization; T lymphocytes; CAS no. 2682-20-4.

Many hundreds of chemicals have been implicated as having the potential to cause skin sensitization and allergic contact dermatitis (ACD) (1, 2). It is apparent that contact allergens vary substantially, and by up to five orders of magnitude, with respect to their relative skin sensitizing

potencies. In practice, potency is best described as being a function of the amount of chemical required for the acquisition of sensitization (3). These differences in potency appear to be reflected in differential thresholds for skin sensitization observed in human repeat insult patch testing, and, collectively, clinical experience broadly matches estimates of skin sensitizing potency based on data from the commonly used toxicology model in this area, the murine local lymph node assay (4, 5).

There is considerable interest in identifying those factors that have the greatest impact on the sensitizing potency of contact allergens. It has been proposed recently that a number of immunological variables may be influential (6). These include the overall vigour of immune

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responses elicited following topical exposure to a contact allergen, and, in particular, the extent of T lymphocyte proliferation and allergen-driven clonal expansion. In addition, the quality of immune responses to contact allergens as reflected by the contribution of discrete functional subpopulations of T lymphocytes, and the promiscuity of responses, are also likely to impact on the effectiveness of sensitization (6). One possible strategy to map immune responses elicited by contact allergens is to employ the lymphocyte transformation test (LTT), a method that seeks to characterize *in vitro* recall responses to chemical allergens by peripheral blood lymphocytes drawn from sensitized subjects (7, 8). Although most experience with the LTT has been derived from studies of nickel allergy (9, 10), there is increasing experience with organic contact allergens, including, for instance, *p*-phenylenediamine (PPD) (11–13) and 2,4-dinitrochlorobenzene (14). Here, we describe the use of the LTT to characterize T lymphocyte responses to a single contact allergen, methylisothiazolinone (MI), an allergen with which there is only limited experience in the LTT (15).

Methylchloroisothiazolinone (MCI)/MI is a preservative mixture at a fixed ratio (3:1). Since MI has found application as a single preservative, it has become apparent that this chemical is an important contact allergen, and may have a greater potential for sensitization than does the MCI/MI mix (16–19). Here, we describe investigations designed to characterize induced changes in the composition of T lymphocyte populations in the peripheral blood of sensitized subjects challenged with MI, and the characteristics of T lymphocyte responses following culture of peripheral blood mononuclear cells (PBMCs) from patients sensitized to MI with free MI, or with MI–protein conjugates.

Materials and Methods

Donor characteristics

Patients were recruited from those attending the Contact Dermatitis Investigation Unit at Salford Royal Hospital for diagnosis (by patch testing) of contact allergy. Peripheral blood samples (20 ml) were drawn from patients ($n = 56$), diagnosed as being sensitized to MI [NRES Ethics Committee North West – Greater Manchester East (12/NW/0602)], into lithium heparin-coated collection tubes (Thermo Fisher Scientific, Waltham, MA, USA) (15 IU/ml). Samples were collected 4 days after patch application, and graded as + (weak; $n = 20$), ++ (strong; $n = 29$), and +++ (extreme; $n = 7$), on the basis of the severity of reaction during patch test challenge with 0.2% MI aq., and following standards established by the ICDRG.

Healthy volunteers

Peripheral blood from healthy volunteers was obtained from the NHS Blood and Transport Service as a buffy coat preparation ($n = 10$). Buffy coat samples were obtained by centrifugation (500 *g*) of anticoagulated whole blood, at 18°C, within 24 h of venepuncture and subsequently received within 48 h.

PBMC isolation and phenotyping by flow cytometric analysis

PBMCs were prepared by density gradient centrifugation with Histopaque-1077 (Sigma-Aldrich, St Louis, MO, USA). Briefly, 20 ml of whole blood was layered onto 15 ml of Histopaque-1077, the samples were centrifuged at 500 *g* for 30 min, and PBMC layers were collected and counted with a haemocytometer. An aliquot (1×10^6 cells) of PBMCs from each sample was stored at -80°C in human AB serum (Sigma-Aldrich) containing 10% dimethyl sulfoxide. The frequencies of different cell populations were analysed in these previously frozen samples by the use of multiparameter flow cytometry. Surface staining for anti-human CD4–allophycocyanin (APC), anti-CD8–APC, anti-CD45RA–fluorescein isothiocyanate and anti-CD27–R-phycoerythrin-Cy5 (PE-Cy5) antibodies was carried out in flow cytometry staining buffer for 30 min at 4°C (all reagents from eBioscience, San Diego, CA, USA). The viability of each sample was determined by the use of forward-scattered light (FSC) and side-scattered light (SSC) parameters for size exclusion, and an unstained control sample and appropriate isotype controls were used for setting gates. Single-stained controls with anti-human CD3 antibodies were used to assess compensation settings prior to acquisition, and to establish a compensation matrix for data analysis. At least 10 000 cells were analysed on a FACS-calibur™ flow cytometer (Becton Dickinson, Mountain View, CA, USA), by the use of FLOWJO software (Tree Star, Ashland, OR, USA). T lymphocyte subsets were identified by CD4 and CD8 expression analysis. Subsequent multi-parameter quadrant analysis, on these T lymphocyte populations, characterized T lymphocyte phenotypes using CD45RA and CD27 (20). Thus, naive cells were defined as CD45RA⁺ CD27⁺, effector memory RA (EMRA)-positive cells as CD45RA⁺ CD27[−], central memory cells as CD45RA[−] CD27⁺, and effector memory cells as CD45RA[−] CD27[−].

Generation of MI–protein conjugate

A solution of human serum albumin (HSA) (Sigma-Aldrich) at 10 mg/ml (150 μM) and 15 mM MI

(Sigma-Aldrich; $\times 100$ M excess) was dissolved in 10 ml of phosphate-buffered saline (PBS). The reaction mixture was stirred at room temperature for 72 h, and the solution was dialysed against PBS for a further 72 h, with the PBS being changed every 4–12 h. The solution was lyophilised, and the conjugate was then stored as a powder at -80°C or dissolved in PBS and stored at -20°C for use in *in vitro* assays. The degree of HSA modification by MI was measured by hapten–protein substitution analysis. Briefly, 200 μl of MI–HSA or HSA at 10 mg/ml in PBS and PBS alone were incubated at room temperature for 20 min with 5 μl of 0.03 M 2,4,6-trinitrobenzenesulfonic acid (Sigma-Aldrich). The optical density was measured at 405 nm on a Biotek reader.

[^3H]Thymidine proliferation assay

The culture medium consisted of RPMI-1640 supplemented with L-glutamine (2 mM), 10% heat-inactivated human AB serum (Sigma-Aldrich), HEPES (1%), penicillin (100 U/ml), and streptomycin (100 $\mu\text{g}/\text{ml}$) (all from Sigma-Aldrich). PBMCs were cultured in 96-well flat-bottomed plates, at 1×10^5 cells/well, in 200 μl of complete medium, in the presence of MI (Sigma-Aldrich) (0.0001–1 $\mu\text{g}/\text{ml}$), MI–HSA (0.01–100 $\mu\text{g}/\text{ml}$) or HSA (0.01–100 $\mu\text{g}/\text{ml}$) for 6 days. In addition, the mitogen phytohaemagglutinin (PHA) was used as a positive control for proliferation at 10 $\mu\text{g}/\text{ml}$. Each well was pulsed at 120 h with 22.2 μBq of [^3H]thymidine; the cells were then harvested, and processed for β -scintillation counting 16 h later. A positive proliferative response was defined as one that resulted in a stimulation index (SI) of ≥ 2.5 as compared with control (medium)-treated cells. Stimulation with irrelevant antigen controls resulted in SI values of < 2.5 . Underlying proliferative responses to HSA were taken into account by representing the changes as fold change relative to HSA alone rather than medium alone, and using the cut-off of a 1.5-fold increase over HSA alone.

Proliferation of T lymphocyte subsets measured according to intracellular Ki-67 expression

PBMCs were cultured in 96-well flat-bottomed plates, at 1×10^5 cells/well in 200 μl of culture medium, in the presence of MI (Sigma-Aldrich) (0.0001–1 $\mu\text{g}/\text{ml}$), MI–HSA (0.01–100 $\mu\text{g}/\text{ml}$) or HSA (0.01–100 $\mu\text{g}/\text{ml}$) for 120 h. Cells were harvested, and the frequencies of proliferating T lymphocytes were determined by multiparameter flow cytometry. Surface staining for anti-human CD4–APC and CD8–APC was followed by intracellular staining for anti-human Ki-67–R-PE-labelled antibodies (eBioscience) with a FoxP3 staining buffer set

(eBioscience), performed according to the manufacturer's instructions. The viability of each sample was determined by the use of FSC and SSC parameters for size exclusion and an unstained control sample, and appropriate isotype controls were used for setting gates. Single-stained controls with anti-human CD3 antibodies were used to assess compensation settings prior to acquisition and to establish a compensation matrix for data analysis. At least 10 000 cells were analysed on a FACS-calibur™ flow cytometer by the use of FLOWJO software. Briefly, T lymphocyte subsets were identified by CD4 and CD8 expression analysis, and Ki-67 $^{+}$ (proliferating) cells were then identified. The proliferation metrics were calculated as either the percentage of proliferating CD8 $^{+}$ (CD8 $^{+}$ Ki-67 $^{+}$) or CD4 $^{+}$ (CD4 $^{+}$ Ki-67 $^{+}$) T lymphocytes in the total viable population, or the % of each T lymphocyte subset proliferating, where positive proliferative responses were defined as Ki-67 positivity of $> 1\%$ in the total PBMC population, and as Ki-67 positivity of $> 2\%$ in T lymphocyte subsets.

Statistical analyses

For comparison of two independent groups between patient samples, the statistical significance of differences was determined by use of the non-parametric Mann–Whitney *U*-test, as appropriate for the data. For comparison of two groups in which PBMCs from the same samples underwent different treatments and where data followed a normal distribution, the paired Student *t*-test was performed. All analyses were performed with PRISM (GraphPad Software, La Jolla, CA, USA). Statistical significance was set at $p < 0.05$.

Results

Characterization of peripheral blood from MI-allergic donors

The relative frequencies of CD4 $^{+}$ and CD8 $^{+}$ T lymphocyte subsets in PBMCs from patients sensitized to MI were $35.9\% \pm 1.2\%$ and $17.3\% \pm 0.9\%$ (mean $\pm$ standard error of the mean), respectively, and showed no correlation with patch test score severity (data not shown). These were similar to the frequencies of CD4 $^{+}$ and CD8 $^{+}$ T lymphocyte subsets in healthy (buffy coat) samples, which were $33.6\% \pm 3.1\%$ and $20.3\% \pm 1.7\%$, respectively. The T lymphocyte subset phenotype with respect to the markers CD45RA and CD27 was analysed, categorized according to patch test score severity, and compared with PBMCs from healthy volunteers, with the caveat

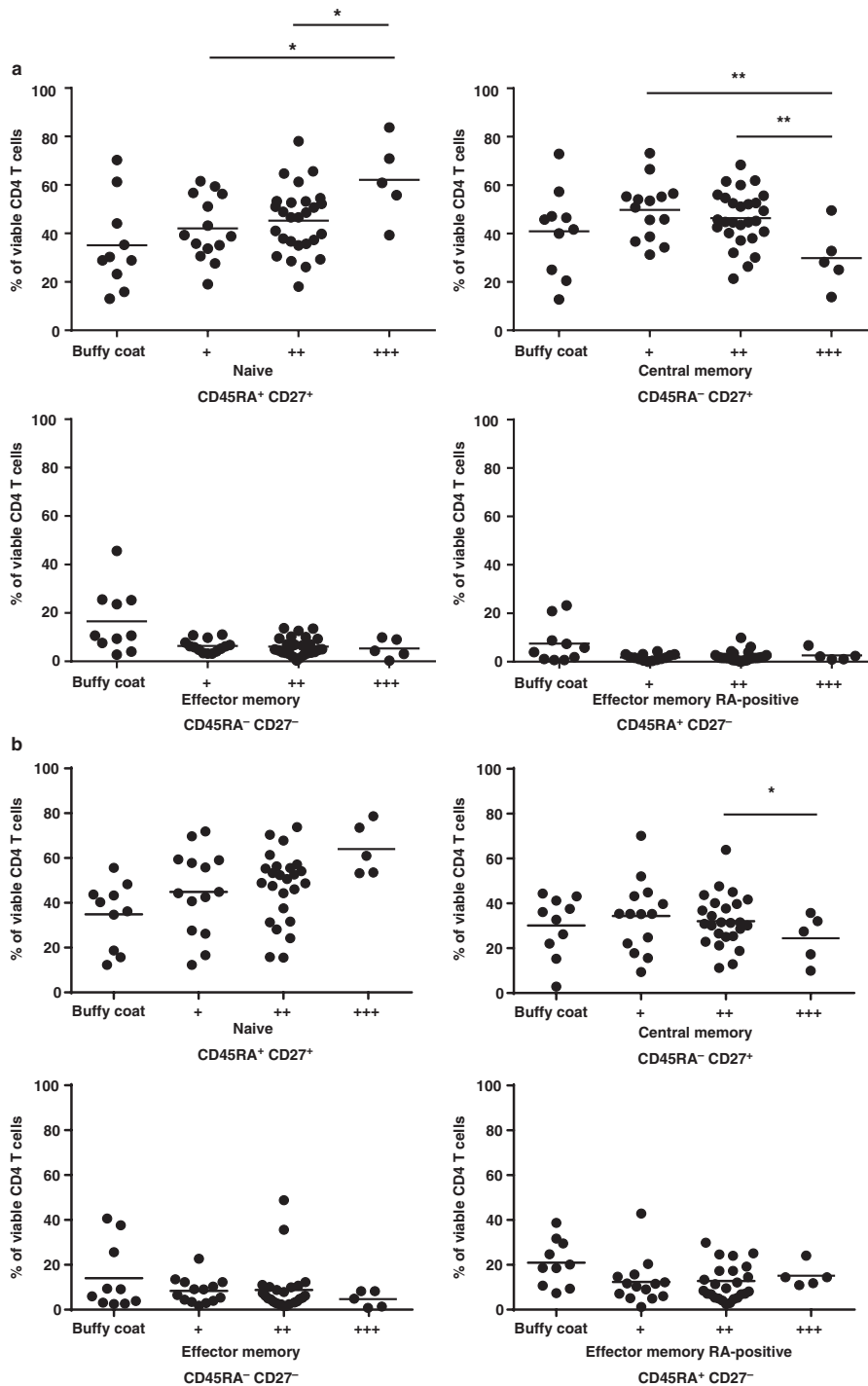


Fig. 1. T lymphocyte phenotype of freshly isolated peripheral blood mononuclear cells (PBMCs) of allergic patients: comparisons with buffy coat donors. PBMCs were isolated from methylisothiazolinone patch test-positive donors or from buffy coat donors by density gradient centrifugation. Cells were stained with anti-human CD4–allophycocyanin (APC), CD8–APC, CD45RA–fluorescein isothiocyanate and CD27–phycoerythrin-Cy5 antibodies. Data are shown as percentage of total viable cells in each phenotype [naive (CD45RA⁺ CD27⁺) (i), central memory (CD45RA⁺ CD27⁺) (ii), effector memory (CD45RA⁺ CD27⁺) (iii), and effector memory RA (CD45RA⁺ CD27⁺) (iv)] within the CD4⁺ (a) or CD8⁺ (b) T lymphocyte populations. Donors are segregated according to patch test status [buffy coat donors (n = 10), + donors (n = 14), ++ donors (n = 27), and +++ donors (n = 5)], and data are shown as group mean (horizontal line) and for each individual donor (●). For comparison of two independent patch tested groups, the non-parametric Mann–Whitney *U*-test was performed (**p* < 0.05; ***p* < 0.01; ****p* < 0.001).

that these donors had not undergone patch testing. This analysis showed significant differences between specific patch test score groups (Fig. 1). The majority of CD4⁺ and CD8⁺ T lymphocyte populations in patch test-positive donor samples showed a naive phenotype (46.1% ± 2.2% and 47.5% ± 2.5%, respectively) or central memory

phenotype (45.7% ± 1.9% and 31.5% ± 1.9%, respectively). Effector memory cells were minority populations (6.1% ± 0.5% of CD4⁺ lymphocytes and 8.1% ± 1.3% of CD8⁺ T lymphocytes), whereas CD4⁺ EMRA cells were a minority population as compared with CD8⁺ EMRA cells (2.1% ± 0.3% and 12.8% ± 1.3%, respectively).

The phenotype of the patch test-positive donors was significantly different between patch test score groups with respect to both CD4⁺ and CD8⁺ T lymphocytes, and showed a trend for the differences to be more pronounced as the strength of the patch test reaction increased from + to +++. Thus, for CD4⁺ T lymphocytes, there was a significant increase in the frequency of naive cells [from ~40% (+) to ~60% (+++)], with corresponding reductions in the frequencies of cells with central memory phenotypes. A similar pattern for naive and central memory cells was recorded for CD8⁺ T lymphocytes, although the frequencies of effector memory and EMRA cells were more variable, and the changes did not tend to reach statistical significance. Indeed, effector memory and EMRA phenotypes were minority populations in all patch test donor samples, particularly for CD4⁺ T lymphocyte populations (<5%).

Lymphocyte proliferation in response to MI

Antigen-specific proliferation in response to 6 days of culture with free MI was measured for PBMCs from a total of 56 MI-sensitized donors across the three patch test-positive classes as a function of radiolabelled thymidine incorporation. A concentration range of 0.001–1 µg/ml MI was utilized, as concentrations of >1 µg/ml resulted in marked toxicity (<20% viable; data not shown). Baseline proliferation varied from 420 to 18 172 cpm, with a trend for lower baseline responses with higher patch test score donors; 4110 ± 908 cpm and 3113 ± 574 cpm were recorded for + and ++ patch test donors, as compared with 2326 ± 663 cpm for +++ patch test donors. Baseline proliferative responses were measured for 9 healthy donors who had not undergone any patch testing and were equivalent to low (+) patch test score donors (5347 ± 1186 cpm). Proliferation in response to free MI for PBMCs from patients representing all three patch test scores is shown in Fig. 2 with respect to SI relative to concurrent baseline control values. A positive proliferative response was defined as one that reached an SI [test cpm/baseline (medium control-treated cells) cpm] of ≥2.5 at one or more concentrations. Regardless of patch test score status, all donors responded to culture with the mitogen PHA, although the extent of the response varied with respect to both absolute cpm and SI recorded. In response to 10 µg/ml PHA, SI values ranged from 2.5 to 200 for both allergic donor and healthy control samples (data not shown). Following culture with free MI, relatively low proliferative responses were recorded for all MI donors, with variable dose–response profiles and no correlation with patch test score. Positive SI values of ≥2.5 were observed for only 14 of 56 donors

(with maximal SI values ranging from 2.5 to 5); 5 in the + group (Fig. 2a), 7 in the ++ group (Fig. 2b), and 2 in the +++ group (Fig. 2c). Hence, MI-induced proliferation was detected in ~25% of donors in each patch test group.

MI–HSA enhances lymphocyte proliferative responses

Given the relatively low response to free MI, in order to evaluate further the activation of MI-specific T lymphocytes, a MI hapten–protein conjugate was synthesized, whereby free chemical MI was bound covalently to the model protein HSA prior to addition to culture. Proliferative responses stimulated by culture for 6 days in the presence of free MI or MI–HSA, or HSA alone, were compared directly in PBMCs from 31 MI patch test-positive donors comprising 11 +, 17 ++ and 3 +++ donor samples (Fig. 3 and Table 1). Because of the degree of cellular toxicity seen at high MI concentrations, the viability of PBMCs following 5 days of culture with the various stimuli was measured for all 31 donors (Fig. 3a). The viability of PBMCs decreased markedly in response to increasing concentrations of MI, from 75% with medium alone to 40% at 1 µg/ml MI. No significant effect on cell death was observed following culture with MI–HSA or HSA at any concentration tested.

In response to free MI, positive SI values of ≥2.5 were observed for 7 of 31 donors (Fig. 3b and Table 1; maximal SI value of 4.0). Similar borderline positive SI values were recorded for 9 of 31 donors when cells were cultured with HSA alone (Fig. 3c and Table 1; maximal SI value of 3.5). No positive proliferative responses were induced in 6 of 6 healthy donor samples in response to free MI, MI–HSA, or HSA (data not shown). In contrast, PBMCs from 17 of 31 donors responded to culture with MI–HSA (Fig. 3d), and these tended to show a typical dose–response profile and were more vigorous (maximal SI value of 12.0), with the level of [³H]thymidine incorporation being higher in 15 of the 17 positive responders after stimulation with MI–HSA than after stimulation with free chemical (detailed in Table 1). Taking into account the underlying proliferative response to HSA alone (representing the changes as fold change relative to HSA alone rather than relative to medium alone), and with the cut-off of a 1.5-fold increase over HSA alone, 14 of 31 donors showed increased proliferation over this baseline.

Allergen-driven proliferation of CD4⁺ and CD8⁺ T lymphocyte subsets

In order to characterize the phenotype of the proliferating cells, allergen-driven proliferation of T lymphocyte subsets in PBMCs from MI-sensitized individuals was analysed by flow cytometry with Ki-67 and T lymphocyte

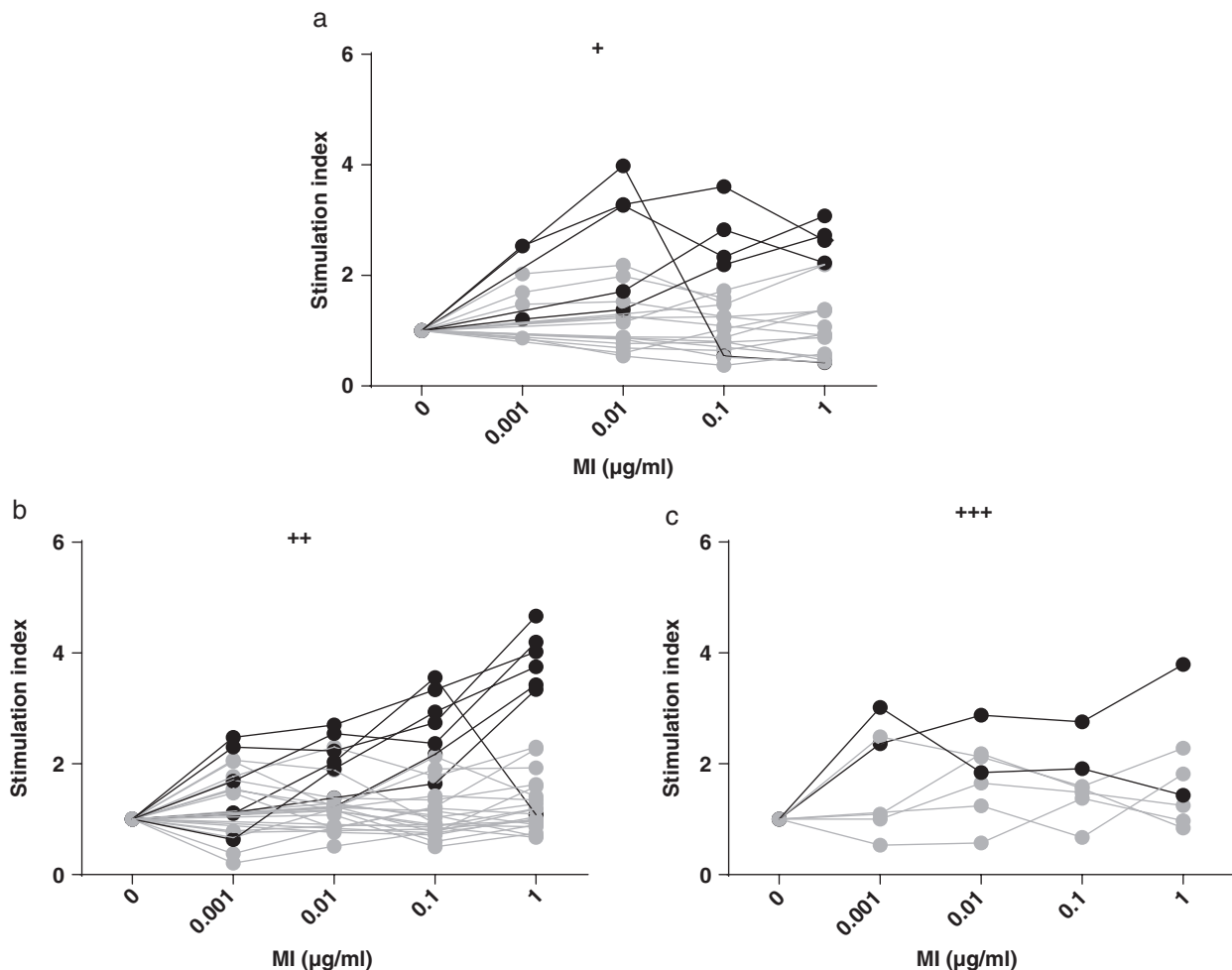


Fig. 2. Peripheral blood mononuclear cell (PBMC) proliferation in response to methylisothiazolinone (MI) measured according to [^3H]thymidine incorporation. PBMCs were isolated from MI patch test-positive donors ($n = 56$) by density gradient centrifugation. PBMCs were cultured in 96-well flat-bottomed plates, at 1×10^5 cells/well, and stimulated with a range of free MI concentrations (0.001–1 $\mu\text{g/ml}$), or cultured in the presence of medium alone (0), and incubated at 37°C ($\geq$ triplicate wells). Cells were pulsed at 120 h with [^3H]thymidine, and harvested and processed for β -scintillation counting 16 h later. Data are shown as a stimulation index (SI) [fold change in proliferation above baseline (medium alone; 0)] per patch test score group for (a) + donors ($n = 20$), (b) ++ donors ($n = 29$), and (c) +++ donors ($n = 7$). Each individual line represents one donor; black lines indicate that an SI of ≥ 2.5 was recorded at least at one concentration; grey lines indicate that an SI of < 2.5 was recorded at all concentrations of MI tested.

markers. Ki-67 as a method to measure cellular turnover was applicable to populations of cells from MI patch test-positive donors that showed an SI of > 2.5 in response to MI–HSA conjugate with respect to [^3H]thymidine incorporation. Quadrant analyses for Ki-67 and CD4 or CD8 staining are shown for one representative +++ donor following 5 days of culture with medium alone, HSA, or MI–HSA (Fig. 4). Of cells cultured with medium alone, $\sim 55\%$ and 15.6% were CD4 $^+$ and CD8 $^+$, respectively, but the frequency of Ki-67 $^+$ cells was $< 0.2\%$. For both CD4 $^+$ and CD8 $^+$ T lymphocytes, there was a clear allergen-dependent increase in the number of cells in the

upper right quadrant, representing proliferating (Ki-67 $^+$) T lymphocytes. Thus, the frequency of Ki-67 $^+$ CD4 $^+$ T lymphocytes increased from 0.1% of total PBMCs with medium alone, to 1.9% and 3.4% with HSA and MI–HSA, respectively. In parallel, the percentage of Ki-67 $^+$ CD8 $^+$ T lymphocytes increased from 0.04% to 1.5% and 2.5% of total PBMCs in response to HSA and MI–HSA, respectively. A total of 17 MI patch test-positive donors (across the range of patch test scores) who showed SI values for MI–HSA of ~ 2.5 were evaluated for T lymphocyte subset Ki-67 expression (the maximal responses achieved are shown in Table 2).

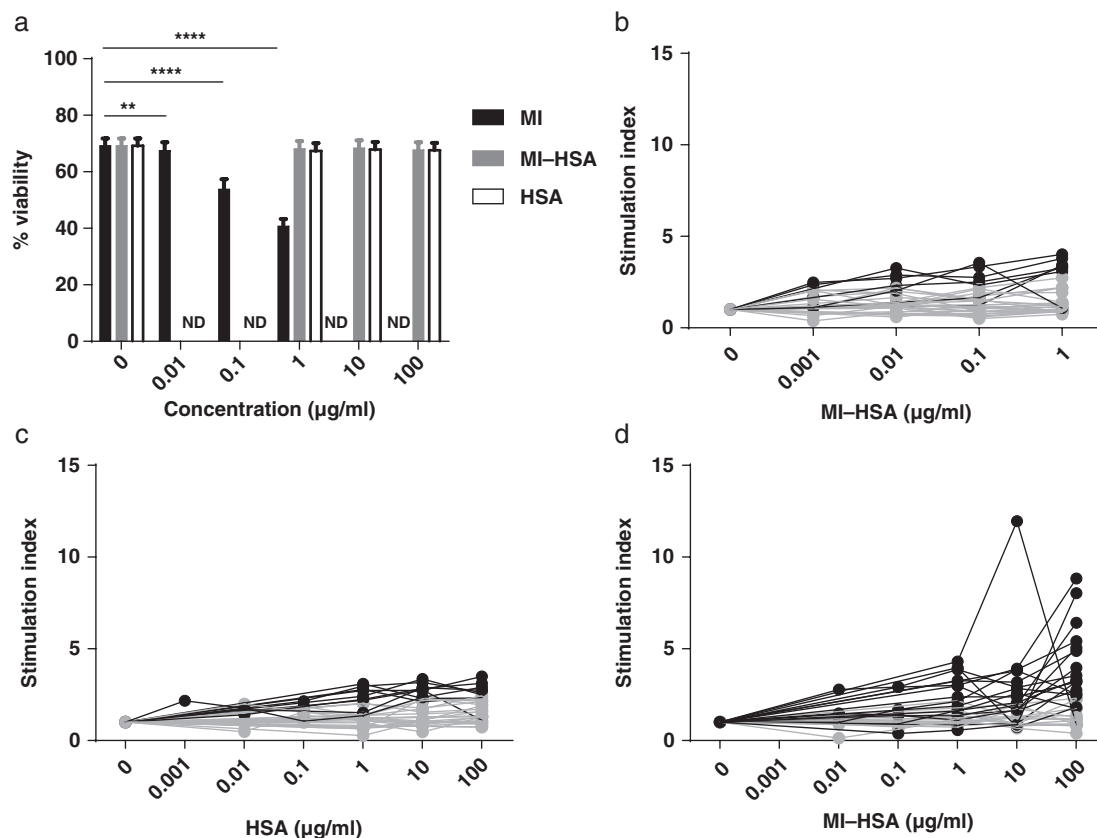


Fig. 3. Allergen-driven proliferation induced by methylisothiazolinone (MI) hapten-protein conjugate. Peripheral blood mononuclear cells (PBMCs) were prepared from MI-allergic donors ($n = 31$; 11 +, 17 ++, and 3 +++), and seeded into tissue culture plates as described in the legend to Fig. 2. PBMCs were stimulated with medium alone (0), free MI (0.001–1 $\mu\text{g/ml}$), human serum albumin (HSA) (0.001–100 $\mu\text{g/ml}$), or MI-HSA (0.001–100 $\mu\text{g/ml}$), and cultured at 37°C. Cells were pulsed at 120 h with [^3H]thymidine, and harvested and processed for β -scintillation counting 16 h later. Viability was assessed by flow cytometry, determined by the use of forward-scattered light and side-scattered light parameters for size exclusion, and recorded as a percentage of total cells (ND, not determined). Results are shown as mean and standard error of the mean for PBMCs in response to stimulation with free MI (0.01–1 $\mu\text{g/ml}$), MI-HSA (1–100 $\mu\text{g/ml}$), or HSA alone (1–100 $\mu\text{g/ml}$) (a). Data on proliferation in response to MI (0.001–1 $\mu\text{g/ml}$) (b), HSA (0.001–100 $\mu\text{g/ml}$) (c) and MI-HSA (0.001–100 $\mu\text{g/ml}$) (d) are shown as stimulation indices (SIs) (fold change in proliferation above baseline). Each individual line represents one donor; black lines indicate that an SI of ≥ 2.5 was recorded at least at one concentration; grey lines indicate that an SI of < 2.5 was recorded at all concentrations of free MI or conjugate. For comparison of two groups, the paired t -test was used (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$).

For 4 representative donors, of the 17 analysed in total, dose responses for [^3H]thymidine incorporation and Ki-67 expression for the same donors are shown in Fig. 5a. The first responded with an SI of 3 to stimulation with MI-HSA at 100 $\mu\text{g/ml}$; however, no detectable proliferating (Ki-67 $^+$) T lymphocytes (CD4 $^+$ and CD8 $^+$) were identified. One donor with an SI value of 5 was characterized by 10% proliferating CD4 $^+$ T lymphocytes and negligible CD8 $^+$ T lymphocytes (Fig. 5b). Baseline proliferating CD4 $^+$ T lymphocytes ($\sim 5\%$) were detected in a third donor (Fig. 5c) with an SI of 5, increasing minimally to 6.4 in response to MI-HSA at 100 $\mu\text{g/ml}$. The same donor was characterized by a baseline level of 10%

proliferating CD8 $^+$ T lymphocytes, increasing to 23% in response to MI-HSA. The final donor, with a relatively high SI of 8 (Fig. 5d), was characterized by 50% of CD4 $^+$ T lymphocytes and 45% of CD8 $^+$ T lymphocytes showing detectable proliferation.

Taking together proliferative responses for all 17 positive responders to MI-HSA (Table 2), donors with a low threshold SI value of 2.5 for [^3H]thymidine incorporation, and $< 1\%$ total Ki-67 expression in PBMCs, were characterized by very few detectable proliferating CD4 $^+$ and CD8 $^+$ (Ki-67 $^+$) T lymphocytes ($< 2\%$). This was representative of 7 of 17 donor responses (Table 2). However, 1 donor with an SI of 2.8 showed baseline proliferation of

Table 1. Summary of proliferative responses recorded in peripheral blood mononuclear cells from methylisothiazolinone (MI)-allergic donors

Patient no.	Patch test score	Maximal SI in response to free MI	Maximal SI in response to MI-HSA	Maximal SI in response to HSA	Fold change in response to HSA
01	+	0.9	0.9	0.7	1.3
02	+	0.9	0.9	1.3	0.7
03	+	1.3	1.2	1.3	0.9
04	+	1.4	1.8	1.1	1.7
05	+	1.4	2.3	1.0	2.3
06	+	2.0	1.4	1.2	1.2
07	+	2.2	2.6	1.6	1.6
08	+	2.2	12	3.4	3.5
09	+	2.2	1.4	1.6	0.9
10	+	2.7	4.9	3.5	1.4
11	+	3.1	5.1	1.9	2.7
12	++	1.0	1.0	1.3	0.8
13	++	1.0	4.0	2.1	1.9
14	++	1.1	1.3	1.3	1.0
15	++	1.2	1.2	1.6	0.8
16	++	1.2	1.7	2.0	0.9
17	++	1.2	2.0	2.3	0.9
18	++	1.2	3.2	1.0	3.2
19	++	1.3	2.8	2.8	1.0
20	++	1.6	8.0	1.2	6.7
21	++	1.6	0.9	0.8	1.1
22	++	2.0	3.3	3.2	1.0
23	++	2.1	5.4	2.9	1.9
24	++	2.1	3.8	1.3	2.9
25	++	3.3	2.9	2.3	1.3
26	++	3.3	3.2	3.1	1.0
27	++	3.6	6.4	2.9	2.2
28	++	4.0	8.8	3.2	2.8
29	+++	1.7	2.2	2.8	0.8
30	+++	2.2	4.0	2.4	1.7
31	+++	3.8	3.9	2.3	1.7

HSA, human serum albumin; SI, stimulation index.

Data represent MI-allergic patients categorized by patch test score at 4 days after patch application, and ranked according to the vigour of the maximal proliferative response to free MI measured according to [³H]thymidine incorporation. Taken from the dose–response data shown in Fig. 2.

~2% of both T lymphocyte subsets. In contrast, 3 donors with SI values of ~4 were characterized by low levels of proliferating CD8⁺ T lymphocytes (2–4%) and no CD4⁺ T lymphocyte proliferation. Four donors with SI values of 4–6 showed similar T lymphocyte responses, characterized by ~7% of CD4⁺ T lymphocytes and 18% of CD8⁺ T lymphocytes expressing Ki-67. The donor with the highest SI of 12 and 66% total Ki-67 expression in PBMCs was characterized by 75% proliferating CD4⁺ T lymphocytes. Interestingly, low patch test score responders (+), in whom more vigorous proliferative responses were induced (SI of >2.5% and >1% total Ki-67 expression), were predominantly characterized by proliferating CD4⁺ T lymphocytes. In the ++ and +++ patch test score groups, both proliferating CD4⁺ and CD8⁺ subsets were present; however, there was a tendency for there to be a higher percentage of proliferating CD8⁺ T lymphocytes.

Discussion

Since the introduction of MI as an individual biocide in products, there has been a substantial increase in the number of individuals diagnosed with ACD as a result of sensitization to MI (17, 19, 21). T lymphocyte activation and the development of allergen-reactive memory T lymphocytes are known to be critical steps during the acquisition of skin sensitization. Thus, for instance, nickel-specific memory T lymphocytes (as defined by CD3⁺ CD45RO⁺ expression) can be identified in nickel-sensitized subjects who have developed ACD (22, 23). The characterization of T lymphocyte responses in patients with ACD, as a result of sensitization to MI, may provide important information that, in turn, will drive an improved understanding of the relationship between the characteristics of such responses and the effectiveness of

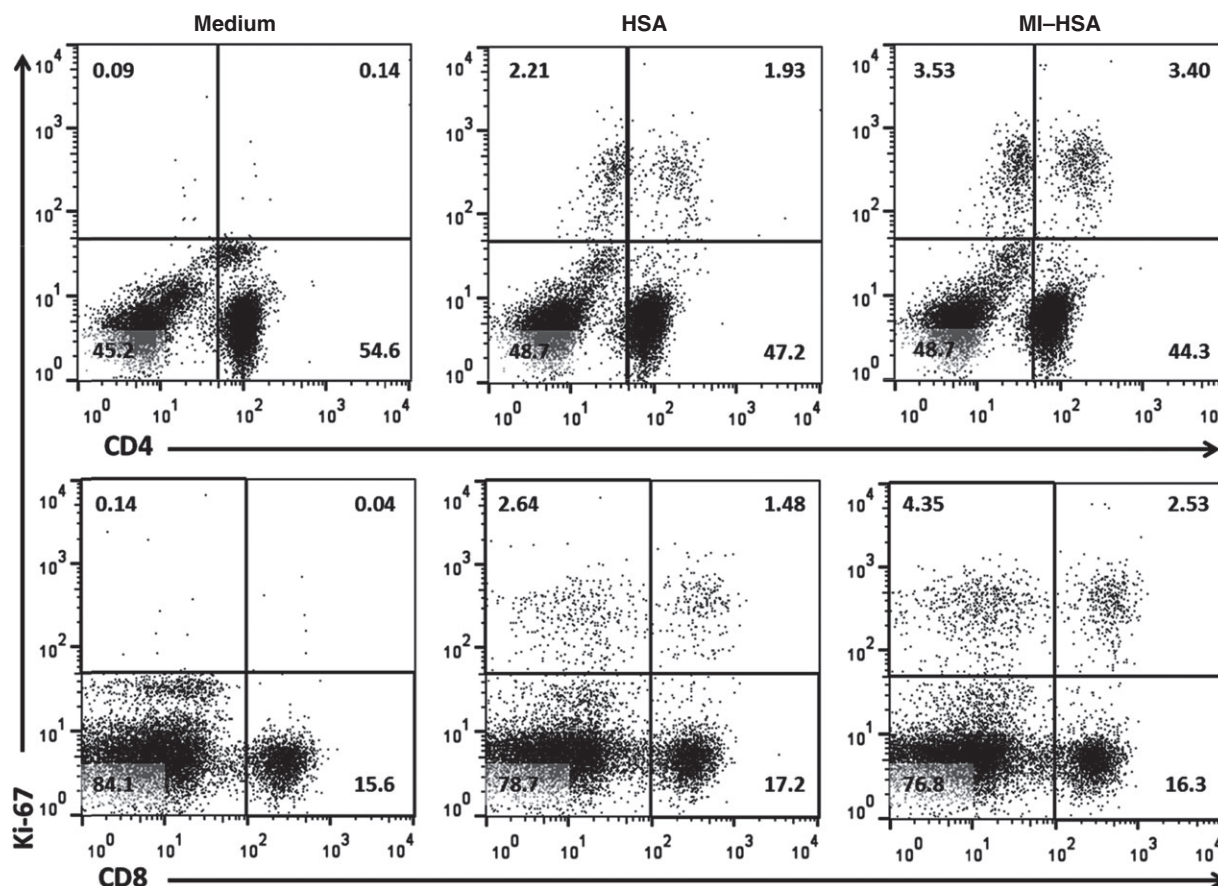


Fig. 4. Allergen-driven proliferation of T lymphocyte subsets in response to methylisothiazolinone (MI) hapten–protein conjugate measured according to intracellular Ki-67 expression. Peripheral blood mononuclear cells isolated from a +++ MI-allergic donor (patient number 30) were stimulated with medium, human serum albumin (HSA) (100 µg/ml) or MI–HSA (100 µg/ml) at 37°C for 120 h as described in the legend to Fig. 3. Cells were stained with anti-human CD8–allophycocyanin (APC), CD4–APC and Ki-67–phycoerythrin antibodies. At least 10 000 cells per group were analysed on a FACS-calibur flow cytometer, by the use of FLOWJOW software. Data are shown as dotplots for an individual donor.

skin sensitization, and thereby facilitate the development of novel paradigms for hazard and risk assessment. Here, the proliferation marker Ki-67 was utilized for the measurement of allergen-driven T lymphocyte responses in patients sensitized to MI. In addition, we have shown that the use of MI hapten–protein conjugate is superior to the use of free chemical in provoking MI-specific proliferation.

The phenotype of T lymphocyte subsets within peripheral blood has been widely studied, and is known to show marked differences, within and between individuals, in the proportion of memory T lymphocytes, depending on vaccination schedules, disease status, and age (24–26). In fact, the proportions of naive and central memory T lymphocyte subsets in a healthy population have both been shown to vary between approximately 20% and 75% of the total lymphocytes, whereby an increase in the proportion of naive T lymphocytes is associated with

a corresponding decrease in the proportion of central memory T lymphocytes and vice versa (27). This is in agreement with our finding that the proportions of both naive and central memory T lymphocytes range from approximately 15% to 75% in the total population of MI-sensitized donors when blood samples are collected 4 days following patch test application. Samples from healthy controls who had not recently undergone patch testing were derived from buffy coat preparations, and the data show that differences exist between samples from healthy controls and samples from allergic patients following patch testing, although it is important to acknowledge that these samples were of different provenance. For future investigations, a more relevant healthy control would comprise blood samples obtained from patients undergoing the same patch testing regime but with negative patch test results.

Table 2. Characteristics of positive proliferative responses to methylisothiazolinone (MI)–human serum albumin (HSA)

Patient no.	Patch test score	Maximal SI in response to MI–HSA ($[^3\text{H}]$ thymidine)	Total Ki-67 expression (% of PBMCs)	% CD4 T cells (% of PBMCs)	% CD8 T cells (% of PBMCs)	% of Ki-67-positive CD4 T cells (proliferating)	% of Ki-67-positive CD8 T cells (proliferating)
07	+	2.6	0.4	42.4	22.6	0.0	0.7
10	+	4.9	3.5	26.8	20.4	11.3	1.3
11	+	5.1	1.4	47.0	19.9	0.9	2.0
08	+	12.0	66.7	42.5	51.3	75.7	0.2
19	++	2.8	2.6	28.3	25.3	2.1	2.8
25	++	2.9	0.1	46.3	13.2	0.0	0.0
26	++	3.2	0.6	49.9	16.5	0.5	1.0
18	++	3.2	2.1	21.5	19.1	1.6	4.2
22	++	3.3	0.4	57.3	14.0	0.1	0.1
24	++	3.8	1.2	30.3	25.7	0.5	0.3
13	++	4.0	0.1	71.3	10.0	0.1	3.6
23	++	5.4	7.6	49.3	17.1	6.4	22.5
27	++	6.4	14.0	45.2	21.8	8.4	14.0
20	++	8	40.5	71.6	12.6	49.1	43.1
28	++	8.8	6.4	51.4	28.9	7.7	23.2
31	+++	3.9	0.9	57.5	13.6	0.1	0.1
30	+++	4	9.6	48.1	18.5	7.1	13.4

PBMC, peripheral blood mononuclear cell; SI, stimulation index.

Data represent MI-allergic patients categorized by patch test score at 4 days after patch application, and ranked according to the vigour of the proliferative response to MI protein conjugate (MI–HSA) measured according to $[^3\text{H}]$ thymidine incorporation. Cells were prepared and treated as described in the legend to Fig. 5.

Surprisingly, categorization of the patients into patch test severity groups (+, ++ and +++) showed significant differences in T lymphocyte phenotype between these groups. The main finding was that the proportion of circulating T lymphocytes (both CD4⁺ and CD8⁺) with a naive phenotype increased in line with patch test score severity. Concurrently, there was found to be a commensurate decrease in the proportion of central memory T lymphocytes. Following sensitization to MI, a memory pool of MI-specific T lymphocytes will be generated. It is hypothesized that subsequent re-exposure to MI during patch testing of sensitized subjects will elicit an inflammatory skin reaction, and that memory/activated T lymphocytes will be induced to traffic to the skin, thereby altering the balance of naive and memory populations within the peripheral blood. Therefore, the more severe the cutaneous reaction, the lower the proportion of central or effector memory T lymphocytes that will be found in the blood. T lymphocyte homing to the skin is mediated via the membrane determinant cutaneous lymphocyte-associated antigen (CLA) (22). This determinant is expressed by ~30% of circulating memory T lymphocytes (28), accounting for 15% of all peripheral blood lymphocytes, and, following skin homing, is expressed subsequently by >90% of T lymphocytes infiltrating inflamed skin. CLA⁺ T lymphocytes have been shown to express receptors (CCR4 and CCR10) for the chemokine ligands

CCL17 (thymus and activation-regulated chemokine), CCL22 (macrophage-derived chemokine), and CCL27 (cutaneous T cell-attracting chemokine) (29, 30). Interestingly, increased serum levels of these chemokines correlate with disease severity of patients with atopic dermatitis, suggesting an increase in T lymphocyte trafficking to the skin (30–33). Evaluation of CLA expression on circulating T lymphocytes in patients previously sensitized to MI, before and after patch test (recall) application, would help to elucidate the kinetics of peripheral T lymphocyte homing in response to allergens such as MI.

Allergen-specific proliferation was investigated with a novel combination of a modified version of the LTT and determination of Ki-67 expression by flow cytometry. To our knowledge, there are no reports in the literature on human T lymphocyte responses measured *in vitro* in response to MI alone. A single study has investigated T lymphocyte responses to MCI/MI (Kathon) by comparing MCI/MI-allergic (++ and +++ patch test-positive; 1–7 years after patch testing) and non-allergic individuals (15). That study also used the LTT to analyse PBMC proliferation, and additionally measured cytokine secretion by the proliferating population. A concentration range of 0.0078–8 µg/ml MCI/MI was utilized; however, decreases in SI values were recorded with concentrations of ≥2 µg/ml, most likely because of chemical-induced cellular toxicity. It was concluded that PBMCs from

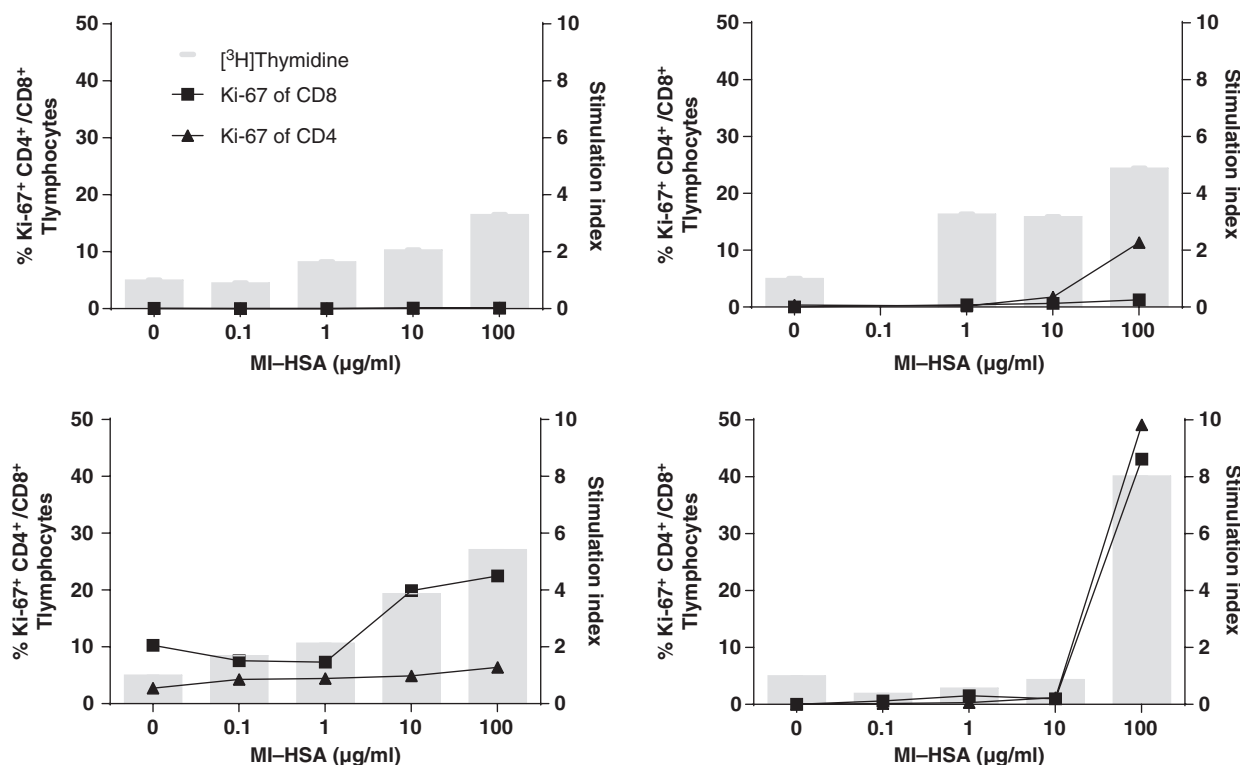


Fig. 5. Allergen-driven proliferation of T lymphocyte subsets in response to methylisothiazolinone (MI) hapten-protein conjugate. Peripheral blood mononuclear cells (PBMCs) from four representative MI-allergic donors (a-d) prepared as described in the legend to Fig. 3 were stimulated with medium alone (0) or with MI-HSA (0.1–100 µg/ml), and incubated at 37°C for 120 h. Separate aliquots of cells were pulsed with [³H]thymidine for 16 h and processed for β -scintillation counting, or were stained with anti-human CD8-allophycocyanin (APC), CD4-APC and Ki-67-phycoerythrin antibodies, and analysed by flow cytometry as described in the legend to Fig. 4. Data are shown for allergen-driven PBMC proliferation in response to MI-HSA measured according to [³H]thymidine incorporation (grey bars), and are represented as a stimulation index (SI) relative to medium alone (0). Proliferating T lymphocyte subsets identified by flow cytometry (Ki-67 positivity) are shown as the percentage of the total CD4⁺ T lymphocytes (▲) or CD8⁺ T lymphocytes (■) that are Ki-67⁺ in each population.

MCI/MI-allergic individuals proliferated in response to restimulation with free MCI/MI, with SI values ranging from 0 to 50 (average of 6), and that this response was characterized by both T-helper 1 and T helper 2 cytokine profiles. These investigations did not identify which cells produced these cytokines. No proliferation was identified in blood samples taken from non-allergic donors. In the present study of patients who were sensitized to MI, attempts to stimulate T lymphocyte responses *in vitro*, even with low concentrations of MI, led to cellular toxicity. The relatively low level of proliferation measured in the donors in the present study in response to free MI could be attributable to the narrow 'dose window', whereby higher concentrations of chemical allergen result in T lymphocyte toxicity. Another factor could be the low frequency of MI-specific T lymphocytes remaining within the peripheral population following trafficking to the skin. In the present study, blood samples were collected from patients 4 days after patch test application,

whereby MI-specific T lymphocytes are likely to have trafficked to the skin, potentially in larger numbers in higher patch test responders. Collection of blood samples from allergic donors before and after patch test application would enable baseline levels of MI specificity within allergic donors to be compared across patch test responder groups. Additional analyses of these blood samples for various pro-inflammatory mediators in serum would elucidate baseline levels of systemic inflammation as compared with healthy donors and allergic donors following re-exposure during patch testing.

A frequent technical challenge for the LTT and variants thereof is to deliver an amount of chemical allergen, in this case MI, to the culture matrix that will stimulate proliferation by responsive T lymphocytes without causing cellular toxicity. In an attempt to address this issue, hapten-protein conjugates have been synthesized. Jenkinson et al. (34) analysed PPD hapten-protein conjugates, and suggested that higher concentrations of

hapten conjugate than of chemical alone were required to stimulate proliferation, but that this proliferative response was more vigorous and without any associated toxicity to cells. Similarly, the MI–HSA conjugate described herein stimulated PBMCs to proliferate more vigorously with no discernible cellular toxicity, and in a higher fraction of MI-allergic donors, than did MI alone.

Ki-67 expression can be used for the measurement of proliferating cells at low frequencies in *in vitro* assay systems (35). Ki-67 is a nuclear protein that is expressed during all active phases of cell division, and has been shown to play a role in the regulation of mitosis. T lymphocytes expressing Ki-67 have been identified in *in situ* skin biopsies from patients with ACD (36). Here, we used Ki-67 in tandem with standard [³H]thymidine incorporation as a means of identifying allergen-specific CD4⁺ and CD8⁺ T lymphocyte subset proliferative responses in patients sensitized to MI. It was shown that allergen-driven proliferation of individual T lymphocyte subsets can be characterized by both [³H]thymidine incorporation and intracellular Ki-67 expression for donors showing vigorous proliferative responses (SI of >2.5) in response to MI–HSA. Ki-67 expression data showed the same dose–response characteristics as those observed with [³H]thymidine incorporation at an individual donor level, and, on that basis, it was possible to show that both CD4⁺ and CD8⁺ T lymphocytes contribute to

the MI-induced response. Importantly, among donors with the same patch test score, and showing similar SI values as those observed with [³H]thymidine incorporation, variable patterns of proliferating CD4⁺ and CD8⁺ T lymphocytes were observed. Indeed, this is in agreement with observations by Coulter et al. (13), wherein purified CD4⁺ and CD8⁺ T lymphocyte subsets from PPD-allergic individuals were stimulated to proliferate *in vitro* to varying degrees within individual patients.

In conclusion, these data show that chemical allergen–protein conjugates are able to induce secondary (recall) responses in lymphocytes drawn from sensitized subjects, and, in some circumstances at least, may provide a more effective source of antigen than free chemical. Furthermore, individual donors show differential activity profiles, with respect to T lymphocyte subsets, in response to the same MI–HSA conjugates. The magnitude of responses was unrelated to patch test score; however, there was a trend associated with CD4⁺ T lymphocyte proliferation in low (+) patch test-positive donors, whereas ++ and +++ donor responses were characterized by both CD4⁺ and CD8⁺ T lymphocyte activation comprising a larger percentage of proliferating CD8⁺ than of CD4⁺ T lymphocytes. Future studies will focus on further refinement of assay systems to delineate in greater detail the specific roles of CD4⁺ and CD8⁺ T lymphocytes in allergic sensitization to MI.

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