ORIGINAL ARTICLE

Investigation of Mechanisms Underlying the T-Cell Response to the Hapten 2,4-Dinitrochlorobenzene

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T-cell mediated contact sensitization by small molecular weight xenobiotics results in significant morbidity and absences from work. To be recognized by T-cells, xenobiotics must act as haptens, becoming protein-bound. At present, the requirement for processing and presentation of xenobiotics, the nature of the T-cell responses to them and the mechanisms that confer individual susceptibility in humans are unclear. We have investigated the T-cell response to the hapten 2,4-dinitrochlorobenzene (DNCB) which can sensitize all immunocompetent people. Fourteen healthy adults were sensitized with DNCB; 11 demonstrated positive T-cell responses to the chemical *in vitro*. Responding cells were of both $CD4^+$ and $CD8^+$ subsets, of Th1 and Tc1 phenotypes, producing high levels of IFN-γ and low levels of IL-10. DNCB-specific T-cell clones were raised from 2 subjects, which in the presence of fixed and unfixed autologous Epstein–Barr virus transformed B cells as antigen-presenting-cells (APC), demonstrated that the chemical requires metabolic processing by the APC in order to initiate the T-cell response. Intracellular-reduced glutathione is consumed in detoxication of DNCB, leaving residual non-detoxified DNCB free to bind to proteins. The results suggest that DNCB forms multiple haptens with intracellular and extracellular proteins leading to Th1 and Tc1 responses in individuals exposed to this compound.

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INTRODUCTION

Contact sensitization involves the reaction of specific T cells with causally relevant small molecular weight environmental xenobiotics. The clinical consequences of allergic contact dermatitis result in great morbidity with loss of time from work. Several fundamental aspects of the immune response to small xenobiotic molecules remain to be elucidated. Firstly, these include the factors that make some individuals susceptible to generating immune responses to xenobiotic compounds that most individuals can encounter with no apparent immunological consequences. Secondly, the mechanisms by which each small molecule become recognizable by T cells are not clear. Thirdly, the nature of the immune response itself in terms of T-cell effector and regulatory mechanisms require further investigation.

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Abbreviations: APC, antigen-presenting-cell; BSO, buthionine sulphoximine; CHS, contact hypersensitivity; CFSE, carboxyfluorescein diacetate succinimidyl ester; DNCB, 2, 4-dinitrochlorobenzene; EBV-B, EBV-transformed B cells; EBV, Epstein–Barr virus; GSH, glutathione; MHC, major histocompatibility complex; PBMC, peripheral blood mononuclear cells; SI, stimulation index; SK/SD, streptokinase/streptodornase; TCC, T-cell clone

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In order for a small molecule or hapten to be recognizable to T cells, it must become protein-bound. The sensitizing potency of small molecules is generally reflected by their chemical reactivity and ability to bind to nucleophiles/polar molecules such as the ε-amino group of lysine and the thiol group of cysteine (Lepoittevin *et al.*, 1995). Although it is supposed that the hapten becomes conjugated to serum or cellular proteins which can be processed and presented to T cells in association with major histocompatibility complex (MHC) molecules on the surface of antigen-presenting cells (APC), very little is known about which proteins are the carriers or the role of the APC in processing and presenting these haptenated proteins. There is evidence that some haptens can associate directly with the MHC–peptide complex itself such that processing by the APC is not required, for example, nickel binds directly to histidine residues and trinitrophenyl binds to lysine residues in MHC-bound peptides (Romagnoli *et al.*, 1991; Moulon *et al.*, 1995; Weltzien *et al.*, 1996). However, it would seem likely that most haptens have to undergo intracellular metabolism-dependent processing in order to become protein-bound and MHC-associated and hence presentable to T cells (Anderson *et al.*, 1995). Indeed, it may be that differences in individual susceptibility to sensitization by a xenobiotic reflect the metabolic detoxication of the chemical, which, in susceptible individuals, either fails to detoxify a reactive metabolite or actively generates a protein-reactive, and hence potentially immunogenic, hapten. 2,4-dinitrochlorobenzene (DNCB) is useful for the study of mechanisms in contact sensitivity

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because it is able to induce contact sensitization in all immunocompetent humans (Friedmann *et al.*, 1983; Friedmann, 1991, 2006). It has been postulated that this is because DNCB is already protein reactive and so does not require metabolic bioactivation. Studies in mice have shown that the immune response to DNCB involves both CD4$^+$ and CD8$^+$ T-cell subsets expressing a Th1/Tc1 cytokine profile reflected by high levels of IFN-γ and little IL-4 and IL-10 (Dearman *et al.*, 1996). However, the immune response to DNCB in humans has not yet been defined. In the present study, we have investigated the nature of the human immune response to DNCB. We have shown that, as in mice, DNCB is recognized by T cells of both CD4$^+$ and CD8$^+$ Th1/Tc1subtypes. In addition, the results demonstrate that the presentation of DNCB in humans is mainly mediated via metabolism-dependent processing. Furthermore, DNCB associates with a great many cellular proteins in both the cytoplasm and nucleus of human cells, an observation which would explain the ability of DNCB to induce contact hypersensitivity (CHS) in all immunocompetent individuals.

RESULTS

Clinical sensitization of volunteers

All the volunteers who received the initial sensitizing dose of DNCB showed positive responses to epicutaneous challenge with DNCB 4 weeks later. The median responses at each challenge dose were: + at 6.25 μg, ++ at 8.8 and 12.5 μg and +++ at 17.7 μg. Peripheral blood mononuclear cell (PBMC) were isolated from these volunteers at between 3 and 6 months after the elicitation challenge.

Both CD4$^+$ and CD8$^+$ T cells respond to DNCB

Culture of PBMCs with DNCB for 6 days resulted in significant proliferative responses in 11 of the 14 sensitized individuals tested. Peak responses occurred at DNCB concentrations between 1 and 5 μM (Figure 1a). The median (interquartile range) c.p.m. for unstimulated- and DNCB-stimulated cultures were 411 (297–731) and 15,818 (3,153–23,607), respectively; median stimulation index (SI) = 23.4, SEM = 6.5. No responses were observed when PBMCs from non-sensitized individuals were cultured with DNCB ($n=7$; Figure 1a).

When CD4$^+$ and CD8$^+$ T cells were enriched by depletion of the reciprocal T-lymphocyte subset, both these subsets of T cells proliferated in response to DNCB (Figure 1a). The responses of the enriched subsets to streptokinase/streptodornase (SK/SD) were examined as a control for a non-contact sensitizing protein antigen. In the six individuals tested, proliferative responses to SK/SD were found exclusively in the CD4$^+$ enriched subset ($P=0.036$; Figure 1a).

These results were confirmed by labeling unfractionated PBMCs with carboxyfluorescein diacetate succinimidyl ester (CFSE). Results from four DNCB-sensitized individuals confirmed that both CD4$^+$ and CD8$^+$ T-cell subsets had proliferated in response to DNCB after 6 days (Figure 1b). This assay also confirmed that the cells responding to SK/SD were almost exclusively CD4$^+$ T cells (Figure 1b).

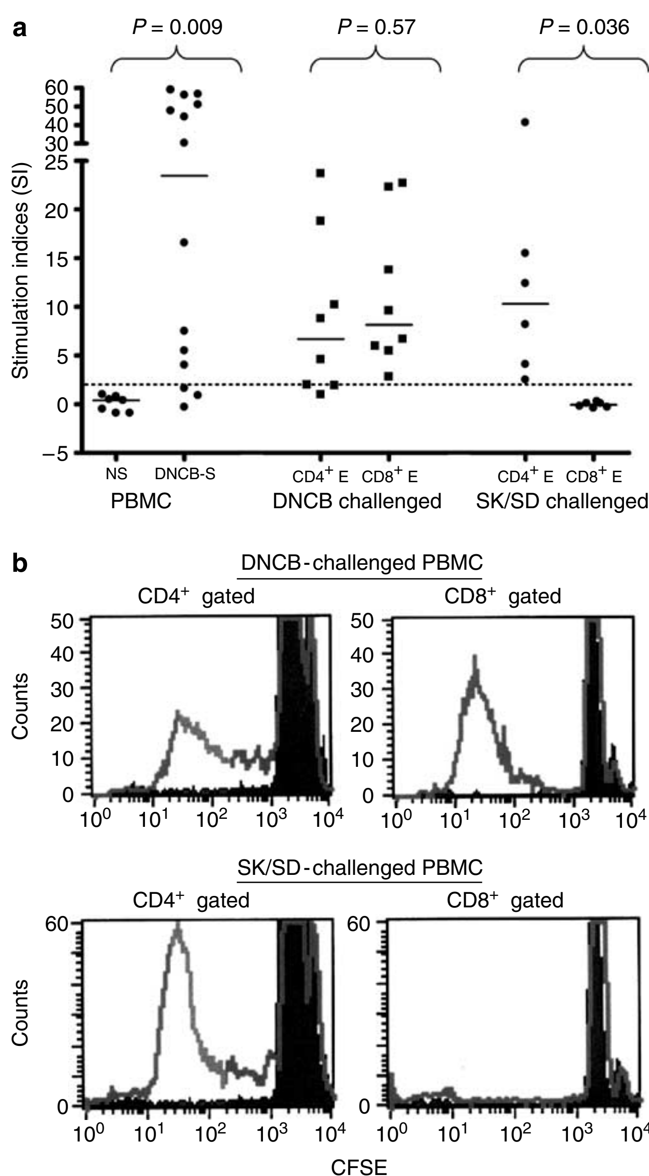

Figure 1. Both CD4$^+$ and CD8$^+$ T cell subsets from pre-sensitized individuals respond to DNCB *in vitro*. (**a**) PBMC from non-sensitized normal controls (NS; $n=7$) and from DNCB-sensitized individuals (DNCB-S; $n=14$), were challenged with DNCB for 6 days in culture. CD4$^+$ and CD8$^+$ subsets were depleted from the PBMC from eight sensitized individuals. The reciprocal enriched subsets (E) were challenged with DNCB and SK/SD. Peak responses are shown for each individual and the horizontal bar represents the mean SI (**b**) Proliferation of both CD4$^+$ and CD8$^+$ subsets was confirmed using the incorporation of CFSE. The filled histogram represents unchallenged CFSE-stained PBMC from a representative pre-sensitized individual. The open histogram represents DNCB challenged PBMC.

The responding T cells exhibit a Th1/Tc1 cytokine profile

Supernatants from CD4$^+$ and CD8$^+$ enriched T cells cultured for 6 days in the presence and absence of DNCB were assayed for production of various cytokines ($N=7$). Both CD4$^+$ and CD8$^+$ enriched T cells produced significant quantities of IFN-γ in response to DNCB ($P=0.03$ and 0.003, respectively; Table 1). Interestingly, production of IL-10 appeared to be actively suppressed in DNCB-containing

Table 1. Cytokine production by DNCB challenged CD4$^+$- and CD8$^+$-enriched subsets

	CD4$^+$ enriched		CD8$^+$ enriched	
	Control	DNCB	Control	DNCB
IFN-γ	665.6 (60.7–1933.0)	3,499.0 (167.4–5000)*	444.0 (60.05–917.8)	4,181.0 (2,589.6–5,000)**
TNF-α	44.0 (24.05–217)	235.0 (87.3–798.8)	101.5 (71.2–167.3)	204.5 (69.1–538.5)
IL-2	3.1 (2.3–7.0)	10.6 (8.4–20.7)	1.2 (0–1.9)	6.3 (2.5–8.8)*
IL-5	1.3 (1.1–1.8)	9.5 (4.1–27.2)	1.8 (0–2.0)	7.3 (4.4–20.7)
IL-10	267.0 (91.9–517.8)	27.0 (21.2–60.2)*	427.4 (208.8–599.3)	32.0 (8.4–54.3)**

DNCB, 2,4-dinitrochlorobenzene.
All values=median pg/ml (interquartile range); *n*=7.
$^*P<0.05$, $^{**}P<0.01$.

cultures compared with DNCB-lacking controls ($P=0.02$ and $P=0.006$, respectively for CD4$^+$ and CD8$^+$ subsets). There was very little IL-5 and no detectable IL-4 release by the DNCB-treated CD4$^+$ and CD8$^+$ subsets, with a nonsignificant increase above baseline in the absence of DNCB ($P=0.3$ and 0.05, respectively).

DNCB requires metabolic processing by viable APC

In order to determine the degree of metabolism required, antigen-specific T-cell clones (TCCs) ($n=14$) from two DNCB pre-sensitized individuals were challenged with DNCB-pulsed autologous, irradiated EBV-transformed B cells (EBV-B) cells. The EBV-B cells were either fixed with glutaraldehyde before antigen challenge, or left unfixed. Fixation did not abrogate T-cell responses to EBV-B presented toxic shock syndrome toxin-1 (Sigma UK; data not shown), indicating that the fixation process did not disrupt the T-cell/MHC synapse. After pulsing with DNCB for 12 hours, the EBV-B cells were washed to remove unbound DNCB. The response to DNCB was abrogated by prior fixation of the EBV-B cells for each of the clones raised from one individual, and for six of seven clones from the other individual. However, one of the clones (TCC#1.5) from this latter individual was able to respond to fixed DNCB pulsed EBV-B cells with responses comparable to those observed with non-fixed antigen-pulsed APC (Figure 2). Fixation of EBV-B cells after pulsing with DNCB resulted in T-cell responses similar to those observed in the presence of unfixed EBV-B cells, providing further support that fixation does not interfere with MHC-T-cell interaction (Figure S1).

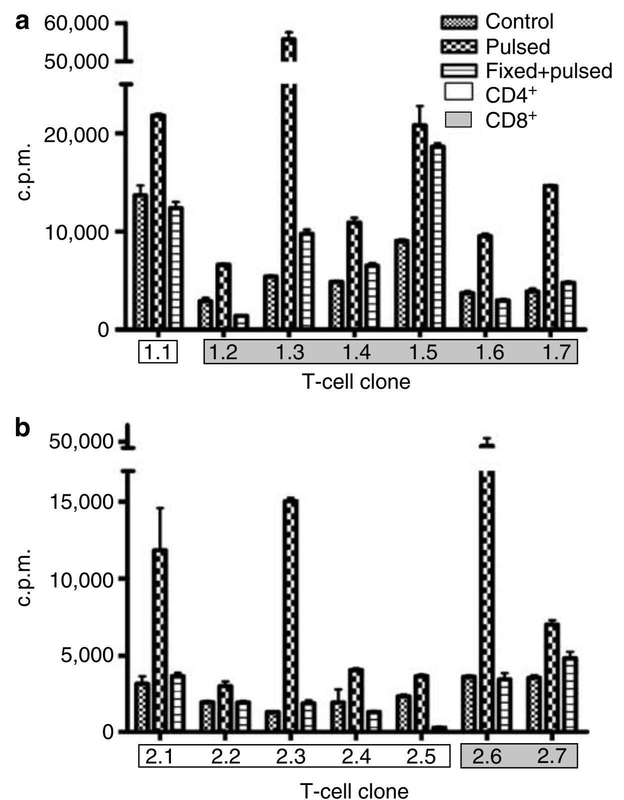

Figure 2. DNCB haptenation of cellular protein requires metabolic processing by viable APCs. DNCB-specific TCCs from two individuals (**a** and **b**) were challenged with both fixed and unfixed irradiated autologous EBV-B cells that had been pulsed for 12 hours with DNCB. Histograms represent the mean c.p.m. of triplicates, with error bars indicating the SEM.

DNCB binds to many intracellular/cellular proteins

PBMC from three individuals were incubated with DNCB for increasing lengths of time (from 5 to 60 minutes) and stained with an anti-dinitrophenyl fluorescein isothiocyanate-conjugated antibody, which recognizes the dinitrophenyl group of DNCB. Cellular localization of DNCB was assessed by confocal microscopy. Results from this analysis showed that after as little as 5 minutes DNCB bound protein extensively on the cell membrane and throughout the cytoplasm and nucleus (Figure 3); the staining intensity at each of these sites increased to a maximum at 1 hour. No staining was observed in the untreated cells. In order to determine the number of proteins associated with the chemical, PBMC isolated from three individuals were incubated with ^{14}C-labeled DNCB and the cellular proteins were extracted and electrophoresed through an SDS-PAGE-gel. This revealed multiple radio-labeled bands suggesting that the DNCB was covalently associated with a large number of proteins (Figure 4a). The pattern of DNCB-labeled proteins generally mirrored the pattern of staining with Coomassie blue in terms of band intensity (Figure 4b). However, a predominant band, running at approximately 75–80 kDa, was seen whereas a band of

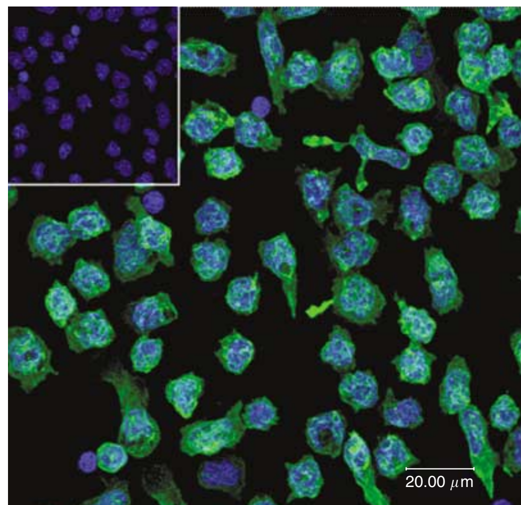

Figure 3. DNCB binds extensively to intracellular protein. Adherent monocytes were incubated with DNCB. Cells were fixed, permeabilized and stained with a goat polyclonal anti-dinitrophenyl antibody and visualized at ×100 original magnification. Green staining shows DNCB bound protein. Blue shows nuclear staining. Inset shows non-DNCB-treated monocytes. Bar = 20 μm.

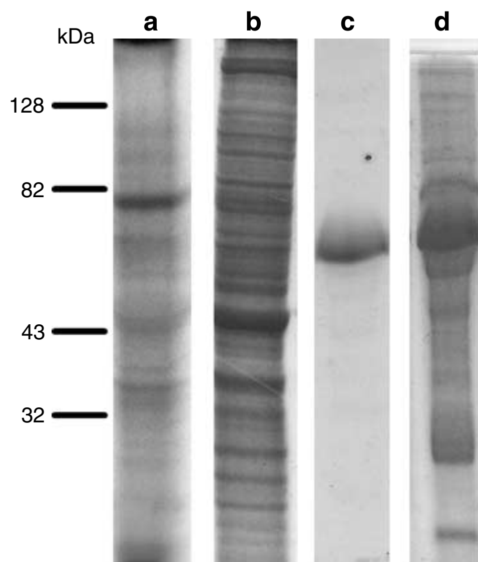

Figure 4. DNCB binds to large number of intracellular proteins and to the albumin component of human serum. (**a**) Total protein was extracted from PBMC incubated with ^{14}C-labeled DNCB for 12 hours, and run on a SDS-PAGE gel. The resulting gel was exposed to X-ray film for seven days. (**b**) Coomassie stain of total protein extracted from PBMC incubated with DNCB. (**c**) ^{14}C-DNCB-labeled human serum. (**d**) Coomassie stain of human serum.

similar intensity was not observed on a Coomassie-stained gel, suggesting that this does not represent an abundant intracellular protein; preliminary attempts to isolate a single protein from this band have been unsuccessful.

DNCB depletes intracellular glutathione

DNCB is detoxified by conjugation to intracellular glutathione (GSH). We hypothesized that depletion of cytoplasmic

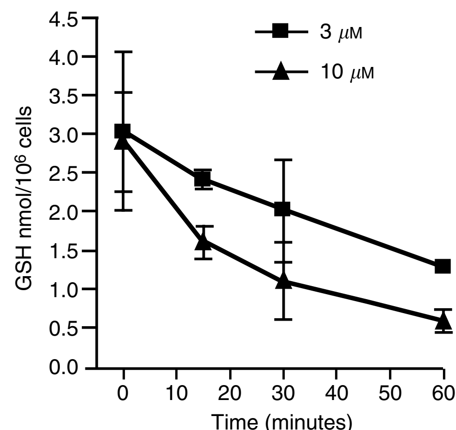

Figure 5. DNCB depletes intracellular GSH. The reduced GSH content of PMBC, pretreated with DNCB, was measured by HPLC. Data points show the mean GSH intracellular content from three individuals, with error bars indicating the SEM.

GSH would allow DNCB to bind to a greater extent to intracellular proteins. To test this, we measured the intracellular levels of GSH in PBMC incubated with 3 and 10 μm DNCB and found that there was a dose- and time-dependent depletion of GSH (Figure 5). Fifteen minutes after addition of DNCB 3 and 10 μm, the intracellular GSH levels had fallen to 79.5 and 55.2% of the baseline, respectively. After 60 minutes GSH levels had fallen to 42.4 and 20.0%, respectively, the latter reaching statistical significance ($P = 0.02$). This reduction in GSH over time correlated inversely with the increase in intracellular DNCB protein binding seen with confocal microscopy above. This reflects the use of GSH as a co-substrate in the detoxication of DNCB and raised the possibility that susceptibility to sensitization by DNCB is determined by intracellular GSH reserves. Hence, depletion of GSH with buthionine sulphoximine (BSO) (Griffith, 1982) should increase the relative potency of a given concentration of DNCB. This was examined by addition of BSO before addition of DNCB. There was indeed a greater lymphocyte proliferation response to each concentration of DNCB (Figure 6): The mean SI elicited by DNCB was 8.3 ± 2.6 (SE), $N = 7$; whereas after depletion of GSH it increased by 54% to a mean of 12.7 ± 3.4; ($P \leqslant 0.05$ paired *t*-test). This was specific for DNCB as there was no effect of BSO on the responses of lymphocytes to a streptococcal-derived antigen mixture (SK/SD).

DISCUSSION

CHS to small xenobiotic molecules is a common clinical problem affecting large numbers of people, sometimes requiring major changes in lifestyle. The nature of the immune response and the reasons why in general, only certain individuals develop such sensitivity, remain to be elucidated. The sensitizing capacity of small molecular weight chemicals is attributed to their ability to act as haptens that, either as the parent compound or following metabolic bioactivation, covalently modify protein nucleophiles (Smith Pease, 2003). The modified protein may then be

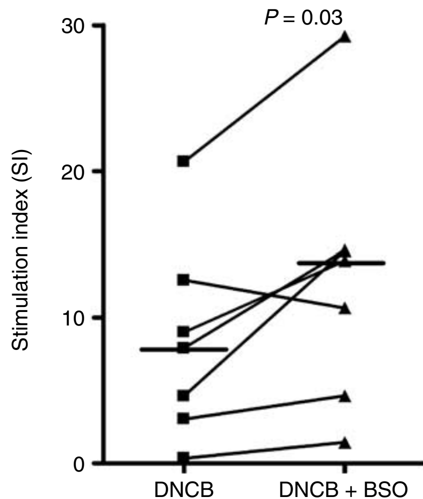

Figure 6. Depletion of intracellular GSH following treatment with BSO increases the proliferative response to DNCB. PBMC from seven DNCB-sensitized individuals were pretreated with BSO for 1 hour, and then cultured for 6 days with DNCB. Peak responses are shown for each individual and the horizontal bar represents the mean.

processed and presented to $CD4^+$ and/or $CD8^+$ T cells in the context of MHC class II and class I molecules, respectively. Upon subsequent exposure to the chemical, the recruitment and activation of memory T cells able to recognize the hapten-modified protein results in the local tissue inflammation characteristic of allergic contact dermatitis.

There has been much controversy regarding the respective roles of the $CD4^+$ and $CD8^+$ T cells in the development of CHS to xenobiotics. In early work in mice it was shown that both $CD4^+$ and $CD8^+$ T-cell subsets appeared to play a role in the inflammatory response to DNCB, with both secreting high concentrations of IFN-γ and only low or undetectable levels of IL-4 and IL-10 (Dearman *et al*., 1996). However, more recent observations in murine systems investigating a range of allergens suggest that the main effector cells in CHS may be $CD8^+$ cells with a Tc1 cytokine profile (high IFN-γ) (Martin *et al*., 2000, 2003) whilst the $CD4^+$ T-cell subset may have a regulatory function typified by a Th2 cytokine profile (high IL-10 and IL-4 production) (Bour *et al*., 1995; Xu *et al*., 1996; Desvignes *et al*., 2000). In humans allergic to nickel, nickel-specific effector $CD4^+$ and $CD8^+$ T cells have been identified (Cavani *et al*., 1998) but in nickel non-allergic individuals an additional subset of $CD4^+$ T cells producing high amounts of IL-10 appear to inhibit the nickel-specific response. More recently, punch biopsies taken from DNCB-treated human skin revealed the presence of $CD4^+$ regulatory cells that produced high amounts of IL-10 and IL-5 (Lecart *et al*., 2001). In our study, both $CD4^+$ and $CD8^+$ subsets demonstrated a Th1/Tc1 phenotype, this finding being more consistent with the earlier murine work carried out by Dearman *et al*. (1996) In addition, the $CD4^+$ response to SK/SD in the absence of a $CD8^+$ reaction confirms that the combined human $CD4^+$ and $CD8^+$ response to DNCB was particular to this antigen and not a nonspecific effect of antigen-induced stimulation in our assay system. Moreover, our results demonstrate that DNCB inhibits the production of IL-10 by $CD4^+$ and $CD8^+$ subsets, which, through removing immunoregulatory restraints by this cytokine, may account for the ability of DNCB to sensitize all immunocompetent subjects.

The question arises as to why and how both $CD4^+$ and $CD8^+$ T-cell subsets are activated. Our results with confocal microscopy and with ^{14}C-DNCB show that DNCB binds avidly to a wide range of intracellular proteins, and that DNCB is also capable of binding to certain extracellular proteins. The binding of DNCB to intracellular proteins which are then processed via the MHC class I pathway would activate $CD8^+$ T cells. To activate $CD4^+$ T cells, DNCB bound to intracellular proteins from other cells (or extra-cellular proteins such as serum proteins) may be cross-presented by the APC via the MHC class II pathway. Our results which showed that the stimulation of DNCB-specific TCCs by DNCB-modified proteins was almost entirely processing dependent (i.e., the APC required to be viable in order to process and present the modified protein in the context of MHC) are consistent with this. Alternatively, DNCB may associate directly with peptides within the MHC grooves of both MHC class I and II molecules, forming either labile coordinate bonds as is the case with nickel, or by covalently modifying peptides as is the case with trinitro-phenyl. The latter has been shown to bind directly to lysine ε-amino groups of MHC embedded peptides in an intra-cellular processing-independent manner (Weltzien *et al*., 1996). Although only one of our $CD4^+$ clones was able to respond in a processing-independent manner, this suggests that, as is the case with trinitrophenyl, DNCB may also bind peptides that already reside within the MHC cleft. The observation that dinitrophenyl modified proteins were also localized on the cell surface on confocal microscopy (in addition to extensive intracellular staining) would support this conclusion. Furthermore, DNCB would appear to have formed a strong association with surface proteins, as vigorous washing of the DNCB-pulsed APC failed to abrogate the subsequent lymphocyte response.

Classically, it has been thought that at physiological pH DNCB binds predominantly to lysine residues of proteins (Lepoittevin *et al*., 1995), although there is evidence to suggest that it also binds efficiently to sulphydryl groups on reduced GSH (Kato *et al*., 2003) and BSA (Parker *et al*., 1983). GSH, the most prevalent non-protein thiol in animal cells, plays a vital role in the detoxication of electrophilic compounds, with the cysteine providing a nucleophilic sulphydryl group to which the reactive chemical can bind via the action of glutathione *S*-transferase (Townsend *et al*., 2003). We have shown that DNCB depletes intracellular levels of GSH and that this correlates inversely with increased binding by DNCB to intracellular proteins (presumably because the depletion of GSH leaves the excess DNCB free to bind to nearby proteins). The corollary of this is that when GSH was depleted by pretreatment with BSO, the given dose of DNCB elicited stronger reactions presumably because less was detoxified by conjugation to GSH. Furthermore, it has

been shown that thiol containing anti-oxidants reduce CHS responses (Becker *et al.*, 2003; Bruchhausen *et al.*, 2003; Mizuashi *et al.*, 2005). This also fits with the idea that DNCB is detoxified by binding to GSH and other thiols so less is available to evoke CHS responses.

The actual protein targets of electrophilic chemicals such as DNCB are largely unknown. The observation that DNCB binds extensively throughout the cytoplasm, nucleus and on the cell membrane after as little as 5 minutes suggests that DNCB is binding to a small number of ubiquitously expressed proteins or that it is binding promiscuously to many proteins. Using ^{14}C-DNCB we were able to show that this chemical binds to a large number of proteins as seen on the SDS-PAGE gel. In contrast, the addition of ^{14}C-DNCB to human serum resulted in a dominant band running at approximately 66 kDa corresponding to human albumin. This suggests that the intracellular environment seems to favor protein binding by DNCB, perhaps through active metabolic processes, and that an unspecified number of these haptenated intracellular proteins may form the antigenic moieties recognized by CD8$^+$ T cells. In addition, the results suggest that one of the CD4$^+$ antigenic targets is serum albumin and further support for the existence of DNCB-serum protein antigen(s) comes from our observations that human serum-DNCB conjugates can stimulate a CD4$^+$ DNCB-specific T-cell response (data not shown). The role of serum albumin in mediating an immune response to DNCB is also supported by a previous finding by Johannesson *et al.*, (2001) who showed that occupational workers and guinea pigs exposed to the sensitizing chemical hexahydrophthalic anhydride developed IgE and IgG antibodies against haptenated serum albumin. Despite extensive binding to intracellular proteins, we did observe a dominant ^{14}C-DNCB-labeled band on the gel at approximately 75–80 kDa; at present the identity of this protein (or proteins) is unknown and further research will be required to determine if it represents one of the immunodominant moieties.

In conclusion, we have characterized the nature of the human T-cell response to DNCB and have shown that both CD4$^+$ and CD8$^+$ cells (Th1 and Tc1) are involved. We showed that metabolism-dependent processing of DNCB is frequently required for presentation of DNCB to T cells, but that processing independent presentation of DNCB can also occur (1 TCC of 14). During metabolic processing of DNCB by APCs, the cellular reserves of GSH are depleted, allowing greater amounts of DNCB to interact with cellular proteins. Furthermore, the suppression of IL-10 by DNCB raises the possibility that DNCB is a potent sensitizer in most individuals because it prevents IL-10-induced inhibition of the immune response against this xenobiotic.

MATERIALS AND METHODS

Subjects and controls

Volunteers were recruited from the dermatology outpatient population and staff in Southampton University Hospitals Trust. All individuals gave signed informed consent and the study was approved by the local research ethics committee in adherence with the Declaration of Helsinki Principles. For investigations on DNCB reactive subjects, volunteers were sensitized to this agent as per our previous studies (Friedmann *et al.*, 1990). Fourteen DNCB allergic subjects were included in the study, of whom 8 were male and 6 were female; ages ranged from 23 to 58 (median 34) years. Non-sensitized controls ($n=12$) included eight males and four females, with ages ranging from 24 to 42 (median 31) years.

Sensitization of volunteers with DNCB

Volunteers received an initial application of 38 μg of DNCB (Sigma, UK) dissolved in acetone (Sigma, UK) on a 1.1 cm diameter paper disc (DNCB concentration 35.4 μg/cm^2) held in place for 48 hours on the upper arm with an aluminum patch test chamber (Finn Chamber, Epitest Oy, Finland). Four weeks later, the degree of sensitization was assessed by application of four epicutaneous patch tests of 6.25, 8.8, 12.5, and 17.7 μg of DNCB on 8 mm Finn chambers. The challenges were removed after 6 hours, according to our previously described method, and read at 48 hours (Friedmann *et al.*, 1990). Reactions were scored according to the International Contact Dermatitis Research Group system (Calnan *et al.*, 1976), where ++ is a definite erythema with palpable infiltration. A positive response (++ or greater) at or below the 12.5 μg challenge site was taken as definite evidence of sensitization.

Lymphocyte culture media

The lymphocyte culture medium used in this study was Rosewell Park Memorial Institute media 1640 with L-glutamine (Gibco-BRL Life Technologies, Paisley, UK) supplemented with 5% human AB serum (Sigma, UK), 1 mM sodium pyruvate and 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco-BRL Life Technologies). For culture of the EBV-B the human serum was replaced by 10% fetal calf serum (Gibco-BRL Life Technologies).

Lymphocyte proliferation assays

PBMCs were isolated from venous blood by centrifugation through a density cushion (Lymphoprep, Nycomed). After washing, cells were cultured at 1.2×10^6 cells/ml in 24-well plates with 0–10 μM DNCB (dissolved in DMSO (Sigma, UK)) at 37°C with 5% CO_2 for 6 days. Lymphocyte proliferation was determined by addition of 1 μCi/ml of ^{3}H-thymidine (Amersham, UK) for the final 6 hours. The cells were harvested and incorporated ^{3}H-thymidine measured with a scintillation counter. The SI was calculated by subtracting the counts per minute (c.p.m.) for untreated cells from those of antigen-treated cells and dividing by the c.p.m. for untreated cells. An SI greater than two was taken as significant.

Depletion of CD4$^+$ or CD8$^+$ cells

CD4$^+$ and CD8$^+$ subsets were removed from whole PBMCs using magnetic microbeads (Miltenyi Biotech, Miltenyi Biotech Ltd, Surrey, UK) to give populations enriched for CD8$^+$ and CD4$^+$ cells, respectively. In all cases, the residual contamination by the reciprocal subset was less than 2%. The enriched subsets were adjusted to 1.2×10^6 cells/ml and cultured for 6 days with 3 μm DNCB. Proliferative responses were determined by measuring incorporation of ^{3}H-thymidine as above. Culture supernatants were collected and quantification of cytokines (IL-2, IL-4, IL-5, IL-10, TNF-α, and IFN-γ) was carried out using a flow-cytometric bead array system (BD Biosciences, Oxford, UK).

CFSE dilution assay

PBMC were adjusted to 1×10^6 cells per ml in Rosewell Park Memorial Institute media and labeled with 5 μM carboxyfluorescein diacetate succinimidyl ester (Molecular Probes, Eugene, OR) for 10 minutes at 37°C. The cells were then washed twice in phosphate-buffered saline, resuspended in culture medium and stimulated with 3 μM DNCB for 6 days. As controls, parallel cultures were stimulated with SK/SD (10/2.5 units/ml, Cyanamid Pharmaceuticals, UK) for 6 days or 1 μg/ml of phytohaemagglutinin (Sigma, UK) for 3 days. The proliferative response of the $CD4^+$ and $CD8^+$ subsets was determined by flow cytometry (described below).

Generation of DNCB-specific TCCs and EBV-B cell-lines

Freshly isolated PBMCs from two DNCB allergic donors were cultured at 1.5×10^6 cells per well in the presence of DNCB (3 μM). After 7 days, the cells were cultured with IL-2 before being stimulated again with DNCB and the addition of irradiated (3,000 Rad) autologous PBMC.

DNCB-specific clones were subsequently generated by limiting dilution assay and expanded with phytohaemagglutinin (Sigma, UK) and IL-2.

Autologous B cell-lines were immortalized with EBV by incubation in supernatant from an EBV-producing marmoset cell line B95-8 (kindly donated by Dr Di-Genova, Cancer Sciences, University of Southampton, UK) as described previously (Moulon *et al.*, 1995).

Effects of fixation of APCs on antigen-induced proliferation of TCCs

To examine the requirement for metabolic processing of DNCB by APCs, the EBV-B were fixed either before or after exposure to DNCB with glutaraldehyde (final concentration 0.05%) for 30 seconds at room temperature. The reaction was stopped by addition of L-lysine (final concentration 0.2 M) and the cells washed three times with phosphate-buffered saline. Fixed and non-fixed EBV-B cells were incubated with 2 μM DNCB for 12 hours, then washed $\times 2$ in large volumes of phosphate-buffered saline to remove excess DNCB. Also, after pulsing with DNCB, EBV-B cells were fixed with glutaraldehyde as above. For the measurement of the antigen-specific proliferation, 10^5 TCC were incubated with 5×10^4 irradiated (6,000 rad) fixed or unfixed autologous EBV-B cells in a U-bottomed 96-well plate for 72 hours. Proliferation was determined by measuring incorporation of ^{3}H-thymidine as above.

Flow cytometry

Cells were stained with allophycocyanin-labeled anti-CD4 (Caltag-Medsystems Ltd, Buckingham, UK) and phycoerythrin-labeled anti-CD8 (Sigma, UK) mABs; cells were analyzed on a FACSCalibur flow cytometer (Becton Dickinson, Plymouth, Devon, UK) and analysis was performed using the Cellquest™ software.

Confocal microscopy

PBMCs (10^6 cells/ml) were plated onto glass coverslips and treated with DNCB (4 μM) at various time points from 5 minutes to 24 hours. The non-adherent fraction was removed and the adherent fraction fixed with 4% paraformaldehyde before being permeabilized with ice cold 100% methanol. The cells were incubated with a polyclonal goat anti-dinitrophenyl antibody (Sigma, UK) (1:2,000) for 1 hour followed by the addition of a fluorescein isothiocyanate-labeled anti-goat secondary antibody (Sigma, UK) (1:500). The cells were counter stained with the anti-nuclear probe Topro-3 (Molecular Probes), and intracellular staining was detected using two-channel microscopy at $\times 100$ original magnification on a Zeiss confocal laser microscope.

Analysis of intracellular protein binding of DNCB

PBMC were incubated with 3 μM of either ^{14}C-labeled (Tocris Cookson Ltd., 56mCi/mmol) or unlabeled DNCB overnight in culture medium. Cells were recovered, washed twice in phosphate-buffered saline and lysed in 1% triton $\times 100$ (Sigma, UK) containing protease inhibitor cocktail (Complete™ Mini, Roche, UK). Cellular protein (100 μg ^{14}C DNCB treated, 30 μg of DNCB treated) and human serum-DNCB conjugate (15 μg) were run on a 10% SDS-PAGE gel under reducing conditions, against a pre-stained protein molecular weight standard (Kaleidoscope Standard, Bio-Rad, UK). Radio-labeled gels were fixed in 1% glycerol, 10% methanol and 10% glacial acetic acid for half-an-hour, dried, and exposed to autoradiograph film (X-OMAT AR film, Kodak, UK) at −80°C for 1–2 weeks. Total protein stains were carried out using Coomassie Brilliant Blue (Bio Rad, UK).

Effects of DNCB on intracellular levels of GSH

The reduced GSH content of PMBC, pretreated with DNCB, was measured by HPLC with fluorescence detection using penicillamine as an internal standard (Anderson, 1985). The GSH: penicillamine peak area ratio was calculated and samples quantitated by comparison to the area ratios of known GSH standards. Intracellular GSH was depleted by addition of buthionine sulphoximine (final concentration 200 μM) for 1 hour (Rouzer *et al.*, 1982).

Statistical analysis

Statistical significance between groups was evaluated using an unpaired, two-tailed Student's *t*-test. Mean differences were considered significant when $P \leqslant 0.05$.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Figure S1: Fixation of EBV-B cells with glutaraldehyde does not disrupt the TCR-MHC synapse.

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