

HLA-DP-unrestricted TNF- α release in beryllium-stimulated peripheral blood mononuclear cells

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ABSTRACT: Berylliosis is a granulomatous disorder of the lung caused by inhalation of beryllium (Be) and dominated by the accumulation of CD4⁺ T-helper (Th)1 memory T-cells proliferating in response to Be in the lower respiratory tract. Two gene markers have been associated with susceptibility to berylliosis: 1) the human leucocyte antigen (HLA)-DP gene whose allelic variants, carrying glutamate in position 69 of the β -chain (HLA-DP_{Glu69}), can bind Be directly and present it to interferon (IFN)- γ releasing Th1 T-cell clones from patients with berylliosis; and 2) the cytokine gene tumour necrosis factor (TNF)- α which has been shown to increase berylliosis risk independent of HLA-DP_{Glu69}.

In order to determine whether TNF- α release was triggered by Th1 T-cell activation by Be stimulation in the context of HLA-DP_{Glu69} molecules, the proliferation of BeSO₄-stimulated blood mononuclear cells and the release of IFN- γ , TNF- α , RANTES (regulated on activation normal T-cell expressed and secreted), granulocyte-macrophage colony-stimulating factor, interleukin (IL)-4, IL-6, IL-8, IL-10 and IL-12 by BeSO₄-stimulated blood mononuclear cells was quantified in 11 individuals with berylliosis using an anti-HLA-DP antibody as a probe for HLA-DP restricted T-cell activation.

While proliferation and IFN- γ release were completely abrogated by HLA-DP inhibition (inhibition with anti-HLA-DP monoclonal antibody (mAb): 88 \pm 16 and 77 \pm 16%, respectively; anti-HLA-DR: 29 \pm 38 and 14 \pm 10%, respectively), the release of TNF- α was not (inhibition with anti-HLA-DP mAb: 8.9 \pm 7.8%). No other cytokine was detected at significant levels. Moreover, Be was able to induce TNF- α production in healthy control subjects not exposed to Be in the absence of T-cell proliferation and IFN- γ production.

In conclusion, these data suggest that the tumour necrosis factor- α response of mononuclear cells is independent of the activation of beryllium-specific human leucocyte antigen-DP restricted T-cells, which is consistent with the finding that the tumour necrosis factorA2 and the human leucocyte antigen-DP_{Glu69} genetic markers are independently interacting in increasing berylliosis risk.

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Berylliosis is a chronic granulomatous disorder of the lung caused by inhalation of beryllium (Be) dusts affecting 1–16% of Be-exposed individuals [1, 2]. The immunopathology of this disorder is dominated by the accumulation of CD4⁺ CD45RO⁺ T-helper (Th)-1 memory T-cells proliferating in response to Be in the lower respiratory tract [3–5]. Previously, the current authors have shown that allelic variants of the human leucocyte antigen (HLA)-DP molecule carrying a glutamate residue in position 69 of the HLA-DP β -chain (HLA-DP_{Glu69}) [6–8] play a central role in disease pathogenesis by directly binding Be in the absence of antigen processing [9] and by restricting the response to Be of Th1 T-cell clones derived from patients with berylliosis [10]. In addition, the current authors have shown that the tumour necrosis factor (TNF)A2 allele of the cytokine gene TNF- α , which is expressed at exaggerated levels by lung and blood mononuclear cells from berylliosis

patients in response to Be [11, 12], is also associated with susceptibility to berylliosis and positively interacts with the HLA-DP_{Glu69} marker to increase berylliosis risk [13].

Since the Th1 cytokines interferon (IFN)- γ and TNF- α , both hyperexpressed by berylliosis patient mononuclear cells in response to Be [12], play a central role in granulomatous reactions [14], one may question whether the expression of TNF- α is the consequence of Be-specific CD4 T-cell activation or is a related but independent event, as the genetic association finding may suggest.

To answer this question, blood mononuclear cells were obtained from individuals with berylliosis and both T-cells and mononuclear phagocytes were stimulated with Be using an anti-HLA-DP monoclonal antibody (mAb) to separate the response to the metal of Be-specific HLA-DP_{Glu69}-restricted Th1 CD4⁺ T-cells from that of the mononuclear phagocytes.

Interestingly, consistent with the finding that the TNFA2 and HLA-DP_{Glu69} genetic markers are independently interacting in increasing berylliosis risk, the data indicate that the TNF- α response of the mononuclear phagocytes is independent of the activation of Be-specific HLA-DP restricted T-cells.

Methods

Study population

Eleven individuals with berylliosis were enrolled in the study after giving informed consent: 10 males and one female, all Caucasians, mean age 45 ± 7 yrs and average time of employment 15 ± 6 yrs. The study protocol was approved by the Cleveland Clinic IRB (Cleveland, OH, USA). As a control, five healthy non-Be-exposed individuals were enrolled: four males and one female, all Caucasians and mean age 32 ± 3 yrs.

Lymphocyte proliferation

Peripheral blood mononuclear cells (PBMCs) obtained from patients with berylliosis were isolated from heparinised whole blood by density centrifugation on a Ficoll Hypaque gradient. PBMCs (2×10^5 cells \cdot well $^{-1}$) were then cultured in 96-well flat-bottomed microtitre plates in Roswell Park Memorial Institute (RPMI) 1640 tissue culture medium supplemented with 2 mM L-glutamine, 10% foetal bovine serum, 100 U \cdot mL $^{-1}$ penicillin, and 100 μ g \cdot mL $^{-1}$ streptomycin in the presence of beryllium sulfate (BeSO₄[4H₂O]) at 10, 50 and 100 μ M (all reagents form Sigma Co., St. Louis, MO, USA). Phytohaemoagglutinin (PHA; 5 μ g \cdot mL $^{-1}$; Sigma) and *Candida albicans* (10 μ g \cdot mL $^{-1}$) were used as positive controls. T-lymphocyte proliferation was measured by thymidylated deoxyribonucleotide thymidine (3 H]TdR) incorporation. After 5 days of culture, cells were pulsed with 1 μ Ci of 3 H]TdR (Amersham International, Amersham, UK) and harvested onto glass-fibre filters 18 h later. Proliferation was measured as 3 H]-TdR incorporation by liquid scintillation spectroscopy. The results are expressed as the mean of triplicate cultures. The stimulation index was calculated as the ratio of mean counts per minute (cpm), in antigen-stimulated wells, to the mean cpm in control wells.

In order to evaluate possible contamination with the bacterial endotoxin product of the BeSO₄ solution used as antigen, the BeSO₄ batch solution used throughout the study was tested with the Limulus Amebocyte Lysate test (CAPE CODE Inc., Woods Hole, MA, USA). The stock solution of BeSO₄ (0.2 M) showed undetectable levels of endotoxin contamination (<0.03 U \cdot mL $^{-1}$ of *Escherichia coli* endotoxin, similar to endotoxin-free water). Similarly, undetectable endotoxin levels were revealed for BeSO₄ at the working concentration in RPMI complete medium.

Measurement of cytokines in supernatants

The levels of IFN- γ , TNF- α , RANTES (regulated on activation normal T-cell expressed and secreted), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-4, IL-6, IL-8, IL-10 and IL-12 release in the culture supernatants of Be-stimulated PBMCs were evaluated with commercially available solid-phase, two-site enzyme-linked immunosorbent assays (CytElisa; CytImmune Science, San Diego, CA, USA). Cell supernatants were collected after 3 days (RANTES, GM-CSF, IL-4 and IL-12) and 5 days (IFN- γ , TNF- α , IL-6, IL-8 and IL-10) of PBMC culture before [3 H]TdR incorporation, and frozen at -80° C until use. Cytokine concentrations were evaluated in triplicate and the results expressed as the mean of triplicate cultures.

Monoclonal antibody inhibition of lymphocyte activation

Protein-A sepharose purified mAb directed against HLA-DR (L243) [10], HLA-DP (B7/21) [10], HLA-DQ (L2) [10], HLA-class I (W6/32) [10] and the 19 kDa *Mycobacterium tuberculosis* protein (HYT6) [15] were used at increasing concentrations (10, 20 and 50 μ g \cdot mL $^{-1}$) to inhibit antigen presentation, lymphocyte proliferation and cytokine production as previously described [5].

HLA typing

HLA-DP and TNFA1/A2 typing was carried out as previously described [13].

Statistical analysis

All the data were expressed as mean \pm SD. Comparisons between groups were made with t-tests. Comparisons between frequencies were made using the Chi-squared test with the Yates and Bonferroni corrections where necessary.

Results

HLA-DP restriction of beryllium-stimulated lymphocyte proliferation

All PBMCs obtained from berylliosis patients (n=11), carrying glutamate-69-positive HLA-DP alleles, showed positive responses to PHA and the recall antigen *C. albicans* (table 1). Of these, nine had a proliferative response to BeSO₄ that was >3 times the mean SI of unstimulated cells (table 1). In all cells the anti-HLA-DP specific mAb induced a marked inhibition of Be-stimulated proliferation ($88\pm 16\%$) that was significantly greater than that obtained with mAbs against HLA-DR ($29\pm 38\%$), HLA-DQ ($2\pm 2\%$), class-I major histocompatibility complex (MHC; $1\pm 2\%$), and

Table 1.—Mitogen (phytohaemagglutinin (PHA))-, *Candida albicans*- and beryllium (BeSO₄)-induced proliferation and cytokine release in fresh peripheral blood mononuclear cells from berylliosis patients

	Subjects n	Cells only	+PHA	+ <i>C. albicans</i>	+BeSO ₄
Proliferation SI	11		120.6±105.2* (11)	26.2±21.5* (11)	8.9±9.8* (9)
IFN-γ U·mL ⁻¹	10	27.9±12.8	858.4±994.0* (10)	626.2±662.1* (10)	163.6±83.3* (9)
TNF-α pg·mL ⁻¹	9	111.8±26.4	1149.6±740.9* (9)	750.1±426.6* (9)	290.7±95.6* (7)
RANTES pg·mL ⁻¹	5	774±186	3718±2671* (5)		1280±673 (1)
GM-CSF pg·mL ⁻¹	5	149.6±98.2	874.2±445.9* (5)	489.3±155.2 (3)	220.6±128.6 (0)
IL-4 pg·mL ⁻¹	5	7.2±3.7	27.8±9.7* (5)		7.4±2.3 (0)
IL-6 pg·mL ⁻¹	5	6.8±5.2	36.2±5.1* (5)		9.8±6.3 (0)
IL-8 pg·mL ⁻¹	5	10550±9086	131522±27928* (5)		18180±11333 (0)
IL-10 pg·mL ⁻¹	5	0.8±1.3	230.8±123.9* (5)	23.0±41.4 (2)	1.0±1.7 (0)
IL-12 pg·mL ⁻¹	5	21.8±34.5	359.0±190.1* (5)	159.3±167.1* (3)	17.4±34.6 (0)

Data are presented as mean±SD (n[#] of positive tests). IFN: interferon; TNF: tumour necrosis factor; RANTES: regulated on activation normal T-cell expressed and secreted; GM-CSF: granulocyte-macrophage colony-stimulating factor; IL: interleukin. #: number of subjects showing a proliferative response ≥3-fold the mean background incorporation of unstimulated cells or a cytokine release ≥3 SD above the mean background release by unstimulated cells. *: p<0.05, paired t-test compared to unstimulated cells.

the *M. tuberculosis* 19 kDa-specific protein (0±1%; p<0.05 for all comparisons with anti-HLA-DP; fig. 1).

Finally, PBMCs from Be-non-exposed healthy subjects did not proliferate in response to BeSO₄ (data not shown).

HLA-DP restriction of interferon-γ beryllium-stimulated release by mononuclear cells

PBMCs from all 10 patients tested released IFN-γ in response to PHA and *C. albicans* (table 1). Be-stimulated IFN-γ release was significantly higher than control in nine of 10 patients (table 1). The release of this cytokine was strongly blocked by the anti-HLA-DP mAb (77±16%) but not by the other mAbs used (anti-HLA-DR, 14±10%; anti-HLA-DQ, 1±2%; anti-class-I, 1±1%; anti-*M. tuberculosis* 19 kDa-specific protein, 0±1%; p<0.05 for all comparisons with anti-HLA-DP; fig. 1).

Finally, none of the five healthy non-Be-exposed controls released IFN-γ in response to BeSO₄ (data not shown).

Lack of HLA-DP restriction of tumour necrosis factor-α beryllium-stimulated release by mononuclear cells

PBMCs from all of the nine berylliosis patients tested released TNF-α in response to PHA and *C. albicans* (750.1±426.6). PBMCs from seven of the subjects evaluated also released TNF-α in response to Be stimulation at levels that were significantly higher than controls (table 1). However, in contrast to IFN-γ, the release of TNF-α was not blocked by the anti-HLA-DP specific mAb (fig. 1). Similarly to berylliosis patients, PBMCs obtained from four of five non-Be-exposed healthy subjects released TNF-α in response to BeSO₄ (195.3±76.2 pg·mL⁻¹, p=0.02 with respect to Be-unstimulated PBMCs (91.9±15.8); p=0.09 with respect to PBMCs from berylliosis patients stimulated with BeSO₄), while anti-HLA class II monoclonal antibodies had no effect on the cytokine release (data not shown).

No significant levels of the cytokines IL-4, IL-6, IL-8, IL-10, IL-12, GM-CSF and RANTES were found in Be-stimulated culture supernatants of

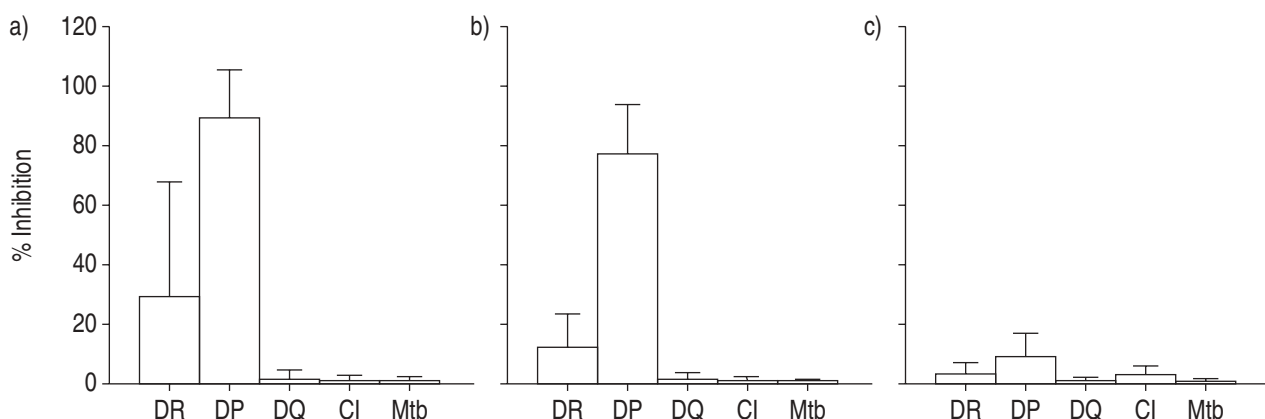


Fig. 1.—Inhibition of beryllium-induced a) proliferation, b) interferon-γ and c) tumour necrosis factor-α release by monoclonal antibodies directed against human leucocyte antigen (HLA)-DR, HLA-DP, HLA-DQ, HLA-class I and the 19 kDa *Mycobacterium tuberculosis* protein in peripheral blood mononuclear cells from berylliosis patients. DR: anti-HLA-DR; DP: anti-HLA-DP; DQ: anti-HLA-DQ; CI: anti-HLA-class I; Mtb: anti-19 kDa *M. tuberculosis*.

PBMCs from any of the patients tested, although all cytokines were released in response to PHA stimulation (table 1). Finally, when TNF- α release levels were analysed in relation to the carriage of the TNFA2 allele (TNFA2/TNFA1 or TNFA2/TNFA2) and those who carried only the TNFA1 allele, no differences were found either in the response to Be (TNFA2-negative, $n=5$, 281 ± 90 pg·mL⁻¹; TNFA2-positive, $n=4$, 312 ± 108 pg·mL⁻¹; $p>0.05$) or to PHA (TNFA2-negative, $1,232\pm 778$ pg·mL⁻¹; TNFA2-positive, $1,385\pm 761$ pg·mL⁻¹; $p>0.05$) and *C. albicans* (TNFA2-negative, 741 ± 469 pg·mL⁻¹; TNFA2-positive, 633 ± 546 pg·mL⁻¹; $p>0.05$).

Discussion

Studies previously carried out by this and other groups [6–8] have suggested that the HLA-DP- β chain allelic variant coding for a glutamate residue in position 69 is the immune response gene associated with Be binding and the induction of the Th1 lymphocyte response that characterises berylliosis [9–11, 16, 17]. In addition, consistent with the observation that TNF- α is released at exaggerated rates in the lungs of individuals with berylliosis [18], the current authors have shown that the TNF- α gene allelic variant TNFA2, implicated in the regulation of TNF- α release [19], is also associated with susceptibility to berylliosis, positively interacting with the HLA-DP β 69 marker in the determination of berylliosis risk [13].

In granulomatous disorders, such as tuberculosis, leprosy and sarcoidosis, IFN- γ -producing Th1 T-cells dominate the disease immune response [20–23]. The observation that proliferation and IFN- γ release by fresh blood mononuclear cells in response to Be are completely abrogated by the anti-HLA-DP specific mAb suggests that in berylliosis-affected individuals the Th1 response to Be is systemic. The fact that HLA-DP glutamate 69-positive molecules are capable of directly binding Be [9], may suggest that Be presentation in the presence of this molecule may itself drive the Th1 response, as has been described in animal models where the Th1 or Th2 polarisation of the immune response is dictated by a specific MHC/peptide combination [24].

TNF- α , together with IFN- γ , is the dominant cytokine produced in the reaction to Be by mononuclear cells from berylliosis patients. Since TNF- α is primarily released by mononuclear phagocytes [25], it is reasonable to question whether cytokine production is driven by, or independent of, Th1 T-cell activation and IFN- γ release. The antibody blocking experiments presented here clearly show that Be-stimulated blood mononuclear phagocytes were able to release TNF- α independently of HLA-DP restricted IFN- γ release. Moreover, the ability of PBMCs from non-Be-exposed healthy subjects to release TNF- α in response to Be strongly support this notion.

Finally, the current authors were unable to demonstrate a correlation between TNF- α levels in Be-stimulated cultures and the TNFA1/TNFA2 genotypes. However, although it has been proposed that

TNF- α release is under the control of a polymorphic sequence with two known alleles (TNFA1 and TNFA2), whereby TNFA2 is associated with higher PHA/phorbol myristate acetate-stimulated cytokine release [19], not all studies have confirmed this finding [26, 27].

The cell function data in this study further support the concept that the tumour necrosis factor- α and human leucocyte antigen-DP genes are immunogenetic independent factors of berylliosis and that they may interact in the development of the granulomatous reaction induced by beryllium in the lung.

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