



HLA-DQ8 is a predisposing molecule for detergent enzyme subtilisin BPN'-induced hypersensitivity

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Abstract

Several million individuals are exposed to agents in the workplace associated with atopy and asthma. Detergent enzymes have been implicated in occupationally induced hypersensitivity. However, the genetic susceptibility and T cell responses to detergent enzymes are undefined. We generated and used HLA-DQ6, -DQ8, -DR2, -DR3, and -DR4 transgenic mice to examine the immune and inflammatory components involved in the response to the detergent enzyme subtilisin BPN'. Based on in vitro and in vivo studies, for the first time, we present evidence that DQ8 is a strong susceptibility marker for BPN'-induced hypersensitivity. Only DQ8 mice showed consistent T cell responses to five immunodominant regions of BPN' comprising peptides #14 to 16, 36–37, 42–43, 62–63, and 80–81. The DQ8 mice also developed allergic eosinophilic inflammatory reactions in the airways following intranasal instillations of this enzyme. The DQ8 mice also responded to BPN' with a significant IgG1 and IgE production. We propose that the HLA Class II tg mice are useful for understanding allergenic responses to enzymes in humans, screening of allergenic and immunogenic properties of detergent enzymes, and for the development of modified enzymes to maintain efficient detergent qualities without allergic properties.

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Introduction

With approximately 9 million individuals exposed and potentially at risk for development of occupational asthma (OA), hypersensitivity to occupational stimuli continues to be a public health concern [1,2]. OA may be caused by high-molecular-weight sensitizers (allergens), low-molecular-weight sensitizers (chemicals), or irritant gases [3]. Washing powders in the detergent industry contain known high-molecular-weight sensitizers enzymes. These enzymes are proteins derived from bacterial or fungal organisms and are present in detergents to

improve their cleansing properties. Allergies and asthma to these powdered enzymes appeared in the late 1960s in the detergent plants where bacterial proteases, subtilisins, were identified as the causative agents. Since the introduction of an advanced technology of enzyme delivery into the final product and Industry's strict control of occupational exposure to respirable enzyme dust, the incidence of immediate hypersensitivity responses and the occurrence of enzyme-associated occupational asthma among detergent workers is currently a rare event [4]. However, new occupational sensitizations in detergent plants have not been totally prevented [5,6].

HLA Class II polymorphism has been found to be associated with OA [7] including asthma and atopy involving isocyanates [8], Western red cedar [9], acid anhydrides [10], and complex platinum salt [11]. Though information has been published on Ab responses in humans, mice, and guinea pigs to detergent enzymes, the association of HLA Class II with sensitization to enzymes is unknown. BPN' (Y217L variant

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herein referred to as BPN'), a bacterial protease from *Bacillus amyloliquefaciens*, was chosen for this study because it is a protease commonly found in liquid laundry detergents. Therefore, in order to further improve our understanding of the BPN' allergenicity, the response to BPN' was evaluated in transgenic (tg) mice expressing HLA-DQ6, -DQ8, -DR2, -DR3, and -DR4 genes. Several routes of Ag administration were evaluated: subcutaneous (s.c.), intranasal (i.n.), and combined intraperitoneal/intranasal (i.p./i.n.). We identified that DQ8 molecules serve as restriction elements for an immune response to BPN'. DQ8 and DQ8/hCD4 tg mice were able to recognize several epitopes on BPN' and developed allergic inflammatory reactions in the airways following i.n. instillations of this enzyme. We show here that the BPN' T cell epitope repertoire in DQ8 mice is not modulated by (1) two non-MHC genetic backgrounds; (2) adjuvant codelivery (but is modulated by adjuvant presence); (3) immunization route and dose of enzyme if adjuvant is used. DR4 mice responded to several epitopes using combined i.p./i.n. BPN' application only. Preliminary data indicate that HLA Class II tg mice have similar epitopes compared to subsets of humans skin prick test positive to BPN'. This work suggests that HLA Class II tg mice can provide information on the allergenicity of enzymes and should be used as potential models to assess the safety of known and new detergent enzymes in humans.

Materials and methods

Mice

Transgenic mice expressing either HLA-DQ8 (DQA1*0301 and DQB1*0302), HLA-DQ6 (DQA1*0103 and DQB1*0601), HLA-DR2 (DRB1*1502), HLA-DR3 (DRB1*0301), or HLA-DR4 (DRB1*0401) in H-2A β ⁰ mice were generated as previously described [12–16]. The H-2A β ⁰ mice have both a spontaneous mutation in the promoter region of the H-2E α gene (in H-2^b haplotypes) and a targeted disruption of the H-2A gene. Neither H-2A α ^b nor H-2E β ^b molecules were detected on peripheral blood leukocytes in tg mice. The surface expression of hybrid molecules formed by H-2A α ^b, DQ β , or DR β chains was not observed. H-2A β ⁰ mice and B10 mice served as transgene negative and background controls. DQ8 transgenic mice were mated to mCD4⁰ (kindly provided by Tak W. Mak, University of Toronto) [17] and then to hCD4 tg mice (kindly provided by Richard A. Flavell, Yale University) [18] to generate the double transgenic double knockout mice [19,20]. All mice used in these experiments were between 8 and 12 weeks of age. Both males and females were equally represented in each experiment. Mice were bred and maintained in the pathogen-free Immunogenetics Mouse Colony at the Mayo Clinic (Rochester, MN). All procedures performed on the mice were in accordance with the Mayo Institutional Animal Care and Use Committee.

Anesthetic agent

Stock avertin solution (10 g of 99% 2,2,2-tribromoethanol; Aldrich Chemical Co., Milwaukee, WI) was dissolved in 10 ml

tert-amylalcohol (Fisher Scientific, Fairlawn, NJ). One hundred microliters of stock solution was diluted in 5 ml PBS. An intraperitoneal injection (1 ml) was used for euthanization, and 0.2–0.3 ml was administered for anesthesia.

BPN'

Subtilisin BPN' (Y217L) is a variant of the bacterial protease derived from *B. amyloliquefaciens*. Purified BPN' was obtained from Genencor International (San Francisco, CA). A pNA assay is used to determine the active enzyme concentration.

Initially, it was determined that active enzyme was detrimental to cells in culture. Therefore, BPN' was inactivated by a trifluoromethylketone inhibitor for in vitro use. This subtilisin inhibitor 2-(*N*-methoxycarbonyl-phenylalanyl-glycyl-alanyl)-amino-5-methyl-1,1,1-trifluoro-2-hexanone was synthesized by Procter & Gamble [21]. The inhibitor is non-cytotoxic at the concentrations used in the proliferation assays, and control wells with inhibitor alone showed no proliferation. In addition, the inhibitor was chosen as it binds to the active site and does not alter the three-dimensional structure of BPN'.

Peptides

A set of 88 overlapping peptides offset by 3 amino acids spanning the length of BPN' was commercially synthesized by Chiron Mimotopes (Raleigh, NC) using the Multi-Pin technique [22]. The peptides were 15 amino acid residues long and had a 12 amino acid residue overlap with adjacent peptides in the panel (Table 1). Peptides were dissolved in DMSO at final concentration of 10 mM. Levels of DMSO in culture were kept below 0.3%.

Antigen immunization protocols

Mice were immunized by three different routes of exposure outlined in Fig. 1.

Subcutaneous immunization

Mice were injected s.c. with a single dose of BPN' (40 μ g) emulsified in CFA (Difco, Detroit, MI) into the tail and footpad. Control mice were dosed s.c. with CFA. Seven days post-injection, draining lymph nodes were removed for epitope mapping studies (Fig. 1A).

Repeated intranasal immunization

For i.n. immunization of transgenic mice, BPN' was instilled (5 μ l/nostril, total 10 μ g/10 μ l/mouse) in sterile endotoxin-free PBS (Gibco BRL) on days 1, 3, 10, 17, and 24 of the study to the restrained anesthetized mice ($n = 4–8$) [23]. Control groups of mice were treated with an equal volume of PBS in similar fashion. On day 29, spleens were obtained for in vitro proliferation assays. BAL fluids were collected as described previously [24] on day 29 of the study (Fig. 1B).

Table 1

Sequences of synthetic overlapping peptides used in this study

1	A	Q	S	V	P	Y	G	V	S	Q	I	K	A	P	A	45	A	A	L	K	A	A	V	D	K	A	V	A	S	G	V	
2	V	P	Y	G	V	S	Q	I	K	A	P	A	L	H	S	46	K	A	A	V	D	K	A	V	A	S	G	V	V	V	V	
3	G	V	S	Q	I	K	A	P	A	L	H	S	Q	G	Y	47	V	D	K	A	V	A	S	G	V	V	V	V	A	A	A	
4	Q	I	K	A	P	A	L	H	S	Q	G	Y	T	G	S	48	A	V	A	S	G	V	V	V	V	V	A	A	A	G	N	E
5	A	P	A	L	H	S	Q	G	Y	T	G	S	N	V	K	49	S	G	V	V	V	V	V	A	A	A	G	N	E	G	T	S
6	L	H	S	Q	G	Y	T	G	S	N	V	K	V	A	V	50	V	V	V	V	A	A	A	G	N	E	G	T	S	G	S	S
7	Q	G	Y	T	G	S	N	V	K	V	A	V	I	D	S	51	A	A	A	G	N	E	G	T	S	G	S	S	S	S	T	V
8	T	G	S	N	V	K	V	A	V	I	D	S	G	I	D	52	G	N	A	E	G	T	S	G	S	S	S	T	V	G	Y	P
9	N	V	K	V	A	V	I	D	S	G	I	D	S	S	H	53	G	T	S	G	S	S	S	T	V	G	Y	P	G	K	Y	
10	V	A	V	I	D	S	G	I	D	S	S	H	P	D	L	54	G	S	S	S	T	V	G	Y	P	G	K	Y	P	S	V	
11	I	D	S	G	I	D	S	S	H	P	D	L	K	V	A	55	S	T	V	G	Y	P	G	K	Y	P	S	V	I	A	V	
12	G	I	D	S	S	H	P	D	L	K	V	A	G	G	A	56	G	Y	P	G	K	Y	P	S	V	I	A	V	G	A	V	
13	S	S	H	P	D	L	K	V	A	G	G	A	S	M	V	57	G	K	Y	P	S	V	I	A	V	G	A	V	D	S	S	
14	P	D	L	K	V	A	G	G	A	S	M	V	P	S	E	58	P	S	V	I	A	V	G	A	V	D	S	S	N	Q	R	
15	K	V	A	G	G	A	S	M	V	P	S	E	T	N	P	59	I	A	V	G	A	V	D	S	S	N	Q	R	A	S	F	
16	G	G	A	S	M	V	P	S	E	T	N	P	F	Q	D	60	G	A	V	D	S	S	N	Q	R	A	S	F	S	S	V	
17	S	M	V	P	S	E	T	N	P	F	Q	D	N	N	S	61	D	S	S	N	Q	R	A	S	F	S	S	V	G	P	E	
18	P	S	E	T	N	P	F	Q	D	N	N	S	H	G	T	62	N	Q	R	A	S	F	S	S	V	G	P	E	L	D	V	
19	T	N	P	F	Q	D	N	N	S	H	G	T	H	V	A	63	A	S	F	S	S	V	G	P	E	L	D	V	M	A	P	
20	F	Q	D	N	N	S	H	G	T	H	V	A	G	T	V	64	S	S	V	G	P	E	L	D	V	M	A	P	G	V	S	
21	N	N	S	H	G	T	H	V	A	G	T	V	A	A	L	65	G	P	E	L	D	V	M	A	P	G	V	S	I	Q	S	
22	H	G	T	H	V	A	G	T	V	A	A	L	N	N	S	66	L	D	V	M	A	P	G	V	S	I	Q	S	T	L	P	
23	H	V	A	G	T	V	A	A	L	N	N	S	I	G	V	67	M	A	P	G	V	S	I	Q	S	T	L	P	G	N	K	
24	G	T	V	A	A	L	N	N	S	I	G	V	L	G	V	68	G	V	S	I	Q	S	T	L	P	G	N	K	Y	G	A	
25	A	A	L	N	N	S	I	G	V	L	G	V	A	P	S	69	I	Q	S	T	L	P	G	N	K	Y	G	A	L	N	G	
26	N	N	S	I	G	V	L	G	V	A	P	S	A	S	L	70	T	L	P	G	N	K	Y	G	A	L	N	G	T	S	M	
27	I	G	V	L	G	V	A	P	S	A	S	L	Y	A	V	71	G	N	K	Y	G	A	L	N	G	T	S	M	A	S	P	
28	L	G	V	A	P	S	A	S	L	Y	A	V	K	V	L	72	Y	G	A	L	N	G	T	S	M	A	S	P	H	V	A	
29	A	P	S	A	S	L	Y	A	V	K	V	L	G	A	D	73	L	N	G	T	S	M	A	S	P	H	V	A	G	A	A	
30	A	S	L	Y	A	V	K	V	L	G	A	D	G	S	G	74	T	S	M	A	S	P	H	V	A	G	A	A	A	L	I	
31	Y	A	V	K	V	L	G	A	D	G	S	G	Q	Y	S	75	A	S	P	H	V	A	G	A	A	A	A	L	I	L	S	
32	K	V	L	G	A	D	G	S	G	Q	Y	S	W	I	I	76	H	V	A	G	A	A	A	L	I	L	S	K	H	P	N	
33	G	A	D	G	S	G	Q	Y	S	W	I	I	N	G	I	77	G	A	A	A	L	I	L	S	K	H	P	N	W	T	N	
34	G	S	G	Q	Y	S	W	I	I	N	G	I	E	W	A	78	A	L	I	L	S	K	H	P	N	W	T	N	T	Q	V	
35	Q	Y	S	W	I	I	N	G	I	E	W	A	I	A	N	79	L	S	K	H	P	N	W	T	N	T	Q	V	R	S	S	
36	W	I	I	N	G	I	E	W	A	I	A	N	N	M	D	80	H	P	N	W	T	N	T	Q	V	R	S	S	L	E	N	
37	N	G	I	E	W	A	I	A	N	N	M	D	V	I	N	81	W	T	N	T	Q	V	R	S	S	L	E	N	T	T	T	
38	E	W	A	I	A	N	N	M	D	V	I	N	M	S	L	82	T	Q	V	R	S	S	L	E	N	T	T	K	L	G	L	
39	I	A	N	N	M	D	V	I	N	M	S	L	G	G	P	83	R	S	S	L	E	N	T	T	T	K	L	G	D	S	F	
40	N	M	D	V	I	N	M	S	L	G	G	P	S	G	S	84	L	E	N	T	T	T	K	L	G	D	S	F	Y	Y	G	
41	V	I	N	M	S	L	G	G	P	S	G	S	A	A	A	85	T	T	T	K	L	G	D	S	F	Y	Y	G	L	K	G	
42	M	S	L	G	P	S	A	G	S	A	A	L	K	A	A	86	K	L	G	D	S	F	Y	Y	G	K	G	L	I	N	V	
43	G	G	P	S	G	S	A	A	L	K	A	A	V	D	K	87	D	S	F	Y	Y	G	K	G	L	I	N	V	Q	A	A	
44	S	G	S	A	A	L	K	A	A	V	D	K	A	V	A	88	F	Y	Y	G	K	G	L	I	N	V	Q	A	A	A	A	Q

Combined intraperitoneal/intranasal immunization

In the combined i.p./i.n. procedure, mice ($n = 4-5$) were injected i.p. with 40 μ g of BPN', absorbed to 1 mg of aluminum hydroxide in 0.5 ml of sterile PBS on days 0 and 7 [25], and challenged i.n. with 20 μ g/50 μ l of BPN' 2 times 6 h apart on day 14. Control mice were treated with PBS. On day 16, spleens were obtained for in vitro proliferation assays. BAL fluids were collected as described previously [24] on day 16 of the study (Fig. 1C).

BAL cell composition, total protein, and cytokine assays

Five days after i.n. or 48 h after combined i.p./i.n. challenge (day 29 and 16, respectively), mice were euthanized with avertin, and BAL fluids were withdrawn from mouse lungs. BALs were characterized for cellularity as described previously [25]. Total protein (TP) contents in BAL was measured

as described previously [25]. IL-2, IL-4, IL-5, IL-9, IL-12p40, IL-13, IFN- γ , and TNF- α in BAL were measured by ELISA [25].

Histologic examination

In separate experiments, the trachea and lungs were removed from euthanized mice ($n = 4-5$) after challenge. Sections were prepared as described [25], using formalin for fixation and paraffin for embedding, and H & E for staining. Evaluation of histologic alterations was done with 4–5 samples from each mouse in a double-blind study. To characterize the intensity of the lung inflammation, a previously described grading scheme was used [20]. Reactions were scored on scale 0–3 (none–high), where grade 0 showed no inflammation and grade 3 showed extension of the inflammatory cells to the lung periphery.

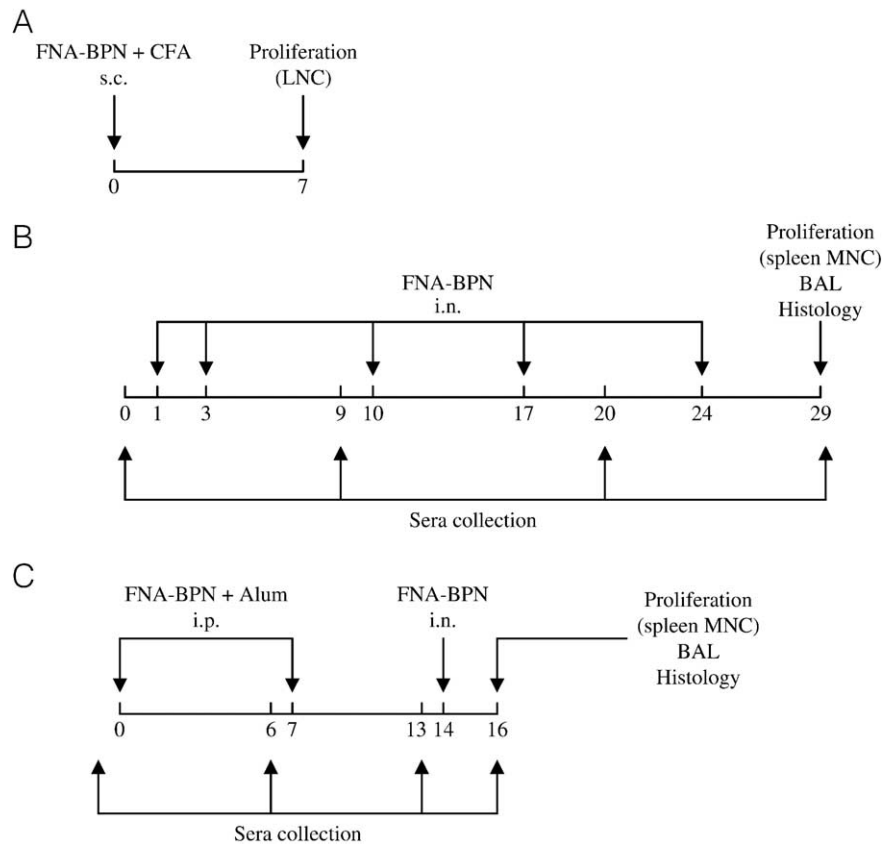


Fig. 1. BPN' immunization protocols. Groups of transgenic and control mice were sensitized to BPN' by the following methods: (A) single subcutaneous injection with CFA; (B) repeated intranasal applications without adjuvant; (C) intraperitoneal injection with adjuvant (alum) with subsequent intranasal application.

T cell proliferation assays

For T cell epitope mapping following s.c. immunization, draining lymph nodes were removed 7 days post-injection and cells were prepared for in vitro cultures as described previously [26]. Following the i.n. and i.p./i.n. immunization protocols, spleens were harvested on day 29 or day 16, respectively, and MNC were prepared for in vitro cultures as described [25]. Either LNC or splenic MNC were placed in triplicate in 96-well flat-bottom plates at a concentration of 10^6 cells/100 μ l. The cells were incubated for 24 h at 37°C with medium alone, 10 μ g/ml of ConA, 200 μ g/ml of inactivated BPN', or 5 μ M/well individual synthetic peptides spanning BPN'. Cultures were pulsed with [^{3} H] thymidine (1 μ Ci/well; Dupont, Boston, MA), and incubated for an additional 24 h. The cells were harvested, and [^{3} H] thymidine uptake was measured as cpm using a liquid scintillation counter (Beckman Instruments, Palo Alto, CA). Results were expressed as Δ cpm. The criteria for a positive response in these assays was Δ cpm >3500 and stimulation index >2. The stimulation index was calculated as the (mean cpm of the test cultures/mean cpm of control cultures).

For the in vitro mAb blocking study, supernatants from the cell lines producing mAb specific for DQ6/8 (TB1), DR

(L227), H-2Abb (7-16-17), H-2Abb (25-5-16), H-2Ea/b (Y-17), CD4 (GK1.5), and CD8 (53-6.72) were used [27].

BPN'-specific Ab levels

In separate experiments, groups of mice ($n = 3-5$) were bled on days 0, 9, 20, and 29 or on days 0, 6, 13, and 16 following repeated i.n. or combined i.p./i.n. immunization, respectively. Sera were collected and stored at -70°C until tested for the presence of BPN'-specific IgG1 and IgE.

BPN'-specific IgG1 ELISA

Enzyme-specific IgG₁ in mouse sera was evaluated using a method previously described [28]. Briefly, plates (Immulon 2, Dynatech, Chantilly, VA) were coated with 1 μ g/100 μ l of BPN' in Dulbecco's PBS (Sigma). After washing and blocking, the plates were incubated with serially diluted sera in wash buffer containing 1% BSA. Bound mouse IgG1 was detected with alkaline-phosphatase-labeled goat anti-mouse IgG₁ Ab (Southern Biotechnology, Birmingham, AL). *p*-Nitrophenyl phosphate substrate dissolved in diethanolamine buffer (Kirkegaard and Perry, Gaithersburg, MD) was added, and plates were read with a Thermomax microplate reader (Molecular Devices, Menlo Park, CA). The OD from normal mouse serum was subtracted from OD values for the

test serum. Ab titer was expressed as the $\log_2$ value of the reciprocal dilution produced at an OD of 0.5.

Passive cutaneous anaphylaxis (PCA) in rats for BPN'-specific IgE

The rat PCA was used to measure BPN'-specific IgE and was conducted according to the method of Ovary et al. [29]. The test serum was diluted 1:2, 1:4, 1:8, 1:16, 1:32, and 1:64. Each rat was evaluated with serial dilutions of two compounds one on each side of the back. Intradermal injections of the test sera (0.1 ml) were made in a straight line approximately 2 cm apart on the shaved back. Two days later, the rats were injected via the tail vein with 2 mg of BPN' in 0.5 ml of a 1% Evans's blue solution in saline. Thirty minutes after the injection, the skin of each animal was evaluated. The highest dilution of serum showing a positive response was the end titer. The PCA antibody titer was expressed as $\log_2$ of the reciprocal of the end point dilution as described above. The data are shown as the reciprocal of the dilution giving lowest positive response.

Statistics

Data are summarized as the mean $\pm$ SEM. To calculate significance levels between treatment groups, Student's *t* test (Sigma Plot and Microsoft Excel) was used. Differences between values were considered significant at $P < 0.05$.

Results

HLA class II tg mice display a dose- and time-dependent proliferative response to in vitro re-challenge with BPN'

To determine whether HLA Class II tg mice were capable of responding to BPN', optimization experiments involving priming and challenging tg mice with varying doses of BPN' were performed. These experiments showed that an in vivo priming dose of 40 μ g/mouse of active BPN' and an in vitro re-stimulation dose of 200 μ g/ml of inactivated enzyme elicited maximum [^{3} H] thymidine incorporation at 48 h LNC cultures (data not shown). Following s.c. priming, LNC of DQ8, DQ6,

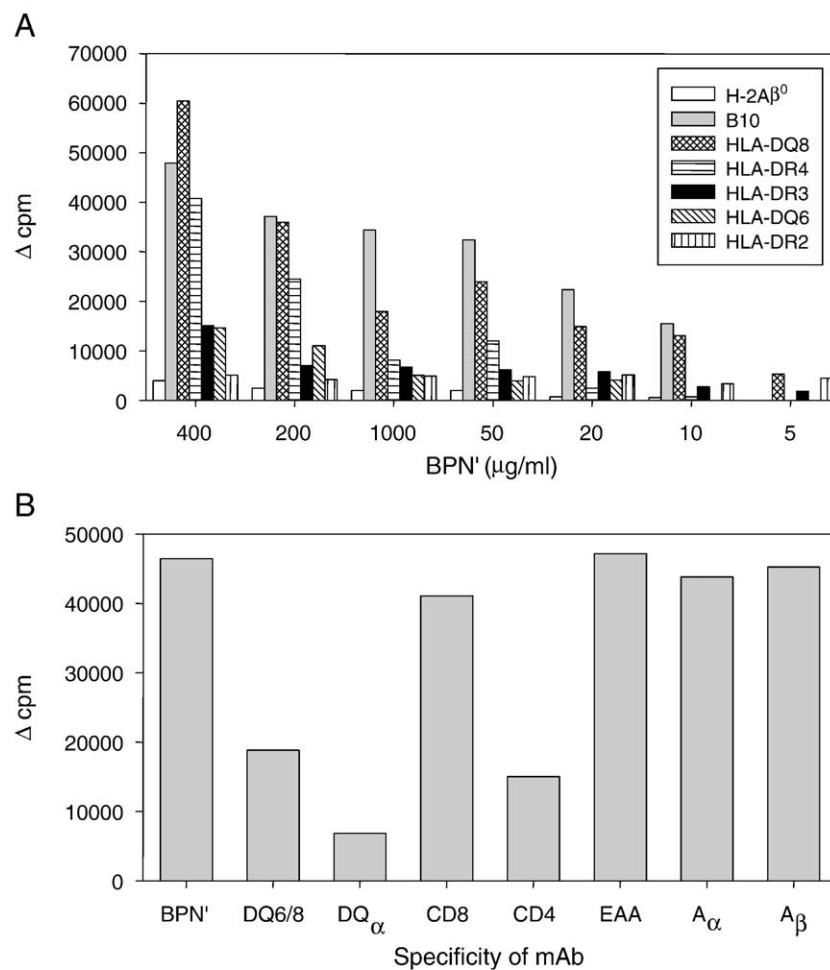


Fig. 2. Comparison of the in vitro proliferation response to BPN' in transgenic mice and control animals (A) and mAb blocking assay in DQ8 mice (B). (A) Mice (four per group) were immunized with 40 μ g of BPN' in CFA. Seven days later, lymph node cells were harvested, pooled within an experimental group, and cultured in the presence of 20 μ g/well of inactivated BPN' or medium alone. B10, DQ8, DR4, DR3, and DQ6 mice display a dose-dependent response to BPN challenge. The response was absent in A β^0 and DR2 mice. (B) Draining LNC were re-stimulated in vitro with BPN' in the presence of the specified mAb. Results are the mean of [^{3} H] thymidine incorporation for one of two representative experiments.

DR4, and DR3 tg mice proliferated to BPN' in a dose- and time-dependent manner with a peak response at day 2 of in vitro culture (Fig. 2A). The highest proliferative responses were seen in DQ8 and DR4 mice. A β ⁰ and DR2 mice did not exhibit significant proliferative responses to BPN'. B10 mice demonstrated a strong proliferative response (Δ cpm, 47894) to BPN'. These studies suggest that DQ8 and DR4 tg mice are able to generate a T cell response to BPN' under the conditions studied.

To identify the genetic restriction of T cells necessary for the response to BPN' in DQ8 tg mice, different mAb were added to the wells containing LNC cultures with BPN'. The proliferative response was significantly inhibited using mAb specific for DQ and CD4 molecules, suggesting that CD4 T cells recognize BPN' in the context of DQ8 (Fig. 2B).

BPN' T cell epitopes recognized by HLA-DQ and HLA-DR tg mice following subcutaneous immunization

To identify the working dose of BPN synthetic peptides, initial proliferation assays were performed based on dose-dependent (kinetic) responses. At first, DR3 and DQ8 tg mice were immunized with varying doses of peptide #24 (from 10 to 75 μ M/mouse), and draining LNC were re-stimulated in vitro with increasing doses (from 0.5 to 10 μ M/well) of this peptide (data not shown). Next, HLA Class II tg mice were immunized with an optimal in vivo dose of peptide #24 (10 μ M/mouse), and LNC were re-stimulated with optimal in vitro concentrations of this peptide (5 and 7.5 μ M/well).

To delineate the immunodominant T cell epitopes in BPN', mice were immunized with enzyme as described above, and their draining LNC were re-stimulated in vitro with either inactivated enzyme or synthetic overlapping peptides at the optimal stimulatory dose. Table 2 summarizes the results. By this route of immunization, no proliferative response to any of the peptides was detected in PBS/CFA-treated HLA Class II tg or BPN'-primed H-A β ⁰, DQ6, DR3, or DR4 mice. DR2 mice were not evaluated as they did not show a T cell response to BPN' in previous studies. Only DQ8 mice showed consistent T cell responses to specific peptides spanning BPN'. The DQ8 mice recognized peptides #14, 15, 16, 36, 37, 42, 43, 62, 63, 80, and 81. In summary, five immunodominant regions were identified comprising peptides #14 to 16, 36–37, 42–43, 62–63, and 80–81. These results were consistently seen and are even more convincing as adjacent peptides in each region-evoked responses. With respect to magnitude of the proliferative response, the greatest response in the DQ8 mouse was seen in the region spanning peptides 14 to 16. LNC cultures stimulated with ConA demonstrated significant in vitro responses measured by [³H] thymidine incorporation with Δ cpm ranging from 98,404 to 246,332 in HLA Class II tg mice and from 48,805 to 80,333 in A β ⁰ knockout mice.

BPN' T cell epitopes recognized by HLA-DQ8 tg mice with different genetic backgrounds

As BPN'-immunized DQ8 mice were able to generate a T cell response to intact BPN' and peptides in the BPN'

Table 2

Immune response of HLA II tg mice to BPN' peptides^a

Mice	Immunization protocol		
	Single subcutaneous	Repeated intranasal	Combination of intraperitoneal and intranasal
B10	nd ^b	nd	nd
B10/A β ⁰	nd	nd	nd
HLA-DQ6	nd	nd	nd
HLA-DQ8	14 ^c (5988) ^d (8192) ^e 15 (10,298) (12,866) 16 (13,087) (9382) 36 (5457) (12,204) 37 (8054) (9526) 42 (7439) (8643) 43 (6277) (8632) 62 (5036) (20,997) 63 (3459) (21,667) 80 (3748) (19,237) 81 (3616) (17,889)	nd	14 (6135) ^d 14 (4597) ^e 15 (7019) 15 (5156) 16 (6268) 16 (5839) 36 (5415) 36 (7020) 37 (10,225) 37 (10,106) 42 (10,404) 42 (7715) 43 (8943) 43 (10,592) 62 (18,445) 62 (19,127) 63 (6564) 63 (9124) 80 (15,721) 80 (4727) 81 (12,145) 81 (5173)
HLA-DR3	nd	nd	nd
HLA-DR4	nd	nd	15 (4668) 23 (5350) 44 (6302) 47 (4891) 51 (5501)
DQ8/hCD4	14 (6022) ^c 15 (9061) 16 (13,485) 17 (18,709) 36 (13,909) 37 (20,925) 42 (15,919) 43 (9280) 62 (14,652) 63 (15,096) 80 (11,162) 81 (6478)	62 (4137) ^c	14 (7028) ^c 15 (5634) 16 (3336) 17 (7491) 36 (3760) 37 (4820) 42 (3000) 43 (4975) 62 (7075) 63 (5211) 80 (3418) 81 (3007)

^a HLA II tg mice treated with adjuvant PBS by similar fashions have not demonstrated any in vitro response to either BPN' or peptide challenge.

^b nd—the response was not detected, mean Δ cpm < 3500 and S.I. < 2.0.

^c Number of peptide corresponds the aa sequences outlined in Table 1.

^d Delta cpm counts in proliferative response to peptide for mice on B10 background.

^e Delta cpm counts in proliferative response to peptide for mice on B6/129J background.

sequence, two other lines of DQ8 mice were evaluated for T cell epitopes. The DQ8 (B10 background) recognized the five immunodominant regions as shown above. DQ8 mice on mixed B6/129J background were also epitope-mapped following s.c. exposure and recognized the same five immunodominant regions (Table 2). Though the five regions identified were similar, the magnitude of the proliferative responses in these mice was greater than those with B10 background. In these mice, peptides 62–63 and 80–81 had the highest proliferative responses. Therefore, these preliminary results suggest that genetic background may not in some cases influence the epitopes identified but may influence magnitude/quality of response to the epitopes.

DQ8/hCD4 mice were also epitope-mapped to determine if the presence of human CD4 can make a difference. DQ8/hCD4 mice also identified the same five immunodominant regions

(Table 2). The only difference noted was that mice with hCD4 recognized one additional peptide (#17), however, it was a peptide adjacent to a region already identified. Therefore, similar T cell epitope regions were identified in all three DQ8 strains and did not appear to be affected by non-MHC genetic background or source of CD4.

BPN' T cell epitopes in HLA-DQ8 tg mice using different immunization protocols and different lymphoid organs for assays

A second immunization protocol was used to prime the tg mice for T cell epitope mapping studies. This method (Fig. 1C) is commonly found in the literature for stimulating Th2 respiratory hypersensitivity/asthma in mice. Splenic MNCs were used for the epitope mapping studies. These studies demonstrated that DQ8 and DQ8/hCD4 mice respond to the same five immunodominant regions (Table 2). DR4 mice recognized several determinants on BPN' using this immunization protocol. Of note, peptide 15 is a common epitope for both DQ8 and DR4 molecules. In contrast, neither DQ6 nor DR3 transgenic mice responded to either BPN' or peptide. DR2 mice were not evaluated in these studies. There was no proliferative response to BPN' or peptides in PBS-treated mice or BPN'-primed control $A\beta^0$ mice.

A third route of immunization was also evaluated in the tg mice. To more closely simulate a relevant route of exposure in humans (without adjuvant), tg and control animals were exposed to repeated i.n. immunizations with BPN' (Fig. 1B), and the proliferative response to peptides and BPN' was evaluated. No response to either intact BPN' or peptides was found in spleen MNC cultures obtained from PBS-treated mice. Splenic MNCs from BPN'-treated B10, DQ8, DR3, and DQ8/hCD4 mice proliferated to the intact BPN' in vitro (Δ cpm, 10,310, 12,947, 9170, and 8877, respectively). With respect to the peptides, no proliferative responses were detected

to any peptide in all strains of the tg mice except DQ8/hCD4 mice. These mice were able to recognize a single epitope in BPN'-peptide #62 (Δ cpm, 4137, Table 2).

BPN'-specific IgG1 and IgE

BPN'-specific IgG1 was detected in both DQ6 and DQ8 mice on days 29 and 16 (Table 3). The repeated i.n. protocol produced higher IgG1 titer in both DQ6 and DQ8 mice compared to the combined i.p./i.n. protocol. It should be pointed out that, for both routes of exposure, the peak IgG1 response to BPN' was reached faster in the DQ8 mice than the DQ6 mice. No IgG1 was detected in DR3, DR4 or $A\beta^0$ mice. Assessment of IgE levels using the rat PCA was done in the DQ8, DQ6, DR3, and DR4 mice (Table 3). As with IgG1, only the DQ8 and DQ6 mice had detectable IgE, and the Ab levels appeared to be developing faster in the DQ8 mice as the IgG1 had done.

Cellular constituents of lung lavage fluid

Sensitization by means of repeated i.n. exposure with BPN' induced a consistent marked increase in BAL lymphocyte counts in both DQ8 and DQ8/hCD4 mice (Fig. 3A). While the predominant cell type in the BAL of DQ8/hCD4 mice remained the macrophage, BPN' induced significant increase in eosinophils in these transgenics (mean $85,350 \pm 51,450$ number of cells). Repeated i.n. treatment with BPN did not affect BAL cellularity in B10, $A\beta^0$, DQ6, and DR4 mice compared to that in PBS-treated counterparts and produced no eosinophilic inflammation. Surprisingly, this treatment induces strong macrophage influx into BAL of DR3 mice.

Sensitization by means of combined i.p./i.n. application of BPN' resulted in inflammation of different severities in different transgenic mice. All transgenic mice as well as control animals demonstrated significant neutrophil influx into BAL (Fig. 3B). This model markedly exacerbated pulmonary

Table 3
Assessment of IgG1 and IgE levels in HLA Class II tg mice immunized with BPN'^a

Transgenic mouse	Route of exposure	Dose of BPN' (μ g)	Study day when blood drawn	IgG1 in sera mean IgG1 titer ($\log_2$) ^b	IgG1 in sera number of BPN' IgG1 positive mice	IgE in sera PCA titer ($\log_2$) ^c
DQ6	Repeated i.n.	10	29	8.0	4/4	2
	Combined i.p./i.n.	40/20	16	3.0	4/4	0
DQ8	Repeated i.n.	10	29	13.0	5/5	>6 ^d
	Combined i.p./i.n.	40/20	16	2.5	2/4	0
DR3	Repeated i.n.	10	29	0	0/3	0
	Combined i.p./i.n.	40/20	16	0	0/4	0
DR4	Repeated i.n.	10	29	0	0/2	0
	Combined i.p./i.n.	40/20	16	0	0/3	0
$A\beta^0$ control	Repeated i.n.	10	29	0	0/5	0
	Combined i.p./i.n.	40/20	16	0	0/4	0

^a Mice dosed by the repeated i.n. route received 10 μ g (5 μ g/nostril) BPN' on days 1, 3, 10, 17, and 24 with sera obtained on day 29.

Mice dosed by combined i.p./i.n. routes received 40 μ g BPN' adsorbed to 1 mg of alum in 0.5 ml of PBS on days 0 and 7 and were challenged i.n. with 20 μ g/50 μ l of BPN' 2 times 6 h apart on day 14. Sera were obtained on day 16.

^b IgG1 Ab titer is expressed as the $\log_2$ value of the reciprocal dilution produced at an OD 0.5.

^c IgE Ab is expressed as the $\log_2$ value of the reciprocal of the end point dilution as described in Materials and methods.

^d PCA titer was not assessed in the same experiment as IgG1 for the DQ8 mice. This is the titer from a separate experiment.

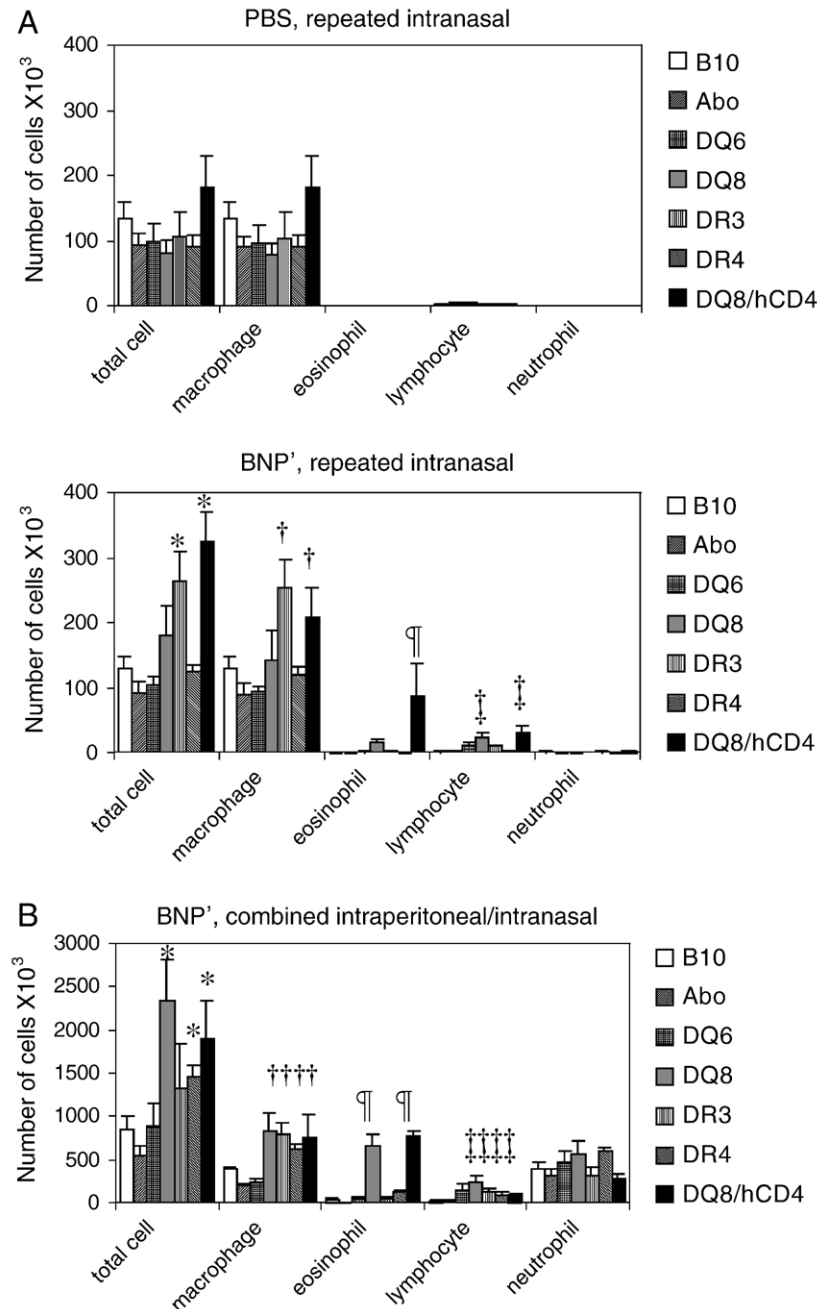


Fig. 3. Bronchoalveolar lavage cell recovery in mice after exposure to (A) repeated intranasal PBS or BPN' (A) or combined intraperitoneal/intranasal BPN' (B) as described in Materials and methods. Mean cell counts $\pm$ SEM are shown. (A) *0.00063 $< P < 0.04$, **0.0032 $< P < 0.029$, super † 0.027 $< P < 0.047$, ‡ 0.012 $< P < 0.05$, marked transgenic mice vs. PBS-treated counterparts, $A\beta^0$ and B10 mice. (B) *0.00017 $< P < 0.016$ vs. $A\beta^0$ and B10 mice; **0.000005 $< P < 0.0005$ vs. $A\beta^0$ mice and 0.0032 $< P < 0.024$ vs. B10 mice; †0.0000005 $< P < 0.0023$ vs. $A\beta^0$ mice and 0.000004 $< P < 0.012$ vs. B10 mice; ‡0.0023 $< P < 0.05$ vs. $A\beta^0$ mice and 0.007 $< P < 0.02$ vs. B10 mice.

allergic inflammation in response to BPN' observed in DQ8 and DQ8/hCD4 mice. Of the BAL fluid cells, 28.5% and 40.6% were eosinophils in the BPN'-sensitized/challenged DQ8 and DQ8/hCD4 mice compared with up to 1.5% of total cells in PBS-treated controls (data not shown) and up to 6.2% in other HLA Class II transgenics. BAL fluids from BPN'-sensitized/challenged DQ8, DR3, and DR4 mice contained from 7% to 10.5% lymphocytes, in contrast to the other experimental groups in which the lymphocyte population was generally $< 5\%$.

Total protein levels in BAL fluids

The changes in lung permeability induced by BPN' instillation were measured by the change in the total protein (TP) content of BAL fluids. In mice dosed with repeated i.n. BPN' administration, a significant increase in TP was observed in DQ8 mice only 48 h after the last i.n. BPN' challenge compared to other BPN'-treated tg mice and PBS-treated counterparts (Fig. 4A). Combined BPN' i.p./i.n. administration induced a significant increase in TP in all tg mice compared to

that in PBS-treated groups including B10 and A β^0 animals (Fig. 4A). There was no significant difference between any of the tg mice. In addition, TP in BAL fluids of mice treated by combination of i.p./i.n. administration of enzyme was signif-

icantly higher than that of corresponding mice treated by repeated i.n. application alone. It should be noted that the combination treatment increased the RBC count in the BAL of all mice that would contribute to the TP index.

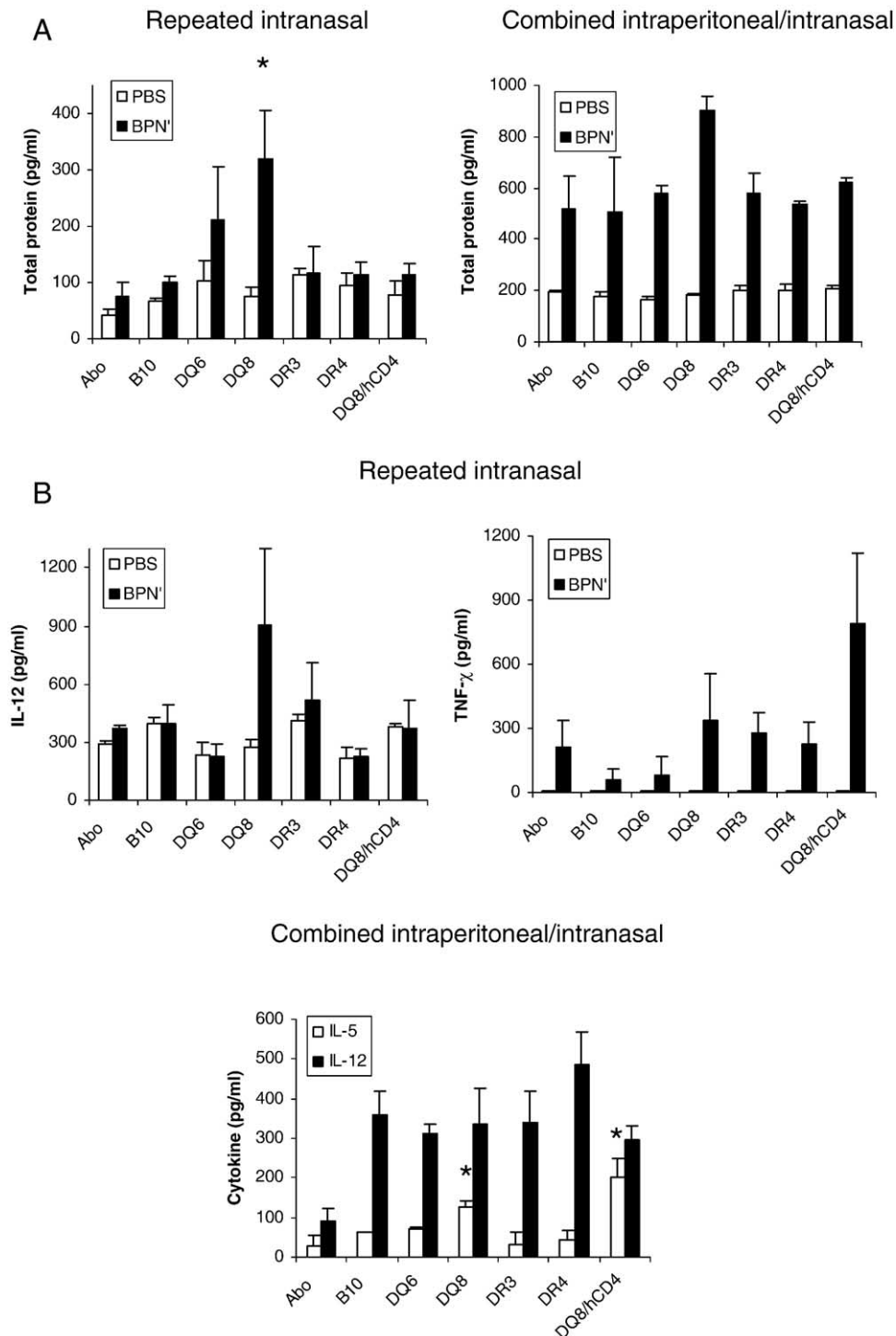


Fig. 4. Total protein (A) and cytokine (B) levels in BAL fluid supernatants taken either 5 days after completion of repeated i.n. BPN' challenge or 2 days after completion of combined i.p./i.n. BPN' administration. (A) Significant increase ($P < 0.03$) in TP was observed in the DQ8 mice dosed i.n. compared to control animals (B10 and A β^0 mice) and PBS-treated counterparts. High levels of TP were detected in all mice after the combined i.p./i.n. immunization ($P < 0.03$) compared to mice treated with PBS by the same protocol. (B) Cytokine levels were measured in BAL fluid supernatants by ELISA. * $P < 0.05$, DQ8 and DQ8/hCD4 mice vs. other groups.

Cytokine levels in BAL fluids

To explore the role of local cytokine expression in BPN'-induced airway inflammation, we quantitated protein levels of cytokines in BAL fluids. Among all the cytokine studied, substantial amounts of IL-12 and TNF- α were detected in mice after repeated i.n. BPN' application (Fig. 4B). IL-12 was detected in both PBS- and BPN'-dosed mice. The only significant effect of BPN' administration was an increase in IL-12 levels in DQ8 mice above control levels. We observed elevated expressions of TNF- α in all the enzyme-treated mice, which was the highest in DQ8/hCD4 mice. Sequestration of eosinophils into BAL of DQ8/hCD4 mice was accompanied by IL-4 (16.3+6.7 pg/ml; data not shown) but not IL-5 production (below detection limit).

Immunization via i.p./i.n. exposure resulted in detection of IL-5 and IL-12 (Fig. 4B). In contrast to repeated i.n. exposure, IL-5 was detected in all tg mice and controls 48 h after final i.n. dose with BPN'. Its concentration was significantly higher in DQ8 and DQ8/hCD4 mice compared to A β^0 controls.

Histologic assessment of lung tissue

The differences observed in the magnitude of BAL inflammatory cell infiltrates were confirmed with pulmonary histology (Fig. 5, Table 4). Following repeated BPN' i.n. administration, histopathological examination of lungs taken from tg and control mice on day 29 demonstrated a significant inflammatory cell accumulation in DQ8 and DQ8/hCD4 mice. These multiple cellular infiltrates extended from the center of the lung to the visceral pleura. The lungs of the PBS-treated tg mice and the BPN'-treated A β^0 mice were without pathology and occasionally showed one or two foci of inflammatory cells consisting predominantly of macrophages. The lungs of enzyme-treated B10 and A β^0 mice appeared normal with no sign of inflammation.

Sensitization of mice with i.p. BPN' in alum and subsequent i.n. challenge significantly increased the severity of cellular infiltration and the mean score of pulmonary inflammation observed in tg mice compared with counterparts treated by

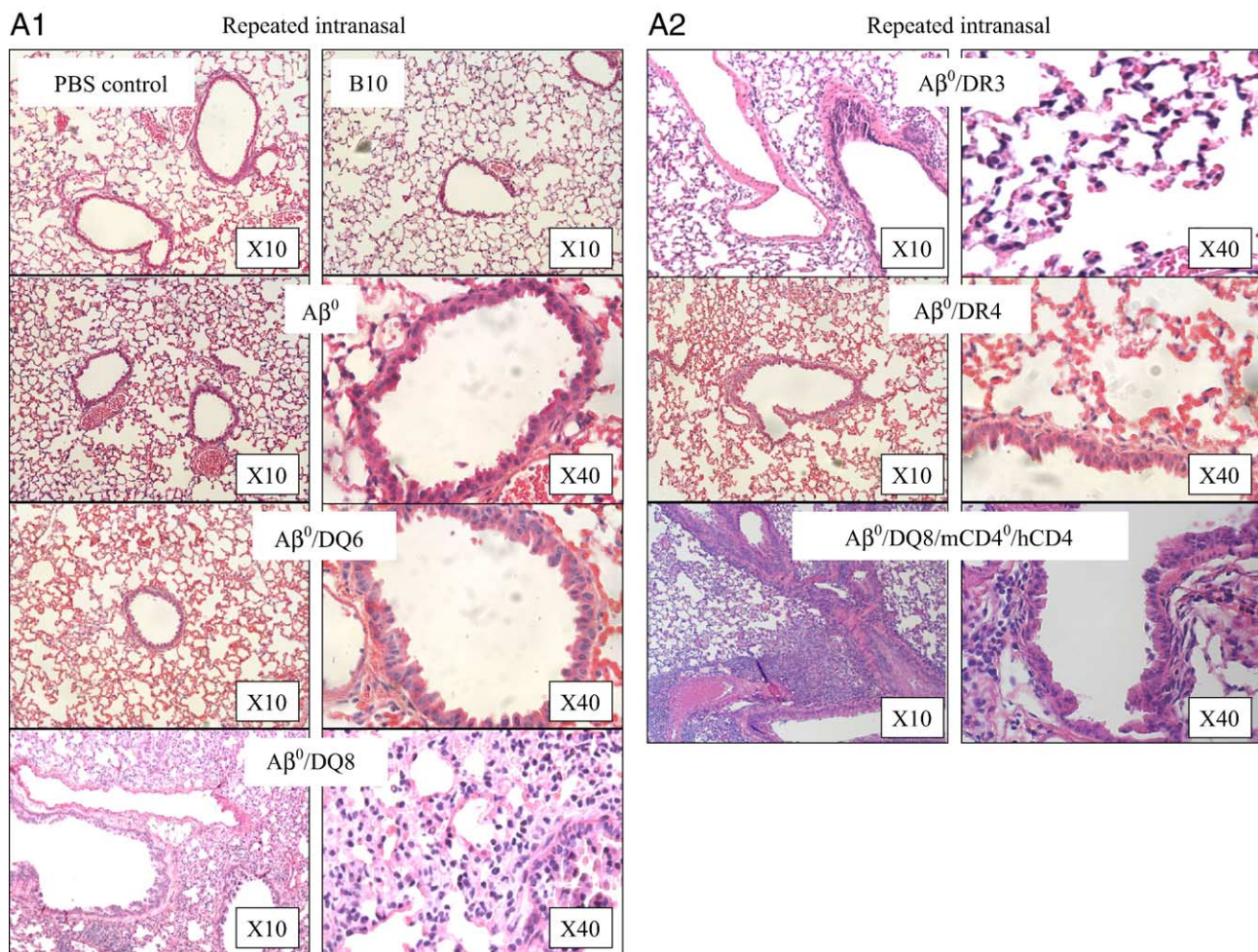


Fig. 5. Histologic examination of lung tissue. The lungs taken from PBS-treated mice represent normal lung histology. No inflammation was observed in A β^0 , B10, DQ6, DR3, and DR4 mice. In contrast, lungs from DQ8 and DQ8/hCD4 mice demonstrated multiple dense perivascular and peribronchial inflammatory infiltrates. Combined i.p./i.n. BPN' immunization resulted in hemorrhagic edema and alveolar wall damage in all HLA Class II tg mice, with DQ8 and DQ8/hCD4 mice being affected the most. The infiltrate in both DQ8 and DQ8/hCD4 mice consisted predominantly of eosinophils, whereas primarily neutrophils were present in the infiltrates found in the lungs of other animals.

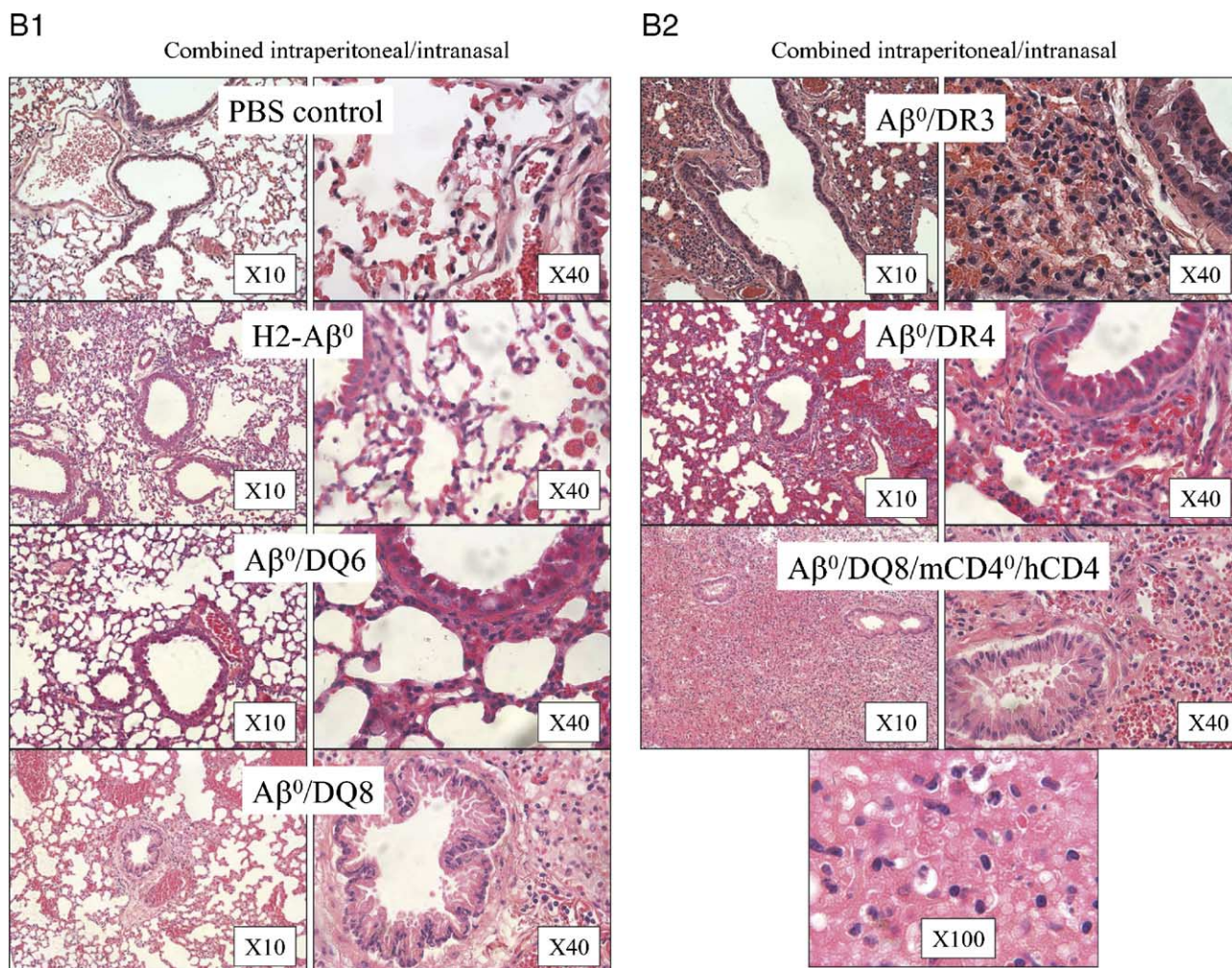


Fig. 5 (continued).

repeated i.n. challenge protocol (Fig. 5, Table 4). Hemorrhagic edema and alveolar wall damage were observed in all HLA Class II tg mice. The severity of this process was different among different tg mice, with DQ8 and DQ8/hCD4 mice being most affected. In DQ8 mice, hemorrhagic alveolitis progressed to pneumonia with desquamation of lining alveolar cells and appearance of scattered neutrophils. In DQ8/hCD4 mice, the altered permeability of capillary walls was accompanied by alveolar wall damage and edema. The inflammatory infiltrate in both DQ8 and DQ8/hCD4 mice consisted predominantly of

eosinophils with a significant number of admixed neutrophils, lymphocytes, and macrophages. In contrast, other tg and control mice did not demonstrate eosinophilic inflammation but contained neutrophils in contrast to i.n. exposure only. In contrast to tg mice, Aβ⁰ mice demonstrated phagocytosis of RBCs by histiocytes but no other signs of inflammation or allergy.

Discussion

We generated and used several lines of HLA Class II transgenic mice to demonstrate, for the first time, that HLA-DQ8 is a susceptible molecule for detergent enzyme BPN'-induced hypersensitivity. Among all the tg mice studied, only DQ8 mice were able to recognize and respond to several determinants on BPN' using three different routes of enzyme application at the time points studied. In addition, three different lines of mice containing the DQ8 transgene identified the same immunodominant T cell epitopes. Only DQ8 mice demonstrated significant BAL and lung tissue eosinophilia in response to mimicked natural ambient exposure to enzyme using only the intranasal route. The DQ8 mice also responded to BPN' with a significant IgG1 and IgE production.

Table 4
Grade assessment of lung tissue histologic changes^a

Mice	Repeated i.n.	Combined i.p./i.n.
Aβ ⁰	0±0	0.5±0.19
B10	0.25±0.16	**
DQ6	0.62±0.18	3±0
DQ8	1.63±0.26	3±0
DR3	0.75±0.16	3±0
DR4	0.62±0.18	3±0
DQ8/hCD4	2.38±0.18	3±0

**Not done.

^a Shown in mean grade of inflammation by grading system described in Materials and methods.

Substitution of mCD4 to hCD4 in the susceptible DQ8 mice potentiated the pulmonary disease outcome as demonstrated by histopathology. DR4 mice responded to several determinants on BPN' using combined i.p./i.n. immunization protocol, however, this immunization did not lead to either local (Th2 cytokines, lung eosinophil sequestration) or general (IgG1 and IgE) Th2 response.

The ultimate goal of these studies was to use HLA Class II transgenic mouse model to determine the HLA restriction of BPN' immunogenicity and allergenicity and compare it to the results obtained for detergent factory employees. The frequency of HLA-DR and HLA-DQ alleles in general population is documented in www.allelefreqencies.net. HLA-DR alleles chosen for generation of tg mice used in this study are representative of 12 common DR types among the worldwide population [30]. The conventional laboratory mice have been extensively used to examine the local lymph node proliferation [31], Ab response [23], and the influence of mouse genetics in response to different detergent enzymes [32]. These animal models have also been used successfully to rank the allergenicity of detergent enzymes for occupational safety. However, in all of these animal models, MHC class II molecules are mouse-derived. The association of HLA with BPN' susceptibility has not been examined to date. Preliminary evidence in detergent plant workers suggests that a number of different haplotypes have developed allergy to BPN' but that DQ8 is a susceptible allele and the haplotype DQ6/DR15 is a protective haplotype (E.S. Finn and A.C. Pursfull, unpublished observation). Therefore, "humanized" HLA Class II mice should provide a useful tool for evaluating allergenic responses to enzymes and examining differences due to MHC Class II molecules.

The immune response to Tri r 2, a subtilase homologue derived from the dermatophyte fungus *Trichophyton rubrum*, was examined in patients with immediate (IH) and delayed hypersensitivity (DTH) to fungal extract and to purified r-Tri r 2 and in normal control subjects [33]. T cell epitopes were identified and compared between groups using 28 overlapping peptides. There was no significant difference in the frequency of HLA-DR and -DQ Ags between subjects with either IH or DTH to Tri r 2. Furthermore, seven subjects with DTH who had the strongest PBMC proliferative responses to one epitope had diverse HLA class II haplotypes. However, authors do not specify if there were some significantly increased HLA phenotype frequencies in patients with IH and DTH compared to those individuals who had neither a delayed nor an immediate skin test response to *Trichophyton* extract and r-Tri r 2.

The genetic background of mice, in particular non-MHC genes, has been shown to significantly influence the nature of the immune response to Ag [34]. Several studies demonstrated that different APC clones/cell types from the same or different non-MHC genetic backgrounds may display different immunogenic peptides from a given Ag [34–36]. In this work, we present evidence that the BPN' immunodominant T cell epitope repertoire recognized by DQ8 tg mice is not modulated by two non-MHC genetic backgrounds, B10 or B6/129J. The same

pattern of response to BPN' in both DQ8 tg mice also indicates a very stable immunogenicity of enzyme. Moreover, there is an indication that the immunogenicity of BPN' is independent of the non-MHC genetic polymorphism, and this may eliminate one possible limit to the use of HLA Class II tg mice for protein immunogenicity screening.

Three different routes of exposure in the HLA Class II mice were evaluated. Studies by Brooks et al. [37] and Todryk et al. [38] have shown that route of immunization can affect T cell epitopes. In addition, it is also important to consider if adjuvants could alter the T cell epitope recognition pattern as they have been shown to alter other aspects of immune responses [39,40]. Similar T cell epitope patterns were found in DQ8 tg mice using a single subcutaneous dose with CFA and combined i.p./i.n. with alum administrations. Therefore, in these mice, the epitope pattern did not appear to be altered under the conditions studied. However, the route of exposure and/or adjuvant may modulate the magnitude/timing of the T cell responses as we demonstrate here for DR4 mice. In addition, the intranasal route of exposure takes longer to induce a maximum antibody and T cell response.

Utilizing HLAII tg mice and repeated i.n. challenge with BPN', we have developed an HLA-DQ8-restricted mouse model of occupational asthma/hypersensitivity characterized by multiple eosinophilic infiltrates around the pulmonary blood vessels and airways as a characteristic feature of allergic airway inflammation. Repeated respiratory exposure to either purified (OVA) or recombinant (recombinant major antigens from different allergens) proteins induces tolerance in murine models. To overcome this, a low dose of LPS acting in a TLR4-dependent manner is required [41]. However, a repeated respiratory exposure to allergen with enzymatic activity such as house dust mite allergen demonstrated a Th2 sensitization and airway eosinophilic inflammation [42]. This response was mediated by endogenous GM-CSF-dependent expansion and activation of local APC. Utilizing HLA Class II tg mice and conventional exposure (i.p./i.n.) protocols to Ag, we observed that hemorrhagic alveolitis progressing to pneumonitis was a characteristic feature of airway inflammation and remodeling observed in DQ8 and DQ8/hCD4 mice. An extensive diffuse parenchymal inflammatory response consisting of eosinophil, neutrophil, and mononuclear cell infiltrate was seen in the lung tissue of BPN-sensitized and challenged DQ8 and DQ8/hCD4 mice compared with PBS-treated counterparts and other BPN-treated animals. Hypersensitivity pneumonitis is considered to be an occupational lung disease, and it is observed in response to the inhalation of different organic and inorganic substances [43]. The immunologic mechanism mediating this illness is still poorly understood. If unrecognized, it can progress to pulmonary fibrosis.

The studies in DQ8 mice have identified five regions containing immunodominant T cell epitopes. These five regions are defined as follows: (1) amino acids 40–63 (peptides #14, 15, 16, and 17), (2) 106–123 (peptides #36 and 37), (3) 124–141 (peptides #42 and 43), (4) 184–201 (peptides #62 and 63), and (5) 238–255 (peptides #80 and 81).

For comparison to humans, we have seen that a common epitope in ~87% of plant personnel (BPN' skin prick test positive) is in the region from #35 to 37, and a similar epitopes was seen in the DQ8 mice, indicating similarity with respect to this region [44]. These studies suggest that these peptides are promiscuous epitopes. Further studies are needed to clarify T cell epitopes with respect to allele typing, but it is encouraging that similarities are being seen between humans and animal models.

It has been shown that some Th2 cytokines (IL-4, IL-5, IL-9, IL-13) and some pro-inflammatory cytokines (TNF- α , INF- γ) contribute to the development of allergic asthma or airway hyperreactivity [45]. To evaluate cytokines involved in the inflammatory and immune process observed in tg mice to BPN' treatment, IL-2, IL-4, IL-5, IL-9, IL-12p40, IL-13, IFN- γ , and TNF- α were measured by ELISA in BAL fluids. Following repeated i.n. BPN' exposure, IL-5 was not detected in any BAL samples. Eosinophilia observed in DQ8/hCD4 mice was accompanied by a slight increase in IL-4. These tg mice also demonstrated increased levels of pro-inflammatory and fibrogenic TNF- α . TNF- α can play multiple roles in occupational asthma such as potentiation of airway mucosal inflammation [46], enhancing T cell transendothelial migration [47], and induction of airway neutrophilia [48]. The presence of TNF- α in BAL fluids obtained from all the experimental mice including control animals may suggest that release of some cytokines may be due to a direct action of the enzyme on the respiratory tract and not a specific immune response. There is some evidence with another enzyme, amylase, that suggests that the enzyme activity itself is able to induce cytokine release (including TNF- α) [49], creating the possibility that the immunogenicity of enzymes may be linked to their enzymatic activity [50]. IL-12 also plays a regulating role in hypersensitivity pneumonitis and was significantly increased only in DQ8 tg mice. The combined i.p./i.n. administration induces greater cytokine response than repeated i.n. exposure. IL-5 is seen in BAL of DQ8 mice after this route of administration.

The results clearly indicate that DQ8 has a selective, although not exclusive, ability to present BPN' to CD4⁺ T cells compared to DQ6, DR2, DR3, and DR4 and is a susceptible allele for BPN' hypersensitivity. Importantly, the DQ8 allele that mediates presentation of BPN' in these tg mouse studies appears to be involved in disease susceptibility in detergent plant workers (E.S. Finn and A.C. Pursifull, personal communication).

Our findings have several important implications for this expanding interest in the allergenicity of enzymes. First, these studies suggest that HLA Class II transgenic mice, particularly DQ8, should be studied further as a model of human allergy/asthma to BPN'. Second, these mice may become potential models to screen newly engineered enzymes which preserve all consumer benefits but lack the allergenic/immunogenic T cell epitopes. Third, these tg mice will be useful to further dissect the genetics and mechanisms of susceptibility to occupational asthma in general and hypersensitivity pneumonitis in particular.

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