



Aspergillus fumigatus Peptides Differentially Express Th₁ and Th₂ Cytokines

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KURUP, V. P., V. HARI, J. GUO, P. S. MURALI, A. RESNICK, M. KRISHNAN AND J. N. FINK. *Aspergillus fumigatus* peptides differentially express Th₁ and Th₂ cytokines. PEPTIDES 17(2) 183–190, 1996.—Relevant allergens from *Aspergillus fumigatus* associated with allergic bronchopulmonary aspergillosis (ABPA) have been cloned and expressed. The pathogenesis of ABPA probably depends on specific cytokines and immunoglobulins secreted by lymphocytes on stimulation with different epitopes of those allergens. In the present study, we synthesized peptides of 12–16 amino acids from the sequence of *Asp f1* and compared their immunological responses in four mice strains (BALB/c, C57BL/6, AKR, and CBA). Of the five peptides studied for their cytokine profile, one showed a clear Th₁, whereas another showed a Th₂ response. The remaining three peptides varied in their immunoreactivity. The results suggest that a number of epitopes of diverse activities are present in individual molecules and may be involved in the pathogenesis of ABPA through differential cytokine secretions.

<i>Aspergillus fumigatus</i>	Ribotoxin	Cytokines	IgE	IgG	T-helper 1	T-helper 2
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Aspergillus fumigatus (Af), a ubiquitous fungus, is implicated in the pathogenesis of a number of clinically different diseases in man. These include allergic asthma, allergic bronchopulmonary aspergillosis (ABPA), hypersensitivity pneumonitis, aspergilloma, and invasive aspergillosis (18). The first three disorders are allergic in nature and are caused by exposure to Af or related species, whereas aspergilloma is a result of saprophytic growth of the fungus in preexisting pulmonary cavities. These diseases caused by Af demonstrate immune responses to antigens of *Aspergillus*. Invasive aspergillosis results from widespread infection by Af in immunosuppression either due to targeted therapy or to existing disease.

The serum IgE response is significantly elevated in allergic asthma and ABPA, whereas both *Aspergillus*-specific serum IgE and IgG are common features of ABPA. Hypersensitivity pneumonitis demonstrates Af-specific serum IgG but not IgE, and both ABPA and hypersensitivity pneumonitis demonstrate antigen-induced lymphocyte activation and other cell-mediated immune responses (10,15–17,28,29,34).

Af antigens are diverse in their physiochemical and immunological characteristics (11,13,18,30). A number of protein and glycoprotein antigens react with specific antibodies in the sera of patients with allergic aspergillosis (18). Studies using lymphocytes from patients with allergic aspergillosis showed considerable diversity in their ability to secrete IgE antibody in vitro cultures in response to Af antigens. Published reports indicate

both stimulatory and suppressive responses of Af on in vitro cultures of lymphocytes (15,16,28,34). Even within a protein molecule, different epitopes exert different responses. In addition, secondary and tertiary structures and protein folding also contribute towards the antigenicity and in the immunoregulatory events (2,5,14,19,27,32,33,37). We have initiated the present study to evaluate the specificity of such epitopes in Af antigen associated with antibody and cytokine synthesis. In the present study, we have synthesized oligo peptides of 12–16 amino acids from mitogillin, a ribotoxin produced by *Aspergillus restrictus*, a species closely related to Af. *Asp f1*, a ribotoxin from Af, shares a high degree of sequence homology with mitogillin (1,20,25). Five Af peptides were selected, based on their T-cell epitope predictability, to immunize mice having different haplotypes to determine their humoral and cell-mediated immunological responses. The results indicate that the cytokine and antibody induction are dependent on the specificity of conformational sequence of the oligo peptides and probably also to their specific interaction with the histocompatibility antigen.

METHOD

Aspergillus Ribotoxins and Synthetic Oligo Peptides

Mitogillin isolated from *A. restrictus* was obtained as a lyophilized powder from Dr. G. F. Cooper, Michigan Department of Public Health. This preparation was used in our previous studies

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TABLE 1
AMINO ACID SEQUENCES OF SYNTHETIC PEPTIDES
OF MITOGILLIN

Peptide No.	Mitogillin Sequence	Amino Acid Sequence (One-Letter Code)
Peptide #1	1-15 (15-mer)	ATWTCINQQLNPKTN
Peptide #5	53-68 (16-mer)	NGYDGNGLIKGRTP
Peptide #9	115-130 (16-mer)	NPGPARVIYTYPNKVF
Peptide #10	128-140 (13-mer)	KVFCGIVAHQRGN
Peptide #11	135-146 (12-mer)	AHQGNQGDLRL

(15,35,36) and the amino acid sequence of mitogillin has been fully elucidated. Synthetic peptides were made based on the primary secondary and tertiary structures and other algorithms including hydrophobicity and amphipathicity of the sequence analyzed using a computer program obtained through the courtesy of Dr. David C. Feller of the MedImmune, Inc. (Gaithersburg, MD) (7). Five peptides were selected for the present study; four showed complete identity to the 18-kDa antigen, *Asp f1* (1,25). The amino acid sequences of these synthetic peptides are shown in Table 1. Peptide #9 differed in one amino acid residue at the 115th (first amino acid in the synthetic peptide) position, which is aspartic acid (D) for *Asp f1* and asparagine (N) for mitogillin. Peptide #11, representing the C-terminal end, was synthesized with an overlap of six amino acids (#135-140) from peptide #10.

The peptides were synthesized manually by a solid-phase peptide synthesis method using Fmoc (9-fluorenylmethoxycarbonyl) chemistry (3,4,12). Briefly, this method is as follows. Fmoc amino acids were purchased from Bachem (Torrance, CA) or

Peninsula Labs (Belmont, CA). The amino acids contained the following side-chain protective groups: N⁹-Mtr for arginine, trityl for cysteine and histidine, *o*-t-butyl for aspartic acid, glutamic acid, serine, threonine, and tyrosine, and boc for lysine.

The C-terminal amino acid of the peptide was attached to *p*-alkoxybenzyl resin cross-linked with divinyl benzene resin (Wang resin) in a 25 × 80 mm glass reaction vessel using dimethylaminopyridine (DMAP) and diisopropylcarbodiimide (DIC). The dried amino acid conjugated resin was allowed to swell overnight in dimethyl formamide (DMF), washed three times with DMF and deprotected by reacting with 50% piperidine in DMF for 20 min, and washed again in DMF prior to coupling with the next amino acid. Amino acid coupling involved the addition of a 4 M excess of the next Fmoc amino acid activated ester freshly prepared by reacting the Fmoc amino acid with HOBT/DIC. Coupling efficiency was monitored by the ninhydrin test (36) and, if necessary, the same amino acid was recoupled using a fresh amino acid activated ester prepared as above. After completion of synthesis, the peptide was cleaved with 95% TFA in the presence of the appropriate scavenger (9). The cleaved peptide was precipitated and washed with ether and dried.

Animals

BALB/c (Hd), C57BL/6 (Hb), AKR (Hk), and CBA (Hk) mice were obtained from Sasco (Omaha, NE) and housed in the Veterinary Medical Unit of the Milwaukee, Wisconsin, VA Medical Center. Ten- to 12-week-old animals were used for exposure to Af. Mice were given food and water ad lib. All the animal studies were carried out according to the guidelines and protocols of NIH and were approved by the Institutional Animal Studies Committee.

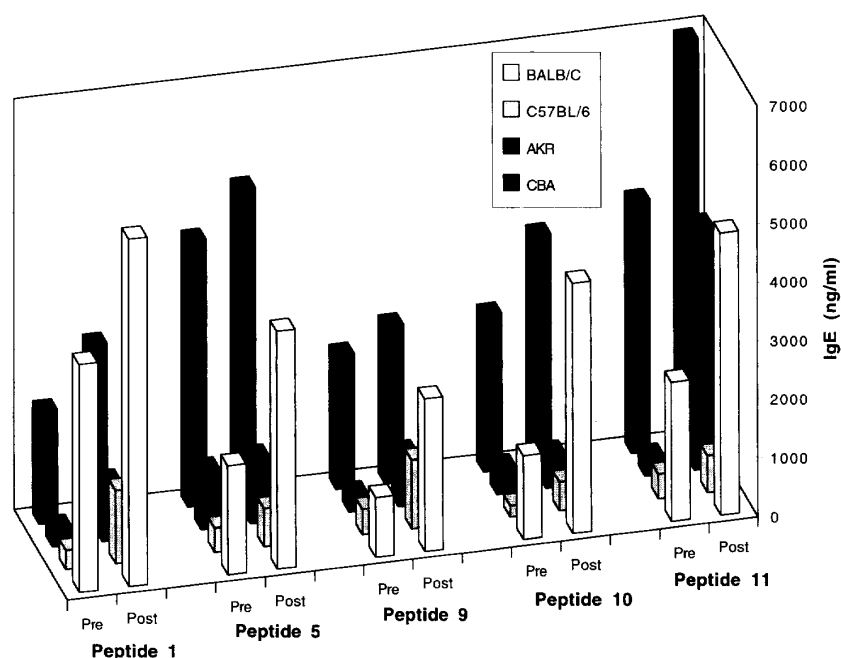


FIG. 1. Total serum IgE (ng/ml) in BALB/c, C57BL/6, AKR, and CBA mice immunized with different peptides; levels before (pre) and after (post) immunization.

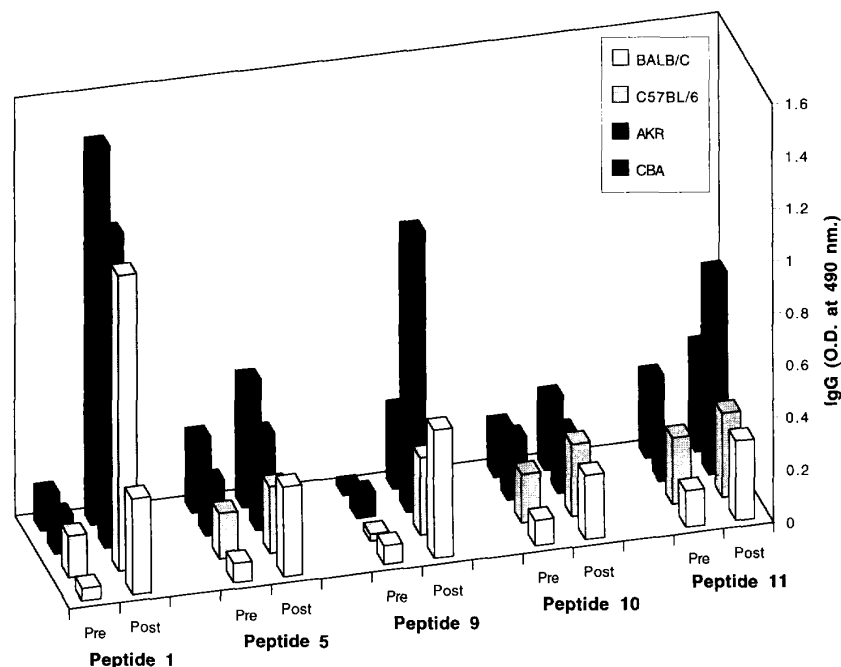


FIG. 2. *Aspergillus*-specific IgG in mice immunized with peptides. Details are the same as in Fig. 1.

Immunization of Animals With the Oligo Peptides

Five peptides synthesized from mitogillin were used to immunize the mice. Approximately 50 mg of peptide in 50 ml of PBS was mixed with equal volumes of Hunter's Titer Max (Cytodex, AL) and injected SC into the gluteal area (38). These injections were repeated once a week for 3 weeks, followed by four weekly IP injections without adjuvant. The animals were sacrificed 1 week after the last injection. Blood was collected from the heart to estimate total serum IgE and IgG antibodies to Af, mitogillin, and synthetic peptides. The spleen cells were prepared and stimulated *in vitro* with antigens and mitogen for antibody and cytokine production. Five animals were used for each peptide. Saline-treated animals from each group of mice served as controls.

Total IgE in Serum

Total serum IgE levels were determined using a monoclonal anti-mouse antibody as a capture antibody, as previously described (22). Briefly, ELISA plates (polystyrene microtiter plates, Immulon-2, Dynatech Laboratories, Inc., Chantilly, VA) were coated with rat anti-mouse IgE in PBS (pH 7.4) at room temperature for 2 h followed by overnight incubation at 4°C. The plates were washed with PBS containing 0.05% Tween-20 (PBS-T) and blocked with PBS-T containing 0.5% BSA (Sigma Chemical Company, St. Louis, MO). After further washing, dilutions of mice sera obtained before (pre) and after (post) immunization with oligo peptides were added to the wells. After incubation for 3 h at room temperature, the wells were washed with PBS-T as before. The wells were then treated with a 1:1000 dilution of biotinylated anti-mouse IgE (Sigma Chemical Company) followed by a 1:1000 dilution of streptavidin peroxidase (Sigma Chemical Company) each for 1 h. After washing, the substrate

(H₂O₂ and orthophenylene diamine) was added to the wells and the color developed was read in an automated ELISA reader (Dynatech Laboratories, Inc.) using 490 nm filter. The optical density (OD) values were converted to nanograms of IgE by comparison with standards run along with the test samples (22).

IgG Antibodies Against the Oligo Peptide and Mitogillin

To evaluate antibody to mitogillin and synthetic oligo peptides, ELISA plates were coated for 24 h at 4°C with 10 µg/ml of the respective peptides dissolved in PBS (20). After washing and blocking as described above, 100 µl of the mouse serum dilutions from pre- and postimmunization were added to each well and incubated for 3 h. The wells were then washed, and biotinylated anti-mouse IgG antibody was added to each well. After incubation, the plates were again washed. The plates were then incubated for 30 min after the addition of streptavidin peroxidase. After washing the plates, substrate was added to the wells and the color developed was read at 490 nm using an ELISA plate reader. Appropriate positive and negative controls were included in each plate. The results are expressed as OD values with the blank subtracted.

Spleen Cell Cultures

Spleen cells were separated from all mice studied as previously described (23). Two million cells/ml were cultured, with Concanavalin A (Con-A), mitogillin, or the oligo peptides, in RPMI 1640 medium supplemented with L-glutamine, antibiotics, and fetal bovine serum. After 60-h incubation, the supernatants were collected and studied for the expression of interleukin-2 (IL-2), IL-4, IL-5, IL-10, and interferon-γ (IFN-γ) by ELISA. The spleen cells from control as well as peptide-immunized mice were stimulated with Con-A, mitogillin, and respective pep-

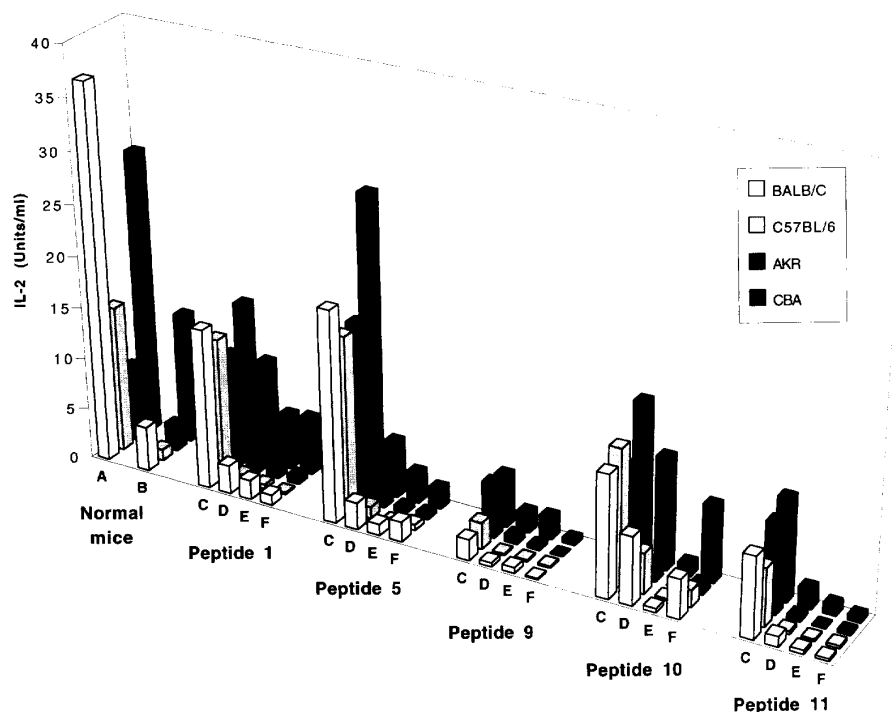


FIG. 3. In vitro IL-2 production by spleen cells of mice immunized with synthetic peptide and normals. A—spleen cells of saline treated mice stimulated with Con-A, and B—with Mitogillin; C—spleen cells from peptide-immunized mice stimulated with Con-A, D—spleen cells from peptide-immunized mice stimulated with mitogillin. E—spleen cells from normal mice stimulated with peptide and F—spleen cells from peptide-immunized mice stimulated with homologous peptide.

tides. The expression of total IgE and IgG antibody to peptides and mitogillin by spleen cells were studied by ELISA using culture supernatants obtained 7 days after incubation of cultures stimulated with various peptides and mitogillin (23). IL-2 was estimated by CTLL (ATCC-TIB-214) assay and the other cytokines were studied by ELISA.

Cytokine ELISA of Spleen Cell Culture Supernatants

Supernatants were assayed for IL-4, IL-5, IL-10, and IFN- γ by a double sandwich ELISA as previously described (22,23). Microtiter plates were coated overnight at 4°C with monoclonal antibodies against IL-4 (11B11), IL-5 (TRFK-5), IFN- γ (XMG1.2), or IL-10 (SXC₂) in PBS. After washing and blocking the wells, the samples were added and incubated for 3 h at room temperature. The wells were then washed and a biotinylated monoclonal antibody to respective cytokine was added and incubated for 1 h. The wells were washed as before, streptavidin peroxidase was added, and the color developed using OPD and substrate as previously described (23). The OD was read at 490 nm in an automatic ELISA plate reader (Dynatech Laboratories, Inc.). Dilutions of standards were run with the samples in each plate and the OD values compared and quantitated. The ELISA reagents were either obtained as a gift from Robert Coffman, Ph.D. from DNAX—Institute of Molecular and Cellular Biology (Palo Alto, CA) or purchased from Pharmingen (San Diego, CA).

IL-2 Production by CTLL Assay

Interleukin-2 (U/ml) expression in response to various peptides were measured using incorporations of [³H]thymidine by the IL-2-dependent cell line CTLL-2 (21). Purified murine-IL-2 (mIL-2) obtained from Pharmacia (Piscataway, NJ) was used as a standard. The CTLL cells were washed three times in Hanks Balanced Salt Solution (HBSS) and plated at 5×10^3 cells/100 μ l to each of the 96 wells of a round bottom tissue culture plate. Supernatants stored at -20°C were thawed and 100 μ l each was added to the wells and incubated for 24 h. The cultures were pulsed with 0.5 μ Ci/well of [³H]thymidine for 12–18 h. The cells were then harvested on glass fiber filter and their radioactivity measured on a beta scintillation counter. The incorporated radioactivity was converted into units using the standard curve. The mIL-2 had a detection limit of 0.15 U/ml.

RESULTS

Antibody Response to Synthetic Peptides

All four strains of mice studied showed only slight increase in total serum IgE levels in response to the oligo peptides from mitogillin (Fig. 1). Peptide #11 showed a fivefold increase of total serum IgE in AKR mice, whereas the IgE levels with sera of C57BL/6 showed no significant increase on immunization with any of the peptides.

Peptide-specific IgG antibody responses also varied among peptides and mouse strains (Fig. 2). All four strains of mice

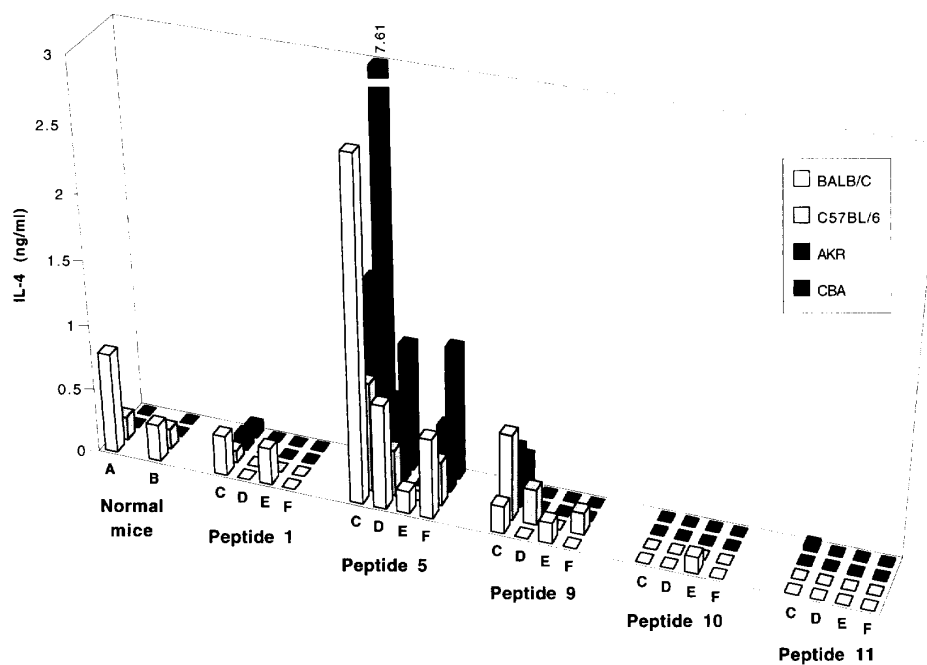


FIG. 4. In vitro IL-4 production by spleen cells of mice immunized with synthetic peptides. The details are the same as in Fig. 3.

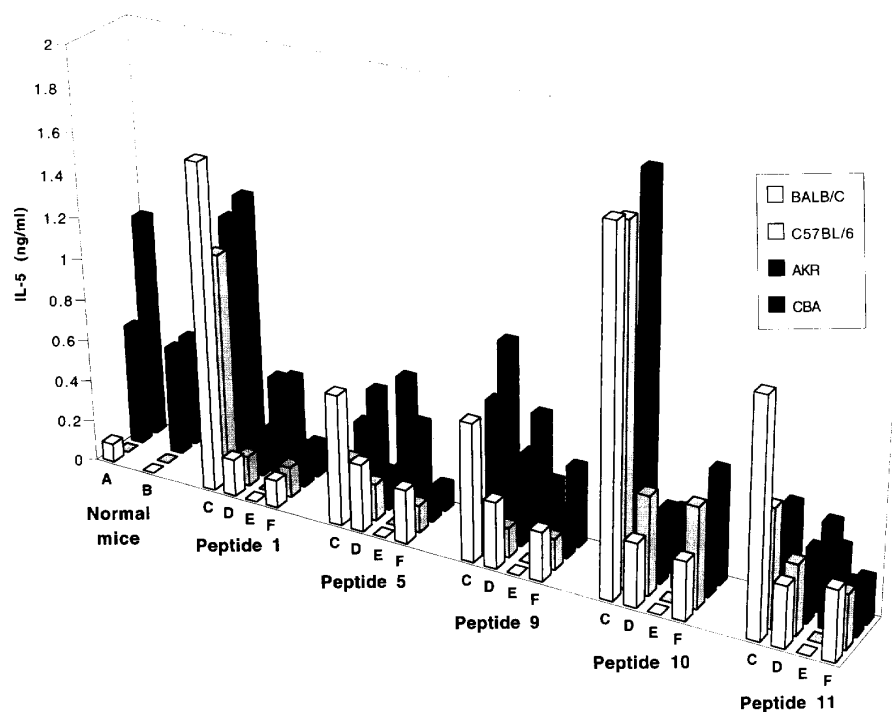


FIG. 5. In vitro IL-5 production by spleen cells of mice immunized with synthetic peptides. The details are the same as in Fig. 3.

produced higher antibody levels to peptides #1 and #9. Peptides #5 and #10 elicited the least antibody response in all four strains of mice studied. AKR mice showed over a fivefold increase in specific IgG antibody to the peptide, similar to that of IgE.

IL-2 Production

The results of IL-2 production in response to peptides are presented in Fig. 3. Spleen cells of all mice strains immunized with peptide #10 produced IL-2 when stimulated with mitogillin and peptide #10. None of the other peptides showed any marked increase in IL-2 production. All except peptide #9 showed enhanced IL-2 production on stimulation with Con-A.

IL-4 Production

Spleen cells from all four strains of mice immunized with peptide #5 produced IL-4 in vitro on stimulation with either the homologous peptide or mitogillin (Fig. 4). These cells also produced IL-4 in vitro in significant quantity when stimulated with Con-A. None of the other peptides produced IL-4 in in vitro cultures of spleen cells.

IL-5 Production

Spleen cells of BALB/c and C57BL/6 mice immunized with all five peptides produced enhanced levels of IL-5 when stimulated with the respective peptide or mitogillin. Spleen cells from normal animals failed to show any IL-5 production from these animals. However, with CBA and AKR mice no significant IL-5 production was detected over the normal controls (Fig. 5). The spleen cells of BALB/c mice showed enhanced IL-5 production

in spleen cultures when stimulated with Con-A compared to normal controls. Peptides #1 and #10 showed more IL-5 with Con-A stimulation compared to similarly stimulated spleen cells of normal mice.

IL-10 Production

No differences were detected in the IL-10 production of animals immunized with different peptides (data not shown). A slight increase was detected in BALB/c and C57BL/6 mice immunized with peptides #1, #10, and #11. The baseline IL-10 was consistently higher in AKR mice (data not shown).

IFN- γ Production In Vitro by Spleen Cells

Elevated levels of IFN- γ production was seen in CBA and AKR mice in response to peptide #10 (Fig. 6). Peptide #9 showed low levels of IFN- γ production in the in vitro spleen cell cultures from all strains of animals (Fig. 6). None of the other synthetic peptides studied or mitogillin showed any significant IFN- γ production. Con-A-stimulated spleen cells from normal animals showed very little IFN- γ in the culture supernates, whereas peptides #1 and #10 showed considerable amounts of IFN- γ .

In Vitro IgE and Specific IgG Antibody

IgE and specific IgG antibodies were not detectable in culture supernatants of spleen cells stimulated with synthetic peptides or mitogillin (data not shown).

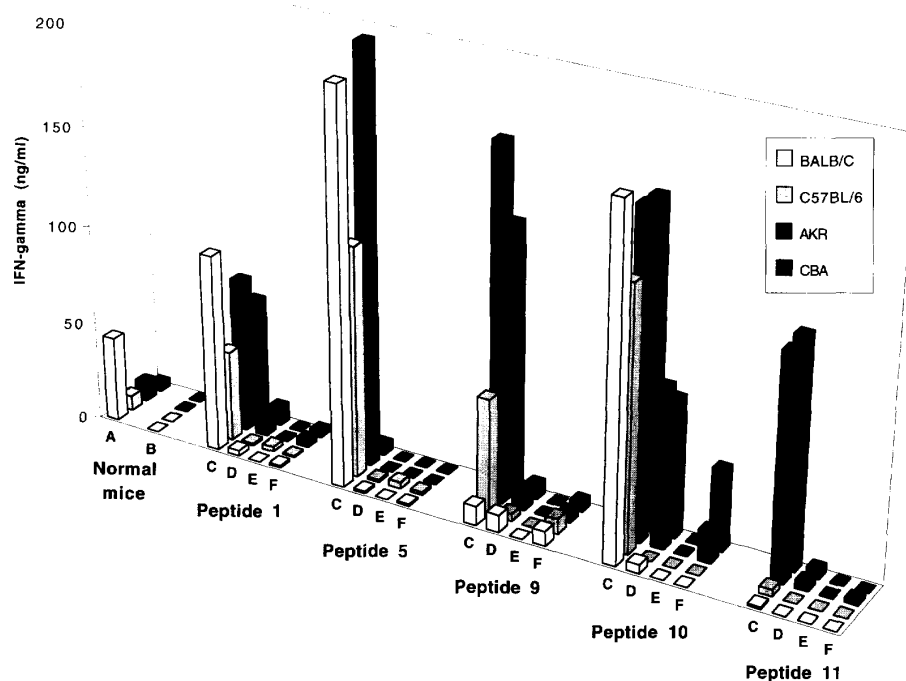


FIG. 6. In vitro IFN- γ production by spleen cells of mice immunized with synthetic peptides. The details are the same as in Fig. 3.

TABLE 2
TH₁ AND TH₂ CYTOKINES IN SPLEEN CELL CULTURES INDUCED IN VITRO BY PEPTIDES #5 AND #10

	IL-2 (U/ml)		IL-4 (ng/ml)		IFN γ (ng/ml)	
	Normal Mice	Peptide-Treated Mice	Normal Mice	Peptide-Treated Mice	Normal Mice	Peptide-Treated Mice
Peptide #5						
Balb/c	1.074 $\pm$ 0.14	1.894 $\pm$ 0.72	0.172 $\pm$ 0.04	0.617 $\pm$ 0.11*	UD	0.94 $\pm$ 0.34
C57BL/6	0.252 $\pm$ 0.03	0.565 $\pm$ 0.15	UD	0.335 $\pm$ 0.03†	3.215 $\pm$ 0.09	1.55 $\pm$ 0.29
CBA	2.895 $\pm$ 0.76	1.921 $\pm$ 0.14	UD	1.028 $\pm$ 0.06†	0.225 $\pm$ 0.04	0.22 $\pm$ 0.09
Peptide #10						
Balb/c	0.405 $\pm$ 0.04	3.786 $\pm$ 0.64*	0.128 $\pm$ 0.12	UD	UD	UD
C57BL/6	0.176 $\pm$ 0.01	1.613 $\pm$ 0.23*	UD	UD	UD	UD
CBA	1.142 $\pm$ 0.52	7.234 $\pm$ 0.94*	UD	UD	0.238 $\pm$ 0.05	40.35 $\pm$ 10.90‡

Spleen cells from normal mice and mice exposed to peptide #5 or #10 were stimulated in vitro with the same peptide. The supernatants from these cultures were tested for cytokines. UD = undetectable. Values are mean $\pm$ SEM.

* $p < 0.01$.

† $p < 0.001$.

‡ $p < 0.02$.

DISCUSSION

Of the several proteins isolated and purified from *Aspergillus fumigatus*, *Asp f1*, a closely related polypeptide of mitogillin, was found to be a major allergen associated with ABPA (6,35). All these ribotoxins, mitogillin, restrictocin, *Asp f1*, and α -Sarcin show marked homology and high toxicity to ribonucleoproteins. These ribotoxins from different species of *Aspergillus* showed reactivity with sera from patients with ABPA (6,35). Only minor differences were detected in the amino acid residues of mitogillin, *Asp f1*, and restrictocin (1,8,22,23,25,34). At the time the oligo peptides used in the present study were synthesized, the sequence of only mitogillin was known. Comparison of the sequences of mitogillin, *Asp f1*, and restrictocin with the synthetic oligo peptides designed indicates that the amino acid residues of four of five peptides, namely: #1, #5, #10, and #11, show complete homology with all three known sequences of ribotoxin, whereas peptide #9 showed a difference of one amino acid residue (i.e., asparagine at the 115th position in mitogillin is replaced by aspartic acid in *Asp f1*). However, a secondary structure assay failed to demonstrate any major differences due to this substitution.

Two major patterns of response were detected with the different peptides investigated. Peptide #10-stimulated spleen lymphocytes of all four strains of mice studied induced IL-2 (Fig. 3). This peptide and mitogillin also produced IFN- γ but not IL-4 in in vitro spleen cell cultures of CBA mice immunized with peptide #10. The results are summarized in Table 2. The cytokine production by spleen cells in response to peptide #10 was comparable to a CD4⁺ T-helper-1 (Th₁) response (26,31). However, additional studies are needed to determine the exact role played by this peptide in regulating the immune responses in allergic aspergillosis.

Peptide #5 induced a Th₂ type of response (Table 2). There was no or little IFN- γ production, whereas IL-4 production was significantly higher in spleen cell cultures of mice immunized with this peptide and stimulated with the peptide and mitogillin, compared to other peptide-immunized animals and controls. The IL-5 and IL-10 production and total serum IgE and *Aspergillus*-specific IgG antibody in the serum was also higher compared to other peptide-immunized mice and controls, indicating that peptide #5 is a responder of Th₂ cells (23,31).

The effect of overlapping amino acid sequences has demonstrated slight shifts in the cytokine profile as evidenced by the responses to peptides #10 and #11. Peptide #10 has six overlapping amino acids with peptide #11 and showed increased IL-2, decreased IL-10, and increased IFN- γ production. Antibody production was also much lower than peptide #11. These results suggest that the Th₁ type of response observed with peptide #11-immunized mice may be due to overlapping amino acids in peptides #10 and #11.

The present study employing synthetic peptides from A. *fumigatus* ribotoxin demonstrated predominant Th₁ and Th₂ epitopes in the same protein molecule. The study also demonstrated that the immune response of the epitope is also regulated by the MHC of the host, such as in inducing IFN- γ by CBA mice.

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