

# The Serpin Secreted by *Brugia malayi* Microfilariae, Bm-SPN-2, Elicits Strong, but Short-Lived, Immune Responses in Mice and Humans<sup>1</sup>

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Understanding the basic immunology of an infectious disease requires insight into the pattern of T cell reactivity and specificity. Although lymphatic filariasis is a major tropical disease, the predominant T cell Ags of filarial species such as *Brugia malayi* are still undefined. We have now identified a prominent T cell Ag from *B. malayi* microfilariae (Mf) as Bm-SPN-2, a serpin secreted exclusively by this stage. Mf-infected mice mounted strong, but short-lived, Bm-SPN-2-specific Th1 responses, measured by *in vitro* production of IFN- $\gamma$ , but not IL-4 or IL-5, 14 days postinfection. By day 35, responsiveness to Bm-SPN-2 was lost despite enhanced reactivity to whole Mf extract. Single immunization with Mf extract also stimulated typical Th1 reactions to Bm-SPN-2, but IgG1 Ab responses dominated after repeated immunizations. Human patients displayed potent humoral responses to Bm-SPN-2 in both IgG1 and IgG4 subclasses. Thus, 100% (20 of 20) of the microfilaremic (MF<sup>+</sup>) patients bore IgG4 responses to Bm-SPN-2, while only 30% of endemic normal subjects were similarly positive. Following chemotherapy, Bm-SPN-2-specific Abs disappeared in 12 of 13 MF<sup>+</sup> patients, although the majority remained seropositive for whole parasite extract. PBMC from most, but not all, endemic subjects were induced to secrete IFN- $\gamma$  when stimulated with Bm-SPN-2. These findings demonstrate that Bm-SPN-2 is recognized by both murine and human T and B cells and indicate that their responses are under relatively stringent temporal control. This study also provides the first example of a stage-specific secreted molecule that acts as a major T cell Ag from filarial parasites and is a prime candidate for a serodiagnostic probe. *The Journal of Immunology*, 2000, 165: 5161–5169.

Parasite genomes are predicted to encode between 5,000 and 20,000 proteins, and consequently, these organisms are likely to present a considerable number of T cell-stimulating Ags to their hosts. As host defenses against most pathogens critically depend on the activation of Ag-specific T cells (1), the identification of the Ags they recognize is a prerequisite for advancing measures to control parasitic diseases. Surprisingly, the major T cell Ags of most parasite species are still undefined due in part to present difficulties in identifying CD4<sup>+</sup> T cell-stimulating epitopes (2, 3).

Lymphatic filariasis is one of the most important human tropical diseases, with an estimated 120 million people infected and a further 900 million at risk of infection (4, 5). It is established that T cell responsiveness in infection is markedly down-regulated (6–11), although there is a heightened level of serum Abs, in partic-

ular IgG4 (12–15). The nature or manner by which filarial Ags elicit these responses, however, is poorly understood.

The filarial nematodes, *Brugia malayi*, *Brugia timori*, and *Wuchereria bancrofti*, have elaborate life cycles that provide a correspondingly complex set of interactions with the host immune system (16, 17). Among the most important issues relating to parasite-host interaction in filariasis is our lack of defined parasitic molecules involved in such interconnections. Particularly relevant molecules are likely to be excretory-secretory (ES)<sup>4</sup> products from parasites (18). A variety of ES molecules from parasitic helminths have been implicated in immune evasion strategies such as suppression of T and B cell proliferation (19–21), alteration of macrophage and granulocyte functions (18, 22), shedding of surface-bound Abs or ligands (23), and modulation of host inflammatory responses (18, 24). Immunopathology and protective immunity may also be induced by ES products (25).

Lymphatic vessel-resident *B. malayi* adult worms produce millions of microfilariae (Mf), which migrate into the bloodstream, surviving for long periods ( $\geq 1$  year). However, little is known of possible filarial ES molecules and mechanisms by which circulating Mf interact with the host immune system. Genes that are expressed in a stage-specific manner have important functional roles in the parasite life cycle, and thus may provide targets for the development of novel immuno- or chemoprophylactic agents. In this report we define a major new T cell-stimulating Ag from *B. malayi*, the serpin Bm-SPN-2, and provide evidence that not only is it stage specific, but it is secreted by Mf into their environment. The results also indicate that human filariasis patients respond

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<sup>4</sup> Abbreviations used in this paper: ES, excretory-secretory; Bm-SPN-2, *Brugia malayi* serpin (serine proteinase inhibitor)-2; BmA, *Brugia malayi* adult worm Ag; DEC, diethylcarbamazine; Mf, microfilariae; MfA, microfilarial Ag; MF<sup>+</sup>, microfilaremic (patients).

strongly to this Ag, and that a high level of Bm-SPN-2-specific Ab may prove a good diagnostic indicator of infection with *B. malayi*.

## Materials and Methods

### Parasites, Ags, and cDNA library

Adults and Mf were obtained from *B. malayi*-infected jirds (*Meriones guiculatus*) purchased from TRS Laboratories (Athens, GA). Somatic extracts of adult worms (BmA) or Mf (MfA) were prepared in PBS by disruption and sonication. Suspensions were centrifuged at  $10,000 \times g$  for 30 min at 4°C before passage through a 0.2- $\mu$ m pore size filter. Mf ES products were obtained by in vitro incubation for 24 h in serum-free medium (26). A *B. malayi* Mf cDNA expression library, SAW94LS-BmMf, was supplied by the Filarial Genome Project (27).

### Mice, immunization, and infection

Female BALB/c mice were immunized with Ags with or without IFA and CFA, purchased from Sigma (St. Louis, MO). Other strains were used for some experiments as indicated in the text. For infection studies, mice were injected i.p. with 300,000 *B. malayi* Mf.

### Study populations

The study population resided in the Rengat District of Sumatra, Indonesia, an area endemic for *B. malayi*. Two groups of 20 subjects were categorized as follows: 1) endemic normal: asymptomatic, Mf negative on Nuclepore (Pleasanton, CA) filtration of venous blood, with no history of filarial disease; and 2) microfilaremic: Mf positive on Nuclepore filtration. Serological and cellular immune responses in these subjects have been described previously (15, 28). PBMC from some of these individuals and from patients with elephantiasis were taken for in vitro cytokine responses. Sera were also obtained from five endemic normal subjects and 13 microfilaremic patients 2 years after beginning chemotherapy with diethylcarbamazine (DEC). Low dose DEC therapy (100 mg/wk) (29) was administered for up to 2 years, with an annual boost of  $3 \times 100$  mg for 10 consecutive days. One individual was drawn from a study in Palu, South Sulawesi, Indonesia, in which asymptomatic microfilaremics were sampled over 2 years before chemotherapy (30).

### SDS-PAGE electroelution

Mf were heated at 100°C for 10 min in SDS-PAGE loading buffer containing 2-ME and were centrifuged at  $14,000 \times g$  for 30 min. The supernatant was loaded onto 15% SDS-PAGE gels. After electrophoresis, slices from each side of the gel were stained with Coomassie blue, and the main gel was cut horizontally into 13 fractions. Gel slices were placed into molecular porous membrane tubing (Spectrum, Laguna Hills, CA), and the proteins were electroeluted on a Western blot transfer apparatus (Hoeffer, San Francisco, CA) for 2 h. After elution, all samples were dialyzed against PBS at 4°C for 48 h. Precipitated SDS and SDS-protein complexes were then removed by centrifugation at  $14,000 \times g$  for 30 min, and the soluble fraction was passed through a 0.2- $\mu$ m pore size filter. Fractions were visualized by SDS-PAGE and silver staining to determine the molecular range of proteins within each fraction. The protein concentration in each fraction was determined by Bradford assay.

### Cell sorting using magnetic beads

The MACS magnetic cell sorting system (Miltenyi Biotec, Bergisch Gladbach, Germany) was used for purification of CD4<sup>+</sup> T cells by positive selection. Splenocytes were stained with MACS CD4 (L3T4) MicroBeads (model 492-01, Miltenyi Biotec) and then added to an MS+ separation column (model 422-01). Positively selected CD4<sup>+</sup> T cells were obtained after removal of the column from the magnet. More than 90% of recovered cells expressed CD4, as determined by flow cytometry.

### T cell proliferation and cytokine assays

Murine popliteal lymph node cells or purified CD4<sup>+</sup> T cells were incubated as previously described (31), except that irradiated (2000 rad) spleen cells were used as APC. After culture for 60 h at 37°C, 100  $\mu$ l of supernatant was removed from each well for cytokine analysis; 1  $\mu$ Ci of [<sup>3</sup>H]thymidine was then added to each well, and plates were incubated for 12–18 h at 37°C before harvesting and counting. For human lymphocyte assays, Ficoll-Hypaque-isolated PBMC were prepared in Iscove's medium containing 20% human serum and incubated at  $1 \times 10^5$  cells/well in 200- $\mu$ l triplicate cultures. After incubation for 5 days at 37°C, 100  $\mu$ l of supernatant was removed from each well for cytokine analysis, and thymidine incorporation was assayed as described for murine cells. Murine (31) and human (32)

IFN- $\gamma$  and IL-5 were measured by capture ELISA, and IL-2 production was assessed by measuring the proliferation of the NK cell line as previously described (31). IL-4 was measured in previous experiments by the NK assay (31) and subsequently by ELISA. Standard curves using recombinant IFN- $\gamma$ , IL-4, IL-5, and IL-2 (Genzyme, Cambridge, MA) were performed to determine cytokine levels in culture supernatants.

### Ag-specific IgG isotype ELISA

Specific murine IgG isotypes were measured by ELISA as previously described (31). For murine IgG isotypes, peroxidase-conjugated goat anti-IgG1 (1/6000; Southern Biotechnology Associates, Birmingham, AL; SBA 1070-05), anti-IgG2a (1/200 for C57BL/6 and 1/4000 for other strains; SBA 1080-05), anti-IgG2b (1/4000; SBA 1090-05), or anti-IgG3 (1/1000; SBA 1100-05) were used. For human IgG isotypes, isotype-specific mouse mAb anti-IgG1 (1/4000; SkyBio, Wyboston, U.K., M15015), anti-IgG2 (1/2000; SkyBio, M10015), anti-IgG3 (1/1000; SkyBio, M74011), or anti-IgG4 (1/4000; SkyBio, M11013) were employed.

### Immune screening of cDNA library and DNA sequencing

The *B. malayi* Mf cDNA expression library was screened using murine antisera (preadsorbed against *Escherichia coli* lysate) diluted at 1/400. Immunoreactive plaques, detected using peroxidase-conjugated rabbit anti-mouse IgG (Dako, Copenhagen, Denmark) and the tetramethyl benzidine membrane peroxidase substrate system (Kirkegaard & Perry Laboratories, Gaithersburg, MD) were purified to homogeneity by two subsequent rounds of low density plaque screening. Bluescript phagemids were then excised from positive clones using the manufacturer's protocols and sequenced. All sequencing was performed on an Applied Biosystems 377 automated sequencer with the ABI PRISM Dye Terminator cycle sequencing method (Perkin-Elmer, Palo Alto, CA). Sequence analysis was performed using MacVector program version 6.0 (Oxford Molecular Group, Oxford, U.K.). Sequence searching was performed with the BLAST algorithm (33) using the National Center for Biotechnology Information BLAST server.

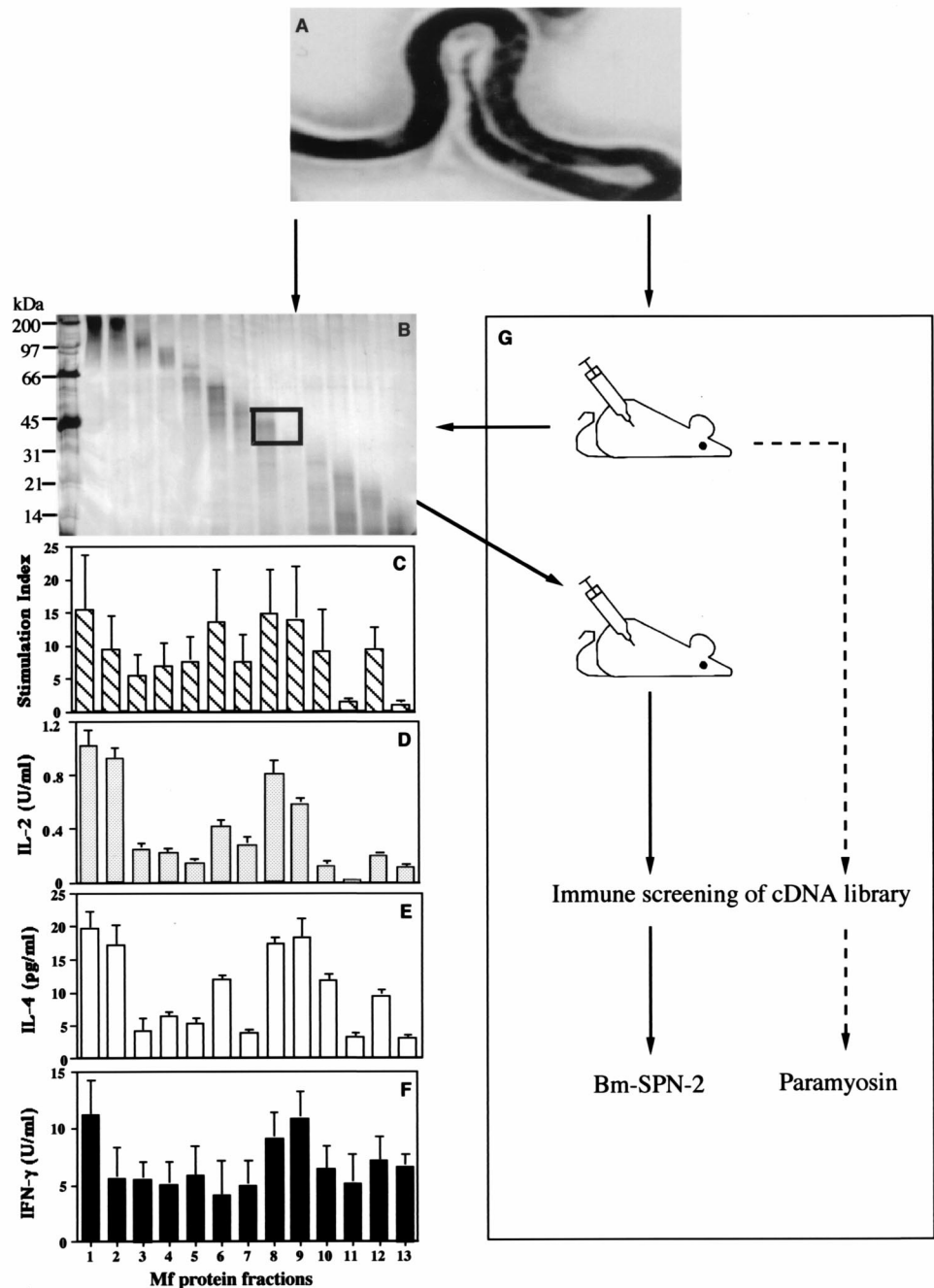
### Construction of mammalian expression vector and DNA immunization

pcDNA3.1(-) (Invitrogen, San Diego, CA) was used to construct a Bm-SPN-2 mammalian expression vector, designated Bm-spn-2/pcDNA3.1(-). Thus, the forward primer PCDNA-SERPIN (5'-CGGG CCCGCCACATG GAGCTTTTCGAAGTA-3') is homologous to bp 31–44 of *Bm-spn-2* cDNA (34) and contains an *Apa*I site and the mammalian Kozak sequence for translation initiation; the reverse primer pcDNA3.1 (2R) (5'-TGGTGAATTCATA GACAATTCGC GTGGATA-3') contains an *Eco*RI site and is homologous to bp 1380–1361. The DNA produced by PCR using primers PCDNA-SERPIN and pcDNA3.1 (2R) was subcloned into the pcDNA3.1(-) *Apa*I and *Eco*RI sites to express Bm-SPN-2. The positive constructs were examined by DNA sequence. Large scale preparations of Bm-spn-2/pcDNA3.1(-) plasmid DNA were prepared using the Qiagen EndoFree Plasmid Mega Kit (Valencia, CA) and eluted in 0.9% sodium chloride solution. The 260:280 ratio of plasmid DNA is >1.8. For DNA immunization, 100  $\mu$ g of Bm-spn-2/pcDNA3.1(-) plasmid or pcDNA3.1(-) alone as a control was injected into the quadriceps muscles of mice. Two weeks later, the mice were boosted with 100  $\mu$ g of plasmid DNA. Blood was taken for Ab measurement 2 wk after the booster.

### Expression and purification of proteins in *E. coli*

Primers serpin-Exp.F (CAACAGTACTTTAAACCATTGTTCTG), corresponding to bases 87–111 of the *Bm-spn-2* cDNA (34), and Serpin-Exp.R (CTAACCTTTGCTTTTTTTTCG GTGTTTCC), complementary to bases 1283–1310, were designed for in-frame expression of the Bm-SPN-2 mature protein (aa residues 21–428) in the pET-29 T-Vector (Novagen, Madison, WI). This construct, designated Bm-SPN-2/pET-29, was sequenced to confirm the fidelity of its reading frame. A control recombinant protein, Bm-MIF-2G (X. Zang et al., manuscript in preparation), was expressed in the same system. Bm-SPN-2/pET-29 and Bm-MIF-2G/pET-29 were transformed into BL21(DE3) *E. coli*. Transformed BL21(DE3) cells were induced with 1 mM isopropyl-1- $\beta$ -D-galactopyranoside (Stratagene, La Jolla,

**FIGURE 1.** Strategy used to isolate T cell-stimulating Ags from *Mf. Microfilariae* of *B. malayi* (A) were solubilized in SDS-PAGE loading buffer, and proteins were fractionated by m.w. on SDS-PAGE followed by electroelution. Thirteen distinct fractions were recovered (B). BALB/c mice were primed s.c. with 20  $\mu$ g of MfA in CFA and 5 wk later were boosted with MfA in IFA injection into the footpads. Popliteal lymph node cells were taken 10 days later, challenged in vitro with the Mf fractions, and assayed for T cell proliferation (stimulation index; C) and cytokine release (IL-2, IL-4, and IFN- $\gamma$ ; D–F). Cytokine concentrations were determined by ELISA for IFN- $\gamma$  and by NK cell bioassay for IL-2 and IL-4. Antiserum was generated against fractions 8 and 9 (boxed), which contained the most potent stimulators of Ag-specific T cell proliferation and cytokine production. This serum was used to screen an Mf cDNA library, from which Bm-SPN-2 was identified as an Ag. In contrast, antiserum raised against whole MfA detected paramyosin, a previously characterized, serologically reactive protein.



CA) for 4 h at 37°C, then harvested, and sonicated. Recombinant Bm-SPN-2 and Bm-MIF-2G, containing six C-terminal histidine residues (His-Tag), were purified by affinity chromatography over His-Bind resin. The purified recombinant proteins were dialyzed in TBS (150 mM NaCl and 20 mM Tris-HCl, pH 7.9) for 48 h at 4°C and then passed through a 0.2- $\mu$ m pore size filter.

#### Western blot

Proteins mixed with an equal volume of 2 $\times$  SDS-PAGE sample buffer were boiled for 10 min. Insoluble debris was removed by centrifugation, and SDS-soluble proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. Blot strips were incubated with mouse anti-recombinant Bm-SPN-2 or control mouse sera, diluted 1/4000, and then with peroxidase-conjugated rabbit anti-mouse IgG (Dako). The bound Abs were detected by chemiluminescence on addition of the luminol-based enhanced chemiluminescence substrate (Amersham, Aylesbury, U.K.).

## Results

### Identification of T cell-stimulating fractions from *B. malayi* Mf

Lymph node cells from BALB/c mice immunized with MfA, when challenged in vitro with MfA, show strong proliferative responses and Ag-specific release of IL-4 and IFN- $\gamma$  (31). We challenged MfA-primed lymph node cell populations with fractionated Mf proteins, separated by SDS-PAGE and recovered by electroelution (Fig. 1). Thirteen distinct fractions were measured for T cell antigenicity, with maximal activity observed in fractions 1 and 2 ( $M_r$  region of 130–220 kDa) and in fractions 8 and 9 (35–55 kDa) as measured by T cell proliferation and IL-2, IL-4, and IFN- $\gamma$  production (Fig. 1). A moderate response was mounted to fraction 6 (50–65 kDa).

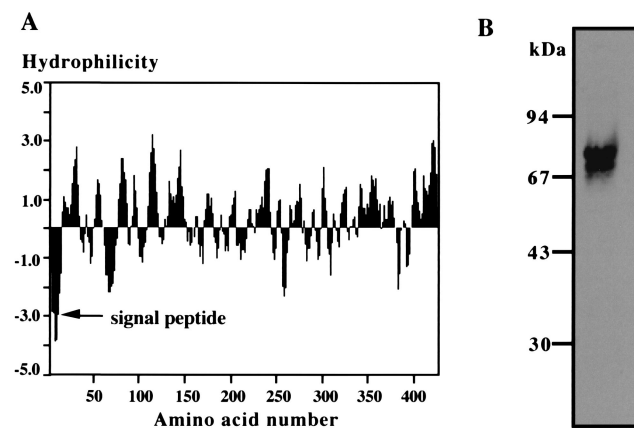
As an alternative approach to identify T cell Ags, MfA were separated into 30 fractions by fast protein liquid chromatography. All fractions were tested, and only one major T cell-stimulating activity was observed eluting at 0.6–0.7 M NaCl (data not shown), suggesting that more T cell Ags were found by SDS-PAGE electroelution, although this is a less refined system than fast protein liquid chromatography.

#### Identification of the serpin, Bm-SPN-2, as an Mf-derived T cell-stimulating Ag

To identify specific proteins in the most strongly T cell-stimulating fraction, murine antisera were raised to Mf fractions 8 and 9 (Fig. 1) and used to screen a *B. malayi* Mf cDNA expression library. Three positive clones were isolated, one of which was found to encode a 428-aa protein of the serpin (serine proteinase inhibitor) family, designated Bm-SPN-2, for which the gene sequence, transcription pattern, and evolutionary homology have recently been described (34). The other two clones were novel. In a control library screen, antisera from mice immunized with unfractionated MfA identified five positive clones, all of which were paramyosin as previously isolated with Abs to whole parasite (35).

#### Bm-SPN-2 is a secreted, stage-specific Mf Ag

Two striking features of Bm-SPN-2 are that the gene is expressed only in the Mf stage, in which it is one of most abundant transcripts (>2% mRNAs), and that recombinant protein specifically inhibits enzymatic activity of human neutrophil-derived cathepsin G and elastase (34). Bm-SPN-2 contains a typical signal peptide (Fig. 2A, residues 1–20), and specific Abs against recombinant Bm-SPN-2 recognized a single 70-kDa component in Mf ES products (Fig. 2B); no reactivity was seen with normal mouse sera. These results suggest that Bm-SPN-2 is a secreted protein. However, the ES band recognized by specific Ab is significantly larger than the Bm-SPN-2 monomer (47.5 kDa) (34). An obvious explanation would be that the ES band represents a complex between Bm-SPN-2 and a protease in the ES products. Indeed, a feature of serpin-serine proteinase interactions is the formation of a stable complex that does not dissociate during SDS-PAGE (36). A previous study with the distantly related larval-specific Bm-SPN-1 indicates that this protein also exists as a complex in L3 ES, but as a monomer in somatic extracts (37).



**FIGURE 2.** Secretion of Bm-SPN-2 by Mf in vitro. *A*, Hydrophilicity plot of Bm-SPN-2 amino acid sequence predicts that residues 1–20 form a typical signal peptide. *B*, Western blot of Mf ES products probed with mouse anti-recombinant Bm-SPN-2. A band at an apparent molecular mass of 70 kDa is specifically recognized. No reactivity was seen with normal mouse sera.

#### Immune responses to Bm-SPN-2 by Ag-immunized mice

To assess whether Bm-SPN-2 was indeed a T cell-stimulating Ag, we examined cellular immune responses to Bm-SPN-2 in BALB/c mice immunized with MfA or Bm-SPN-2. Popliteal lymph node cells from MfA-immunized mice, when challenged in vitro with MfA, produce substantial amounts of IFN- $\gamma$  and IL-4 (Fig. 3A). However, an exclusive IFN- $\gamma$  response without accompanying IL-4 was seen on challenge with Bm-SPN-2 in vitro (Fig. 3A), implying that Bm-SPN-2-specific T cells primed in vivo were predominantly of the Th1 phenotype. It was noted that cell proliferation was dramatically depressed by high concentrations of Bm-SPN-2, which is in agreement with previous observations that some proteinase inhibitors inhibit T cell proliferation (20, 38, 39). In mice immunized with recombinant Bm-SPN-2, only IFN- $\gamma$  responses were mounted on challenge with cognate Ag (Fig. 3B).

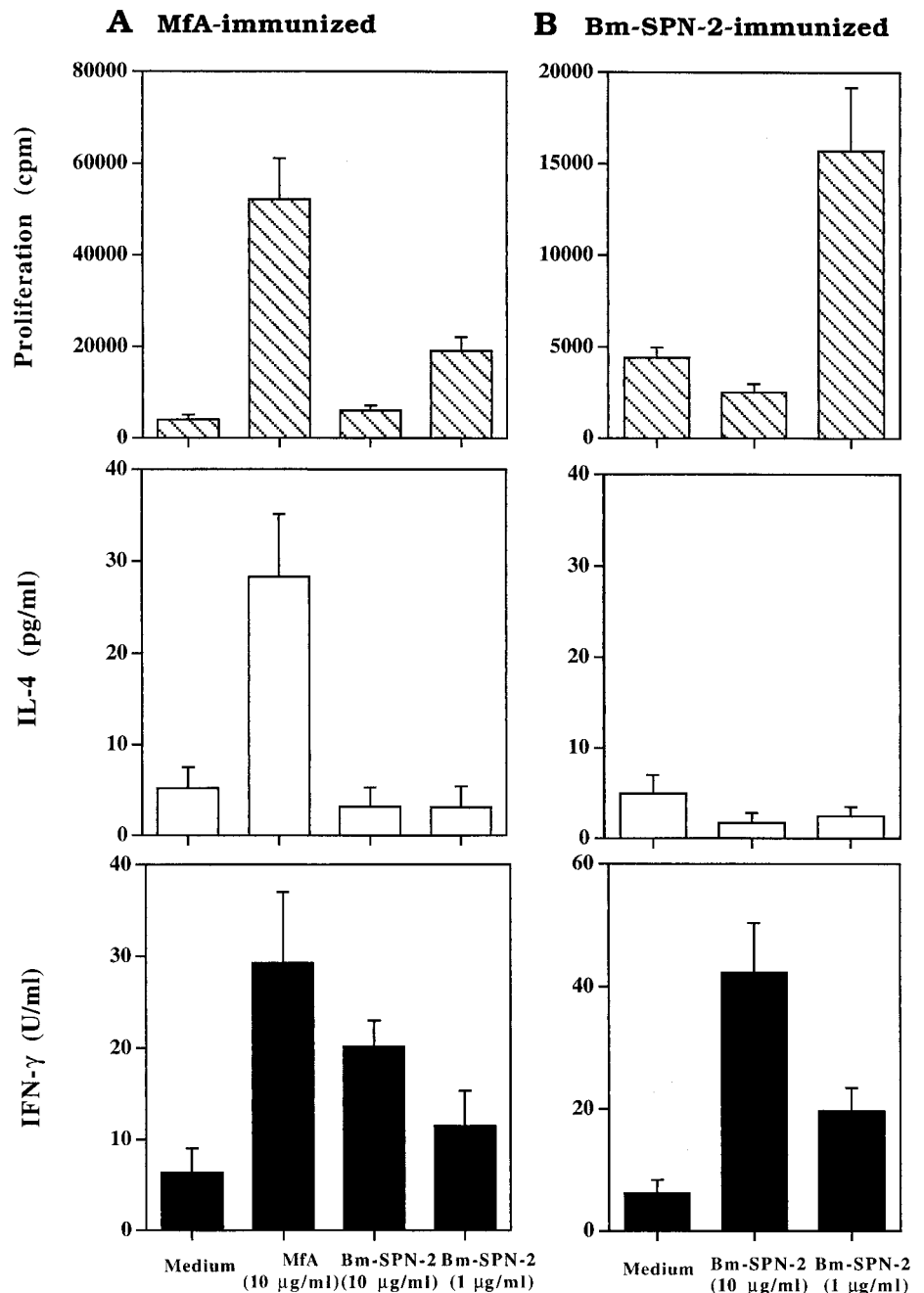
Bm-SPN-2-specific Ab responses were also measured in mice immunized with Mf, comparing strains with three different MHC and background genotypes, BALB/c (H-2<sup>d</sup>), C57BL/6 (H-2<sup>b</sup>), and CBA/Ca (H-2<sup>k</sup>). Mice were injected s.c. with MfA or PBS as a control and then challenged with Mf, and serum was recovered for isotype-specific Ab analyses. All mice had strong humoral responses to total Mf Ag, and both BALB/c and C57BL/6 strains produced anti-Bm-SPN-2 Abs (Fig. 4A). CBA/Ca mice, however, were poorly responsive to the Bm-SPN-2 Ag. Despite multiple immunizations and a strong response to total Mf Ag, no specific IgG was evident on day 20 after the final immunization or at any later time assayed up to day 60. There was, however, some Bm-SPN-2-specific IgM production (data not shown). The IgG subclass profiles of responder mice showed a bias toward IgG1, particularly in C57BL/6 animals. However, when DNA immunization with a mammalian vector expressing Bm-SPN-2 was employed, BALB/c mice produced high levels of Bm-SPN-2-specific IgG2a and IgG2b and moderate IgG1 responses (Fig. 4B).

#### Bm-SPN-2 stimulates the production of IFN- $\gamma$ and IgG1 in Mf-infected mice

Recognition of Bm-SPN-2 was then evaluated in mice receiving live Mf by i.p. transplantation, a procedure known to elicit an early Th1-type response (31). CD4<sup>+</sup> splenic T cells from Mf-infected BALB/c mice responded to Bm-SPN-2 with strong production of IFN- $\gamma$ , but no IL-4 or IL-5, within 14 days of infection (Fig. 5). At this time, the level of the IFN- $\gamma$  response to Bm-SPN-2 was approximately equal to that elicited by MfA. Interestingly, 3 wk later (on day 35) the Bm-SPN-2-specific IFN- $\gamma$  response had disappeared, while both IFN- $\gamma$  and IL-4 responses to MfA rose significantly (Fig. 5). Likewise, MfA-specific IL-5 was produced by day 35, but this cytokine was produced at no time in cultures stimulated with Bm-SPN-2 (data not shown). An unrelated control protein, Bm-MIF-2G, expressed and purified using the exactly the same vector system (X. Zang et al., in preparation), stimulated no T cell responses in Mf-infected animals. These data show that Bm-SPN-2 stimulates a strong, but short-lived, Th1 response in Mf-infected mice. Ab responses in these mice indicated some type 2 stimulation had occurred, as a weak Bm-SPN-2-specific IgG1 response was detectable on day 35, but not on day 14 (data not shown). In contrast, Mf-infected mice displayed high levels of all IgG isotypes to MfA at both time points (data not shown).

#### Human IgG isotype response to Bm-SPN-2

To demonstrate the antigenicity of Bm-SPN-2 in human filariasis, the four isotypes of filarial-specific IgG Abs from 40 subjects (20 microfilaremic and 20 Mf-negative endemic normal subjects) were determined for reactivity against BmA, MfA, and Bm-SPN-2 (Fig.



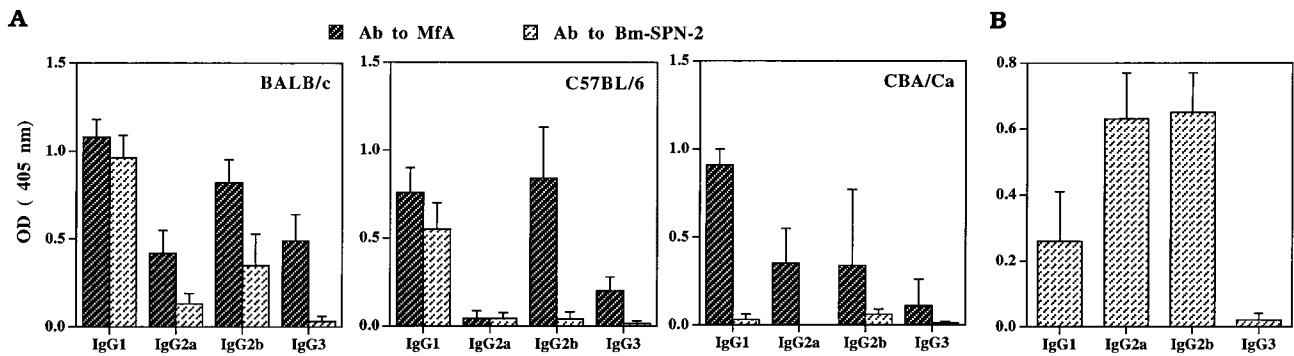
**FIGURE 3.** T cell stimulation by Bm-SPN-2 in MfA- or Bm-SPN-2-immunized mice. Proliferative and cytokine release responses of popliteal lymph node cells from BALB/c mice, primed with MfA (A) or Bm-SPN-2 (B) and challenged in vitro with MfA and Bm-SPN-2 at the indicated concentrations, are shown. Priming consisted of a primary dose in CFA and a secondary immunization in IFA. Popliteal lymph node cells were taken 10 days following the boosting dose. Proliferation was measured by tritiated thymidine uptake at 72 h in culture. Supernatants were taken after 60 h in culture, and cytokines were measured by ELISA for IFN- $\gamma$  or by NK cell bioassay for IL-4 (31). Results are expressed as the mean  $\pm$  SD of four mice individually assayed from each group.

6). One striking feature of this subclass analysis is the predominance of IgG1 and IgG4 Abs to both parasite extracts and recombinant protein. All microfilaremic patients generated BmA- and MfA-specific IgG1 and IgG4 responses above the threshold level for positivity, calculated as the mean of European normal control values + 3 SD, while most endemic normal subjects were also seropositive for specific anti-filarial IgG1 and IgG4.

Interestingly, every microfilaremic patient was positive for Bm-SPN-2-specific IgG1 and IgG4 (Fig. 6). While most endemic normal subjects were IgG1 positive, far fewer (30%) showed positive IgG4 recognition of Bm-SPN-2. The three endemic normal subjects with very high IgG4 levels against Bm-SPN-2 also expressed high IgG4 Ab responses to MfA and BmA, suggesting that these individuals might harbor a cryptic infection.

*IgG isotype level in microfilaremic patients after drug treatment*

Antifilarial Ab levels are known to decline following effective drug treatment, and IgG4 decays more rapidly than other isotypes (40). We therefore tested specific Ab levels in a set of 13 microfilaremic patients and five endemic normal subjects, for whom samples were available from before and 2 years after commencement of long term, low dose DEC therapy. Both IgG1 and IgG4 to BmA and MfA decreased after treatment (Fig. 7). However, many patients remained seropositive even 2 years following treatment when assayed for IgG1 and IgG4 against MfA (5 and 7 of 13 positive, 38–54%) or against BmA (10 of 13 positive, 77%). In the endemic normal subjects, whose infection status was ambiguous, there was also a slight decrease in the level of Abs after treatment.



**FIGURE 4.** Serum isotype responses in mice immunized with Mf and Bm-SPN-2 plasmid DNA. **A**, BALB/c, C57BL/6, and CBA/Ca mice were injected s.c. four times in 14 days with 10  $\mu$ g of MfA or PBS as a control and then challenged with  $2.5 \times 10^5$  Mf i.v. Twenty days after the final immunization, MfA- and Bm-SPN-2-specific serum IgG isotype levels were measured by ELISA. **B**, BALB/c mice were injected with 100  $\mu$ g of Bm-spn-2/pcDNA3.1(-) or pcDNA3.1(-) as a control and were boosted 2 wk later. Sera were collected 2 wk after the final immunization, and Bm-SPN-2-specific IgG isotypes were measured by ELISA. Results are expressed as the mean  $\pm$  SD of four mice in each group, with control OD values subtracted.

The level of Abs to Bm-SPN-2 changed much more dramatically, and all patients except one converted to seronegativity for anti-Bm-SPN-2 IgG1 and IgG4 following treatment (Fig. 7). The levels of IgG2 and IgG3 were also determined before and after treatment, and no significant changes were found, except for IgG2 to BmA, which slightly decreased 2 years after treatment (data not shown).

In filariasis, microfilaremic patients occasionally clear their Mf without drug treatment. During the course of a longitudinal study, serum samples had been taken from an individual before and after such spontaneous clearance (30). We determined the IgG isotype kinetics of serum Abs to filarial Ags over a 2-years period (Fig. 8). High levels of IgG1 and IgG4 to BmA, MfA, and Bm-SPN-2 were found at the initial time point at which the patient was Mf positive (100 microfilariae/ml blood). Within 6 mo the patient had become Mf negative, and IgG4 to Bm-SPN-2 was undetectable. Bm-SPN-2 specific IgG1 decreased dramatically, but remained at a low level for the duration of the study period. In contrast, in response to crude BmA and MfA extracts, IgG1 and IgG4 were maintained at high levels for 2 years after clearance. This result concurs well with the IgG isotype analysis from microfilaremic patients and endemic normal subjects (Fig. 6), suggesting that high titers of Ab against Bm-SPN-2, especially IgG4, are diagnostic of an active filarial infection.

#### Human T cell response to Bm-SPN-2

We then tested the level of responsiveness to Bm-SPN-2 of peripheral T cells from 15 endemic patients of different status. As shown in Fig. 9, most individuals showed enhanced IFN- $\gamma$  release on stimulation with Bm-SPN-2, displaying increases up to 5-fold

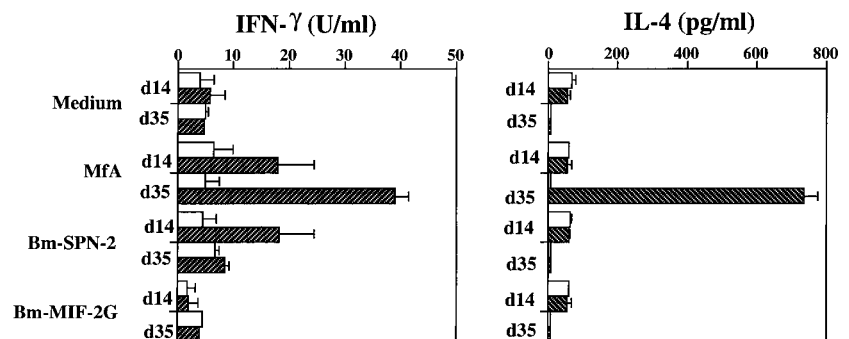
the uninduced levels. This response was not restricted to any disease category. In only one of the same patients did Bm-SPN-2 induce a rise in IL-5 production, to little more than double the background level (data not shown).

#### Discussion

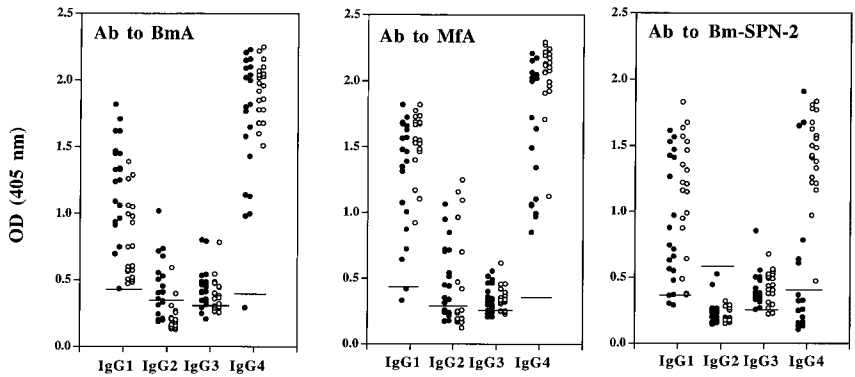
The complexity of parasite genomes and the diversity of proteins they encode have yet to be matched by a thorough analysis of immune recognition of any eukaryotic pathogen. The disparity is accentuated in the case of T cell specificities, because of the obstacles in identifying class II-restricted T cell-stimulating epitopes (2, 3). A small number of parasite T cell Ags, such as Ldp23 from *Leishmania donovani* (41), LACK from *Leishmania major* (42), and Sm-p40 from *Schistosoma mansoni* (43), have recently been identified by directly screening T cell determinants. In filarial parasites, T cell-stimulating activity segregates, with certain Ag fractions containing as yet unidentified proteins (44, 45), while a separate study has identified the gp15/400 protein as an inducer of T cell responses (46).

There is an urgent need to develop comprehensive approaches for direct identification of CD4<sup>+</sup> T cell Ags from parasites. Using a biochemical strategy, we report here that the *B. malayi* Mf stage-specific secreted serpin, Bm-SPN-2, is a prominent T cell Ag. However, this approach requires relatively large quantities of parasite material and is suited only to identifying prominent T cell-stimulating proteins such as Bm-SPN-2. Bacterial expression libraries can provide exogenous proteins for MHC class II presentation by APC (47, 48), but screening libraries directly by expression in class II-positive mammalian APC would provide the most systematic and sensitive strategy (49).

**FIGURE 5.** Cytokine responses in T cells from Mf-infected BALB/c mice. CD4<sup>+</sup> T cells isolated from splenocytes at early (14-day) or late (35-day) phases of infection with 300,000 Mf (■) were compared with control uninfected mice (□) upon stimulation with MfA, Bm-SPN-2, or Bm-MIF-2G at 10  $\mu$ g/ml in the presence of APCs. Supernatants were taken after 60 h in culture, and cytokines (IL-4 and IFN- $\gamma$ ) were measured by ELISA. Results are expressed as the mean  $\pm$  SD of five mice in each group.



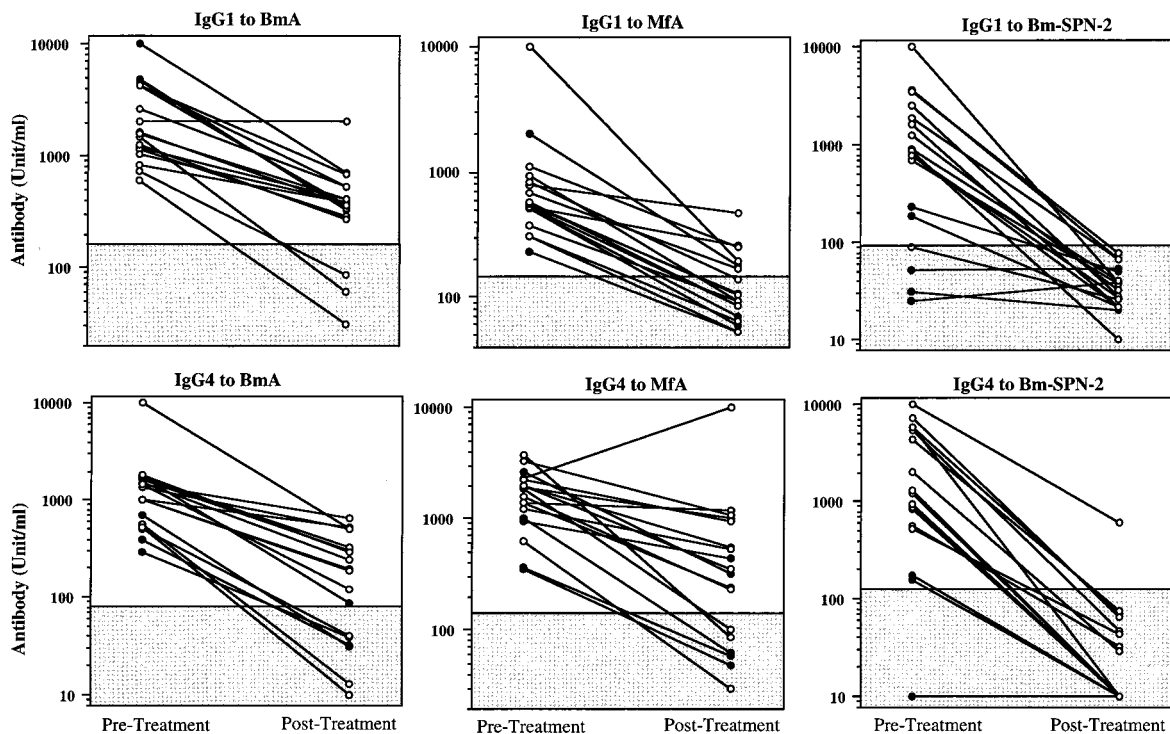
**FIGURE 6.** Analysis of specific human Ab levels. Ab levels specific for BmA, MfA, and Bm-SPN-2 in the sera of 20 Mf-negative endemic normal subjects (●) and 20 microfilaremia patients (○) were measured by ELISA for IgG1, IgG2, IgG3, and IgG4 using plates coated with Ags. The cutoff values are represented by short, horizontal lines and are derived from the mean of 10 European normal control values + 3 SD.



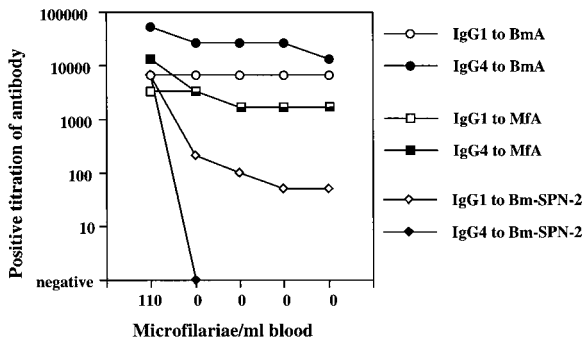
The *Bm-spn-2* gene is one of the most highly expressed transcripts in the Mf stage, representing >2% of the mRNA (34), and its protein product is secreted by blood stage parasites. Because Mf are continually produced even in low level infection, it is perhaps not surprising that Bm-SPN-2 is also one of the most stimulatory Ags from *B. malayi* for both Ab and T cell responses in rodents and human patients. However, in contrast to whole parasite Ag preparations, Bm-SPN-2 does not elicit a typical Th2-dominated profile in Mf-infected mice, and this one protein can stimulate as much Th1 cytokine production as can whole Mf Ag (MfA). Moreover, the Bm-SPN-2-specific response is remarkably short-lived for such a potent Ag, unless multiple immunization with protein extracts is employed. Although Mf may cease Ag secretion and/or die in rodents, the early termination of the response suggests that a specific down-regulatory mechanism comes into play. For ex-

ample, the overall response to Mf, as measured by responses to MfA, switches from an early Th1 pattern, to a more mixed Th1/Th2 reaction after 28 days (31, 50). Thus, this switch could result in or depend upon the termination of the Th1-restricted response to Bm-SPN-2.

Similar to the mouse model, the human response to Bm-SPN-2 shows both type 1 and type 2 characteristics. When peripheral T cell populations were challenged in vitro, Bm-SPN-2 induced more consistent IFN- $\gamma$  responses, and yet there were universally high titers of Bm-SPN-2-specific IgG4, an isotype that is known to be IL-4 dependent and associated with a Th2 response in humans (51, 52). In this respect, the Bm-SPN-2 response is consistent with the general nature of the human immune response to filarial infections, in that the IgG4 isotype is unusually predominant, representing 50–95% of the total IgG response (14, 15, 53) in contrast



**FIGURE 7.** Decline in IgG1 and IgG4 Abs following chemotherapy. Changes in IgG1 and IgG4 responses to BmA, MfA, and Bm-SPN-2 in five Mf-negative endemic normal subjects (●) and 13 microfilaremic patients (○) before and 2 years after DEC treatment. The serum sample with the highest Ab level was designated 10,000 U/ml and used to calibrate a standard curve. Each serum sample was measured by ELISA for IgG1 and IgG4 using plates coated with BmA, MfA, or Bm-SPN-2, and Ab concentrations were calculated according to a regression line. The cutoff values are represented by horizontal lines and are derived from the mean of 10 European normal control values + 3 SD.

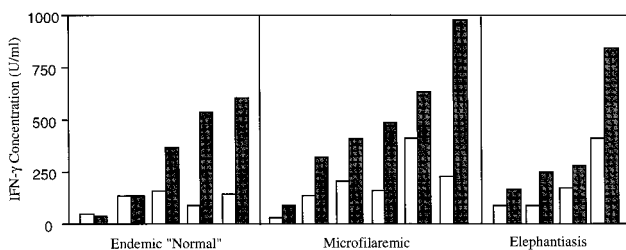


**FIGURE 8.** Kinetics of IgG1 and IgG4 responses in a microfilaremic patient for whom serum samples before and after natural parasite clearance are available. Blood samples were taken every 6 mo for Nuclepore filtration assays to verify amicrofilaremic status. Ab levels were measured by ELISA for IgG1 and IgG4 using plates coated with BmA, MfA, or Bm-SPN-2 Ags.

to a normal serum proportion of 4% (54). Recent work has found that  $\alpha_1$ -antitrypsin, a physiological serpin that neutralizes neutrophil elastase, selectively up-regulates human B cell differentiation into IgE- and IgG4-secreting cells (55). This effect was not observed with other mammalian serpins. The possibility that Bm-SPN-2, which also neutralizes human neutrophil elastase (34), may act as a costimulus for IgG4 switching remains open.

The most widely used method for diagnosis of filarial infection is examination of blood for Mf, which, due to Mf periodicity, requires nocturnal blood sampling (29). However, there are practical and biological limitations to this approach. Ideally, the night blood smear test should be replaced by a simple serologic assay for lymphatic filarial infection. Novel Ag detection assays hold great promise, but as yet are unreliable for *Brugia* infection (56). More broadly, the assessment of community-based disease management strategies, such as mass chemotherapy and vector control, and the evaluation of new drug protocols would be enhanced with known molecular markers that decline with the removal of parasite infection or transmission. High levels of IgG4 Ab responses have led to the suggestion that high titers of this isotype are diagnostic of filarial infection (14, 15, 57), even as measured against a complex mixture of parasite Ags. For example, a recent study reported that about four times more positive cases of *B. malayi* infection could be detected by IgG4 ELISA than by the conventional microscopic method (58).

Our new results now demonstrate that single Ags can offer even greater definition. Thus, all 13 MF<sup>+</sup> patients were positive for Bm-SPN-2-specific IgG4 and IgG1, and the levels of these Abs



**FIGURE 9.** IFN- $\gamma$  responses to Bm-SPN-2 by PBMC from filariasis patients. Production of IFN- $\gamma$  by PBMC of five endemic normal subjects (age range, 18–54 years), six microfilaremic patients (age range, 25–63 years; microfilaremia range, 50–331 Mf/ml), and four elephantiasis patients (age range, 35–70 years) is shown for cultures incubated in medium alone (▨) and those induced by Bm-SPN-2 (□).

declined sharply after DEC therapy, with most patients reverting to seronegativity. In agreement with this observation, previously high levels of Bm-SPN-2-specific IgG4 and IgG1 were lost in a case of natural elimination of Mf, although both isotypes remained measurable against BmA and MfA over a 2-year period. These results suggest that a high level of Bm-SPN-2-specific Ab, especially IgG4, is a diagnostic indicator of infection with *B. malayi*. Therefore, this Ag could well be used to generate new diagnostic tests for both individual infection and community parasite loads.

The wide distribution of serpins and their ability to regulate a variety of divergent proteinase-dependent physiological functions, such as blood coagulation and complement activation (36), prevention of apoptosis (59, 60), suppression of tumor cells (61, 62), and regulation of the Toll signaling pathway (63), show that they are intimately involved in a host of biological processes. In several systems, serpins from viruses have been implicated in pathogen evasion of the host immune system (64, 65). The ability of Bm-SPN-2 to specifically inhibit two human neutrophil-derived serine proteinases, cathepsin G and elastase, provides the first example of functional serpin from nematodes that may be important in this context (34). Our finding that Bm-SPN-2 is a prominent target of both T and B cell responses may seem at odds with the proposition that the same protein is an important component of parasite survival strategy. However, clear precedents exist, such as the HIV gp120 glycoprotein (66) and the *Plasmodium falciparum* var Ags (67). Such molecules are likely to be at the center of the evolutionary “arms race” between the host and pathogen.

There are few effective vaccines against helminth pathogens (25), and while chemotherapy is often curative, it provides no protection from reinfection. One of the remarkable features of the Mf stage of *B. malayi* is its longevity, which is likely to be many months in the bloodstream. The survival of Mf provides a reservoir of infection in an endemic community, but also a clear target for intervention. We suggest that Bm-SPN-2 is an important component of the parasite’s survival strategy, and as an Mf stage-specific Ag recognized by T and B cells, it may prove to be an appropriate target for vaccination or pharmaceutical attack. Bm-SPN-2 vaccination trials, including DNA immunization, which provoked strong immune responses to Bm-SPN-2, are now in progress.

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