

## A 70-Kilodalton Recombinant Heat Shock Protein of *Candida albicans* Is Highly Immunogenic and Enhances Systemic Murine Candidiasis

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The 70-kDa recombinant *Candida albicans* heat shock protein (CaHsp70) and its 21-kDa C-terminal and 28-kDa N-terminal fragments (CaHsp70-Cter and CaHsp70-Nter, respectively) were studied for their immunogenicity, including proinflammatory cytokine induction in vitro and in vivo, and protection in a murine model of hematogenous candidiasis. The whole protein and its two fragments were strong inducers of both antibody (Ab; immunoglobulin G1 [IgG1] and IgG2b were the prevalent isotypes) and cell-mediated immunity (CMI) responses in mice. CaHsp70 preparations were also recognized as CMI targets by peripheral blood mononuclear cells of healthy human subjects. Inoculation of CaHsp70 preparations into immunized mice induced rapid production of interleukin-6 (IL-6) and tumor necrosis factor alpha, peaking at 2 to 5 h and declining within 24 h. CaHsp70 and CaHsp70-Cter also induced gamma interferon (IFN- $\gamma$ ), IL-12, and IL-10 but not IL-4 production by CD4<sup>+</sup> lymphocytes cocultured with splenic accessory cells from nonimmunized mice. In particular, the production of IFN- $\gamma$  was equal if not superior to that induced in the same cells by whole, heat-inactivated fungal cells or the mitogenic lectin concanavalin A. In immunized mice, however, IL-4 but not IL-12 was produced in addition to IFN- $\gamma$  upon in vitro stimulation of CD4<sup>+</sup> cells with CaHsp70 and CaHsp70-Cter. These animals showed a decreased median survival time compared to nonimmunized mice, and their mortality was strictly associated with organ invasion by fungal hyphae. Their enhanced susceptibility was attributable to the immunization state, as it did not occur in congenitally athymic nude mice, which were unable to raise either Ab or CMI responses to CaHsp70 preparations. Together, our data demonstrate the elevated immunogenicity of CaHsp70, with which, however, no protection against but rather some enhancement of *Candida* infection seemed to occur in the mouse model used.

Microbial heat shock proteins (Hsp) are major targets of host immune responses. In particular, members of the 70-kDa Hsp (Hsp70) family are among the most immunogenic proteins of human pathogenic microorganisms (6, 16, 17, 19, 22). In the host-*Candida* relationship, the only Hsp which has received substantial attention is the 90-kDa Hsp (Hsp90), which has been shown to be an immunodominant target of protective antibody responses (23, 24). In addition, a 49-kDa fragment of Hsp90 has also been proposed as a diagnostic antigen (23). To our knowledge, no specific studies on the immunogenicity of and host response modulation by *Candida albicans* Hsp70 (CaHsp70) have been performed.

Because of the wide interest in these molecules as potential transdisease candidate vaccines (10, 17, 26) and because of some contrasting data about the protective nature of microbial Hsp70 against fungal infections (11, 12, 15, 22, 24), we have assessed the capacities of recently obtained recombinant CaHsp70 and of some peptide fragments thereof to induce both humoral and cell-mediated immunity (CMI) responses in mice, as well as the potential of CaHsp70 to confer protection in a systemic mouse infection. Unexpectedly, high immunogenicity, in particular that shown by a 21-kDa C-terminal frag-

ment of CaHsp70 (CaHsp70-Cter), not only induced no protection against but instead induced some apparent enhancement of the acute systemic infection by the fungus.

### MATERIALS AND METHODS

Unless otherwise specified, *C. albicans* ATCC 20955 was used throughout this study. It was grown in YPD (2% glucose, 1% yeast extract, 2% Bacto Peptone [Difco, Detroit, Mich.]). Construction of a *C. albicans* cDNA library, molecular cloning, and sequencing of the CaHsp70 gene were done as previously described (18).

**Preparation and purification of recombinant CaHsp70 and C- and N-terminal fragments.** CaHsp70 sequences were generated by *EcoRI* restriction of plasmid cDNA inserts or by PCR-assisted amplification as described elsewhere (18). The *EcoRI* fragments were cloned into the *EcoRI* site of the expression vector pDS56/RBSII6xhis/E<sup>-</sup>. The PCR product, after digestion with *Bam*HI and *Eco*RI, was cloned into the *Bam*HI/*Eco*RI polylinker sites of pDS56/RBSII6xhis/E<sup>-</sup>, resulting in a fusion of six histidines at the amino termini of CaHsp70 peptides (27). The expression of recombinant six-His-tagged CaHsp70 and its C- and N-terminal fragments was obtained by use of *Escherichia coli* M15 carrying the *lac* repressor-producing pUHA1 plasmid. Induction in Luria-Bertani medium containing kanamycin and ampicillin was performed by adding isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG; Boehringer GmbH, Mannheim, Germany) at a final concentration of 1 mM to a culture at an optical density at 600 nm (1-cm-diameter cuvette) of 0.6, followed by 4 h of incubation at 37°C. Figure 1a shows the size and genetic locations of the C- and N-terminal fragments used throughout.

**Purification of recombinant proteins.** Recombinant six-His-tagged CaHsp70 proteins were purified by nickel chelate affinity chromatography in accordance with the manufacturer's (Qiagen, Hilden, Germany) instructions (denaturing conditions). Fractions containing the purified polypeptides were pooled, precipitated with 3 volumes of absolute ethanol, resuspended in water, and stored at -20°C.

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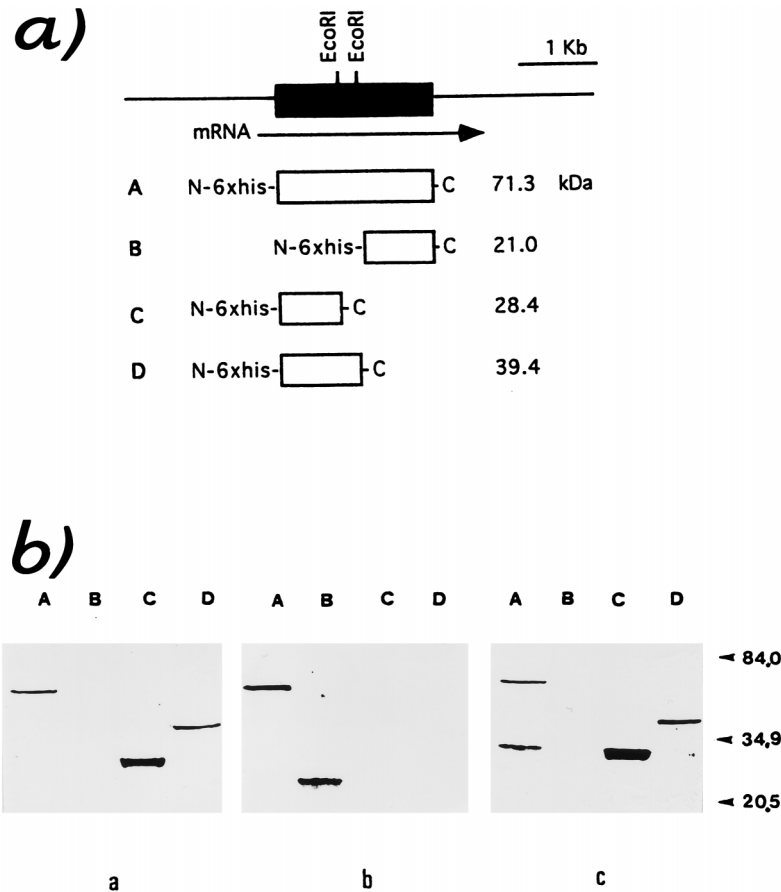


FIG. 1. (a) Molecular mass, definition, and location of the gene fragments encoding the CaHsp70 products used as immunogens throughout this study. A, CaHsp70; B, CaHsp70-Cter; C, CaHsp70-Nter; D, 39.4-kDa N-terminal fragment. The arrow indicates the 5'→3' direction of transcription. N-6xhis, six-histidine tag. (b) Immunoblot reaction with the CaHsp70 recombinant products indicated in panel a of serum from mice immunized with CaHsp70, CaHsp70-Cter, and CaHsp70-Nter (blots a, b, and c, respectively). One microgram of each recombinant product was electrophoresed on an SDS-polyacrylamide gel and electrotransferred to a nitrocellulose membrane. The reaction was performed with a 1:1,000 dilution of each antiserum and visualized with a phosphatase-conjugated second antibody as detailed in Materials and Methods. Arrowheads indicate molecular mass markers (in kilodaltons).

**Mouse strains, immunization, and immunogenicity assays.** Unless otherwise specified, hyperimmune serum against CaHsp70 and its fragments was raised in CD2F1, CD1, and C3H/HeJ mice (18 to 21 g) by four intraperitoneal injections at weekly intervals of 100  $\mu$ l of a 100- $\mu$ g/ml solution of the recombinant products in complete (the first two injections) and incomplete (the last two injections) Freund's adjuvant. Other immunizations were performed with the mannoprotein antigen fraction MP-F2 under the same immunization schedule as that described above or with the live *Candida* vaccine PCA-2 as described elsewhere (2, 25, 31).

Antibody titers were measured by an enzyme-linked immunosorbent assay (ELISA). Briefly, polystyrene microtiter plates (Dynatech, PBI, Milan, Italy) were coated overnight at 4°C with 200 ng of antigen dissolved in 100  $\mu$ l of 0.05 M sodium carbonate (pH 9.6). After a wash in bovine serum albumin-phosphate-buffered saline (PBS) blocking solution, 100  $\mu$ l of twofold dilutions in PBS-0.05% Tween 20 of serum from immunized animals was diluted and incubated at 37°C for 2 h.

Pooled serum (diluted 1:2) from nonimmunized mice was used as a negative control. After three washes with 400  $\mu$ l of PBS-Tween 20, a 1:20,000 dilution in PBS of alkaline phosphatase-conjugated rabbit anti-mouse immunoglobulin (Sigma) as the secondary antibody was added to wells (1 h at 37°C), and the reaction was developed with nitrophenyl phosphate disodium (Sigma) as the substrate. Titers were defined as the highest dilution of mouse serum which gave an optical reading of at least twice the reading of the negative control.

For the immunoblot (Western blot) assay, recombinant CaHsp70 and its fragments in sample buffer were boiled for 10 min and subjected to sodium dodecyl sulfate (SDS)-5 to 15% gradient polyacrylamide gel electrophoresis. The electrophoresed materials were electroblotted onto nitrocellulose filters in buffer containing 25 mM Tris, 192 mM glycine, 0.1% SDS, and 20% methanol. Filters were incubated with antibodies as described for single experiments. In all cases, nonspecific binding of antibodies to nitrocellulose was prevented by blocking of the filters with 1% bovine serum albumin in PBS for 2 h at room temperature. After extensive washing with PBS, bound antibodies were detected with suitable alkaline phosphatase-conjugated secondary antibodies.

For CMI assessment, splenocyte proliferation assays were performed. Normal or immunized mice were sacrificed immediately before dissection. Spleens were removed, and single-cell suspensions were obtained by grinding the spleens in a potter containing 3 ml of lysis buffer (0.16 M Tris-buffered  $\text{NH}_4\text{Cl}$  [pH 7.2]). After 3 min, the lysis of erythrocytes was stopped by the addition of 9 ml of RPMI 1640 with 2% fetal calf serum (Gibco BRL, Life Technologies, Milan, Italy). Cells were centrifuged for 8 min at  $600 \times g$ . Cell pellets were resuspended in complete medium (RPMI medium; GIBCO, Grand Island, N.Y.) to  $10^6$  cells per ml and then incubated at a volume of 0.2 ml/well in triplicate in the presence of the relevant stimulator as described for single experiments. The plates were incubated at 37°C in 5%  $\text{CO}_2$ , and cells were harvested after 2 days for concanavalin A (ConA) (Sigma) stimulation and after 4 days for all other stimuli. [methyl- $^3\text{H}$ ]thymidine (0.5  $\mu\text{Ci}$ ) (TRK120; Amersham, Milan, Italy) (specific activity, 2.5 Ci/mmol) was added to the cultures 18 h before cell harvesting with a semiautomatic harvester (Skatron, Oslo, Norway), and DNA synthesis was evaluated by measuring tritiated precursor incorporation. The data were expressed as mean counts per minute ( $10^3$ ) of triplicate values  $\pm$  standard deviation. Before the addition of the tritiated precursor, the plates were checked for growth under a light microscope.

**PBMC isolation from and proliferation in humans.** Peripheral blood mononuclear cells (PBMC) were obtained from heparinized venous peripheral blood samples from healthy donors by centrifugation on density gradients (Lymphoprep; Nyegaard, Oslo, Norway). PBMC were washed twice and resuspended in RPMI medium (GIBCO) supplemented with 5% pooled AB serum and antibiotics (penicillin, 100 IU/ml; streptomycin, 0.1 mg/ml [both from GIBCO]), hereafter referred to as complete medium. In a few experiments, mononuclear cells from cord blood were used after isolation and purification as reported for PBMC. PBMC proliferation was measured with  $2 \times 10^5$  cells in 0.2 ml of complete medium per well in triplicate in 96-well flat-bottom microwell trays (Falcon 3072; Becton Dickinson, Lincoln Park, N.J.) in the presence of the relevant stimulants. The trays were incubated at 37°C in 5%  $\text{CO}_2$ , and cells were harvested after 7 days. PBMC proliferation was measured as described above for

TABLE 1. Antibodies against CaHsp70 products and their isotypes in mice following immunization<sup>a</sup>

| Antigen              | Mouse strain | Total IgG titer <sup>c</sup> | ELISA reading <sup>b</sup> for: |       |       |      |
|----------------------|--------------|------------------------------|---------------------------------|-------|-------|------|
|                      |              |                              | IgG1                            | IgG2a | IgG2b | IgG3 |
| CaHsp70              | CD1          | 1:10,000                     | 0.32                            | 0.12  | 0.25  | 0.11 |
|                      | CD2F1        | 1:12,800                     | ND <sup>e</sup>                 | ND    | ND    | ND   |
| CaHsp70-Cter         | CD1          | 1:32,000                     | 0.47                            | 0.34  | 0.48  | 0.28 |
|                      | CD2F1        | 1:30,000                     | ND                              | ND    | ND    | ND   |
| CaHsp70-Nter         | CD1          | 1:5,000                      | 0.21                            | 0.14  | 0.17  | 0.12 |
|                      | CD2F1        | 1:12,800                     | ND                              | ND    | ND    | ND   |
| Control <sup>d</sup> | CD1          | None                         | ND                              | ND    | ND    | ND   |

<sup>a</sup> CD1 and CD2F1 mice were immunized by four intraperitoneal injections at weekly intervals of 10 µg of antigen in 100 µl of adjuvant.

<sup>b</sup> Determined from measurements of the optical density of a 1:1,000 serum dilution.

<sup>c</sup> End-point positive dilution, i.e., twice the reading of the control ELISA with nonimmune mouse serum.

<sup>d</sup> Recombinant six-histidine-tagged mouse DHFR was used instead of CaHsp70 preparations as the coating antigen in the ELISA.

<sup>e</sup> ND, not done.

murine splenocyte proliferation. The data were expressed as mean counts per minute ( $10^{-3}$ ) of triplicate values  $\pm$  standard deviation.

**Purification and culturing of murine CD4<sup>+</sup> T cells.** CD4<sup>+</sup> T lymphocytes from naive or CaHsp70-immunized mice were positively selected from pools of spleen cells by means of sequential adherence on anti-immunoglobulin-coated plates three times, followed by adherence on anti-murine CD4 monoclonal antibody (MAb) GK1.5; this procedure resulted in >95% pure populations, as determined by fluorescence-activated cell sorter analysis (Becton Dickinson & Co., Mountain View, Calif.). CD4<sup>+</sup> T lymphocytes ( $5 \times 10^6$ /ml) were cultured in the presence of accessory macrophages ( $5 \times 10^6$ /ml), which were obtained after 2 h of adherence to plastic, and different stimuli, which included 10 µg of ConA per ml,  $5 \times 10^5$  heat-inactivated *C. albicans* cells (HCA) per ml, 10 µg of CaHsp70 or CaHsp70-Cter fragment per ml, or 10 µg of mannoprotein fraction MP-F2 of *C. albicans* per ml (12, 25).

**Measurement of cytokine production in vivo and in vitro.** The levels of interleukin-6 (IL-6), IL-1 $\beta$ , tumor necrosis factor alpha (TNF- $\alpha$ ), and IL-10 in mouse sera were assayed by an ELISA with the Quantikine Murine Kit (RD Systems, Abingdon, United Kingdom) in accordance with the manufacturer's instructions. Levels of gamma interferon (IFN- $\gamma$ ), IL-12, IL-4, and IL-10 in murine splenocyte culture supernatants were measured after 48 h of incubation. The sources and characteristics of the anticytokine antibody reagents used in IFN- $\gamma$ , IL-4, and IL-10 ELISAs were previously described in detail (31, 32, 35). Briefly, supernatants were tested for IFN- $\gamma$  concentrations with rat anti-murine IFN- $\gamma$  MAb R4-6A2 as the primary antibody and biotinylated MAb AN-18.17.24 as the secondary antibody. For IL-4 and IL-10 measurements, two-site ELISAs involved the use of MAb 11B11 in combination with biotinylated MAb BVD6-24G2 and the use of MAb SXC-2 plus biotinylated MAb SXC-1, respectively. Levels of circulating IL-12 p70 were determined by a modified antibody capture bioassay as described previously (35).

All cytokine titers were calculated by reference to standard curves constructed with known amounts of recombinant IFN- $\gamma$ , IL-4 (Genzyme, Boston, Mass.), IL-10 (ParMigen, San Diego, Calif.), and IL-2 (Genetics Institute, Cambridge, Mass.). The detection thresholds for the assays were 5 pg/ml for TNF- $\alpha$ , 3 pg/ml for IL-6 and IL-1 $\beta$ , 0.1 ng/ml for IFN- $\gamma$  and IL-12, 0.5 ng/ml for IL-4, and 2 ng/ml for IL-10.

**Mouse systemic infection with *C. albicans*.** Nonimmunized or immunized mice were subjected to intravenous challenge with *C. albicans* cells. To this end, the fungal cells were grown to the stationary phase at 28°C under slight agitation in Winge medium (0.2% glucose, 0.3% yeast extract). After centrifugation ( $1,000 \times g$ ) and two washes in saline, the cells were resuspended at a density of  $2 \times 10^6$ /ml. A 100-µl portion of this suspension was injected intravenously into mice. Previous experiments served to establish that, under our experimental conditions, this cell concentration corresponded to approximately two 50% lethal doses (LD<sub>50</sub>). The animals were observed for 30 days, and mortality was assessed as the number of dead animals out of the total number of animals challenged and as median survival time (in days).

**Histopathological observations.** Mice were sacrificed by cervical dislocation, and the left kidneys were removed and immediately fixed in 10% (vol/vol) neutral buffered formalin. After dehydration in ethanol, clearing with xylene, and paraffin embedding, 8-µm-thick sections were stained with periodic acid-Schiff-van Gieson stain and observed under a light microscope.

## RESULTS

**Ab induction in mice.** Mice immunized with CaHsp70 preparations produced high Ab titers, with the expected specificity. As shown in Table 1, the most immunogenic protein in both

outbred CD1 and inbred CD2F1 animals was CaHsp70-Cter. Immunoglobulin G1 (IgG1) and IgG2b were the most represented serum Ab isotypes, and no IgM or IgA was found in any group of animals at the end of the immunization protocol. The specificity of the Ab response was demonstrated by a lack of recognition of another recombinant six-histidine-tagged protein (dihydrofolate reductase [DHFR]; Quiagen), expressed and purified in the same manner as the CaHsp70 protein. Immunization with each recombinant protein fragment raised Abs which recognized both the specific fragment and the whole protein. However, mice immunized with the whole protein produced very low levels of Ab against CaHsp70-Cter (Fig. 1b). In the immunoblot experiments, a 39.4-kDa N-terminal fragment was included as an additional control. Antisera against both CaHsp70 and the 28-kDa N-terminal fragment of CaHsp70 (CaHsp70-Nter) recognized this additional fragment (Fig. 1b).

**CMI induction in mice.** CD2F1 mice were immunized against CaHsp70, CaHsp70-Cter, or CaHsp70-Nter, and their splenocytes were assessed for their capacity to mount a CMI response against each specific immunogen in vitro.

The data in Fig. 2 are from two of five independent experiments performed, with qualitatively similar results. They show the elicitation of a CMI response against each specific immunizing antigen. In particular, at equal doses of in vitro stimulant, the highest lymphoproliferative response, almost equaling the proliferation induced by the mitogen, was that of the splenocytes of animals immunized with CaHsp70-Cter. All animals immunized with CaHsp70-Cter or CaHsp70-Nter also recognized the whole protein as a CMI target (data not shown), and all responded to the polyclonal stimulant ConA as a positive proliferation control. DHFR-immunized control animals, while showing a specific CMI response to DHFR, did not show a CMI response to any CaHsp70 constituent, demonstrating the specificity of the CMI response to CaHsp70 (data not shown).

**CMI induction in humans.** As previously shown (18), normal human subjects have appreciable levels of serum Ab against CaHsp70, mostly directed against the highly conserved CaHsp70-Nter moiety and possibly originating from both *Candida* and non-*Candida* Hsp70 stimulation (17, 19). On this basis, we determined whether PBMC from these subjects also recognized CaHsp70 as a CMI target in vitro. Figure 3 shows that CaHsp70-stimulated PBMC from two randomly selected, normal adults showed a low but appreciable degree of lymphocyte proliferative responses against the whole protein and, in

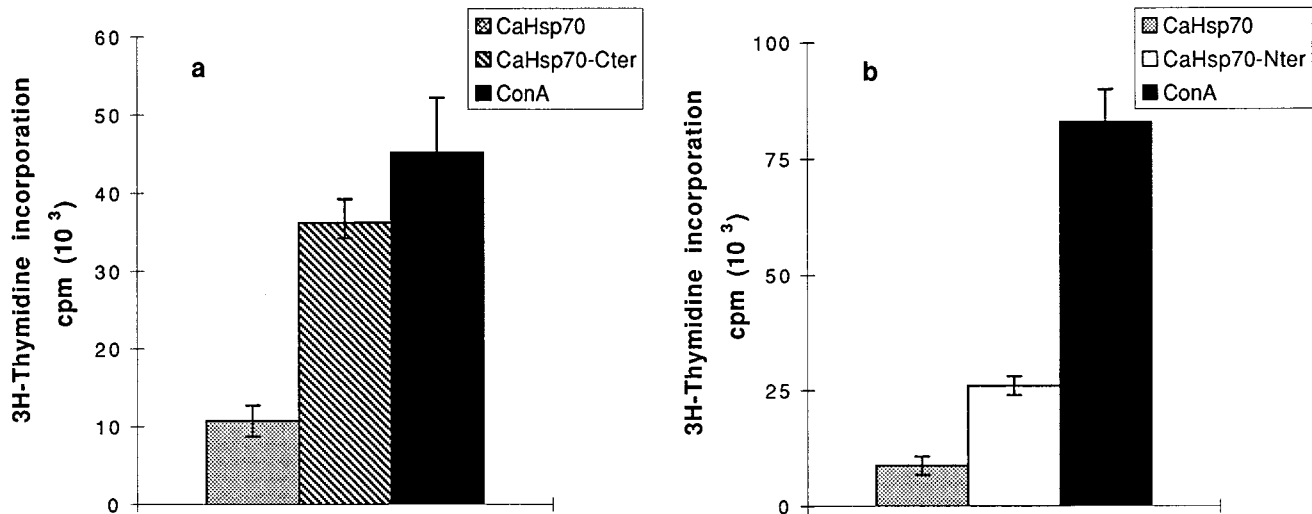


FIG. 2. Proliferation of splenocytes from CD2F1 mice not immunized or immunized with CaHsp70, CaHsp70-Cter, and CaHsp70-Nter and stimulated in vitro with the respective antigen (10  $\mu$ g/ml) or ConA (0.1  $\mu$ g/ml). (a and b) Two independent experiments. Nonstimulated splenocyte cultures never incorporated more than 800 cpm, and these values were subtracted. Error bars indicate standard deviations.

one donor, against the N-terminal fragment. CaHsp70-induced proliferation was significantly lower than that achieved by stimulation in vitro with a major CMI antigen target, such as the *C. albicans* mannoprotein (MP-F2) or the polyclonal stimulant IL-2 (Fig. 3). That the cell proliferation in response to CaHsp70 constituents, although not particularly intense, was of an antigenic rather than a polyclonal or mitogenic type was demonstrated by the substantial lack of proliferative response to the same constituents by naive, human cord blood lymphocytes (data not shown).

**Response of CaHsp70-immunized mice to *C. albicans* challenge.** The high immunogenicity of CaHsp70 and its products led us to assess the resistance of CaHsp70-immunized animals to a lethal *C. albicans* challenge. To this aim, two independent

experiments were performed whereby CD2F1 mice receiving a fully immunogenic dose (10  $\mu$ g four times at weekly intervals) of CaHsp70 products in an adjuvant were challenged with a lethal dose of *C. albicans*. Control mice received the adjuvant only or (in one experiment) the protective mannoprotein antigen MP-F2 (25) or the irrelevant recombinant six-histidine-tagged protein DHFR. In both experiments, mice immunized with CaHsp70 or its fragments were not protected by the immunization. Instead, their susceptibility was significantly enhanced, in terms of median survival time, as compared to that of nonimmunized animals (which received adjuvant only), after immunization with the whole recombinant protein (in both experiments) or the N-terminal fragment (in one experiment). No mortality enhancement, yet no protection, was conferred

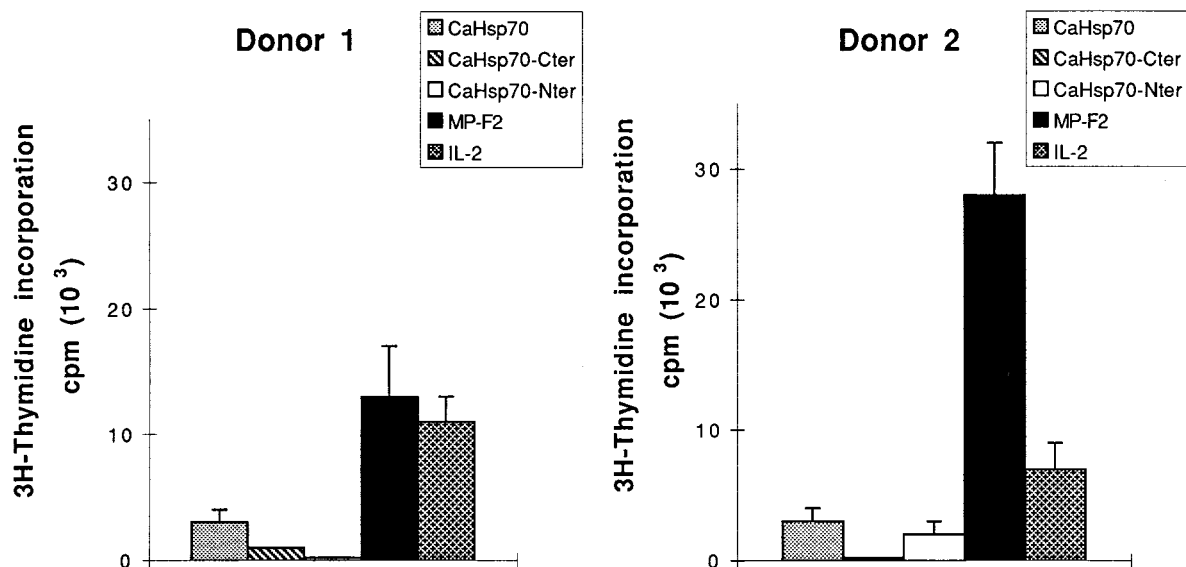


FIG. 3. Proliferation of human PBMC from two independent donors following in vitro stimulation with the indicated antigen or IL-2. The concentrations of CaHsp70 stimulants were as in the legend to Fig. 2. The mannoprotein fraction (MP-F2) was used at 10  $\mu$ g/ml, and IL-2 was used at 100 U/ml. Nonstimulated PBMC cultures never incorporated more than 350 cpm, and these values were subtracted. Error bars indicate standard deviations.

TABLE 2. Mouse mortality after challenge with a lethal *C. albicans* dose<sup>a</sup>

| Immunization <sup>b</sup> | Mortality <sup>c</sup> in expt: |     |                  |                  |
|---------------------------|---------------------------------|-----|------------------|------------------|
|                           | 1                               |     | 2                |                  |
|                           | MST                             | D/T | MST              | D/T              |
| Adjuvant only             | 27                              | 4/6 | 20               | 5/6              |
| MP-F2                     | ND                              | ND  | >30 <sup>d</sup> | 2/6 <sup>d</sup> |
| DHFR                      | ND                              | ND  | 22               | 5/6              |
| CaHsp70                   | 10 <sup>d</sup>                 | 6/6 | 8 <sup>d</sup>   | 6/6              |
| CaHsp70-Cter              | 29                              | 6/7 | 20               | 5/6              |
| CaHsp70-Nter              | 9 <sup>d</sup>                  | 5/5 | 20               | 3/6              |

<sup>a</sup> Mice were injected intravenously with  $2 \times 10^5$  *C. albicans* cells, corresponding in our model to approximately two LD<sub>50</sub> over a 30-day period.

<sup>b</sup> All immunogens were given at a dose of 10 µg four times at weekly intervals (in complete Freund's adjuvant for the first two doses and incomplete Freund's adjuvant for the last two doses).

<sup>c</sup> MST, median survival time (days); D/T, number of dead animals/total number of animals after 30 days.

<sup>d</sup> Statistically significant difference ( $P < 0.05$ , as determined by the Mann-Whitney U test) between these data and those for mice given adjuvant only.

by immunization with CaHsp70-Cter, as well as with the irrelevant protein DHFR. In the same experiments, immunization with the mannoprotein antigen of *C. albicans* partially protected against the lethal challenge, confirming previous observations (25) (Table 2).

The above-described pattern of mortality was equally pronounced when the animals were given a suboptimal immunogenic dose of CaHsp70 and its products (four 1-µg doses in an adjuvant), which was capable of inducing both Ab and CMI responses in mice. Necroscopic examination of mouse organs showed that mortality was attributable to massive organ invasion by *C. albicans* hyphae (Fig. 4).

To determine whether the enhancement of mortality was specifically attributable to immunization with CaHsp70 products, athymic *nu*<sup>+</sup>/*nu*<sup>+</sup> mice, which were unable to raise an Ab or a CMI response against the antigen, were challenged with *C. albicans*, and their mortality was assessed within a 30-day period. As shown in Table 3, nude mice were similarly susceptible to *C. albicans*, irrespective of CaHsp70 administration. In this experiment, the control euthymic animals showed a shorter survival time after immunization with CaHsp70 or CaHsp70-Nter.

**Serum cytokines in mice.** It has been reported that Hsp are strong stimulators of proinflammatory and immunomodulatory cytokine production (9, 28). Because some of these cytokines are essential to up- or down-regulate antifungal protection in murine models (29, 33–36), we assayed for IL-1β, TNF-α, IL-6, and IL-10 production in the serum of CD2F1 mice, either immunized or not immunized against CaHsp70 constituents, shortly after in vivo administration of the different CaHsp70 constituents. These experiments were performed with various mouse strains, including non-lipopolysaccharide (LPS)-responsive (C3H/HeJ) mice. In immunized CD2F1 animals, CaHsp70 and its fragments, but not the control recombinant protein DHFR or the irrelevant antigen MP-F2, induced rapid production of high circulating IL-6 and TNF-α levels. These levels usually persisted for up to 5 h, returning to the baseline values of untreated animals after 24 h (Table 4). On a weight basis, CaHsp70-Nter appeared to be a stronger cytokine inducer than the whole recombinant protein. When the animals were not immunized or when they were immunized with subimmunogenic antigen doses, IL-6 levels close to the baseline values of control (unstimulated) mice were detected (data not shown). No IL-10 was detected in any animals, with the exception of CaHsp70-Cter-immunized or MP-F2-immunized mice,

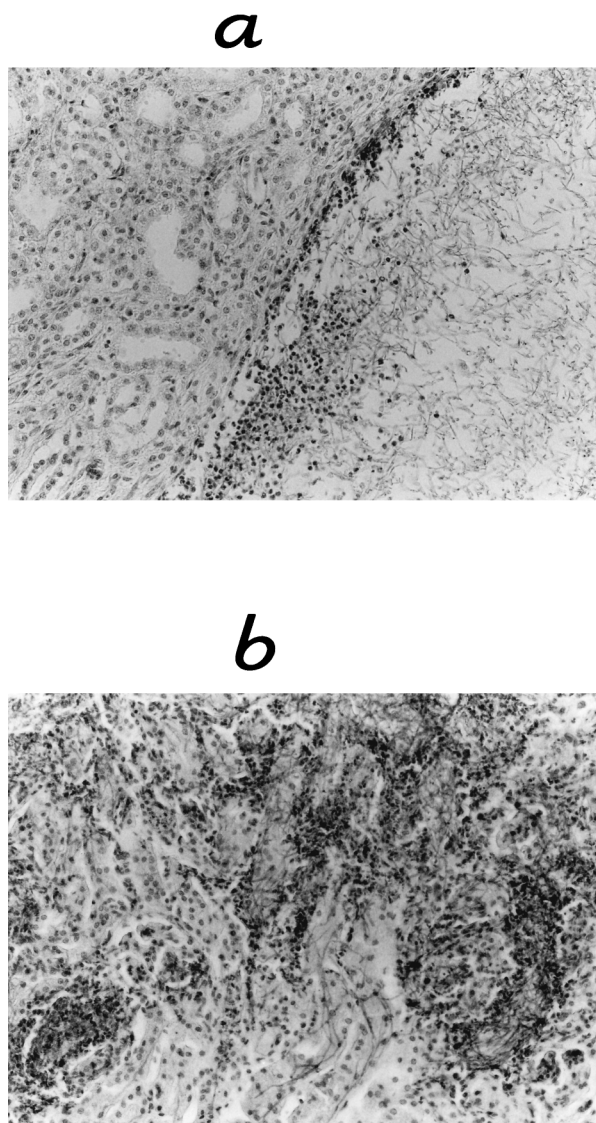


FIG. 4. Kidney histopathology of mice immunized with CaHsp70 (a) or not immunized (adjuvant) and challenged with *C. albicans*. In both cases, hyphal cells were seen clustering with inflammatory cells both in cortical tissue and outside parenchymal tissue.

which showed some IL-10 present in the serum at 5 or mostly 24 h, respectively, after a CaHsp70-Cter boosting injection (Table 4).

Because LPS, even in very small amounts (smaller-than-nanogram levels), is a strong in vivo inducer of proinflammatory cytokines and we could not rule out in vitro subliminal (undetectable), yet in vivo effective, LPS contamination in our CaHsp70 preparations, experiments with IL-6 and TNF-α production were performed with non-LPS-responsive C3H/HeJ mice. Both nonimmunized and CaHsp70-immunized animals were assessed for cytokine production 2 h after in vivo administration of CaHsp70-Cter or CaHsp70-Nter. Table 5 shows that nonimmunized C3H/HeJ mice responded to the administration of CaHsp70 products with low serum IL-6 levels, which were, however, significantly higher than the baseline values detected after saline or LPS stimulation. No TNF-α (or IL-1β; not shown) was produced by these animals. When C3H/HeJ mice were preimmunized with the relevant stimulant, they were extremely responsive to CaHsp70-Cter, producing in 2 h

TABLE 3. Mortality of athymic and euthymic mice challenged with a lethal inoculum of *C. albicans*<sup>a</sup>

| Immunization <sup>b</sup> | Mortality <sup>c</sup> of mice |     |                |     |
|---------------------------|--------------------------------|-----|----------------|-----|
|                           | Athymic                        |     | Euthymic       |     |
|                           | MST                            | D/T | MST            | D/T |
| Adjuvant only             | 8                              | 6/6 | 27             | 4/6 |
| DHFR                      | 10                             | 6/6 | 23             | 3/4 |
| CaHsp70                   | 8                              | 6/6 | 9 <sup>d</sup> | 8/8 |
| CaHsp70-Cter              | 7                              | 6/6 | 29             | 6/7 |
| CaHsp70-Nter              | 8                              | 6/6 | 9 <sup>d</sup> | 5/5 |

<sup>a</sup> Mice were injected intravenously with  $2 \times 10^5$  *C. albicans* cells, corresponding in our model to approximately two LD<sub>50</sub> over a 30-day period.

<sup>b</sup> All immunogens were given at a dose of 10 µg four times at weekly intervals (in complete Freund's adjuvant for the first two doses and incomplete Freund's adjuvant for the last two doses).

<sup>c</sup> MST, median survival time (days); D/T, number of dead animals/total number of animals after 30 days.

<sup>d</sup> Statistically significant difference ( $P < 0.05$ , as determined by the Mann-Whitney U test) between these data and those for mice given adjuvant only.

about 1 ng of IL-6 (as compared to 33 pg after LPS or saline stimulation) (Table 5). Appreciable levels of TNF-α were also detected after CaHsp70 stimulation. No IL-12 or IFN-γ was detected in the serum of these as well as CD2F1 animals (see above) at any time during the 2-h experimental period following the administration of CaHsp70 products.

**Cytokines produced by CD4<sup>+</sup> cells from naive or CaHsp70-immunized mice following in vitro stimulation with CaHsp70 products.** To evaluate the ability of CaHsp70 preparations to stimulate the production of immunomodulatory cytokines by

TABLE 4. Serum cytokine levels<sup>a</sup> in CaHsp70-immunized CD2F1 mice after in vivo stimulation with CaHsp70 constituents

| Stimulant <sup>b</sup> | Time of assay (h) | IL-6 (pg/ml) in expt: |     | TNF-α (pg/ml) in expt: |     | IL-10 (pg/ml) in expt 1 |
|------------------------|-------------------|-----------------------|-----|------------------------|-----|-------------------------|
|                        |                   | 1                     | 2   | 1                      | 2   |                         |
|                        |                   |                       |     |                        |     |                         |
| CaHsp70                | 2                 | 240                   | 220 | 100                    | 10  | 0                       |
|                        | 5                 | 126                   | 80  | 70                     | 35  | 0                       |
|                        | 24                | 76                    | 48  | 50                     | 70  | 0                       |
| CaHsp70-Cter           | 2                 | 280                   | 140 | 100                    | 100 | 18                      |
|                        | 5                 | 260                   | 190 | 70                     | 80  | 18                      |
|                        | 24                | 13                    | 13  | 10                     | 10  | 30                      |
| CaHsp70-Nter           | 2                 | 400                   | 360 | 210                    | 820 | 0                       |
|                        | 5                 | ND                    | 65  | ND                     | ND  |                         |
|                        | 24                | 40                    | 12  | 10                     | 10  | 0                       |
| DHFR                   | 0                 | 46                    | 16  | 70                     | 50  | 0                       |
|                        | 5                 | 64                    | 44  | 10                     | 10  | 0                       |
|                        | 24                | 54                    | 40  | 70                     | 50  | 0                       |
| MP-F2                  | 0                 | 28                    | ND  | 130                    | 110 | 0                       |
|                        | 5                 | 78                    | ND  | 30                     | 0   | 34                      |
|                        | 24                | 82                    | ND  | 30                     | 70  | ND                      |
| None                   | 2                 | 51                    | 45  | 64                     | 54  | 0                       |
|                        | 5                 | 60                    | 48  | 72                     | 70  | 0                       |

<sup>a</sup> As assayed by an ELISA in two independent experiments with different groups of five mice each. Sera from each animal were pooled, and the amount of cytokine in a single well was measured. ND, not determined.

<sup>b</sup> Each stimulant was used as a single intraperitoneal injection of 100 µl of a 100-µg/ml solution 1 week after the last immunization (fourth injection) with the respective material. For all other details, see Materials and Methods.

TABLE 5. Serum IL-6 and TNF-α levels in C3H/HeJ mice after CaHsp70 stimulation

| Stimulant <sup>b</sup> | CaHsp70-immunized mice <sup>c</sup> |               | Naive mice   |               |
|------------------------|-------------------------------------|---------------|--------------|---------------|
|                        | IL-6 (pg/ml)                        | TNF-α (pg/ml) | IL-6 (pg/ml) | TNF-α (pg/ml) |
| Saline                 | 33                                  | 15            | 13           | 0             |
| DHFR                   | 43                                  | 15            | 43           | 0             |
| LPS <sup>d</sup>       | 33                                  | 10            | 12           | 2             |
| CaHsp70-Cter           | 983                                 | 85            | 99           | 5             |
| CaHsp70-Nter           | 103                                 | 95            | 83           | 2             |

<sup>a</sup> As assayed by an ELISA 2 h after stimulant inoculation.

<sup>b</sup> Each stimulant was given as an injection of 100 µl of a 100-µg/ml solution in saline.

<sup>c</sup> The schedule of immunization used for CD2F1 mice in Table 2 was also used for these animals.

<sup>d</sup> Used at 100 ng/100 µl.

CD4<sup>+</sup> T cells from naive or immunized mice, the levels of IFN-γ, IL-4, and IL-10 production were measured in the culture supernatants of cells stimulated with the relevant fractions in the presence of accessory macrophages. The production of IL-12 was also measured in these cultures. The results were comparatively analyzed with those obtained in response to such powerful primary cytokine production stimulants as ConA (in experiments of primary stimulation) and HCA (24, 30) or with MP-F2, a protective *C. albicans* mannoprotein antigen (12, 25) (in experiments of secondary in vitro stimulation of cells from immunized mice). Both CaHsp70 and CaHsp70-Cter were able to induce IFN-γ, IL-12, and IL-10 production by cells from naive mice to levels that were comparable to (IFN-γ) or even higher than (IL-12 and IL-10) those observed in response to mitogen or fungal cells (Fig. 5). Interestingly, stimulation with CaHsp70-Cter induced levels of IL-12 and IL-10 that were higher than those obtained in response to the CaHsp70 whole protein. No IL-4 was detected in response to any stimulant but ConA.

In experiments performed with cells from immunized mice (Fig. 6), IFN-γ but not IL-12 was produced after in vitro stimulation with all immunizing antigens, and again, higher levels of IFN-γ were present in supernatants of cultures from mice immunized with CaHsp70-Cter than in those from animals receiving CaHsp70. Interestingly, the cultures from the former mice produced appreciable quantities of both IL-4 and IL-10, while some IL-4 but no IL-10 was present in the cultures from the animals immunized with CaHsp70 (Fig. 6). Altogether, these results demonstrate that (i) both recombinant products are endowed with the ability to induce the production of immunoregulatory cytokines by CD4<sup>+</sup> T cells in vitro; (ii) the overall production of T-helper type 2 (Th2) cytokines IL-4 and IL-10 is higher in immunized than in naive mice, and the highest level of IL-4 production occurs in animals immunized with CaHsp70-Cter; and (iii) neither Th2 cytokine, and only IFN-γ, is produced by cells from mice immunized with the protective soluble antigen (MP-F2) or the live *Candida* vaccine (PCA-2) (see also references 2, 25, 31, and 32).

## DISCUSSION

We recently cloned and expressed in *E. coli* one of the genes of the CaHsp70 family (18). Other authors studied the same gene or other genes of this family, and López-Ribot et al. (20, 21) proposed a classification scheme for CaHsp70 genes. These authors also demonstrated that members of the Hsp70 family are components of fungal cell wall proteins (20). Thus, cell wall-located, major immunogens of *C. albicans*, such as Hsp70

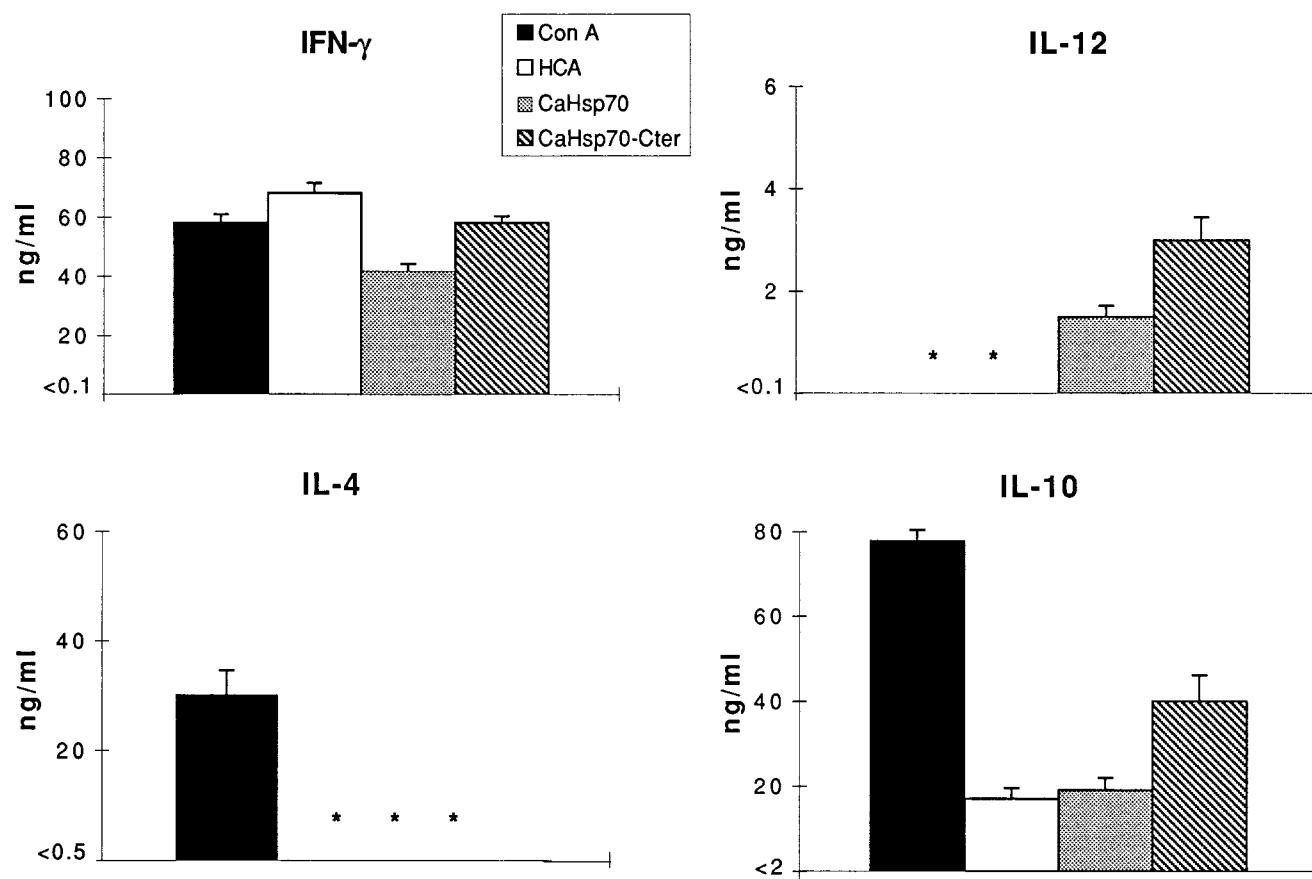


FIG. 5. Cytokine production by purified CD4<sup>+</sup> splenocytes cultured in vitro with splenic adherent macrophages and incubated in the presence of ConA (0.1  $\mu$ g/ml), HCA ( $5 \times 10^5$  cells), CaHsp70 (10  $\mu$ g/ml), or CaHsp70-Cter (10  $\mu$ g/ml). Cytokine levels were determined by cytokine-specific ELISAs. \*, below the detection limit of the assay, indicated by a less-than symbol on the y axis. Error bars indicate standard deviations.

itself, Hsp90 (24), mannoprotein (12, 25, 39), and enolase (37, 38), currently are being considered for a potentially protective role against disease, with some contrasting reports (1, 5, 11, 12, 22; reviewed in reference 8). Highly immunogenic proteins are obvious candidates for antifungal protection but, while immunogenicity has been well documented in most cases, protection has rarely been satisfactorily shown. Moreover, the mechanisms linking immunogenicity with or translating immunogenicity into protection have not yet been elucidated. Overall, the evidence for protection in mice immunized by definite antigens and in stringent models of lethal infection by *C. albicans* is quite limited (8, 13, 24, 25).

This study shows that immunization with CaHsp70 products not only does not protect against systemic candidiasis but may accelerate animal death. In a similar approach, Allendoerfer et al. (1) demonstrated that Hsp70-immunized mice were not protected against pulmonary histoplasmosis, although they generated *Histoplasma*-specific CMI, a major mechanism of anti-*Histoplasma* protection.

Because of the high immunogenicity of previously studied Hsp70 from other microorganisms, there was a clear expectation that CaHsp70 would also be highly immunogenic. This study confirms and strengthens that expectation. In particular, we found that CaHsp70 not only induces elevated Ab titers and CMI responses in mice but also is expressed as a CMI target in healthy subjects colonized by *C. albicans*. However, the possibility that these findings are the result of cross-antigen immunization cannot be excluded because of the high level of homology of all microbial Hsp (17).

Cultures of CD4<sup>+</sup> and splenic accessory cells from naive mice were able to produce consistent amounts of IFN- $\gamma$  and IL-12, some IL-10, but no IL-4 when stimulated in vitro with both whole recombinant CaHsp70 and CaHsp70-Cter. Interestingly, the same cultures from specifically immunized mice produced no IL-12 but some IL-4 after stimulation with both CaHsp70 and CaHsp70-Cter. In parallel experiments of immunization with protective *Candida* antigens (whole cells or MP-F2), no Th2 cytokines, and only IFN- $\gamma$ , were detected.

IFN- $\gamma$  and IL-12 were recently shown to be relevant for a protective host response against *C. albicans* (29–32). In addition, Romani and collaborators demonstrated a remarkable enhancement of *C. albicans* infections in IL-6 gene-deficient animals, owing to the impaired neutrophil response and type 1 CD4<sup>+</sup> T-helper-cell development (29, 30). The rapid and abundant production of IL-6 after in vivo administration of CaHsp70 products suggests that cells with natural immunity (e.g., macrophages and polymorphonuclear leukocytes) may be involved. The capacity of Hsp70 and other Hsp from various microbial sources to induce the rapid production of potentially protective proinflammatory cytokines by macrophages of naive animals has been reported by various authors (9, 28). Although these reports are clearly consistent with our findings, it should be noted that IL-6 and TNF- $\alpha$  production in vivo did not occur in naive, nonimmunized animals, suggesting that, if involved, cells with natural immunity are probably preactivated in vivo by CaHsp70-specific lymphocyte-derived products.

Besides macrophages, polymorphonuclear leukocytes also produce IL-6 and TNF- $\alpha$  upon suitable stimulation in vitro and

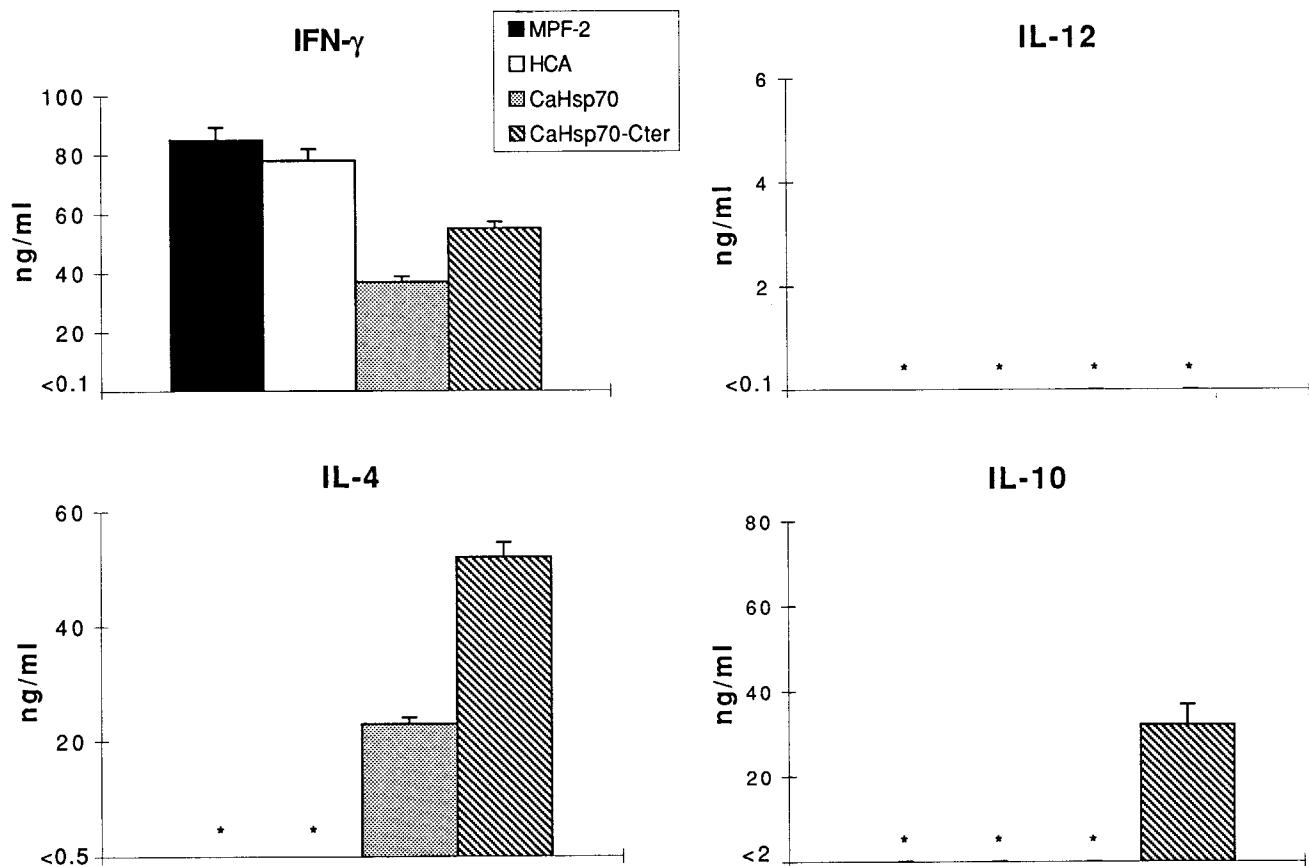


FIG. 6. Cytokine production by purified CD4<sup>+</sup> splenocytes of immunized mice, cultured in vitro with splenic adherent macrophages and incubated in the presence of HCA ( $5 \times 10^5$  cells), mannoprotein fraction MP-F2 (25) (10  $\mu$ g/ml), CaHsp70 (10  $\mu$ g/ml), or CaHsp70-Cter (10  $\mu$ g/ml). The animals were immunized against the CaHsp70 products as described in Materials and Methods, and their spleens were removed 3 days after the last immunizing antigen dose. The animals whose CD4<sup>+</sup> cells were stimulated in vitro with HCA or MP-F2 had been immunized with a low-virulence nongerminating variant of *C. albicans* (PCA-2) or MP-F2, respectively, following previously published methods (25). Cytokine levels were determined by cytokine-specific ELISAs. \*, below the detection limit of the assay, indicated by a less-than symbol on the y axis. Error bars indicate standard deviations.

in vivo (3, 29, 30, 40), and this production has been assumed to favor the induction of a Th1 cytokine protective pattern (29, 30). However, as emphasized above, splenic CD4<sup>+</sup> cells produced IL-10 and some IL-4 mostly after stimulation with CaHsp70-Cter and in immunized animals. Moreover, a small amount of IL-10 was found in the serum of mice immunized with CaHsp70-Cter, although this finding occurred relatively late with respect to IL-6 or TNF- $\alpha$  production. IL-4 and IL-10 have been shown to decrease the protective efficacy of Th1 cytokine pattern activation, and both anti-IL-4 and anti-IL-10 treatments enhanced the activation of the Th1 cytokine pattern induced by protective *Candida* antigens (25, 29, 31).

Overall, the interpretation of our data on cytokine production in the context of anticandidal protection is not easy, owing to the complexity of the cytokine patterns elicited by highly immunogenic proteins such as CaHsp70 and its fragments. Nonetheless, our data suggest that putatively protective cytokines produced during primary and secondary responses ex vivo and in vivo are not, per se, decisive inducers of a protective response. While immunization with CaHsp70 products also elicited the production by CD4<sup>+</sup> cells of nonhealing, anti-inflammatory cytokines such as IL-4 and IL-10, larger quantities of these cytokines were produced by animals immunized with CaHsp70-Cter (with which no enhancement of infection occurred) than by those immunized with the susceptibility-enhancing CaHsp70 whole protein.

The most immunogenic CaHsp70 product was CaHsp70-Cter, which contains the most variable region of CaHsp70 (17, 18, 21). At least theoretically, therefore, the responses directed against the C-terminal regions are those most specific to the fungus and not to the host Hsp70. This finding was particularly evident for the CMI response. Less evident was the dominance of CaHsp70-Cter in the antibody response, as the antibody titers were equally high against both N- and C-terminal fragments in animals immunized with the CaHsp70 whole protein. These dominance data should be interpreted cautiously with regard to the natural situation, as the recombinant protein and its fragments cannot fold and express the conformational B-cell epitopes the way that the natural CaHsp70 protein can.

There is renewed interest in the use of immunological tools to prevent or cure candidiasis (4, 8), so truly protective *C. albicans* antigens and immunomodulators are intensely sought. However, the evidence for their expression during natural commensalism or infection may be particularly elusive (4). Recent data support the view that immune responses to a particular mannoprotein and mannan adhesin or to aspartyl proteinase may indeed induce a degree of protection against systemic or mucosal experimental infection by *C. albicans* (4, 7, 13, 14). Studies by Matthews and Burnie (24) indicated a protective antigen in *C. albicans* Hsp90. Of interest is that all of these antigens appear to exert their protective effects by antibody induction, in apparent contrast to the protective

mechanisms elicited by immunization with whole cells of a low-virulence *C. albicans* strain (2, 29–32). Whatever the immunoregulatory mechanisms, the present study demonstrates that high B- and T-cell immunogenicity of a cell surface-expressed, immunodominant antigen of *C. albicans* may be irrelevant if not detrimental for protection. This finding further highlights the need to finely discriminate among the redundant immunogenicity constituents in the search for protective *Candida* antigens, as well as the need for more in-depth dissection of the immunoregulatory mechanisms in anti-*Candida* protection.

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