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# Effect of Systemic Candidiasis on Blastogenesis of Lymphocytes from Germfree and Conventional Rats

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Germfree and conventional rats were challenged (intravenously) with *Candida albicans* and sacrificed at various times after infection, and their spleen cells were harvested to examine the effect of disseminated candidiasis on in vitro lymphocyte hypersensitivity to *Candida* antigens (CA). Results showed that conventional rat splenocytes, initially responsive in vitro to stimulation by CA, manifested a depression in CA-specific responsiveness after challenge with viable *C. albicans* (days 3 to 6 postchallenge). In addition, the latter splenocyte response to phytohemagglutinin (PHA) and concanavalin A (ConA) was suppressed by 3 to 6 days after challenge with *Candida*. In contrast to conventional rats, the response of germfree rat splenocytes to CA was insignificant before challenge with *C. albicans*, and it was increased at 9 days after infection. The response of uninfected germfree rat splenocytes to PHA and ConA was significantly lower than that of unchallenged conventional rats. Challenge with viable *C. albicans* did not result in a suppression of gnotobiotic rat splenocyte responses to PHA and ConA, but rather, the disseminated infection resulted in as much as fivefold increases in PHA or ConA-induced blastogenesis. These findings suggest that disseminated candidiasis is capable of suppressing blastogenesis in immunologically mature conventional rats and of improving lymphocyte blastogenesis from immunologically immature germfree rats.

Recent reports have suggested that cell-mediated immunity (CMI) may not play a vital role in the defense of experimental animals against the disseminated form of candidiasis (8, 26). In contrast, defense against human mucocutaneous candidiasis is believed to be largely dependent on an intact cellular immune system (14, 15, 33). Recent studies suggest that infections by certain microorganisms may cause a depression in the CMI responses of the host (7, 10, 21-23, 27). The studies described in this report were initiated to examine the possibility that experimental infection by *Candida albicans* may lead to depressed CMI function. It is possible that a suppression of CMI, induced by experimental candidiasis, may be responsible for the failure of investigators to observe a role for CMI in defense against the systemic form of this infection, and it may explain the lack of in vivo and in vitro correlates of CMI in patients with serious candida disease (14, 15, 28, 33). In addition to studies with conventional rats (which are likely to have had previous stimulation by *Candida* sp. or similar yeasts in the gut flora), this report also describes the effect of experimental candidiasis on the in vitro cellular immune function of germfree rats.

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## MATERIALS AND METHODS

**Microorganisms.** *C. albicans* strain B311 (type 1) was originally obtained from H. F. Hasenclever (National Institutes of Health, Bethesda, Md.). All experiments were carried out with organisms that were grown on Sabouraud dextrose agar (GIBCO, Madison, Wis.) for 24 h at 37°C, washed from the slants, and stored frozen at −70°C in 0.3 ml of Sabouraud dextrose broth at a concentration of $5 \times 10^8$ viable units/ml.

**Rats.** Conventional and germfree Sprague-Dawley rats (between 3 and 4 months of age at the time of infection), of both sexes, were used in these studies. All germfree rats were bred and housed in flexible-film germfree isolators at the University of Wisconsin Gnotobiotic Laboratory and were fed a crude, pelleted, autoclaved L5010C diet (Ralston Purina Co., St. Louis, Mo.). Conventional animals of the same strain were obtained from Sprague-Dawley (Madison, Wis.).

**Challenge procedure.** Frozen cultures of each organism were thawed on the day of use, and proper dilutions (in saline) were carried out to achieve a desired challenge inoculum. Rats were anesthetized with sodium pentobarbitol, restrained with a metal rat holder, and injected via cardiac puncture with a 0.1-ml dose of *C. albicans*.

**Vaccination with Freund adjuvant.** Vaccination with Freund complete and incomplete adjuvant (CFA and IFA; Difco Laboratories, Detroit, Mich.) was carried out according to the method of Campbell et al. (6). Briefly, adjuvant was mixed, in equal volumes, with a suspension of Formalin-killed organisms ($10^6$

viable *C. albicans* per ml) and emulsified by repeated drawing and ejection of the mixture in a 20-ml syringe (Jellco Laboratories, Raritan, N.J.) fitted with an 18-gauge needle (Jellco). Once emulsification took place, the material was used immediately for subcutaneous administration to rats in two locations on the back (2 ml, total, per rat). For animals receiving only adjuvant, the suspension of killed organisms was replaced with sterile saline.

**Antigens.** *C. albicans* antigen (CA) was prepared by growing the microorganisms in Sabouraud dextrose broth for 24 h at 37°C in a shaker-incubator (100 rpm). The organisms were then washed three times by centrifugation at 3,000 × *g* for 10 min and resuspended in saline. The organisms were then broken under high pressure by passing three times through a modified (20) French pressure cell (American Instrument Co., Inc., Silver Spring, Md.) at 10,000 lb/in$^2$. Particulate and soluble fractions were separated by centrifugation at 12,000 × *g* for 10 min. The particulate fraction was subjected to 70°C for 30 min, and the soluble fraction was passed through a membrane filter (0.22-μm pore size; Millipore Corp., Bedford, Mass.) to obtain sterile preparations. Whole-cell antigen was prepared as above, except that the organisms were not subjected to high-pressure breakage. A heavy suspension of yeast cells was washed, as above, and resuspended in 10% Formalin for 1 h at 4°C. The fixed cells were then washed four times by centrifugation at 12,000 × *g* for 10 min and resuspended in saline. The sterility of each antigen preparation was checked with Sabouraud dextrose broth, brain heart infusion broth, and thioglycolate broth.

Purified protein derivative (PPD) prepared from *Mycobacterium tuberculosis* was obtained from the National Institute of Allergy and Infectious Diseases (Bethesda, Md.). The PPD was obtained in a lyophilized state and was reconstituted with saline. With the exception of PPD, the concentration of protein in each of our antigen preparations was estimated by the method of Lowry et al. (18).

**Mitogens.** Concanavalin A (ConA; Calbiochem, San Diego, Calif.), phytohemagglutinin M (PHA; Difco), and pokeweed mitogen (PWM; Grand Island Biological Co. [GIBCO], Grand Island, N.Y.) were obtained in a lyophilized form and reconstituted with saline. All mitogens were assumed to have a 2-week stable lifetime in the reconstituted state. After 2 weeks, all reconstituted mitogens were discarded.

**Preparation of spleen cell cultures.** Isolation of individual spleen cells was carried out according to a procedure described elsewhere in detail (17). Briefly, animals were sacrificed, and the entire spleen, with all visible adipose tissue removed, was placed in a sterile disposable 60-mm petri dish (on ice) (Falcon Plastics, Los Angeles, Calif.) containing 5 ml of sterile phosphate-buffered saline (GIBCO). The spleen was then placed on top of two layers of sterile stainless-steel screen mesh inside a 60-mm petri dish with 5 ml of phosphate-buffered saline. The spleen was gently pushed through the screen with a 10-ml plastic syringe plunger (Jellco), and the resulting cell suspension was collected in a 15-ml glass centrifuge tube (Matheson Scientific, Elk Grove Village, Ill.). After 10 min, to allow large clumps to settle, the supernatant suspension was then drawn into a second centrifuge tube, washed by centrifuging at 180 × *g* for 10 min, and resuspended in 1 ml of phosphate-buffered saline. Erythrocytes in the latter suspension were lysed through osmotic shock by adding 9 ml of sterile water. Five to 10 s later, 1 ml of a concentrated (10×) solution of phosphate-buffered saline was added to return the spleen cells to normal osmotic pressure. This suspension was then washed twice, as above, and finally resuspended in 5 ml of RPMI 1640 medium supplemented with 1.5% glutamine, 2,500 U of penicillin per ml, 2,500 μg of streptomycin per ml, and 2.5 μg of amphotericin B (GIBCO) per ml. A sample of cells was placed in 0.1% eosin (1:20 dilution of cells in eosin solution) for enumeration of viable cells by counting in a hemacytometer. Dilution of the cell suspension to $10^6$ cells per ml was then carried out with RPMI 1640 medium supplemented as above and 20% autologous serum (unless otherwise noted).

**Blastogenesis assay.** The blastogenesis assay was carried out according to a method described elsewhere (9). Briefly, the cell suspension ($10^6$ viable cells per ml) was dispensed in 0.1-ml samples into Falcon microtiter plates (Falcon Plastics, Los Angeles, Calif.). Various antigens or mitogens, at different concentrations in 0.1 ml, were then added to the cells, in quadruplicate for each antigen concentration, in the individual wells of the microtiter plate. Appropriate controls were also included, consisting of wells with supplemented RPMI 1640 medium substituted for antigens or mitogens or wells with only the antigens or mitogens present.

Plates were incubated for either 30 h or 4 days at 37°C in a humidified 5% $CO_2$–95% air atmosphere before adding 2 μCi of [$^3$H]thymidine (specific activity, 119 Ci/mmol; New England Nuclear, Boston, Mass.) in a volume of 0.05 ml of supplemented RPMI 1640 medium. Since the deoxyribonucleic acid (DNA) synthesis of 4-day cultures was found to be greater than that of 30-h cultures, only the 4-day cultures are reported in the results. Cultures were incubated for an additional 18 h, and harvested with a MASH II automatic cell harvestor (Microbiological Associates, Bethesda, Md.) onto glass fiber filter paper (Microbiological Associates). The filter papers were air dried for at least 24 h and placed in glass scintillation vials (Research Products International, Elk Grove Village, Ill.) with 3 ml of a scintillation cocktail that consisted of 47.9% toluene (Mallinckrodt, Inc., St. Louis, Mo.), 45.5% ethyl alcohol, and 4.2% Liquifluor (New England Nuclear). The vials were then counted in a model 3375 liquid scintillation spectrometer (Packard Instrument Co., Inc., Downer's Grove, Ill.).

**Calculations and statistical analysis.** Calculations were carried out according to a procedure described elsewhere (11). The stimulation index (SI) was calculated according to the following equation: counts per minute in stimulated cultures/counts per minute in unstimulated cultures = SI, where in each case the counts per minute are corrected for background radiation detected by the scintillation counter. The stimulated cultures consisted of splenocytes incubated with antigens or mitogens. The unstimulated cultures consisted only of splenocytes in culture medium. The variation among cultures was consistently less than 10%, and the variation (standard error) reported is a function of the variation among animals, not of that among cultures. The blastogenesis response was ex-

pressed as the mean summed SI (11). This is calculated by summing the SI for three antigen (or mitogen) concentrations and obtaining a "summed SI" for a given animal. The mean summed SI ± the standard error was then calculated for a given group of animals. The mean summed SI was chosen for use as an assessment of the overall response of splenocytes to stimulation by a given antigen or mitogen. We feel it more adequately reflects the stimultion of cells than a simple "SI" at only one antigen or mitogen dose.

Due to the unequal variance sometimes obtained when comparing two groups of animals, values for counts per minute were transformed to $\log_{10}$ values, and further statistical analysis was then carried out. Statistical analysis consisted of a comparison of the SI for one group with the SI of a second animal group by using Student's *t* test. All calculations were done through the use of the Univac 1110 computer at the Madison Academic Computing Center, Madison, Wis. A program to perform all transformations, *t* tests, and other mathematical manipulations was written specifically for these experiments by T.J.R.

## RESULTS

**In vitro blastogenesis of splenocytes from germfree rats vaccinated with FKCA.** Since an analysis of germfree rat lymphocyte blastogenesis has not been previously reported in the literature, a number of critical independent variables had to be defined in preliminary experiments. Germfree rats vaccinated with Formalin-killed *C. albicans* (FKCA) in CFA were used to provide the splenocytes for these preliminary experiments, since normal germfree rats were not found to be sensitive to antigens from *C. albicans*.

Several CA were tested in the blastogenesis assay in order to choose the optimal antigen preparation for later studies (Table 1). The latter results demonstrated that the Formalin-killed, whole-cell antigen gave the best stimulation of blastogenesis for both conventional and vaccinated germfree rat splenocytes and that the optimal antigen concentration was 23 μg/culture.

In another set of preliminary experiments, the optimal concentration of each nonspecific mitogen was also determined. The results of these experiments are shown in Table 2. The lymphocytes were cultured in the presence of the mitogen for 1 or 4 days before adding [$^3$H]thymidine (for 18 h). A 114-h incubation of lymphocytes was better (larger and more consistent incorporation of [$^3$H]thymidine) than a 24-h incubation period. The optimal concentration of PWM at 114 h was 4.2 μg/culture (Table 2). In a similar fashion, the optimal concentrations of PHA and ConA were found to be 50 and 5 μg/culture, respectively. PPD, at a concentration of 5 μg/culture, was found to provide maximum stimulation of splenocyte cultures (Table 2).

TABLE 1. *Blastogenesis of rat splenocytes cultured with various CA preparations*

| Antigen prepn | Concn (μg of protein) | Vaccinated germfree rats[a] | | Unvaccinated conventional rats[b] | |
|---|---|---|---|---|---|
| | | SI | SE[c] | SI | SE |
| Soluble fraction (supernatant) | 0.1 | 0.71 | 0.21 | 0.75 | 0.17 |
| | 0.2 | 0.78 | 0.19 | 0.78 | 0.36 |
| | 1.0 | 0.98 | 0.14 | 0.82 | 0.31 |
| | 20.0 | 0.91 | 0.28 | 0.88 | 0.22 |
| | 40.0 | 1.41 | 0.40 | 1.01 | 0.22 |
| Insoluble fraction (cell wall and membranes) | 0.1 | 0.92 | 0.19 | 0.90 | 0.42 |
| | 0.2 | 0.89 | 0.14 | 1.09 | 0.19 |
| | 1.0 | 0.95 | 0.25 | 1.10 | 0.14 |
| | 20.0 | 1.14 | 0.48 | 1.20 | 0.50 |
| | 40.0 | 2.26 | 1.01 | 0.81 | 0.31 |
| Formalin-killed whole cells | 0.1 | 1.14 | 0.70 | 0.92 | 0.31 |
| | 0.2 | 1.20 | 0.42 | 1.10 | 0.18 |
| | 2.3 | 4.40 | 1.26 | 1.01 | 0.18 |
| | 23.0 | 5.21 | 1.49 | 3.30 | 0.41 |
| | 230.0 | 1.02 | 0.42 | 1.19 | 0.36 |

[a] Results represent the mean SI of five germfree rats vaccinated with Formalin-killed *C. albicans* yeast cell antigen in CFA 2 weeks before sacrifice.
[b] Results represent the mean SI of five conventional rats.
[c] SE, Standard error of the mean.

TABLE 2. *Blastogenesis of rat splenocytes cultured with various mitogenic agents*

| Mitogenic agent | Concn (μg of dry wt/culture) | Vaccinated germfree rats[a] | | Conventional rats[b] | |
|---|---|---|---|---|---|
| | | SI | SE[c] | SI | SE |
| PHA | 5 | 37.18 | 7.85 | 1.11 | 0.39 |
| | 25 | 22.70 | 5.70 | 6.41 | 1.86 |
| | 50 | 49.34 | 12.27 | 12.81 | 2.40 |
| | 250 | 5.31 | 1.41 | 3.19 | 0.82 |
| | 500 | 2.11 | 1.01 | 1.06 | 0.33 |
| ConA | 0.5 | 40.60 | 9.66 | 6.85 | 1.51 |
| | 5.0 | 64.29 | 5.80 | 8.61 | 1.22 |
| | 25.0 | 3.18 | 0.74 | 2.29 | 0.63 |
| | 50.0 | 1.81 | 0.44 | 1.45 | 0.40 |
| PWM | 2.0 | 1.02 | 0.41 | 2.12 | 1.04 |
| | 4.2 | 12.38 | 4.26 | 5.83 | 1.41 |
| | 6.6 | 11.46 | 4.15 | 1.51 | 0.10 |
| | 8.4 | 10.45 | 3.17 | 1.04 | 0.34 |
| | 10.0 | 2.61 | 1.03 | 1.08 | 0.13 |
| PPD[d] | 1.0 | 1.21 | 0.28 | | |
| | 5.0 | 3.91 | 0.51 | | |
| | 10.0 | 2.52 | 0.16 | | |
| | 50.0 | 2.73 | 1.55 | | |

[a] Results represent the mean SI of five germfree rats vaccinated with Formalin-killed *C. albicans* yeast cell antigen in CFA 2 weeks before sacrifice.
[b] Results represent the mean SI of five conventional rats.
[c] SE, Standard error of the mean.
[d] Rats in this group received only CFA, 2 weeks before sacrifice.

**In vitro blastogenesis of splenocytes from rats infected with *C. albicans*.** To assess the degree of antigen-specific cellular hy-

persensitivity present in splenocytes after *C. albicans* challenge, lymphocytes were taken from the spleen of germfree or conventional animals at various times after challenge and cultured in vitro in the presence of the specific CA. The results of this experiment are shown in Fig. 1. Splenic lymphocytes from germfree animals did not show a statistically significant blastogenic response to CA until day 9 after challenge (when splenocytes cultured with antigen were compared with spleen cells cultured without antigen). It is interesting that the response of conventional rat spleen cells to CA (although significant on day 0; $P < 0.01$) dropped to insignificant levels (Fig. 1) on days 3 and 6 postinfection and then returned to a statistically significant level on day 9 ($P < 0.001$).

A previous report showed that germfree rats treated with FKCA in CFA, CFA alone, or IFA were more resistant than nonvaccinated animals to an intravenous challenge with $10^5$ viable *C. albicans* (25a). It would be interesting to determine if *C. albicans*-vaccinated germfree rats, when challenged with *C. albicans,* could manifest blastogenic response to CA in the same manner as nonvaccinated germfree animals. The results of such an experiment are shown in Fig. 2. The CA-specific blastogenic response of lymphocytes from FKCA in CFA-vaccinated germfree rats was significant before challenge with viable *C. albicans.* Six days after intravenous infection with *C. albicans,* however, splenocytes from the FKCA in CFA-vaccinated germfree rat group manifested a depressed blastogenic response to CA similar to that observed with conventional rat splenocytes (cf. Fig. 2 and 1). On the other hand, 6 days after challenge, splenocytes from rats treated with IFA or CFA, when cultured with CA (Fig. 2), manifested a significantly greater level of DNA synthesis than the splenocytes from prechallenged controls. The latter CA-specific blastogenic responses, however, were not statistically different from the CA-specific blastogenesis of germfree rat splenocytes 6 days after *C. albicans* challenge (Fig. 1, SI of 2.8).

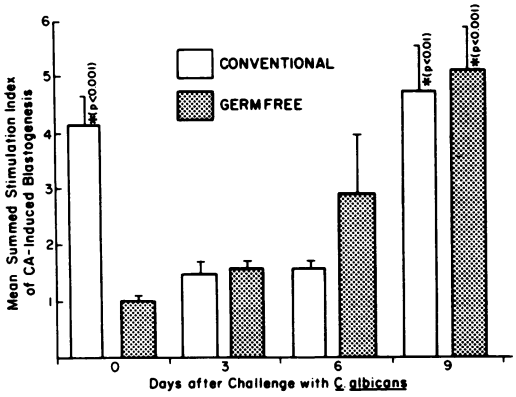


FIG. 1. *CA-specific cellular hypersensitivity of splenocytes from conventional and germfree rats sacrificed at various times after challenge with $10^5$ viable C. albicans. The summed SI represents the mean sum of responses with 2.3, 23.0, and 230 μg of CA per culture. The bars and vertical lines represent the mean and standard error of five rats. The asterisk (*) indicates a CA-specific response in which the DNA synthesis of antigen-stimulated splenocytes is statistically higher than the DNA synthesis of control splenocytes.*

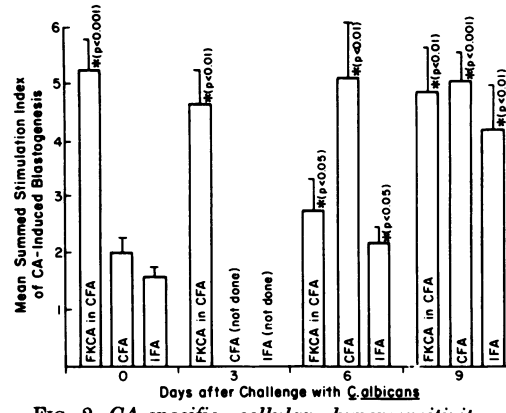


FIG. 2. *CA-specific cellular hypersensitivity of splenocytes from germfree rats vaccinated with FKCA in CFA, CFA alone, or IFA 2 weeks before infection with $10^5$ viable C. albicans. The bars and vertical lines represent the mean and standard error of at least five rats. The asterisk (*) indicates a CA-specific response in which the DNA synthesis of antigen-stimulated splenocytes is statistically higher than the DNA synthesis of control splenocytes.*

Since there was some indication, both in the literature and from the studies reported herein, that infection by *C. albicans* may lead to a suppression of in vitro CMI responses, the lymphocytes from rats infected with *C. albicans* were cultured with various nonspecific polyclonal mitogens, and the resulting DNA synthesis was measured. In the first of these experiments, germfree and conventional rats were sacrificed at various times after challenge with *C. albicans,* and the blastogenic responses of splenocytes cultured with PHA, ConA, or PWM were measured. The results of these experiments (Table 3) show that the DNA synthesis of germfree rat splenocytes cultured with PHA increased significantly by day 3 postchallenge and then dropped on days 6 and 9 to values close to that seen on day 0. The pattern seen in the response of conventional rat splenocytes to PHA differed from that of germfree rats since there appeared to be a suppression of DNA synthesis, on days 6 and 9 postinfection, below the level observed on day 0. The DNA synthesis of germfree rat splenocytes cultured with ConA showed an in-

crease on days 3, 6, and 9 after challenge with *C. albicans*. Conversely, 3 days after *C. albicans* challenge, conventional rat splenocytes showed a significant suppression in the level of ConA-induced blastogenesis; however, an increase, to levels above that observed on day 0, was observed on days 6 and 9 postchallenge. The PWM-induced response of conventional rat splenocytes manifested a suppression on days 3, 6, and 9 after challenge, whereas the DNA synthesis of germfree rat splenocytes stimulated with PWM showed a small increase 3 through 9 days after *C. albicans* infection.

In general, these latter blastogenesis studies (Table 3) indicated that splenocytes from germfree rats manifested no suppression of in vitro cellular blastogenesis after intravenous infection with *C. albicans*, but there did appear to be some blastogenesis depression, albeit inconsistent, on days 3 and 6 in conventional rats similarly challenged with viable organisms.

TABLE 3. *DNA synthesis in splenocytes from conventional and germfree rats infected with C. albicans*[a]

| Mitogen | Days after *C. albicans* challenge | Conventional rats | | Germfree rats | |
|---|---|---|---|---|---|
| | | MSSI[b] | SE[c] | MSSI[b] | SE |
| PHA[d] | 0 | 19.54 | 2.32 | 5.00 | 1.01 |
| | 3 | 24.00 | 3.62 | 18.58 | 2.33 |
| | 6 | 10.66 | 1.14 | 9.03 | 1.81 |
| | 9 | 8.54 | 0.89 | 7.81 | 1.68 |
| ConA[e] | 0 | 15.01 | 1.67 | 3.03 | 0.42 |
| | 3 | 5.32 | 0.92 | 8.83 | 1.09 |
| | 6 | 24.21 | 3.42 | 12.11 | 2.06 |
| | 9 | 22.08 | 1.01 | 15.93 | 2.43 |
| PWM[f] | 0 | 12.14 | 2.64 | 3.01 | 0.62 |
| | 3 | 3.85 | 0.82 | 5.09 | 1.71 |
| | 6 | 6.13 | 1.01 | 5.14 | 0.98 |
| | 9 | 8.02 | 1.67 | 5.01 | 0.81 |

[a] Rats were infected with $10^5$ viable *C. albicans* and sacrificed at various times thereafter.

[b] MSSI, Mean summed stimulation index; values represent the mean of at least five rats.

[c] SE, Standard error of the mean.

[d] Splenocytes were cultured with 25, 50, and 250 μg of PHA per culture.

[e] Splenocytes were cultured with 0.5, 5.0, and 25 μg of ConA per culture.

[f] Splenocytes were cultured with 2.0, 4.2, and 6.6 μg of PWM per culture.

The data in Fig. 1 and 2 and Table 3 show that the level of DNA synthesis of nontreated germfree rat splenocytes stimulated with PHA, ConA, or PWM before infection by *C. albicans* was significantly lower than the level seen in conventional animals before challenge. The results of studies on the blastogenesis of splenocytes from germfree and conventional rats (treated and nontreated) before challenge with *C. albicans* are summarized in Table 4. Once the rats were infected or treated (with FKCA in CFA, CFA, or IFA), the level of DNA synthesis in lymphocytes from germfree rats approached that observed in splenocytes from conventional rats and eventually became even greater; e.g., the DNA synthesis of germfree rat splenocytes cultured with PHA or ConA 21 days after *C. albicans* infection (data not shown) reached an SI of 46 or 89, respectively, as compared with about 19 and 15 in conventional rats.

An assessment of the in vitro CMI responses of germfree rats vaccinated with FKCA in CFA, with CFA alone, or treated with IFA and then challenged with viable *C. albicans* was also carried out. The results of these experiments are reported in Table 5. The data in Table 5, with splenocytes from rats vaccinated with FKCA in CFA, show that infection with *C. albicans* has a suppressive effect on the PHA-induced DNA synthesis. At the same time, the DNA synthesis of the splenocytes cultured with ConA initially increased after challenge (day 3), and then on day 6 dropped below the level observed on day 0. A suppression of the PHA- and ConA-induced DNA synthesis after challenge with *C. albicans* was also observed in germfree rats treated with CFA or IFA alone. The PWM-stimulated re-

TABLE 4. *PHA-, ConA-, PWM-, and CA-induced blastogenesis of splenocytes from conventional and both vaccinated*[a] *and nonvaccinated germfree rats*[b]

| Mitogen or antigen | Nonvaccinated rats | | | | Vaccinated germfree rats | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Germfree | | Conventional | | FKCA in CFA | | CFA | | IFA | |
| | MSSI | SE | MSSI | SE | MSSI | SE | MSSI | SE | MSSI | SE |
| PHA | 5.00 | 1.01 | 19.54 | 2.32 | 49.34 | 4.02 | 35.38 | 4.56 | 22.94 | 3.22 |
| ConA | 3.03 | 1.09 | 15.01 | 1.67 | 81.99 | 12.82 | 16.61 | 2.02 | 14.31 | 1.66 |
| PWM | 3.01 | 0.62 | 12.14 | 2.64 | 12.38 | 2.71 | 2.82 | 0.36 | 2.97 | 0.73 |
| CA | 0.92 | 0.16 | 4.10 | 0.52 | 5.18 | 0.58 | 2.00 | 0.24 | 1.52 | 0.20 |

[a] Germfree rats were vaccinated with FKCA in CFA, CFA alone, or IFA 2 weeks before sacrifice.

[b] Results represent the mean summed responses of at least five rats per treatment group. MSSI, Mean summed stimulation index; SE, standard error of the mean.

TABLE 5. *Blastogenic response of splenocytes from vaccinated germfree rats infected with C. albicans*[a]

| Mitogen or antigen | Days after *C. albicans* challenge[b] | Vaccination | | | | | |
|---|---|---|---|---|---|---|---|
| | | FKCA in CFA | | CFA | | IFA | |
| | | MSSI[c] | SE | MSSI[c] | SE | MSSI[c] | SE |
| PHA[d] | 0 | 49.34 | 4.02 | 35.38 | 4.56 | 22.94 | 3.22 |
| | 3 | 17.66 | 2.34 | 15.25 | 2.09 | 22.50 | 2.15 |
| | 6 | 19.61 | 1.81 | 11.82 | 1.34 | 13.50 | 1.50 |
| ConA[d] | 0 | 81.99 | 12.82 | 16.61 | 2.02 | 14.13 | 1.66 |
| | 3 | 106.15 | 20.81 | 4.45 | 0.39 | 5.92 | 0.77 |
| | 6 | 31.77 | 3.44 | 5.92 | 0.88 | 4.61 | 0.54 |
| PWM[d] | 0 | 12.38 | 2.71 | 2.82 | 0.36 | 2.97 | 0.73 |
| | 3 | 11.64 | 2.22 | 4.62 | 0.41 | 13.23 | 2.01 |
| | 6 | 11.85 | 1.26 | 3.01 | 0.68 | 4.09 | 0.41 |
| PPD[e] | 0 | 4.22 | 0.81 | 5.43 | 1.14 | 2.07 | 0.34 |
| | 3 | 3.13 | 0.54 | 3.01 | 0.68 | 2.68 | 0.39 |
| | 6 | 1.59 | 0.20 | 2.81 | 0.52 | 2.91 | 0.68 |

[a] Germfree rats were vaccinated 2 weeks before infection with *C. albicans*. Rats were sacrificed at various times after infection, and the splenocytes were assayed for their mitogen- or antigen-induced DNA synthesis. MSSI, Mean summed stimulation index; SE, standard error of the mean.

[b] $10^5$ viable *C. albicans*.

[c] Results represent the mean of at least five rats.

[d] The doses of PHA, ConA, and PWM used in these experiments are given in Table 3.

[e] Splenocytes were cultured with 5.0, 10.0, and 50.0 μg of PPD per culture.

sponses, on the other hand, were not suppressed after challenge in any of the treated rat groups, suggesting that, as in mice (T. Rogers and E. Balish, submitted for publication), mainly T-cell and not B-cell responses are affected by the *C. albicans* infection.

The PPD-induced response of splenocytes from germfree rats vaccinated with FKCA in CFA (Table 5) or CFA alone also appeared to be suppressed by the infection with *C. albicans*. In germfree animals vaccinated with mycobacterial antigens (present in the CFA), there was an initial SI (before infection with *C. albicans*) of 4.2 (for the FKCA in CFA-vaccinated group) and 5.4 (for the CFA alone). Six days after *C. albicans* infection the SI dropped to 1.5 and 3.0, respectively. Although this suppression was not dramatic in terms of absolute values, the DNA synthesis of PPD-stimulated splenocytes from infected rats was only 38 to 52% of the values observed before infection ($P < 0.05$).

A comparison of the results in Tables 3 and 5 for vaccinated and nonvaccinated germfree rats before infection with *C. albicans* (summarized in Table 4) shows that the mitogen-induced DNA synthesis of spleen cells was amplified by the vaccination with FKCA in CFA, or CFA alone, or treatment with IFA. The greatest increase in mitogen-induced DNA synthesis of the germfree rat splenocytes occurred when rats were vaccinated with FKCA in CFA. The DNA synthesis of lymphocytes from the latter group of germfree rats was even higher than that observed in conventional rats (Table 4).

The data in Table 4 also indicate that germfree rats vaccinated with FKCA in CFA exhibit a greater response to PHA, ConA, and PWM than germfree rats vaccinated with CFA alone. It appears that the incorporation of FKCA into the CFA had a greater effect on the T- and B-cell populations than the CFA alone. The vaccination with CFA (alone) or treatment with IFA did not stimulate the PWM-induced blastogenic response of the splenocytes from germfree rats above that seen with splenocytes from nonvaccinated germfree rats (Table 4). The vaccination with FKCA in CFA, however, did induce a significant increase in the PWM-stimulated blastogenesis of germfree rat splenocytes.

**Effect of various sera on the response of splenocytes in vitro.** All of the data reported thus far are reported for splenocyte cultures containing 20% autologous rat sera. It is possible that the suppression in blastogenesis observed in this paper was due to a "factor" present in the rat sera. To determine if the sera used in the splenocyte cultures were responsible for the suppression in blastogenesis reported herein, splenocytes from uninfected (*C. albicans*) germfree or conventional rats were cultured with various sera, and the response to PHA was determined. The data (not shown) indicate that sera from *C. albicans*-infected rats (with depressed PHA and ConA responses) were incapable of suppressing the responses of normal splenocytes to PHA. Sera collected from animals after their responses to PHA andConA had returned to normal (day 9 postchallenge) also had no suppressive effect

on the response of normal splenocytes to PHA. The responses (to PHA) of rat splenocytes cultured with fetal calf sera were not significantly different than those of splenocytes cultured with autologous sera. The results of this experiment also show that sera from conventional rats had no influence on the response of germfree rat splenocytes to PHA, and vice versa. This indicates that the suppressed PHA response of splenocytes from conventional rats challenged with *C. albicans* cannot be explained by enhancing or suppressive factors in the respective rat sera.

## DISCUSSION

The development of cellular hypersensitivity in germfree and conventional rats infected with *C. albicans* was assessed in these studies. The development of CA-specific cellular hypersensitivity in germfree animals occurred about 9 days after intravenous infection with viable cells. This was also the time during which the peak of infection by *C. albicans* in germfree rats was found to take place (Rogers and Balish, submitted for publication). A kinetic (but not causal) relationship between this in vitro correlation of CMI and resistance to *C. albicans* growth in the kidneys was indicated by these experimental conditions.

The CA-specific cellular hypersensitivity of splenocytes from conventional animals showed a suppression during the first 6 days after challenge with *C. albicans*. The same phenomenon was observed in germfree rats vaccinated with FKCA in CFA 2 weeks before challenge. The reason for this suppression is not clear, but suppression of cellular immunity by *C. albicans* has been reported previously by other investigators (5, 16, 19). Suppression of cellular immunity by a variety of other microorganisms has also been reported (4, 7, 10, 21, 23, 27). Several explanations for a microbially induced suppression of CMI have been offered, and they include direct microbial-derived toxic effects on lymphocytes or macrophages, the induction of tolerance, the stimulation of suppressor T-cell populations, and the synthesis of blocking antibodies (27). The suppression of immunity (as manifested by this in vitro correlate of CMI) early in the infection by *C. albicans* may be an important virulence factor for this microorganism.

The T-cell sensitivity of each of the rat groups after challenge with *C. albicans* was also studied using PHA, ConA, and PWM. PHA and ConA are thought to stimulate only T-cells, whereas PWM is believed to stimulate both T- and B-cells (T-cells are believed to be only weakly activated by PWM). Germfree rats showed a very dramatic increase in T-cell reactivity after the *C. albicans* infection (day 3). On the other hand, conventional rat T-cell responses showed a very dramatic decrease after infection (day 3), followed by a reversal to normal response levels by day 9. The suppression of T-cell reactivity may partially explain the drop in CA-specific cellular hypersensitivity after *C. albicans* infection in conventional rats. Recent reports (2, 12) have shown that the PHA response of spleen cells from mice and rats is suppressed after vaccination with large doses of antigen. Moreover, the latter investigators (12) found that a discrete subpopulation of splenic lymphocytes is responsible for this suppression, indicating that the immune stimulation by antigen may induce suppressor cells and inhibit the response of splenocytes to PHA. It is possible that a stimulation of antigen-sensitive suppressor cells by the *C. albicans* infection was responsible for the drop in PHA- and ConA-induced blastogenesis reported in the present study. A recent report by Stobo et al. (28) has shown that infections by a number of fungi (including *C. albicans*) may all stimulate populations of suppressor T-cells, and that these T-suppressor cells were capable of inhibiting normal lymphocyte responses to both PHA and ConA, as well as *Candida* antigen.

Splenocytes from noninfected and nonvaccinated germfree rats manifested significantly lower responses to CA, PHA, and ConA than any of the other rat groups tested (see Table 4). It has been suggested that lymphoid tissues in germfree animals are populated by greater numbers of immature cells than lymphoid organs in conventional animals (3, 24, 25), and larger numbers of these immature cells may have been responsible for the less intense blastogenic response of the germfree rats. Once vaccinated with Freund adjuvant, or infected with *C. albicans*, however, the splenocyte blastogenic responses of the latter animals increased to levels very near those of conventional animals. It is very likely that the infection by *C. albicans*, or the Freund adjuvant vaccination, provided more mature lymphocytes to the spleen, and these more immunocompetent cells were capable of greater responses in the blastogenesis assay.

The infection of nonvaccinated germfree rats did not lead to a depression in the T-cell responses. It is possible that because of the overall immaturity of lymphocytes in germfree animals (13, 24, 29–32), the infection by *C. albicans* in germfree rats did not stimulate suppressor cell populations. Germfree rats vaccinated with either FKCA in CFA or CFA alone, or treated with IFA, and challenged with *C. albicans*, all

showed a suppression of T-cell responses (days 3 and 6 after challenge) similar to that of conventional rats. This indicates that prior sensitization by *Candida* antigens is not a requirement for the establishment of this T-cell suppression, since animals immunized with *Candida* antigens (conventional rats in addition to the FKCA-in-CFA vaccination group), as well as those just treated with the adjuvants alone, exhibited the suppression.

It is interesting that a disseminated infection by *C. albicans* would lead to a suppression in the in vitro cellular immune responses of vaccinated germfree and conventional rats. The role of CMI in protection against disseminated candidiasis is not clearly understood at this time. Rogers et al. (26) have recently shown that congenitally athymic mice are more resistant to a disseminated infection by *C. albicans* than their phenotypically normal littermates. Further evidence (Rogers and Balish, submitted for publication) indicated that defense against renal candidiasis may be primarily dependent on the expression of the innate immune systems. The present report suggests the possibility that expression of cellular immunity in defense against disseminated candidiasis may be inhibited by the *Candida* infection itself. The mechanism of this suppression is unclear, but the time course of its expression suggests the possibility that suppressor T-cells (1, 2, 12) or macrophages may be involved.

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