Medical Mycology 2002, **40**, 169–178 Accepted 7 August 2001

Experimental *Paracoccidioides brasiliensis* infection in mice: influence of the hormonal status of the host on tissue responses

B. H. ARISTIZÁBAL*, K. V. CLEMONS†‡§, A. M. COCK*, A. RESTREPO*, AND D. A. STEVENS†‡§

Corporación para Investigaciones Biológicas[1], Medellín, Colombia; †Division of Infectious Diseases, Department of Medicine, Santa Clara Valley Medical Center and ‡California Institute for Medical Research, San Jose, California 95128; §Division of Infectious Diseases and Geographic Medicine, Department of Medicine, Stanford University Medical School, Stanford, California 94305

We have previously proposed that 17β-estradiol may be responsible in part for the decreased frequency of clinical paracoccidioidomycosis in females via a blocking of the initial morphological transformation necessary to initiate infection. Here we examined the course of infection in male and female mice in relation to their hormonal status. After pulmonary infection with conidia, normal males showed progressive infection, whereas normal females restricted proliferation and progressive disease. In contrast, castrated animals exhibited lesser capacity to restrict disease progression. Castrated male mice reconstituted with 17β-estradiol initially restricted proliferation, but showed disease progression later in infection, whereas castrated female mice reconstituted with testosterone were unable to restrict disease. Quantitative histological analyses demonstrated that only normal male and castrated reconstituted mice developed granulomas, which decreased in number and size with time correlating with increasing numbers of CFU in the lungs. Greater numbers of chronic inflammatory foci did not correlate with higher CFU. These results further support a role for 17β-estradiol during early innate resistance of females to paracoccidioidomycosis.

Keywords *Paracoccidioides brasiliensis,* murine models, estrogens, granuloma

Introduction

Paracoccidioidomycosis is one of the most important systemic mycoses in Latin America, especially in countries such as Brazil, Colombia and Venezuela. The etiological agent, *Paracoccidioides brasiliensis,* is a dimorphic fungus that grows as a mold at temperatures below 26 °C and as a yeast, characterized by multiply budding cells, both at 37 °C and *in vivo* [1–3]. Its natural habitat is probably the soil [4]. The infection is likely to be acquired through inhalation of the infectious propagules, which are probably mycelial fragments and conidia [5]. The clinical manifestations, which vary by patient, range from asymptomatic pulmonary infection to systemic generalized disease [2,3,6,7].

One of the most intriguing characteristics of paracoccidioidomycosis is its unequal gender distribution, with males more frequently manifesting clinical disease than females in a ratio of about 15:1 [8]. Early *in vitro* studies showed that the female hormone 17β-estradiol (E_2) was capable of inhibiting the transformation of the mycelial or the conidial propagules into yeast cells, thus suggesting a connection with the increased resistance of females to paracoccidioidomycosis [9,10]. More recently, *in vivo* studies corroborated the previous *in vitro* findings demonstrating that in normal male BALB/c mice over the course of the first 96 hours of infection, conidia progressively transformed in the lung into the tissue-form yeast cells, while during the same time no transformation of conidia into yeast occurred in normal females [11]. Furthermore, no CFU were recovered from

Correspondence: Dr K.V. Clemons, Division of Infectious Diseases, Santa Clara Valley Medical Center, 751 South Bascom Avenue, San Jose, CA 95128-2699. Tel.: (408) 998-4557; fax: (408) 998-2723; e-mail: clemons@cimr.org.

the lungs of normal female mice after two to six weeks of infection, whereas CFU were recovered from the lungs of normal male mice [11]. Thus, the blocking of the conidia to yeast transformation in female mice hindered the progression of the infection, allowing the female host to be more resistant.

In our initial study, we also noted differences in the histological profile between male and female mice after two to six weeks of infection [11]. In this study we chose to further examine the differences in the histological response of BALB/c mice infected with the conidia of *P. brasiliensis*, and the possible role that the hormonal status of the animals might have in this response, and how the hormonal status of the host affects the longer-term course of infection. The experiments included normal males and females as well as castrated and castrated inversely hormone-reconstituted animals. Our results indicate that gender, castration and castration accompanied by inverse hormonal reconstitution influenced the histopathological pattern of response of the host induced by the *P. brasiliensis*.

Materials and methods

Fungus and inoculum

P. brasiliensis, isolate ATCC 60855, in the M form was used to produce conidia as described previously [12]. The viability of the conidia was determined by ethidium bromide fluorescence staining [13], and the inoculum was adjusted so that 4×10^6 viable conidia were contained in 0.06 ml. The inoculum was administered by intranasal instillation in two 30-µl volumes instilled approximately one hour apart. Instillations were done while the animals were under methoxyflurane anesthesia [11,14].

Animals

Specific pathogen-free male and female BALB/c mice obtained from Simonsen Laboratories (Gilroy, Calif.) were used. The animals were 4–6 weeks old, and were supplied with sterilized food and acidified water *ad libitum*. They were placed in six groups: normal males (NM), normal females (NF), castrated males (CM), castrated females (CF), castrated males reconstituted with 17β-estradiol (CM+E) and castrated females reconstituted with testosterone (CF+T). Gonadectomies of male and female mice were performed when the animals were two weeks of age by personnel at Simonsen Laboratories.

Hormonal reconstitution began 48 h prior to infection by subcutaneous injection. Castrated males were given 17β-estradiol valerate, 50 µg in 0.1 ml of sesame oil (Delestrogen, 17β-estradiol valerate injection USP, Squibb & Sons, Princeton, NJ), and castrated females received testosterone propionate, 62.5 µg in 0.1 ml of sesame oil (testosterone propionate for injection USP, Steris Laboratories, Phoenix, Arizona). Hormone treatments were given once daily, for 6 weeks [15].

Histopathology

At 2, 4 and 6 weeks post-challenge, three mice from each group were killed and their lungs removed for analysis. The right lung was removed, insufflated, fixed in 10% neutral buffered formalin and embedded in paraffin. Serial sections (5-µm thick) were prepared and stained either with hematoxylin and eosin (H&E) or by the silver-methenamine Gomori technique (GMS stain). The latter served to detect and count fungal cells and assess their morphology.

H&E stained sections were used to observe the host's cellular response and the type of host cells present in the tissues. Using a ×10 objective, 10 different fields in the lung, selected at random, were chosen for further study. The numbers of inflammatory or granuloma foci in the fields, as well as the total area of each focus, were determined using the NIH Image 1.61/ppc program for Macintosh Performa 6400/180. This program uses pixels^2 as the unit of measurement for area (using a ×10 objective, 1 µm is equal to 0.14 pixels^2) [16]. The following measurements were made: (a) total area of the 10 lung fields selected, once the empty spaces represented by major bronchi and arteries had been excluded; (b) area of all inflammatory foci. The numbers of foci, total area of the affected area and the area per focus are presented as the mean ± SD. The cellular response was differentiated into a chronic inflammatory response or a granulomatous response for purposes of scoring. A chronic inflammatory response was defined as foci consisting primarily of mononuclear cells, whereas the granulomatous response showed a distinctive pattern of chronic inflammation in which activated macrophages were the predominant cell type, characterized by an epithelioid appearance (giant cells could be present).

CFU determination

At the same sample times of 2, 4 and 6 weeks postinfection used for examining the histopathological response of the animals, the number of CFU in the left lungs were determined as described previously [11].

Statistical analyses

Statistical analyses were done with the aid of the GraphPad Prism Software (version 2.01, GraphPad Software, San Diego, California) or GB-STAT (ver. 6,

Dynamic Microsystems, Silver Spring, Maryland). Comparisons among the different groups were made by ANOVA followed by a Newman-Keuls test to determine which groups differed. Three replicas of each experiment per group of mice at each time point were done. A value of $P<0.05$ was considered to indicate statistical significance in all cases. Regression analyses were done to observe the correlation between inflammatory foci and presence of granulomas or number of CFU recovered from the lungs.

Results

Presence of yeast cells in lung

A GMS stain was used to recognize in sections of lung tissue the three types of *P. brasiliensis* propagules; conidia, intermediate cells (conidia in transition to yeast) and yeast cells. At 2 weeks post-challenge, yeast cells were found in the lungs of only three of the six groups of mice. These were the NM, CM+E and CF+T. The NF, CM and CF mice had no yeast cells at this time point ($P<0.05$). Observations later in the course of infection (4 and 6 weeks post-challenge) showed the same pattern. NM, CM+E and CF+T mice had yeast cells present in the lungs, whereas none were observed in NF, CM or CF. Although well developed yeast cells were abundant in the sections taken from NM, none were observed in sections taken from NF but instead granular methenamine silver deposits previously described as 'fungal dust' [17] were observed.

The histological observations of the presence or absence of yeast in the lungs were corroborated only in part by the determination of the number of CFU of *P. brasiliensis* recovered from the various animals at the same time points. These data are presented in Table 1 and show that viable organisms were recovered at all times from animals in all groups except the NF. These results are similar to the histological observations by GMS staining of the presence of yeast cells in NM, CM+T and CF+E, as well as the absence of yeast in the NF. However, viable *P. brasiliensis* were recovered from CM and CF even though no organisms were observed in the tissue sections stained with GMS.

Comparisons of the 95% confidence intervals around the means showed that NF had significantly lower mean burdens at all time points, and that CF+T carried significantly higher burdens at week 2 than did other groups; the burdens in NM and CF were equivalent and significantly lower than those in CM. By weeks 4 and 6, CM+E carried significantly higher mean burdens than did any other group; the next highest burdens were carried by the CF+T group, which were significantly higher than those recovered from NM, NF, CM or CF. Both hormonally reconstituted groups showed increasing numbers of CFU in the lungs with time. The fungal burdens in the CF showed a progressive decline from

Table 1 Recovery of viable *Paracoccidioides brasiliensis* from the lungs

	Log_{10} geometric mean number of cfu ± 95% confidence interval in the lungs at week post infection		
Group	2	4	6
NM	2.41†	2.09	2.55
	2.37–2.45	2.06–2.12	2.52–2.59
CM	2.83	2.86	2.36**
	2.69–2.98	2.72–3.00	2.23–2.48
CM+E	0.71†	4.49§	4.93§
	0.68–0.74	4.40–4.58	4.88–4.98
NF	0*‡	0	0
CF	2.47†	2.37	2.25**
	2.41–2.52	2.29–2.45	2.18–2.31
CF+T	3.47	3.99¶	4.02¶
	3.44–3.50	3.95–4.02	3.97–4.07

NM, normal males; CM, castrated males; CM+E, castrated maled administered estradiol; NF, normal females; CF, castrated females; CF+T, castrated females administered testosterone.
*A value of 0 indicates that any viable cfu in the lungs were below the detectable limit of the assay of approximately 5 cfu.
†cfu significantly lower than CM.
‡cfu significantly lower than all other groups at all time points.
§cfu significantly higher than all other groups.
¶cfu significantly higher that those in NM, NF, CM or CF.
**cfu significantly decreased with time within the group.

week 2 through 6 (significantly lower only by week 6), whereas CM had declined only between weeks 4 and 6, with the CFU significantly lower at week 6. The burden in NM declined between week 2 and 4, but showed a significant progressive increase by week 6. No CFU were recovered from NF at any time point.

Histopathology of lungs sections (H&E stain)

A quantitative scoring system for differences in histological response was developed using the type and the area of response (e.g., chronic inflammation or granulomatous). This scoring system included the numbers of foci present, the total area of the foci and a normalization of the data to give the mean area per focus. We defined chronic inflammation with histological features differently from those manifested in acute inflammation of vascular changes, edema and a largely neutrophilic infiltration. Chronic inflammation was characterized by cellular infiltration with mononuclear cells, including macrophages, lymphocytes, and plasma cells, which is a reflection of a persistent reaction to injury, tissue destruction, largely induced by the inflammatory cells, evidence of healing with connective tissue replacement of damaged tissue, accomplished by proliferation of small blood vessels (angiogenesis) and, in particular, fibrosis. It should also be noted that angiogenesis and fibrosis are components of wound healing and repair. Granuloma formation was defined by the typical appearance of macrophage groups, as well as by epithelioid and giant cells.

The overall histological picture differed by group. In NM, there was a chronic inflammatory reaction with predominance of lymphocytes and macrophages (Fig. 1a); few plasmocytes were also observed. Granuloma formation was apparent with yeast cells present in the middle of the granuloma. This formation was composed of macrophages with an eosinophilic cytoplasm indicative of their epithelioid transformation. In contrast, in NF the chronic inflammatory reaction was scarce, although some small mononuclear cells (lymphocytes) were regularly observed. In these animals, there were few chronic inflammatory foci or granulomas, and yeast cells were absent (Fig. 1b).

CM mice had a mild-to-moderate chronic inflammatory reaction. There were increased numbers of lymphocytes, macrophages, as well as some plasmocytes. Granulomas were absent and there were no yeast cells apparent (Fig. 2a). Similarly, there was no chronic inflammatory reaction, nor were granulomas or yeast cells observed in CF mice (Fig. 2b).

Both groups of castrated inversely hormone-reconstituted animals presented a strong, chronic inflammatory reaction with lymphocytes, PMN and macrophages as predominant cells; small numbers of giant cells and some plasmocytes were also seen. Granulomas were also observed at all sample times. These were loosely organized and composed of lymphocytes, macrophages, epithelioid and giant cells. Yeast cells were also present and were regularly seen in the center of the granulomas (Fig. 3a, b).

Some chronic inflammatory foci were present in all groups at all sample times. However, there were differences in the number of foci present and the size of the infiltrates (Fig. 4a, b). CM+E and CF+T animals had significantly greater numbers of chronic inflammatory foci at all sample times ($P<0.01$) than did all other groups, with the CM+E animals having significantly greater numbers than CF+T animals ($P<0.01$). By week 4 of infection, CM had greater numbers of inflammatory foci than NM, NF or CF ($P<0.05$ or 0.01). Week to week changes in the number of chronic inflammatory foci in each group were minimal.

Interestingly, comparisons of the total area of chronic inflammation by group showed that CM had significantly larger total areas at week 2 than did all other groups ($P<0.01$). No differences in total area were found at week 4 of infection. By week 6, NF and CF had significantly smaller areas of chronic inflammation that did CM, CM+E or CF+T ($P<0.05$) (Fig. 4b). The total mean area of chronic inflammation within each group showed some change, with NM and CF+T having the largest mean areas at week 4, whereas areas decreased or remained unchanged for CM, NF and CF. Only the CM+E group showed a progressive increase in the total mean area of chronic inflammation with time (Fig. 4b). Comparisons of the mean area of chronic inflammation per inflammatory focus demonstrated that at week 2 CM had significantly greater areas per focus than all other groups ($P<0.01$). Although no significant differences were noted at week 4, by week 6 NM had significantly greater areas of chronic inflammation per focus than did any other group ($P<0.01$) (Fig. 4c).

Similar to the parameter of chronic inflammation, granuloma formation also differed among groups. The number of granulomas formed and the total area of the granulomas were significantly higher in the CM+E and CF+T groups at all three sampling times than in all other groups ($P<0.01$) (Fig. 5a, b); it was higher in CM+E than in CF+T ($P<0.01$). However, analyses of the mean area per granuloma (Fig. 5c) showed that the NM had significantly larger granulomas than all other groups at weeks 2 and 6 ($P<0.01$) than all other groups; no differences were noted at week 4. Although smaller than those in NM, the CF+T group had larger mean areas per granuloma than did CM, CM+E (week 6 only), NF and

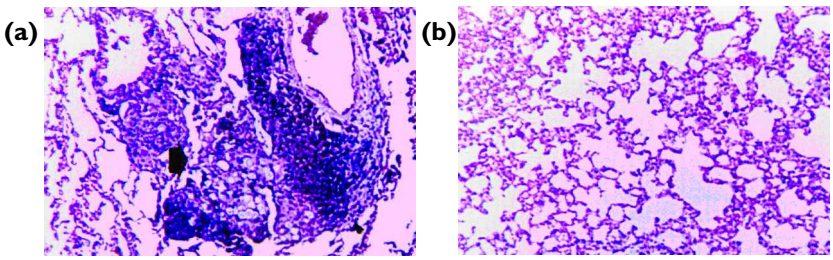

Fig. 1 Histopathology of the lungs in normal mice 4 weeks after infection with conidia of *P. brasiliensis* (H&E, ×100). a, Normal males (NM) showing an intense chronic inflammatory reaction also with granuloma formation and presence of yeast cells. b, Normal females (NF) showing a slight inflammatory reaction, with no yeast cells present.

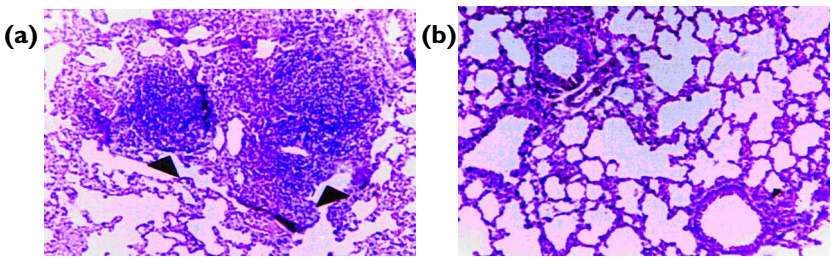

Fig. 2 Histopathology of the lung in castrated mice 4 weeks after infection with conidia of *P. brasiliensis* (H&E, 100). a, A lung section from a castrated male (CM) showing a slight inflammatory reaction with presence of lymphocytes, histocytes and plasma cells similar to the description above except for the intensity of inflammation. Note the absence of granulomas and of yeast cells. b, A lung section from a castrated female (CF) similar to the CM above, but with minimal inflammatory reaction. Note the absence of granulomas and yeast cells.

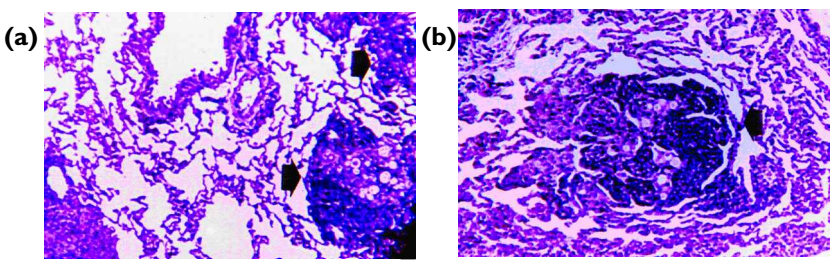

Fig. 3 Histopathology of the lung in castrated inversely hormone-reconstituted mice 4 weeks after infection with conidia of *P. brasiliensis* (H&E, ×100). a, A lung section from a castrated male administered estradiol (CM+E) showing the presence of an intense chronic inflammatory reaction characterized by the presence of loose granulomas with epithelioid and multinucleated giant cells. There are abundant yeast cells. b, A lung section from a castrated female administered testosterone (CF+T) showing the presence of an intense inflammatory reaction characterized by the presence of compact granulomas with epithelioid and multinucleated giant cells. Few yeast cells are present.

CF at weeks 2 and 6 ($P<0.01$). Table 2 presents the frequency with which granulomas were formed according to the gender profile of the mice studied. There was an association between hormonal profile and the presence of granuloma, as this formation was present in NM, CM+E and CF+T but not in NF, CM or CF. There were statistically significant differences ($P<0.00001$) among these groups.

Further regression analyses showed an overall significant correlation between the number of chronic inflammatory foci and the number of granulomas with all groups of animals considered ($r = 0.92265$). Similarly, there was a significant correlation between the number of granulomas and their area ($r = 0.86$), but not between the number of chronic inflammatory foci and their area. The number of CFU and number of granulomas in normal animals showed a strong correlation ($r = 0.99319$), but not in reconstituted animals. Similarly, the area of granuloma in normal animals showed a moderate positive correlation with CFU ($r = 0.71340$) and a negative correlation in reconstituted animals ($r = -0.7654$). No analyses could be done on CM or CF as no granulomas were observed.

Discussion

Previous *in vitro* and *in vivo* studies had shown that gender-related hormones seem to influence the outcome of *P. brasiliensis* infection. This is thought to occur primarily through the action of 17β-estradiol in the organism by inhibiting dimorphism in tissues, similarly to that demonstrated *in vitro* [9,10], which would block the

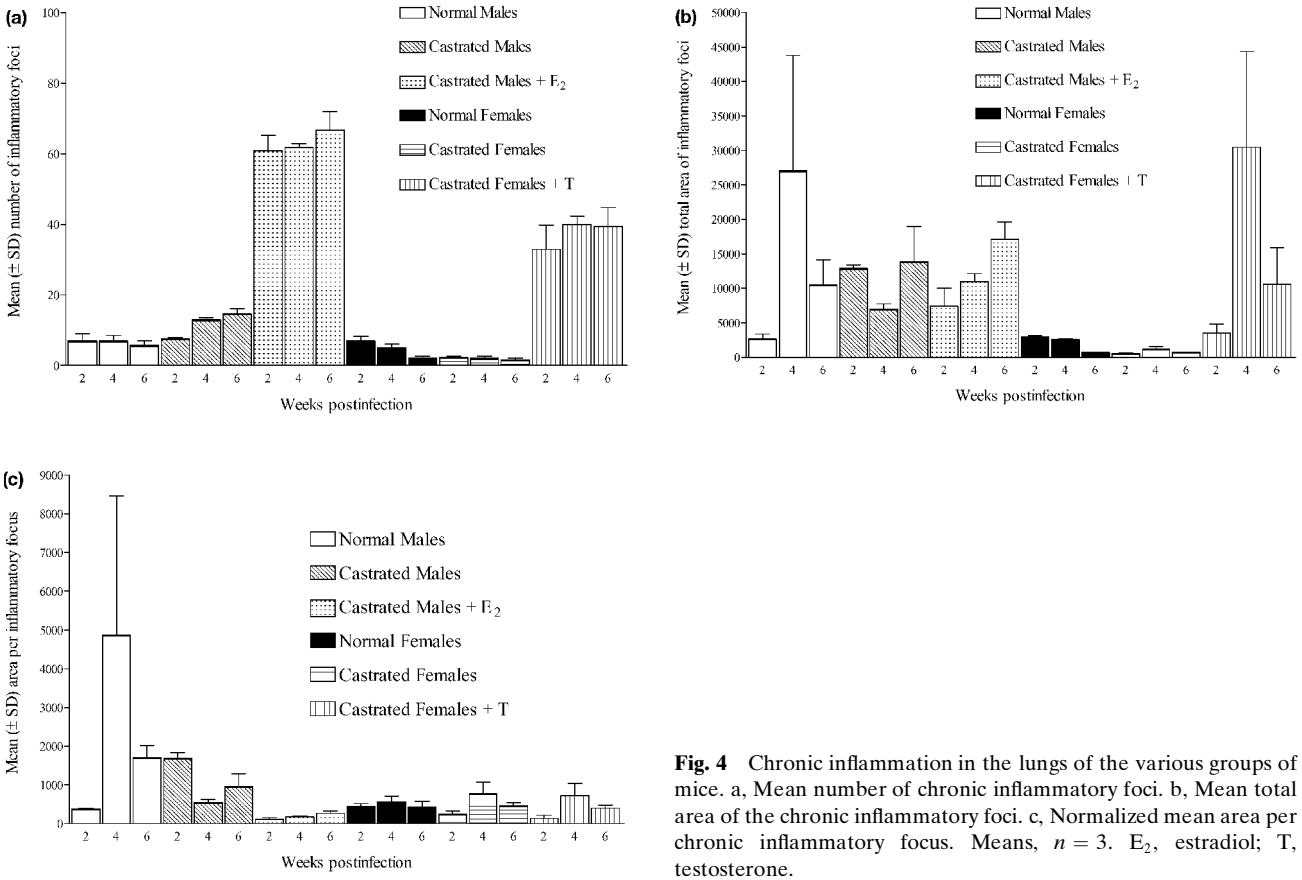

Fig. 4 Chronic inflammation in the lungs of the various groups of mice. a, Mean number of chronic inflammatory foci. b, Mean total area of the chronic inflammatory foci. c, Normalized mean area per chronic inflammatory focus. Means, $n = 3$. E_2, estradiol; T, testosterone.

establishment of infection [8,10,18]. However, not all investigators have found gender-related differences in resistance to infection with *P. brasiliensis* [5]. Similarly, previous investigations with mice and rats infected with yeast cells by the intravenous or intraperitoneal routes showed no clear differences according to gender [17,19,20]. As the yeast form of *P. brasiliensis* is not known to respond to 17β-estradiol [9], it is likely that

Table 2 Analyses of the frequency of granuloma formation according to number and gender profile of affected mice

Groups	Granulomas		
	Present No. animals	Absent No. animals	Total no. of mice with granuloma (%)
Testosterone present*			
Normal males	9	0	
Castrated females + T	9	0	18/18 (100)
17β-estradiol present*			
Normal females	0	9	
Castrated males + E_2	9	0	9/18 (50)
Reduced hormonal levels*			
Castrated males	0	9	
Castrated females	0	9	0/18 (–)

E_2, estradiol; T, testosterone.
* By Chi square, $P < 0.0001$ for an overall comparison of the three groups (numbers in far right column), $P = 0.0005$ for the comparisons of the testosterone present or reduced hormonal levels versus 17β-estradiol present group (numbers in far right column) and $P < 0.0001$ for the comparison of testosterone present versus reduced hormonal levels group (number in far right column).

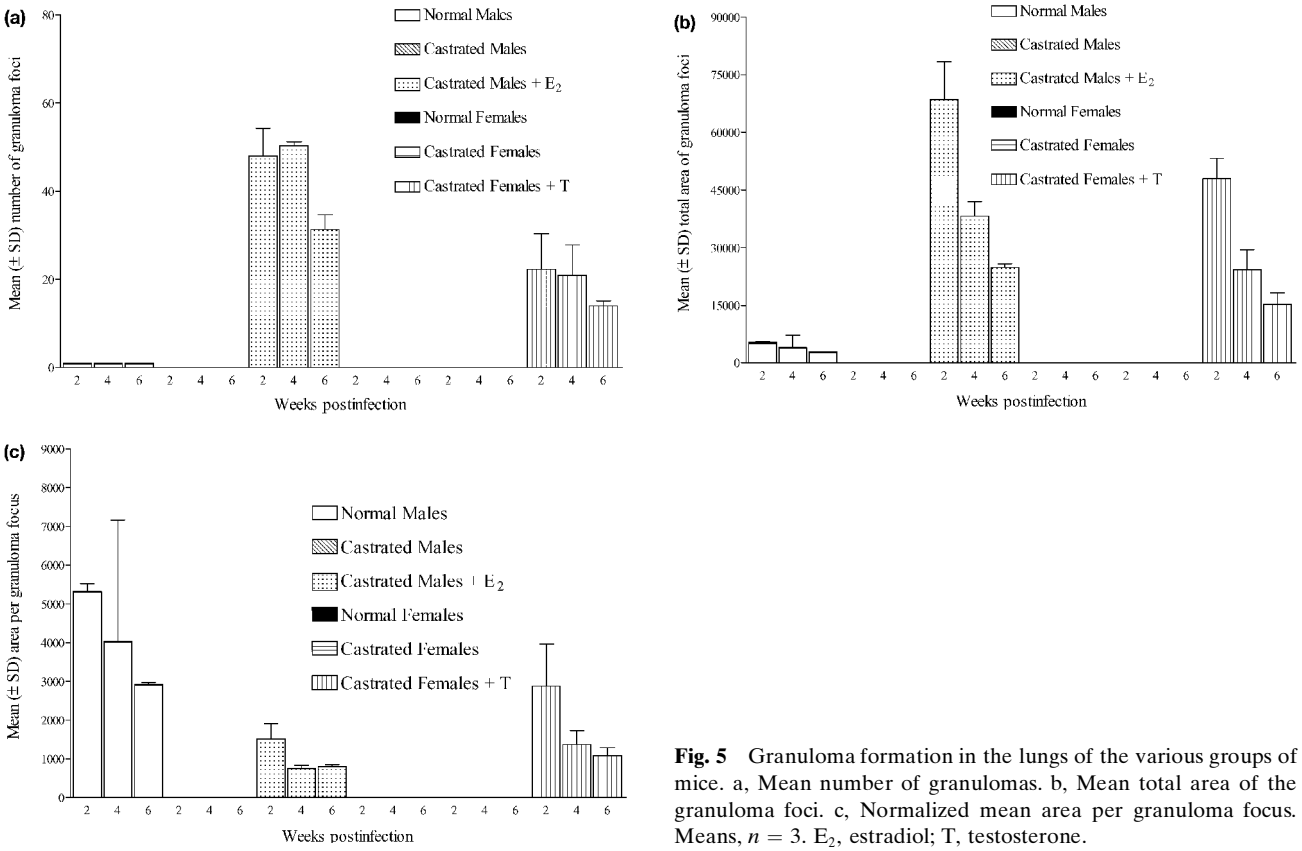

Fig. 5 Granuloma formation in the lungs of the various groups of mice. a, Mean number of granulomas. b, Mean total area of the granuloma foci. c, Normalized mean area per granuloma focus. Means, $n = 3$. E_2, estradiol; T, testosterone.

hormonal influences on the organism become less important when yeast cells are used as the infecting inoculum [8]. Thus, the hypothesis that 17β-estradiol provides a beneficial innate resistance to paracoccidioidomycosis for females is likely to be relevant only for infection initiated by a mycelial or conidial cell.

Numerous authors have indicated that hormones may have a direct influence on the host immune responses [21–25]. These effects have been described as both enhancing and suppressive and likely are concentration-dependent. At pharmacologic concentrations, 17β-estradiol has been reported to suppress T cell-mediated, but not B cell-mediated immunity, whereas testosterone suppresses both [24–28]. Our results show differences among the various groups of mice with differing hormonal status. The NM host responded with an intense large chronic inflammatory and granulomatous reaction throughout the experimental period. Progressive disease with yeast cells was observed histologically and CFU of *P. brasiliensis* were recovered at all time points. In contrast, NF had no progression of disease. In this group, there was an absence of yeast cells observed histologically or by recovery of CFU, and only a minor chronic inflammatory reaction; the lack of granuloma formation is probably due to the absence of viable organisms. These results corroborate and extend our previous data on differences between NM and NF during the course of infection by quantifying the histological response reported previously [11].

Although inhibition of conidial transformation to yeast by 17β-estradiol, and subsequent phagocytosis and killing of the conidia in NF may largely explain the lack of an observable histological response several weeks after infection, the difference in response might also be related to the anti-inflammatory properties of estradiol [26,27,29]. Furthermore, exogenous 17β-estradiol treatment of ovariectomized rats suppresses granuloma formation in the lungs in response to bacillus Calmette-Guerin [30], and reduces polymorphonuclear neutrophil infiltration in lungs in a model of carrageenan-induced pleurisy [29].

In our studies, the castration of mice resulted in an increase in the intensity of the tissue response; the area involved was even larger than in NM, which is consistent with previous reports of an increased inflammatory response after castration [28]. Although no yeast cells were observed in the lungs during the early periods post-challenge, they appeared later indicating that CM and CF mice were unable to control the infection. Furthermore, these animals had no granulomas in their lungs.

Although granuloma formation is considered to be the prime element of defence during the early stages of the host-parasite interaction [20], they tend to become non-effective with time and end by perpetuating the inflammatory reaction without controlling fungal growth [31,32]. Consequently, castration did not increase the resistance of mice to this infection. This seemed particularly relevant in females, in which castration abrogated resistance, further supporting the role of 17β-estradiol in the innate resistance of the female.

The overall response of the castrated animals might be due to adrenal gland compensation for the synthesis of testosterone or to the possible involvement of other androgenic compounds, such as androstenedione or dihydrotestosterone [25,33,34]. Castrated male rats have been shown to convert androstenedione into testosterone in adrenal glands, and to have levels of androstenedione and dihydrotestosterone that recover with time to become equal to those in sham-operated controls [34,35]. Furthermore, androgen synthesis by lymphocytes may also serve to modulate the immune response [36]. Thus, a mechanism of suppressing cellular control of the proliferation of the organism, such as dihydrotestosterone down-regulation of lymphokine production [33], but not sufficient to induce granuloma formation, may explain in part our observations. Whether or not testosterone prompts the host to respond with this type of cellular formation remains to be determined, but the association shown here indicates that such a role may exist.

Castration followed by inverse hormonal reconstitution resulted in data more difficult to interpret. At two weeks post-challenge, CM+E mice were able to restrict fungal multiplication, and appeared similar to NF. In contrast, CF+T were unable to restrict multiplication and had CFU in numbers equal to or greater than NM. These results are in accord with our hypothesis on the importance of 17β-estradiol in innate resistance. However, both hormonally reconstituted groups had significantly greater numbers of CFU in the lungs at week 4 and 6, indicating an inability to control the infection. This was also reflected in the decrease in granuloma formation with time, of which both results are more similar to the NM group. These data indicate that at least for a short time, 17β-estradiol contributed to the early resistance of the CM+E animals. However, yeast cells arose and persisted in spite of the administration of 17β-estradiol. The rise in CFU from week 4 to 6 in the CM+E mice might be reflective of a down-regulation of tumor necrosis factor-α and interferon-γ due to administration of estrogen [26]. Furthermore, an inability to kill the organism might well be related to estrogen depression of interferon-γ-dependent nitric oxide production [29], which is important in the killing of the organism by activated macrophages [37]. Furthermore, immunological modulation by compensatory androgens might also be involved in reducing resistance [34].

In the CF+T group, treatment was deleterious, as the tissue response exceeded the one observed in the NM group, increased the numbers of inflammatory foci and loose granulomas, and was associated with significantly greater numbers of CFU in the lungs than in CF animals. The increasing numbers of CFU in CF+T compared with NM or NF animals is likely to be the result of pharmacological rather than physiological levels of testosterone (i.e., higher than would be found in NM animals), which result in down-regulation of the immune response. Although these levels are probably the primary cause of immunosuppression, the conversion of testosterone to 17β-estradiol or other estrogens might have a role in the lesions. With high concentrations of substrate (i.e., testosterone) available, aromatase activity in the adipose tissue or in macrophages [38] may contribute to localized estrogen levels sufficient to contribute to immunosuppression and reduced killing as discussed above. Furthermore, the decrease in granuloma formation in both groups of hormonally reconstituted mice may be due in part to 17β-estradiol effects, first with induction of a chronic response of granuloma [39] followed by reduction of the inflammatory response. Regardless, alteration of the normal hormonal milieu by either castration or inverse reconstitution resulted in lack of protection against infection with *P. brasiliensis*.

Our results may be indicative of differences in the efficacy of the immune system between genders. It could well be that in females the hormonal environment facilitates the synthesis and release of cytokines by both T and B cells, where physiological levels of 17β-estradiol contribute to the activation of macrophages [40–44]. Thus, the estrogens could influence not only the fungus, but also the immune response of the host by activating macrophages and increasing their fungicidal activity. Suggestive of this were the granular silver methenamine deposits observed in lung macrophages, in the absence of yeast cells in NF. To this effect, direct estrogenic influence on *P. brasiliensis*, as well as hormonal modulation of macrophage response based on concentration of estrogens [42–45], seem to be involved and would serve to make the female host much less susceptible to *P. brasiliensis*.

It seems to be that 17β-estradiol alone is sufficient to inhibit transformation from conidia to yeast and for resistance to progressive infection through the first two weeks of infection. That resistance declines with time in hormonally altered mice is indicative of the many factors

involved in the complex mechanisms governing the interactions between host and fungi. However, the results of the present study extend our previous findings, suggesting that 17β-estradiol is important against primary infection in the female.

References

1 Brummer E, Castaneda E, Restrepo A. Paracoccidioidomycosis: an update. *Clin Microbiol Rev* 1993; **6:** 89–117.
2 Londero AT, Ramos CD. Paracoccidioidomicose: estudo clinico-micologico de 260 casos observados no interior do Estado do Rio Grande do Sul. *J Pneumol* 1990; **16:** 129–132.
3 Negroni R. Paracoccidioidomycosis (South American blastomycosis, Lutz's mycosis). *Int J Dermatol* 1993; **32:** 847–859.
4 Restrepo A. The ecology of *Paracoccidioides brasiliensis*. In: Franco M, Lacaz CS, del Negro G, Restrepo A, editors. *Paracoccidioidomycosis*. Boca Raton: CRC Press; 1994, p. 121–127.
5 McEwen JG, Bedoya V, Patino MM, Salazar ME, Restrepo A. Experimental murine paracoccidioidomycosis induced by the inhalation of conidia. *J Med Vet Mycol* 1987; **25:** 165–175.
6 San-Blas G, Restrepo A, Clemons K, Stevens DA, San-Blas F, Puccia R, *et al.* Paracoccidioidomycosis. *J Med Vet Mycol* 1992; **30 Suppl 1:** 59–71.
7 Wanke B, Londero AT. Epidemiology and paracoccidioidomycosis infection. In: Franco M, Lacaz CS, del Negro G, Restrepo A, editors. Paracoccidioidomycosis. Boca Raton: CRC Press; 1994. p. 109–120.
8 Restrepo A, Salazar ME, Clemons KV, Feldman D, Stevens DA. Hormonal influences in the host interplay with *Paracoccidioides brasiliensis*. In: Vanden Bossche H, Stevens DA, Odds FC, editors. *Host-Fungus Interplay*. Bethesda: National Foundation for Infectious Diseases; 1997, p. 125–133.
9 Restrepo A, Salazar ME, Cano LE, Stover EP, Feldman D, Stevens DA. Estrogens inhibit mycelium-to-yeast transformation in the fungus *Paracoccidioides brasiliensis*: implications for resistance of females to paracoccidioidomycosis. *Infect Immun* 1984; **46:** 346–353.
10 Salazar ME, Restrepo A, Stevens DA. Inhibition by estrogens of conidium-to-yeast conversion in the fungus *Paracoccidioides brasiliensis*. *Infect Immun* 1988; **56:** 711–713.
11 Aristizabal BH, Clemons KV, Stevens DA, Restrepo A. Morphological transition of *Paracoccidioides brasiliensis* conidia to yeast cells: in vivo inhibition in females. *Infect Immun* 1998; **66:** 5587–5591.
12 Restrepo A, Salazar ME, Cano LE, Patino MM. A technique to collect and dislodge conidia produced by *Paracoccidioides brasiliensis* mycelial form. *J Med Vet Mycol* 1986; **24:** 247–250.
13 Calich VL, Purchio A, Paula CR. A new fluorescent viability test for fungi cells. *Mycopathologia* 1979; **66:** 175–177.
14 Restrepo S, Tobon A, Trujillo J, Restrepo A. Development of pulmonary fibrosis in mice during infection with *Paracoccidioides brasiliensis* conidia. *J Med Vet Mycol* 1992; **30:** 173–184.
15 Beamer WG, Wilson MC, Leiter EH. Endocrinology. In: Foster HL, Fox JG, Small JD, editors. *The Mouse in Biomedical Research*. New York: Academic Press; 1983, vol. 3, p. 223–224.
16 Seebacher T, Bade EG. Quick and easy molecular weight determination with Macintosh computers and public domain image analysis software. *Electrophoresis* 1996; **17:** 1573–1574.
17 Burger E, Miyaji M, Sano A, Calich VL, Nishimura K, Lenzi HL. Histopathology of paracoccidioidomycotic infection in athymic and euthymic mice: a sequential study. *Am J Trop Med Hyg* 1996; **55:** 235–242.
18 Sano A, Miyaji M, Nishimura K. Studies on the relationship between the estrous cycle of BALB/c mice and their resistance to *Paracoccidioides brasiliensis* infection. *Mycopathologia* 1992; **119:** 141–145.
19 Franco M, Mendes RP. Paracoccidioidomycosis. *Balliere's Clin Trop Med Commun Dis* 1989;**4:** 185–220.
20 Montenegro MR, Franco M. Pathology. In: Franco M, Lacaz CS, del Negro G, Restrepo A, editors. *Paracoccidioidomycosis*. Boca Raton: CRC Press; 1994, p. 131–150.
21 al-Afaleq AI, Homeida AM. Effects of low doses of oestradiol, testosterone and dihydrotestosterone on the immune response of broiler chicks. *Immunopharmacol Immunotoxicol* 1998; **20:** 315–327.
22 de Brito T, Franco MF. Granulomatous inflammation. *Rev Inst Med Trop Sao Paulo* 1994; **36:** 185–192.
23 Da Silva JA. Sex hormones, glucocorticoids and autoimmunity: facts and hypotheses. *Ann Rheum Dis* 1995; **54:** 6–16.
24 Da Silva JA. Sex hormones and glucocorticoids: interactions with the immune system. *Ann NY Acad Sci* 1999; **876:** 102–117.
25 Grossman CJ. Regulation of the immune system by sex steroids. *Endocrine Rev* 1984; **5:** 435–455.
26 Salem ML, Hossain MS, Nomoto K. Mediation of the immunomodulatory effect of beta-estradiol on inflammatory responses by inhibition of recruitment and activation of inflammatory cells and their gene expression of TNF-alpha and IFN-gamma. *Intl Arch Allergy Immunol* 2000; **121:** 235–245.
27 Miyamoto N, Mandai M, Suzuma I, Suzuma K, Kobayashi K, Honda Y. Estrogen protects against cellular infiltration by reducing the expressions of E-selectin and IL-6 in endotoxin-induced uveitis. *J Immunol* 1999; **163:** 374–379.
28 Castro JE. Orchidectomy and immune response. *Ann Royal Col Surg Engl* 1976; **58:** 359–367.
29 Cuzzocrea S, Santagati S, Sautebin L, Mazzon E, Calabro G, Serraino I, *et al.* 17β-estradiol anti-inflammatory activity in carrageenan-induced pleurisy. *Endocrinol* 2000; **141:** 1455–1463.
30 Shirai M, Sato A, Chida K. The influence of ovarian hormones on the granulomatous inflammatory process in the rat lung. *Eur Respir J* 1995; **8:** 272–277.
31 Mariano M. The experimental granuloma. A hypothesis to explain the persistence of the lesion. *Rev Inst Med Trop Sao Paulo* 1995; **37:** 161–176.
32 Silva CL, Alves LM, Figueiredo F. Involvement of cell wall glucans in the genesis and persistence of the inflammatory reaction caused by the fungus *Paracoccidioides brasiliensis*. *Microbiol* 1994; **140:** 1189–1194.
33 Araneo BA, Dowell T, Diegel M, Daynes RA. Dihydrotestosterone exerts a depressive influence on the production of interleukin-4 (IL-4), IL-5, and gamma-interferon, but not IL-2 by activated murine T cells. *Blood* 1991; **78:** 688–699.
34 Ando S, Canonaco M, Beraldi E, Valenti A, Maggiolini M, Piro A, *et al.* The evaluation of androgen circulating levels following castration in adult male rats. *Exp Clin Endocrinol* 1988; **91:** 311–318.
35 Ando S, Canonaco M, Valenti A, Aquila S, Tavolaro R, Maggiolini M, *et al.* The in vitro conversion of ^{3}H androstenedione to testosterone and dihydrotestosterone in the adrenal gland of castrated male rat: influence of gonadal steroid administration. *Exp Clin Endocrinol* 1989; **93:** 83–89.
36 Zhou Z, Shackleton CH, Pahwa S, White PC, Speiser PW. Prominent sex steroid metabolism in human lymphocytes. *Mol Cell Endocrinol* 1998; **138:** 61–69.

37 Gonzalez A, de Gregori W, Velez D, Restrepo A, Cano LE. Nitric oxide participation in the fungicidal mechanism of gamma interferon-activated murine macrophages against *Paracoccidioides brasiliensis* conidia. *Infect Immun* 2000; **68:** 2546–2552.

38 Schmidt M, Kreutz M, Loffler G, Scholmerich J, Straub RH. Conversion of dehydroepiandrosterone to downstream steroid hormones in macrophages. *J Endocrinol* 2000; **164:** 161–169.

39 Fonseca MM, Rins de David ML, Gendelman H. Experimental sialadenitis in castrated rats administered estrogens. *Revista de La Facultad de Odontologia* 1989; **17:** 43–53.

40 Mock BA, Nacy CA. Hormonal modulation of sex differences in resistance to *Leishmania major* systemic infections. *Infect Immun* 1988; **56:** 3316–3319.

41 Ruh MF, Bi Y, D'Alonzo R, Bellone CJ. Effect of estrogens on IL-1beta promoter activity. *J Steroid Biochem Mol Biol* 1998; **66:** 203–210.

42 Deshpande R, Khalili H, Pergolizzi RG, Michael SD, Chang MD. Estradiol down-regulates LPS-induced cytokine production and NFkB activation in murine macrophages. *Am J Reprod Immunol* 1997; **38:** 46–54.

43 Chao TC, Van Alten PJ, Walter RJ. Steroid sex hormones and macrophage function: modulation of reactive oxygen intermediates and nitrite release. *Am J Reprod Immunol* 1994; **32:** 43–52.

44 Chao TC, Van Alten PJ, Greager JA, Walter RJ. Steroid sex hormones regulate the release of tumor necrosis factor by macrophages. *Cell Immunol* 1995; **160:** 43–49.

45 Chao TC, Phuangsab A, Van Alten PJ, Walter RJ. Steroid sex hormones and macrophage function: regulation of chemiluminescence and phagocytosis. *Am J Reprod Immunol* 1996; **35:** 106–113.