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Mechanisms of Resistance to Infection with *Coccidioides immitis* in Mice

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Serum from vaccinated mice was ineffective in neutralizing the infectivity of arthrospores of *Coccidioides immitis* for recipient mice. However, a T-cell-enriched lymphocyte population was effective in preventing a lethal infection. Spleen cells from immune mice were passaged through nylon wool columns resulting in a T-cell-enriched, B-cell-depleted population as shown by the susceptibility of the cell population to anti-theta serum and the inability of the cells to transfer adoptively an immune response to ovalbumin. Whereas transfer of 5×10^7 unfractionated immune spleen cells was required to protect 100% of the recipients against a lethal infection with *C. immitis*, 7×10^6 T-cell-enriched immune spleen cells were sufficient for the same level of protection. Thus, transfer of resistance to infection was achieved with fewer cells after the removal of B cells from the transferred spleen cells. The results confirm that T cells are crucial in transferring resistance against infection with *C. immitis* in mice.

The production of serum antibody specific for coccidioidin does not appear to be critical for resistance to infection with *Coccidioides immitis* in mice. Thus, the intramuscular injection of spherule vaccine induces resistance to infection but no complement-fixing antibody and only low titers of precipitating antibodies, whereas the intravenous injection of spherule vaccines induces the production of higher titers of precipitating antibodies specific for coccidioidin but poor protective immunity to infection (7). Kong et al. (5) found that the transfer of spleen cells from mice immune to *C. immitis* seemed to confer upon recipients some protection against infection with the fungus. However, passive transfer of serum from immune mice to infected mice did not change the outcome of an established infection. This indicated that the serum from immune mice either did not affect the spherules or endospores found in vivo or that it did not come in contact with the tissue forms of the organism. It is not clear whether serum from immune mice might neutralize the infectivity of arthrospores for mice. Beaman et al. (1) found that spleen cells containing both T and B lymphocytes from immunized mice could adoptively transfer resistance to infection with *C. immitis* and that the ability of the spleen cells to transfer resistance was eliminated by incubation of the spleen cells with anti-theta serum plus complement, which destroys the T cells. Because the surviving B cells present in the transferred spleen cells did not provide protection, it was concluded that B cells were not effective in transferring resistance to infection.

To further define the role of antibodies and of lymphocyte subsets in resistance to murine coccidioidomycosis, serum from vaccinated mice was used to treat arthrospores to determine the effect on their infectivity. Also, adoptive transfers of normal spleen cells, immune spleen cells, and immune spleen cells depleted of B cells were performed to compare their protective effect on mice infected with *C. immitis*. The present communication describes the results of these investigations.

MATERIALS AND METHODS

Animals. Female DBA/2 mice (Jackson Laboratories, Bar Harbor, Maine) weighing 18 to 22 g were used throughout the reported experiments.

Cultures and vaccine. Arthrospores of *C. immitis* strain Silveira (ATCC 28868) harvested from slants of glucose-yeast extract agar were stored in distilled water at 4°C until use. Spherules were obtained from cultures grown in modified Converse medium (6). Mature endosporulating spherules were harvested after 40 h of incubation. For preparation of vaccines, formaldehyde was added to the spherule suspensions to a final concentration of 0.18%. The suspensions were kept at 4°C for 8 days. The spherules were then washed and suspended at 6 mg (dry weight)/ml of phosphate-buffered saline, pH 7.2 (PBS), for use as a vaccine. Merthiolate was added to a final concentration of 1:10,000 (wt/vol).

Infection and immunization of mice. Mice were infected by intraperitoneal injection of arthrospores diluted in PBS. Mice were immunized with three

intramuscular injections of 1.2 mg (in 0.2 ml) of the Formalin-killed spherule suspension at days 1, 7, and 14. Thirty days after the last injection a sample group of vaccinated mice was challenged with live arthrospores to test the effectiveness of the vaccine.

Preparation of spleen cell suspensions. Spleen cell suspensions were prepared from individual organs by mincing and pressing the tissue through a 40-gauge, stainless-steel screen. The cells were suspended in PBS containing 5% fetal bovine serum, washed, and resuspended in PBS with 5% fetal bovine serum at the desired cell concentration.

Removal of B cells was accomplished by filtration through nylon wool columns as described by Julius et al. (4). The cells were resuspended to a concentration of 5×10^7 cells per ml in PBS with 5% fetal bovine serum. Nylon wool (Fenwal Laboratories, Morton Grove, Ill.) was packed into the barrels of 12-ml plastic syringes to the 6-ml mark, and the packed syringe barrels were autoclaved. The columns were rinsed in PBS with 5% fetal bovine serum and placed at 37°C for 1 h before use. Two milliliters of the cell suspension (5×10^7 cells per ml) was added to the column, followed by 1 ml of warm PBS with 5% fetal bovine serum. The columns were held at 37°C for 45 min and then washed slowly. The first 25 ml of effluent was collected and centrifuged, and the cells were resuspended to the appropriate concentration. In some experiments the filtration was repeated. The viability of cells was determined by dye exclusion (erythrosin B). The proportion of theta-bearing cells or immunoglobulin-bearing cells in spleen cell preparations before and after passage of the spleen cells through nylon wool columns was determined by the use of anti-theta and anti-immunogobulin serum as follows: spleen cells (10^7) in 0.1 ml were incubated in the presence of a 1:40 dilution of rabbit anti-theta serum (0.1 ml) prepared as described by Golub (3) (which killed 96 to 98% of thymic lymphocytes and 45% of splenic lymphocytes as shown by dye exclusion). Another sample of spleen cells, consisting of 0.1 ml containing 10^7 cells, was incubated with rabbit anti-mouse immunoglobulin G serum (Miles Laboratories, Kankakee, Ill.) for 30 min at 37°C. The rabbit serum was absorbed with mouse liver cells before use. The cells were then washed and incubated in the presence of guinea pig complement (0.1 ml) diluted 1:10 for 30 min, and their viability was demonstrated by dye exclusion (erythrosin B).

Adoptive transfer of immunity. Recipients of immune or normal spleen cells were irradiated in Lucite boxes, each containing 14 mice. The mice were given 500 rads under the following conditions: 29 rads/min; 250 kV; 15 mA; anode-to-target distance of 60 cm. A General Electric Maximar 250 type II with Thoris II filter was used. All irradiated mice were maintained on acid hypochlorite water (pH 2.8). The recipients were given intravenously 0.5 ml containing various numbers of spleen cells from immune or normal mice. Twenty-four hours later the mice were given an intraperitoneal booster injection of 15 μg of Formalin-killed spherules.

As a marker of successful transfer of immune cells, the immune mice were given one intraperitoneal injection of 100 μg of chicken ovalbumin in Freund adjuvant 30 days before removal of the spleen cells. The recipients of the spleen cells were given a booster injection of 100 μg of chicken ovalbumin 24 h after cell transfer. Five days later, representative mice from each group were bled, and the individual sera were tested for the presence of chicken ovalbumin antibody or antibody specific for coccidioidin. Chicken ovalbumin was labeled with ^{125}I, using lactoperoxidase as previously described (2). The serum diluted 1:10, 1:100, and 1:1,000 was mixed in 0.1-ml portions with 0.1 ml of the labeled antigen (1 μg/ml) for 30 min at room temperature. The antibody was precipitated by the addition of 0.2 ml of rabbit anti-mouse immunoglobulin for 30 min. After centrifugation the supernatant was removed, and the precipitate was counted in a gamma counter (Nuclear-Chicago Corp., Chicago, Ill.). The concentration of labeled antigen found in the precipitate, indicating antibody-bound antigen, was then calculated. The serum was also tested for the presence of precipitating or complement-fixing antibodies specific for coccidioidin, using the procedures previously described (8, 9).

Seven days after the cell transfer, 10 mice of each experimental group were infected intraperitoneally with 400 arthrospores in PBS, and the mice were observed for 40 days. Survivors were sacrificed and autopsied, and sections of spleen, liver, lung, or other lesions were cultured on Mycosel (Baltimore Biological Laboratory, Cockeysville, Md.) agar for 3 weeks.

RESULTS

Role of antibody in immunity to coccidioidomycosis. Vaccinated mice were bled 30 days after the last injection of spherules. Although such mice could withstand challenge with a lethal dose of arthrospores (1,000 arthrospores), no precipitating or complement-fixing antibodies reactive with coccidioidin were detected in the serum. However, since these tests may not have detected antibodies induced by spherules or antibodies which may be effective in resistance to infection, attempts were made to determine if serum from immune mice could neutralize the infectivity of arthrospores for mice. For this purpose 1 ml of undiluted serum from vaccinated or normal mice was mixed for 30 min at room temperature with 1 ml of PBS containing 4,000 or 1,000 arthrospores. Then, 0.2 ml, containing 400 or 100 arthrospores, was injected per mouse intraperitoneally. The donors of the serum were infected with 0.2 ml of PBS containing 1,000 arthrospores. The mice were observed for 40 days. The vaccinated serum donors could resist the lethal dose of 1,000 arthrospores (Table 1). In contrast, the survival of the recipients of arthrospores that had been incubated in immune serum was not higher than that of recipients of arthrospores that had been incubated in normal serum. These results indicated a lack of detectable neutralizing antibody in the serum of vaccinated mice. The experiment was repeated except that mice were injected

TABLE 1. *Capacity of serum from vaccinated mice to neutralize the infectivity of C. immitis for mice*

Mice	Serum source used for neutralization	No. of arthrospores used for infection	Survivors in days postinfection:			
			10	20	30	40
Normal	Immune mice[a]	100	10/10	10/10	7/10	2/10
Normal	Normal mice[a]	100	16/16	14/16	12/16	8/16
Normal	Immune mice	400	10/10	0/10	0/10	0/10
Normal	Normal mice	400	10/10	1/10	0/10	0/10
Serum donors						
Vaccinated[a]	Saline	1,000	10/10	10/10	10/10	10/10
Normal	Saline	1,000	11/11	2/11	0/11	0/11

[a] The donors of immune mouse serum had received three weekly injections of 1 mg of Formalin-killed spherules 30 days before bleeding. The undiluted serum (1 ml) was mixed for 30 min at room temperature with 1 ml of PBS containing 4,000 or 1,000 arthrospores, and then 0.2 ml (containing 400 or 100 arthrospores) was injected intraperitoneally per mouse.

with 400 or 100 arthrospores in 1.0 ml of serum after 30 min of incubation at 37°C. The results were essentially the same as those shown in Table 1, again indicating no significant difference between the survival rate of mice receiving arthrospores in immune or normal serum. These results were obtained with either 0.2 or 1 ml of immune serum, representing a fivefold range of serum. It was impractical to incubate and inject arthrospores in more than 1 ml of serum because of the limit of serum volume which can be passively transferred to mice.

Effect of depletion of B cells in immunity to coccidioidomycosis. Spleen cells from immune mice were passed through nylon wool columns to deplete the number of B cells. The spleen cells, before and after passage through nylon wool columns, were incubated with anti-theta serum or anti-mouse immunoglobulin G serum in the presence of complement to ascertain the percentage of theta-bearing or immunoglobulin G-bearing cells, respectively. Data in Table 2 represent results of two to four separate experiments in which the proportion of cells susceptible to anti-immunoglobulin G serum with complement was 42 to 57% of the total population before passage through a nylon wool column, whereas in the effluent of the columns, 0% of the cells were susceptible to anti-immunoglobulin G with complement. In contrast, the number of cells susceptible to anti-theta serum with complement increased from 44 to 58%, before passage through nylon wool, to 80 to 95% in the effluent from the column. The decrease in B cells present in the effluent was also shown by the decreased ability of spleen cells to adoptively transfer immunity to chicken ovalbumin to recipient mice. A 10-μl amount of serum from the recipients of cells passed through nylon wool could bind approximately as much labeled antigen as 1 μl of the serum from the recipients of

TABLE 2. *Effectiveness of nylon wool columns in removing B cells*

Spleen cells	% Killed by:[a]	
	Anti-IgG + complement	Anti-theta + complement
Before fractionation on nylon wool	42, 57	44, 45, 50, 58
In effluent from nylon wool columns	0, 0	80, 85, 91, 95

[a] Rabbit anti-mouse immunoglobulin G (IgG) (Miles Laboratories) was incubated with 1×10^7 cells in 0.1 ml for 30 min. The cells were washed and incubated with 0.1 ml of guinea pig complement diluted 1:10 for 30 min. The viable cell count was determined by dye exclusion (erythrosin B). The same procedure was repeated with rabbit anti-mouse brain antibody prepared by methods described by Golub (3). A total of 5×10^7 spleen cells per ml was added to nylon wool columns as described by Julius et al. (4) in four separate experiments. In two experiments the proportion of cells killed by anti-IgG was not determined.

the unfractionated immune spleen cells (Table 3). Since the use of the nylon wool columns had resulted in a significant decrease in B cells and an increase in T cells, this procedure was used to determine the numbers of effective cells required for transfer of resistance to *C. immitis*.

Recipient mice were given various numbers of immune spleen cells ranging from 1×10^6 to 2×10^8 cells per mouse. Results in Table 4 indicate that 5×10^7 cells from immune mice protected 100% of the mice against a lethal infection, whereas 10^7 cells protected 50% of the mice against a lethal infection. In contrast, 7×10^6 immune spleen cells in the nylon wool effluent protected 100% of the recipient mice from a lethal infection. Thus, the relative increase in

theta-bearing cells and the decrease in immunoglobulin-bearing cells in the effluent were correlated with greater protective potency. Apparently the B cells removed by passage through nylon wool were not required for transfer of resistance to infection with *C. immitis*. Autopsy of the surviving mice and cultures of the spleen, liver, lungs, and of visible lesions showed that all of the surviving normal cell recipients had lesions and viable *C. immitis* in the organs. *C. immitis* was found only in the spleens of 50 to 60% of the surviving recipients of immune cells either unfractionated or fractionated, whereas *C. immitis* was found in 5% of the immune cell donors surviving infection, indicating that resistance to lethal infection does not require complete elimination of the fungus as has been previously reported (5).

TABLE 3. *Adoptive transfer of the ability to produce antibodies to chicken ovalbumin, using immune spleen cells passaged through nylon wool*

Serum source	% Antigen bound[a] by varying amounts of serum:		
	10 μl	1 μl	0.1 μl
Immune donors[b]	75	70	41
Recipients of immune cells[c]	57.7 ± 3.9	19.3 ± 4.7	1.3 ± 1.4
Recipients of cells in nylon wool effluent[d]	15.4 ± 3.2	4.6 ± 4.8	0.3
Recipients of normal cells	0	0	0

[a] The percentage of ^{125}I-labeled chicken ovalbumin (0.1 μg) bound by varying amounts of serum diluted to 0.1 ml, with the standard deviation given between individual bleedings.

[b] Mice were immunized with 100 μg of chicken ovalbumin in Freund adjuvant and bled 30 days later.

[c] The recipients of immune spleen cells were injected with 100 μg of chicken ovalbumin 24 h post-transfer and bled 5 days later.

[d] The immune spleen cells were passaged through nylon wool columns as described by Julius et al. (4).

DISCUSSION

It was shown by Kong et al. (5) that serum from mice vaccinated with spherules and given a booster injection 2 months later had precipitating antibodies for coccidioidin or spherule antigen. Nevertheless, injecting infected mice 4 to 10 times with this serum did not change the outcome of the infection. These investigators did not ascertain whether or not such immune serum could affect the infectivity of arthrospores. In the investigations reported herein no precipitating or complement-fixing antibodies for coccidioidin were found in mice 30 days after intramuscular immunization with spherules or in the serum of recipients of immune spleen cells, although both groups of mice could resist an infection with *C. immitis* which is lethal to immunized mice. As with normal serum, the serum from the immunized mice did not neutralize the infectivity of 100 or 400 arthrospores. The data indicate that serum from resistant (immunized) mice did not prevent progression of infection. This observation is compatible with Kong's observation that passive transfer of antibodies did not affect an established infection.

The inability of B cells to confer resistance to *C. immitis* has been previously demonstrated by showing that recipients of immune spleen cells treated with anti-theta serum and complement resulting in the killing of T cells could not resist infection with *C. immitis*. A method to separate T and B cells, which does not involve killing of

TABLE 4. *Survival of mice receiving immune spleen cells, T-cell-enriched and B-cell-depleted spleen cells, or normal spleen cells and subsequently infected with C. immitis*[a]

Cell source	No. of cells	Ratio of surviving mice at days postinfection:[b]			
		10	20	30	40
Immune spleen	1×10^7	10/10	10/10	6/10	5/10
	5×10^7	10/10	10/10	10/10	10/10
	1×10^8	10/10	10/10	10/10	10/10
	2×10^8	10/10	10/10	10/10	10/10
Nylon wool effluent	1×10^6	10/10	7/10	4/10	3/10
	7×10^6	10/10	10/10	10/10	10/10
	1×10^7	10/10	10/10	10/10	10/10
	5×10^7	10/10	10/10	10/10	10/10
Normal spleen	1×10^7	10/10	0/10	0/10	0/10
	1×10^7	10/10	3/10	2/10	2/10
	2×10^7	10/10	8/10	6/10	4/10
	5×10^7	8/8	1/8	0/8	0/8

[a] Spleen cells, 10^8 cells in 2 ml, were added to nylon wool columns as described by Julius et al. (4).

[b] The mice were infected with 400 arthrospores intraperitoneally and observed for 40 days.

cells, is to pass the spleen cells through nylon wool (4). This procedure was used in the present studies. As the data indicate, this procedure resulted in the removal of the majority of the B cells as evident by the removal of immunoglobulin-bearing cells (Table 2) and by the highly reduced titer to ovalbumin used as an antigenic marker (Table 3). The nylon wool effluent yielded a cell population enriched for theta-bearing cells. Seven million cells which were primarily theta-bearing lymphocytes were required for effective transfer of resistance to infection, whereas a minimum of 5×10^7 of the original spleen cells were required to protect 100% of the recipients. Obviously the decrease in B cells does not affect the transfer of protective immunity, since fewer B-cell-depleted spleen cells are required for transfer of immunity than whole spleen cells. The data presented in this communication demonstrate that resistance to a lethal infection with *C. immitis* can occur despite a significant depletion of B cells. Indeed, in the absence of B cells, T cells appear to be more effective in conferring protection. These findings may imply that B cells adversely affect resistance to the fungus.

The mechanism(s) by which T cells confer immunity to infection is not yet clear. T cells have not yet been shown to exert a direct fungicidal effect on *C. immitis*. Experiments are in progress to ascertain whether T cells mediate the killing of the fungus or whether they mediate suppression of its growth.

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