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INFLUENCE OF AGGLUTINATING ANTIBODY IN EXPERIMENTAL CRYPTOCOCCAL MENINGITIS

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Summary.—A model for chronic *Cryptococcus neoformans* meningitis in corticosteroid-treated rabbits was used to determine the influence of pre-formed agglutinating antibody to cryptococcal polysaccharide on the progress of this infection. Immunized rabbits developed serum agglutinating antibody with a geometric mean titre of 1:32, but none was detected in cerebrospinal fluid. Prior immunization did not enhance immunity to infection, had no effect on the number of viable cryptococci in cerebrospinal fluid, and did not prevent dissemination outside the central nervous system. Future investigations in this field should focus on cellular rather than humoral defence mechanisms.

IT IS NOT KNOWN whether possession of specific antibody provides any immunity against cryptococcal infection. Diamond and Allison (1976) demonstrated antibody-dependent killing of *C. neoformans* by human peripheral-blood mononuclear cells *in vitro*, suggesting that antibody might play a role in host defence against cryptococci. Gadebusch (1958) showed limited protection when antiserum was injected i.p. with cryptococci in mice. Also, clinical observations in humans with cryptococcal meningitis suggest that the outcome is more likely to be favourable in patients with measurable titres of serum antibody (Diamond and Bennett, 1974). Specific anti-cryptococcal immuno-globulin has been detected in the cerebrospinal fluid (CSF) of a patient with cryptococcal meningitis (Porter, Sinnamon and Gillies, 1977). On the other hand, passive immunization of mice conferred no detectable protection against challenge with these yeasts (Louria and Kaminski, 1965; Goren, 1967). Mice with a genetically determined B-cell deficiency are not more susceptible to cryptococcal infection than normals (Monga *et al.*, 1980).

In the present study, we tested the effect of pre-immunization on the natural history of meningitis and dissemination of yeast outside the central nervous system in rabbits with chronic cryptococcal meningitis.

MATERIALS AND METHODS

Organism and method of inoculation.—A strain of *C. neoformans* Serotype A (Strain DP) which had been used in a previous study (Perfect, Lang, and Durack, 1980), was used in all experiments. Strain DP was streaked on Columbia blood-agar base containing 100 μg/ml of chloramphenicol and incubated at 35° for 4–5 days. Yeasts were taken up on a swab, suspended in 0·015M phosphate-buffered saline (PBS) pH 7·4, and the suspension adjusted to give approximately 5×10^7 c.f.u. in 0·3 ml. After sedation with 0·3 ml/kg of fentanyl and droperidol (Innovar®, McNeil Laboratories, Irvine, CA.), 0·3 ml of the yeast suspension was injected intracisternally into New Zealand White male rabbits. One group of rabbits was treated with 2·5 mg/kg/day i.m. cortisone acetate, which was generously supplied by Merck, Sharp and Dohme, West Point, PA.

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Measurement of cryptococcal antigen and antibody.—All serum and CSF specimens were centrifuged at 200 *g* for 10–20 min to sediment leucocytes, erythrocytes, and yeasts. One drop of supernatant from each aspirate was cultured on agar to ensure that all viable yeast cells had been removed.

To determine antigen titres, serial dilutions of 0·025 ml of each specimen were made with Crypto LA diluent (International Biological Labs, Rockville, MD) in microtitre plates, and 0·025 ml of Crypto LA antibody reagent was added to each well. The plates were agitated for 10 min and read using a mirror. High, low, and negative antigen standards (Meridian Diagnostics, Cincinnati, OH) were run in parallel to check for false positives. The sensitivity of the test was 0·02 μg/ml of cryptococcal polysaccharide antigen.

To determine antibody titres a micro-tube agglutination method was used. Serial dilutions of CSF and serum specimens (0·025 ml) in normal saline were made in microtitre plates. To each of these wells was added an equal volume of PBS containing 10^8 c.f.u./ml methylene-blue-stained yeast cells; the wells were then incubated for 2 h at room temperature and refrigerated overnight (4–8°). Wells were read as positive if visible agglutination was apparent after gently tapping to resuspend cryptococci. Negative CSF and serum controls were included on each plate, to exclude false-positive reactions.

To determine whether the agglutinating antibody was primary IgM or IgG, we tested the ability of serum specimens to agglutinate cryptococci after treatment with mercaptoethanol. After incubation of serum samples for 1 h at 37° with equal volumes of normal saline or 0·1M 2-mercaptoethanol (Scott and Gershon, 1970), agglutinating antibody was determined as above.

Preparation of antigen and immunization procedure.—Strain DP was grown for 48–72 h at 35° in air on Sabouraud agar (Difco). Colonies were wiped off with a dry swab and suspended in 10% formalin for 24 h. The yeasts were then washed 3× and suspended in PBS at a concentration of approximately 2×10^8 yeast cells per ml, determined by counting in a haemacytometer chamber. Cultures were made to confirm that no viable yeasts remained in this suspension, which was then stored in 10 ml tubes at −70°. One tube was removed each week and kept at 4° to provide the antigen for daily injections. One millilitre of this suspension containing approximately 2×10^8 killed yeast cells was injected into the marginal ear vein of the rabbit on each of 5 successive days for 2 weeks, followed by a 1-week rest period. This schedule was then repeated twice. Rabbit CSF and serum agglutinating antibody titres were determined 1 week later.

Dissemination studies and quantitative CSF yeast counts.—After intracisternal inoculation of yeast to produce cryptococcal meningitis, CSF was withdrawn on Days 4 and 7. CSF leucocyte counts were performed using Turk solution in standard haemacytometer chambers. 100μl samples of CSF were diluted serially in PBS, incubated for 48–72 h at 35° on Columbia blood-agar base with chloramphenicol, and colonies counted. Seven days after inoculation, the rabbits were killed with pentobarbitone. Approximately 10 ml of heart blood from each animal was cultured in vented bottles of Columbia broth with added sucrose (Difco) at 35°. Portions of lung, liver, kidney, and spleen were weighed and homogenized in a tissue grinder in order to make a suspension of each tissue in PBS. This suspension was serially diluted in PBS, plated on Columbia agar and incubated at 35°. Colonies were counted after 48–72 h.

Statistical methods.—Significant differences between groups were determined by applying the *t* test for unpaired means.

RESULTS

No detectable agglutinating antibody was found in either serum or CSF from 6 normal rabbits. After immunization but before infection, the geometric mean anti-cryptococcal antibody titres in the serum of immunized rabbits was 1:32 (range 1:4–1:512). No detectable antibody was found in the CSF of 3 of these immunized animals before infection. Titres did not change after the sera were treated with 2-mercaptoethanol, indicating that the agglutinating antibody produced by our immunization procedure was primarily IgG, not IgM.

Four and 7 days after inoculation of cryptococci, CSF was again obtained from all rabbits and tested for antibody. None of 10 non-immunized, and only 1 of 14 immunized rabbits had detectable antibody. The single positive specimen showed agglutination in undiluted CSF only.

Soluble cryptococcal polysaccharide antigen was present in CSF of all rabbits at 4 and 7 days at titres of 1:32–1:128. The antigen titres were similar in all treatment groups. To determine whether antigen may have masked the antibody response, we followed 5 normal rabbits inoculated with cryptococci but not treated with cortisone for 5 weeks, until

TABLE I.—*Quantitative yeast counts in CSF at 4 and 7 days in immunized and non-immunized rabbits with cryptococcal meningitis*

Treatment	No.	Log_{10} c.f.u. yeast/ml CSF (mean ± S.E.)	
		4 days	7 days
Immunized, no cortisone	(5)	3·6 ± 0·2	2·9 ± 0·3
Non-immunized, no cortisone	(5)	3·5 ± 0·2	2·1 ± 0·4
Immunized, cortisone*	(9)	4·9 ± 0·2	6·2 ± 0·3
Non-immunized, cortisone*	(5)	4·6 ± 0·5	5·0 ± 0·8
		N.S.	N.S.
		$P < 0·001$	$P < 0·001$

* 2·5 mg/kg/d cortisone acetate i.m.

TABLE II.—*Quantitative yeast counts in lung, spleen, liver, and kidney in immunized and non-immunized rabbits with cryptococcal meningitis at 7 days after inoculation*

Treatment	No.	Log_{10} c.f.u. yeast/g tissue (mean ± S.E.)			
		Lung	Spleen	Liver	Kidney
Immunized, no cortisone	(5)	0·4 ± 0·4	sterile	0·4 ± 0·4	sterile
Non-immunized, no cortisone	(5)	0·4 ± 0·4	0·3 ± 0·3	0·6 ± 0·6	sterile
Immunized, cortisone*	(8)	4·2 ± 0·4	2·6 ± 0·6	1·9 ± 0·4	2·1 ± 0·5
Non-immunized, cortisone*	(5)	2·9 ± 1·3	2·8 ± 1·0	1·5 ± 1·1	1·5 ± 1·0

* 2·5 mg/kg/d cortisone acetate i.m.

TABLE III.—*Mononuclear cell counts in CSF of rabbits with cryptococcal meningitis at 4 and 7 days*

Treatment	No.	Mononuclear cells/mm^3 CSF (mean ± S.E.)	
		4 days	7 days
Immunized, no cortisone	(5)	1031 ± 248	4470 ± 592
Non-immunized, no cortisone	(5)	276 ± 102	2823 ± 883
Immunized, cortisone*	(8)	73 ± 49	355 ± 98
Non-immunized, cortisone*	(5)	13 ± 4	371 ± 130
		$P < 0·02$	N.S.
		$P < 0·001$	$P < 0·001$

* 2·5 mg/kg/d cortisone acetate i.m.

all viable yeasts and detectable polysaccharide antigen (0·02 μg or greater) had disappeared from the CSF. No agglutinating antibody was detected in the CSF of these rabbits at any time.

The effect of pre-formed serum antibody on the counts of cryptococci in CSF is shown in Table I. The number of viable yeasts rose in cortisone-treated rabbits and fell in non-cortisone-treated rabbits between Days 4 and 7. Total counts were significantly higher in both the cortisone-treated groups at both 4 and 7 days ($P < 0·001$). Prior immunization had no discernible effect on yeast counts in either treatment group. Blood cultures were positive at 7 days in 4/7 of the immunized, cortisone-treated group and 1/5 treated with cortisone but not immunized, while all 10 blood cultures from rabbits not receiving cortisone were sterile. These differences were not statistically significant.

We had shown previously that cortisone strikingly reduced the number of mononuclear cells in the CSF of infected rabbits (Perfect *et al.*, 1980). These experiments confirmed that observation in the group of non-immunized rabbits (Table III). The same effect was observed in the

immunized group. Even though total cell counts were always lower in cortisone-treated animals, leucocyte counts in CSF were higher in immunized than in non-immunized rabbits at 4 days ($P < 0{\cdot}02$). This trend persisted at 7 days in the no-cortisone group, but the difference was no longer statistically significant. In all groups there was an inverse relationship between leucocyte counts and yeast counts in CSF.

Dissemination from CSF to other organs was not prevented by pre-immunization (Table II). All 4 groups had at least 1 rabbit with positive organ cultures. The lungs usually contained more yeast than did liver, spleen, or kidney. Again, the concentration of yeast in tissue was related to treatment with cortisone, not to the presence or absence of serum antibody.

DISCUSSION

The role of antibody in infections of the central nervous system (CNS) is not well understood. In particular, the significance of anticryptococcal antibody in the CNS response to cryptococcal infection is unknown.

We have used the rabbit model to determine the effect of anticryptococcal antibody on cryptococcal meningitis. This is a convenient model for chronic meningitis, the course of which can be followed by repeated analyses of CSF and serum. We found that pre-formed serum antibody did not prevent dissemination of *C. neoformans* to organs outside the CNS, and had no effect on CSF yeast counts over the first week of infection. Even though immunized rabbits had significantly more mononuclear cells in their CSF at 4 days, yeast counts were no different to those found in non-immunized rabbits receiving the same cortisone regimen. Thus pre-formed antibody conferred no immunity and had no effect on the early course of cryptococcal meningitis.

Normal, non-immunized rabbits were followed for several weeks during and after recovery from cryptococcal meningitis. Despite successful eradication of all yeasts and disappearance of polysaccharide antigen (which theoretically could have interfered with the assay) from CSF, no antibody was detected in the CSF. This suggests that the highly effective defence mechanisms of the normal rabbit against cryptococcal infection of the CNS are not dependent upon humoral immunity, at least as measured by agglutinating antibodies.

These experiments confirmed the major potentiating effect of cortisone on cryptococcal meningitis and dissemination to other organs, as previously reported (Perfect *et al.*, 1980). The striking reduction in mononuclear cells at the site of infection in cortisone-treated rabbits seems to be a more important determinant of the ability of these rabbits to eradicate cryptococci from their CNS and other organs than presence of antibody. Lack of a good cellular inflammatory response in the CNS is also characteristic of some human cryptococcal infections, especially in patients treated with corticosteroids (Schroter *et al.*, 1976).

Our demonstration of cryptococcaemia in some cortisone-treated rabbits illustrates the ability of cryptococci to seed the bloodstream and other organs from a meningeal site. Perhaps cryptococcaemia in steroid-treated humans with meningitis (in whom yeast counts in CSF may be as high as 10^4–10^7 c.f.u./ml, unpublished data from this laboratory) could arise directly from the meninges rather than from bone, lung, or other sites outside the CNS. Cryptococcaemia in humans, as in rabbits, usually occurs in individuals having a large burden of organisms, corticosteroid treatment, or both.

In conclusion, the presence of pre-formed agglutinating antibody to cryptococcal polysaccharide capsule had no detectable influence on resistance to cryptococcal meningitis in normal and cortisone-treated rabbits. Future experiments should therefore focus on the role of CSF mononuclear cells in host defence against *C. neoformans* and other fungi. We hope that such investigations will reveal

whether susceptibility to CNS infection with fungi is due primarily to quantitative or qualitative deficiencies of the mononuclear response.

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