Interaction between *Salmonella enteritidis* and *Candida albicans* (37338)

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Candida albicans is often a normal inhabitant in the gastrointestinal tract of man. Under conditions where alterations occur in the host tissue, in the defense mechanisms, or in the microbial community, this organism may become a focus for systemic invasion. The fact that coincidence of *C. albicans* and *Salmonella* species has been recognized in a few cases seems to encourage examination of the possible interaction of these organisms in causing disease. One reported case was cited in an eighteen-month-old girl in Maimonides Hospital in Brooklyn, NY (1). Both *C. albicans* and *S. typhimurium* were implicated as the etiology of the enteritis. The diarrhea persisted after elimination of *S. typhimurium* and subsided only after course of nystatin therapy. Lack of mycological data at time of admission prevented determination of the "primary" or "secondary" role of *C. albicans* in the etiology of the severe gastroenteritis in this patient. Therefore, this investigation was conducted to study the possible *in vitro* and *in vivo* interaction of *S. enteritidis* and *C. albicans*.

Materials and Methods. Cultures. S. enteritidis and *C. albicans* strain A 311 (obtained from Dr. William H. Chew, Department of Medicine, Medical College of Georgia, Augusta, GA), maintained on tryptic soy agar (TSA-Difco) at 37°, were used. Growth rates of pure cultures were determined at intervals by both plate counts on TSA and spectrophotometrically (640 nm) after inoculating prewarmed (37°) tryptic soy broth (TSB) with each test culture and incubating at 37° in an incubator-shaker. Direct counts with a Levy hemacytometer were also made to check relative accuracy of plate counts for *C. albicans*.

Mouse toxicity test. Adult (Webster strain), pathogen free, white Swiss mice weighing 18–25 g were used. The mice were housed in air-conditioned quarters (23°) in isolated cages and offered *Salmonella*-free commercial laboratory pellets and water *ad libitum*. Cells of test cultures, harvested by centrifugation after 4–5 hr incubation at 37°, were washed 3 times and suspended in sterile saline. The LD_{50} for *C. albicans* was determined using both intracerebral (ic) and intraperitoneal (ip) injection of mice, whereas the LD_{50} for *S. enteritidis* was conducted by ic injection only. A group of mice were injected with saline suspension containing decreasing amounts of cells under test; the number of which was confirmed by plate count using TSA. Five mice were injected with each dilution of the test culture. Five mice were also injected with sterile saline and served as controls. The mice injected ic received 0.03 ml inoculum, while those injected ip received 0.5 ml. Deaths occurring within 14 days were scored, and the LD_{50} was calculated according to the methods of Reed and Muench (2) and Brown (3). The latter is a version of Spearman's procedure (4).

In vitro and in vivo interaction. Three methods were used to examine the *in vitro* interaction between mixed cultures of the test organisms. In the 1st, prewarmed tubes of TSB were each inoculated with test cultures alone or in mixtures using a 2% inoculum, and the tubes incubated at 37°. At intervals ranging from 0 to 72 hr, samples were obtained for viable counts, using brilliant green agar (BGA) for *Salmonella* and TSA containing 100 μg streptomycin sulfate (Upjohn) and 100 μg cephaloridine (Lilly)/ml medium for *C. albicans*. Growth of *Candida* was totally inhibited in BGA, whereas *S. enteritidis* would not grow on medium containing the anti-

biotics. The 2nd method was designed to detect the effect of a bacteria-free culture filtrate of *S. enteritidis* against *C. albicans* and vice versa. Eighteen-hour TSB cultures of *C. albicans* and *S. enteritidis* as well as uninoculated control medium were each filtered through Millipore filters, and 0.05 ml of each filtrate was added to sterile discs placed on surface of TSA plates (seeded with viable test organism). The plates were incubated and examined after 48 hr for growth stimulation or inhibition surrounding the discs. In the 3rd procedure, 0.05 ml of an 18-hr broth of each test culture, was added to sterile discs placed on surface of TSA plates that were seeded with either *S. enteritidis* or *C. albicans* to determine the inhibitory or stimulatory effect of one organism on growth of the other. The three previous methods were tested under aerobic, microaerophilic, and anaerobic conditions. To maintain aerobiosis, the plates were incubated at 37°; broth cultures were kept on a metabolic shaker, in a candle jar for microaerophilic, and in a "GAS PAK" anaerobic jar (Baltimore Biological Lab) for securing anaerobiosis.

Two methods were used to examine the *in vivo* interaction. The first involved five groups of 25 mice each. Four of the groups were injected ip with *S. enteritidis* and the fifth with saline (control). Each mouse of the first group received log 8.57 salmonella cells, the second group log 7.46, the third log 6.51, the fourth log 5.59, and the fifth received 0.5 ml of saline. Twenty-four hours after the salmonella infection, these mice were infected with *C. albicans* by injecting (ic) five different levels of *C. albicans* to each 25 mice (5 from each group). The levels used were log 5.46, 4.49, 3.43, 2.44, and 0.00 (saline only). The number of mice dead (per total number inoculated) within 14 days of candida infection was recorded, and the LD_{50} doses of *C. albicans* at various preinjected concentrations of *S. enteritidis* were calculated using the Brown (3) version of the Spearman method (4). In the second method, 4 groups of 30 mice each were used. The first and second groups were injected ip with 0.5 ml saline suspension containing log 6.89 *S. enteritidis* cells, and the third and fourth groups received 0.5 ml saline ip. Twenty-four hours after *Salmonella* infection, the first and third groups were injected ic with 0.03 ml saline suspension containing log 5.10 *C. albicans* cells, whereas the second and fourth group of mice received 0.03 ml saline ic. Mice of the fourth group (receiving saline broth ic and ip) were used in order to ascertain that no *Candida* or *Salmonella* were present prior to test inoculation. At definite intervals post-candida-infection, 0.5 ml of blood was obtained from each of 5 animals of the four groups. The mice were then sacrificed; and kidneys, spleen, liver, and cecum were aseptically excised, placed in sterile petri dishes, chilled immediately, and homogenized by the method of Pierce *et al.* (5). Each homogenate and/or blood sample was quantitated on TSA containing 100 μg streptomycin sulfate (Upjohn) and 100 μg cephaloridine (Lilly)/ml medium to determine *C. albicans* population and also on Brilliant Green Agar (BGA) to enumerate the *S. enteritidis*. The plates were examined after incubation for 48 hr at 37°. Enrichment techniques were employed whenever necessary to detect the presence of very small numbers of *Salmonella* in samples. In this technique, 0.2 ml of tissue homogenate or blood was inoculated into Tetrathionate Broth (TB) to selectively enrich the *Salmonella*, and the tubes were incubated aerobically at 37° for 24 hr. Growth from TB tubes was then streaked on BGA plates incubated for 48 hr and typical *Salmonella* colonies were isolated, examined on other media, and serotypes (when necessary) as previously described (6).

Results. Mortality of mice. The LD_{50} at 14 days for *C. albicans* injected ip in mice was log 7.2 ± 0.5 cells ($p < 0.05$), whereas that injected ic was 5.2 ± 0.5 cells ($p < 0.05$). These values were calculated by the Spear Method (4) and were almost identical to those obtained by the Reed and Muench (2) method. The LD_{50} at 14 days for *S. enteritidis* injected ic in mice was log 6.6 + 0.5 cells ($p < 0.05$) as determined by both methods.

Growth curves. Rapid growth of *S. enteritidis* and *C. albicans* at 37° using an incubator-shaker was noted reaching log phase after a very short lag (Fig. 1A) prob-

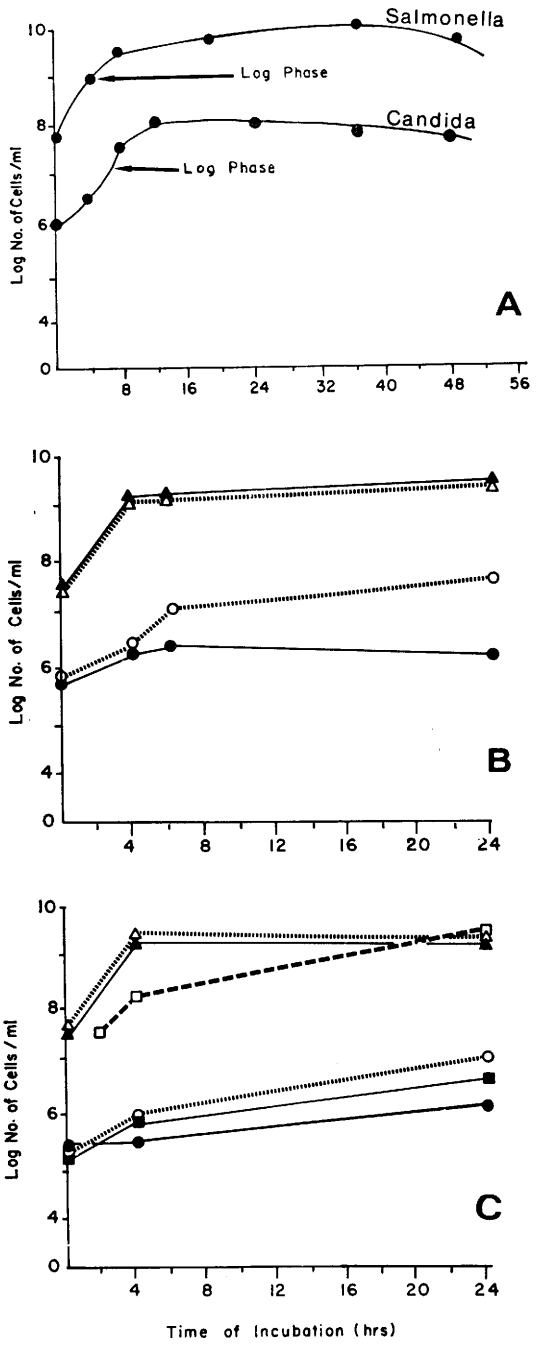

FIG. 1. (A) Growth curves of pure *S. enteritidis* and *C. albicans* during incubation at 37° in an incubator-shaker. (B) Growth of pure and mixed cultures of *S. enteritidis* and *C. albicans* under microaerophilic conditions as a function of time of incubation at 37°: (△) population of pure *Salmonella* culture, (▲) *Salmonella* when grown in mixed culture with *Candida*, (○) population of pure *Candida* culture, (●) *Candida* when grown in mixed culture with *Salmonella*. (C) Changes in pure and mixed populations of *S. enteritidis* and *C. albicans* during incubation at 37° and under anaerobic conditions without oscillation: (△) pure *Salmonella* culture, (▲) *Salmonella* in mixed culture, (○) pure *Candida* culture, (●) *Candida* in mixed culture, (□) *Salmonella* in medium where *Candida* was allowed to grow 2 hr prior to the inoculation of *Salmonella*, (■) *Candida* population in mixed culture but given 2 hr incubation.

ably due to use of prewarmed medium and shaking during incubation. The stationary phase of both cultures was long and extended from 12 to 45 hr. Absorbance measurements at 640 nm correlated well with plate counts throughout both the positive acceleration and the log phases. The number of *Salmonella* cells after 4 hr was 10^9/ml TSB and that for *C. albicans* was 10^7/ml TSB after 6 hr. The consistency in the number of cells/ml media during the log phase made it possible to maintain similar inocula for the experiments throughout this study.

Environmental conditions affecting in vitro interaction. Growth of *S. enteritidis* under aerobic conditions was not altered in presence of *C. albicans*, whereas marked reduction in growth of *C. albicans* was observed when mixed with *S. enteritidis* (Table I). A tenfold or greater disparity was noted after incubation for 6 hr and tended to increase thereafter. Under microaerophilic conditions, the growth curve of *Salmonella* in mixed cultures was similar to that of pure, whereas a decrease in maximal growth of *C. albicans* occurred in the presence of *S. enteritidis* (Fig. 1B). The data (Fig. 1C) depicts the changes in pure and mixed populations of *C. albicans* and *S. enteritidis* under anaerobic conditions and without oscillation. While growth of *Salmonella* pattern in pure and/or mixed culture was not affected by the anaerobic conditions, growth of candida both in pure and in mixed cultures was markedly reduced at all intervals. Again the presence of *Salmonella* inhibited the growth of the *Candida* culture. Since growth of *C. albicans*

TABLE I. Changes in Mixed and Pure Culture Populations during Incubation at 37° under Aerobic Conditions in a Metabolic Shaker Incubator.

Time of incubation (hr)	Population (log no. of viable cells/ml)[a]			
	S. enteritidis		*C. albicans*	
	Pure culture	Mixed culture	Pure culture	Mixed culture
0	7.9	7.9	5.9	5.8
2	8.4	8.0	6.0	5.9
4	9.2	9.1	6.5	6.1
6	9.3	9.3	7.2	6.3
24	9.7	9.7	7.7	6.4
48	9.8	9.7	7.9	6.6
72	9.3	9.5	7.7	6.2

[a] All values are means, and SEM ranged from ± 0.10 to ± 0.072.

was slower than that of *S. enteritidis* in reaching maximal growth level, *C. albicans* was allowed to grow 2 hr prior to the inoculation of *S. enteritidis*. The rate of growth of *S. enteritidis* was slow particularly between the 2nd and 16th hr of incubation (Fig. 1C). However, the growth pattern of *Candida* given a head-start over *Salmonella,* had less inhibition than that of *Candida* grown simultaneously with *S. enteritidis*.

The bacteria-free filtrates of each test culture were tested for their effects using disc sensitivity procedure. These filtrates were obtained from TSB cultures after 18 hr incubation under aerobic, microaerophilic, and anaerobic conditions. Repeated attempts failed to detect growth inhibitory substances in any of the filtrates of *S. enteritidis* against *C. albicans* or vice versa. This was evidenced by the lack of measurable zones surrounding the discs where the filtrates were added. Likewise, no zones of inhibition were noted on plates where both viable organisms were grown together. Both *S. enteritidis* and *C. albicans* cultures grew commensally on all plates incubated aerobically, as well as under microaerophilic or anaerobic conditions.

In vivo interaction. The data on the LD_{50} at 14 days of *C. albicans* at various preinjected levels of *S. enteritidis* are summarized in Table II. The number of mice dead per total number injected with log 8.57 and/or 7.46 cells *S. enteritidis* indicated that these animals died within 14 days. The majority of mice receiving the large numbers of *Salmonella,* and/or dually infected with either level of salmonella and with various numbers of candida cells died within 14 days. The high level of *Salmonella* cells probably contributed to the lethality of the dose injected. The LD_{50} at 14 days (of candida) at these levels of *Salmonella* was almost ½ the value noted when smaller numbers of *Salmonella* cells were injected. It was also observed that the majority of mice preinjected with log 6.51 salmonella cells (the LD_{50} of *S. enteritidis*) and with a lethal dose of *C. albicans* (log 5.46 cells) survived both lethal doses injected. When the number of salmonella cells in the injected dose was log 5.59, the titration of candida yielded results similar to those where only saline was injected ip. These results indicated that the net effect due to the interaction of the two organisms leading to the survival of the infected mice depended on the relative number of cells of

TABLE II. The Protective Effect of *C. albicans* in Mice Infected with *S. enteritidis.*[a]

Log no. *Salmonella* injected	No. dead/total mice within 14 days; Log no. of *C. albicans* infected					LD_{50} at 14 days
	0.00	*2.44*	*3.43*	*4.49*	*5.46*	
8.57	5/5	5/5	4/5	5/5	3/5	2.62 (0.65)[b]
7.46	3/5	4/5	4/5	4/5	5/5	2.55 (0.68)
6.51	1/5	1/5	1/5	1/5	0/5	4.96 (0.79)
5.59	0/5	0/5	0/5	0/5	4/5	5.15 (0.39)
0.00	0/5	0/5	0/5	0/5	5/5	4.95 (0.00)

[a] Mice were infected with *Salmonella* 24 hr prior to *Candida* injection.

[b] Number in parentheses are 95% confidence limits.

S. enteritidis injected rather than the number of *C. albicans*.

Recovery of test cultures from organ tissues. Figure 2 depicts the relationship between the average number (reported as log) of *Salmonella* and/or *Candida* recovered from various organ tissues at intervals after injection with the test organism(s) alone or in combination. The spleen did not support populations of *C. albicans,* while the renal (kidney) tissues were invaded by *C. albicans* as evidenced by their progressive increase in population. On the other hand, the candida recovered from kidneys of dually infected mice failed to increase at the rate noted in mice infected only with candida. A decrease in population, however, did not occur after the usual salmonellosis peak of 72 hr had elapsed. In the ceca of infected mice, a dramatic difference in candida populations occurred. The ceca of mice receiving only *C. albicans* supported vast populations of the fungi as indicated by greater recovery of more than log 7.0 organisms/g tissue after 168 hr of infection, whereas mice preinfected with *S. enteritidis* did not support invasion by the candida. The population of *Candida* in the liver maintained a steady level in animals infected with pure candida, whereas candida counts decreased within 6 hr in mice dually infected with both cultures. Analysis of blood of mice infected with candida showed the presence of low numbers of cells (log 1.98) during the 1st 24 hr postinfection, increasing thereafter to a maximal level of log 3.38 after 72 hr. No *Candida* was recovered from the blood of these animals after 168 hr. In mice dually infected with both test cultures, log 1.37 and 1.51 *Candida* cells were recovered only after 1 and 6 hr postinfection, respectively, but not thereafter. In general, *Candida* was recovered for a more extensive period and in larger quantities from tissues of mice infected only with candida than from animals dually infected. It should be pointed out that several mice of the *Candida*-infected group, not sacrificed, died after 38 hr of infection.

The same animals which received both *Candida* and *Salmonella* were further analyzed for the number of viable *S. enteritidis* in blood and in each tissue. The data showed

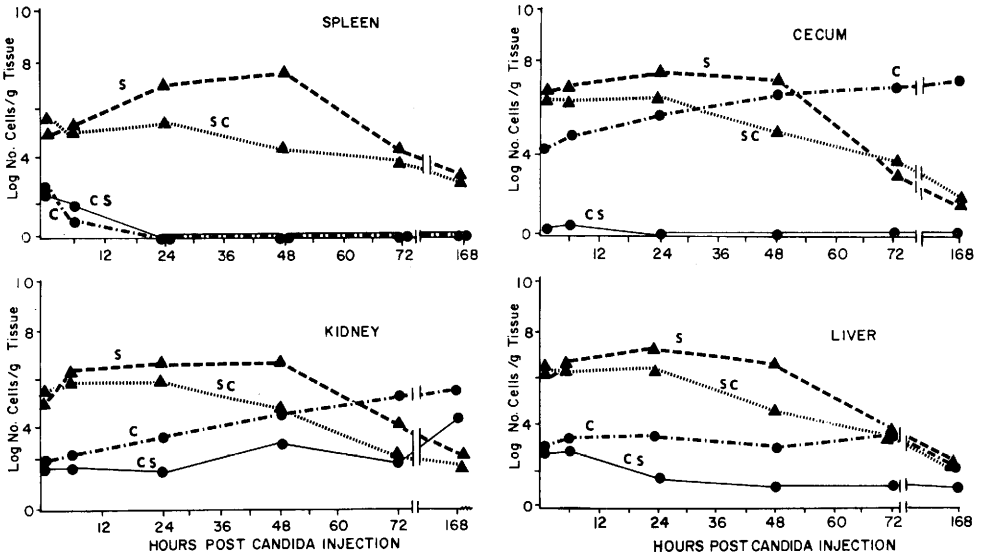

FIG. 2. Changes in population of *Salmonella* and *Candida* in various organ tissue of mice following infection with pure or mixed cultures. The mice were infected with log 6.89 *Salmonella* and/or 5.10 *Candida* cells. The data are recorded following *Candida* injection. (S) *Salmonella* counts in mice infected with pure *Salmonella,* (C) *Candida* counts in mice infected with pure *Candida,* (SC) *Salmonella* counts in mice infected with both *Salmonella* and *Candida,* and (CS) *Candida* counts in mice infected with both cultures.

the absence of *Salmonella* in blood and the presence of high levels of salmonella in spleens, livers, kidneys and ceca at early stages of examination in all test animals followed by a gradual decline thereafter (Fig. 2). Maximal level of *Salmonella* in all tissues was noted 48 hr postinfection in mice infected only with *Salmonella*. There was a slight variation in the number of *Salmonella* recovered from all four tissues of these mice at that time. On the other hand, it was noted that the presence of *C. albicans* affected the tissues of these animals as judged by the lower level of *S. enteritidis* recovered at any time. By 168 hr postinjection, the surviving animals began to eliminate the *Salmonella*. Several mice infected only with *Salmonella* died within 48–72 hr post-*Salmonella*-infection, while none of the mice infected with both test cultures succumbed to disease. No *Salmonella* was recovered from blood of mice infected with *Salmonella* alone or in combination with *Candida*. Again, the interaction between these organisms was noted as evidenced by the change of population pattern of organisms recovered from tissues of dually infected mice compared to those infected with either organisms alone. For example, the population of *Salmonella* declined within 24 hr in mice infected with both cultures compared to mice infected with pure *Salmonella*. Pathological examination revealed enlarged spleens and livers with lesions in animals infected with salmonella alone or dually infected with *Salmonella* and *Candida*. Kidneys with bleached discoloration and occasional lesions were also observed in mice which were only infected with *C. albicans*.

Discussion. Increased virulence of *S. enteritidis* above that reported previously (6) was achieved using cells grown in prewarmed and agitated media as well as by harvesting cells from early log phase (4–5 hr). The present study also showed that when log 8.0 *S. enteritidis* cells were given ip, the infection caused 100% mortality within 5 days. Miller and Bohnhoff (7) reported that a much higher death rate occurred in mice injected ip with *S. enteritidis* compared to those in which the bacteria were administered *per os*. Sluggishness, diarrhea, and enlarged spleens and livers were frequently detected in mice infected with log 6.0 cells or with higher numbers of cells. Virulence of *C. albicans* in Webster strain of mice was also enhanced appreciably by ic compared to ip infection. Salvin *et al.* (8) showed that mice resisted *C. albicans* infection and that virulence of this organism was increased by addition of 2.5% mucin. Since a significant increase in virulence of *C. albicans* was acquired by the ic route, it was decided to avoid addition of gastric mucin.

Usually the net effect of a mixed bacterial population is often smaller or greater than the simple sum of activities of each organism growing in pure culture. Our data revealed that mixed cultures of *S. enteritidis* and *C. albicans* led to reduced growth of the latter organism probably due to exhaustion of critical nutrients by *S. enteritidis*. However, the actual limiting factor was not ascertained under the three different environmental conditions tested *in vitro*. The *in vivo* interaction of the test cultures on the other hand revealed several observations. The first concerns the virulence of test cultures, the second is related to protection and the third to proliferation in organ tissues. When mice received log 7.46 and 8.57 *S. enteritidis* cells, the lethal effect of *Salmonella* was not appreciably altered by the presence of *C. albicans* injected ic 24 hr later. At these levels of *Salmonella*, there was a masking of any resistant effect against candida invasion in the mice. The protective effect occurred when log 6.51 or 5.59 *S. enteritidis* cells were injected 24 hr prior to injection of either log 2.44, 3.43, or 4.49 *C. albicans* cells. None of the infected animals succumbed to disease by either organism. Supporting data from organ tissues showed the failure of *C. albicans* to proliferate in tissues from mice receiving both organisms compared to those injected with *C. albicans* only. Another variation due to interaction occurred in the intestinal tissue of animals injected with both organisms where the ceca of animals did not support growth of *C. albicans*. Gale and Sandoval (9) reported that prior inoculation of a nonlethal concentration of viable *Escherichia coli* protected mice from a lethal concentration of *C. albicans*. Furthermore, the protective phenomenon was exerted by *E. coli* within a narrow range of concentration. Paine

(10) stated that Gram-negative bacilli generally were more effective in inhibiting candida than were Gram-positive cocci. Evidence from present study seems to indidate that previous establishment of high concentrations of *S. enteritidis* in the intestinal tract of mice prevents invasion of tissues by *C. albicans,* which also has a strong affinity for that body tissue. When concentration of *S. enteritidis* is approximately log 6.0 cells, the normal lethal effect of log 5.0 *C. albicans* cells was hampered. This evidence indicated that the lethal effect of *C. albicans* depended both on its ability to invade and to proliferate in intestinal tract and in kidney. Wright *et al.* (11) indicated that enhanced resistance to candidiasis was observed one day following administration of bacterial endotoxin of *E. coli.* It is generally accepted that endotoxin contributes to the pathogenesis of Gram-negative infections, although it is not believed to be the most important factor in the ability of the bacteria to invade a host and establish infection (12). The 24 hr preinfection of mice with *S. enteritidis,* a Gram-negative known to produce endotoxin, may be a contributing factor to the increased resistance of the host to lethal *C. albicans* infection.

Summary. Injection of *Candida albicans* ic facilitated establishment of disease as evidenced from the LD_{50} studies. The LD_{50} at 14 days for mice injected ic with *C. albicans* was log 5.2 $\pm$ 0.5 cells and log 7.2 $\pm$ 0.5 cells when injected ip. The LD_{50} at 14 days for *Salmonella enteritidis* injected ic was log 6.6 $\pm$ 0.5. Interaction between mixed cultures of *C. albicans* and *S. enteritidis* was studied under various environmental conditions. Retardation of growth of *C. albicans* was noted in broth in presence of actively growing *S. enteritidis.* Cell-free culture filtrates of *S. enteritidis* or of *C. albicans* failed to inhibit proliferating cells of competing organisms. Also, test cultures grew commensally on all plates incubated aerobically as well as under anaerobic or microaerophilic conditions.

Proliferation of *S. enteritidis* in the spleen, ceca, kidneys, and liver depended upon number of cells injected. Several organ tissues showed the presence of large populations of *S. enteritidis* when infected ip with log 6.0 or more *S. enteritidis* cells. No deaths occurred among animals receiving log 5.0 *S. enteritidis* cells. The spleen and blood of mice infected with *C. albicans* were able to eliminate the organisms rapidly. When mice received only *C. albicans* (log 5.0 cells), large populations of the organism were noted in both kidney and cecum. Lower concentrations of *C. albicans* were readily eliminated by these organs. Mice infected with *S. enteritidis* 24 hr prior to infection with *C. albicans* exhibited three conditions depending upon the numbers of cells of *S. enteritidis.* High numbers (above log 7.0) of *S. enteritidis* cells produced greater mortality rates in infected animals similar to those where no *C. albicans* were injected. Log 6.0 *S. enteritidis* cells plus log 5.0 *C. albicans* cells (lethal dosages) prevented mortality to mice. Lower numbers of *S. enteritidis* (log 5.0 or less) did not produce disease syndrome in mice and did not alter lethal effect of *C. albicans.* Organ tissues of mice preinfected with log 6.0 *S. enteritidis* and then injected with log 5.0 *C. albicans* cells showed a decrease in growth of *C. albicans.* The cecum of these mice did not support growth of *Candida.* Evidence indicated that interaction between these test cultures prevented invasion of the tissues by *C. albicans.*

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