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Effect of Hyperimmune Rabbit Serum on Intracellular Growth of *Mycobacterium bovis.** (30528)

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Experimental evidence of humoral immunity in tuberculosis is inconclusive(1,2). Although the presence of a phagocytosis-promoting factor in sera from tuberculous animals(3) and patients(4) has been demonstrated, opsonization as a positive role in resistance to tuberculosis is controversial(5). Fong *et al*(6) reported that immune serum increased the resistance of immune histiocytes to the toxic effect of virulent tubercle bacilli. However, this resistance factor was nonspecific. Recently, Hsu(7) found that, although the growth of *M. tuberculosis* in macrophages of susceptible rabbits was not suppressed in the presence of anti-BCG serum, the parasitized cells were somewhat protected from the damaging effect of the contained bacilli.

Reports by Shepard(8) and from our laboratory(9) indicated that established cell lines may be useful in studying the host-parasite relationship in tuberculosis. As part of a study of the mechanism of resistance and immunity to tuberculosis, experiments were carried out on the influence of hyperimmune anti-BCG serum on (a) phagocytosis of virulent *Mycobacterium bovis* by the J-111 cell line of human leukemic monocytes(10), (b) growth of the phagocytosed mycobacteria and (c) survival of infected cells.

Materials and methods. For hyperimmune anti-BCG serum, commercial New Zealand albino rabbits were given 3 consecutive monthly intravenous injections of 20×10^6 to 40×10^6 viable units of BCG-IV (Rosenthal)† as described by Fong *et al*(11). BCG agglutinins were observed at a serum dilution of 1:640. Antityphoid serum was obtained from animals one week after an immunization regimen described by Fong *et al* (6). Agglutination titers against typhoid O and H antigens were 1:1280 or greater. Uninjected rabbits served as the normal serum source. All sera were stored at —10°C. Where indicated, sera were inactivated at 56°C for 30 minutes.

Stock cultures of J-111 cells were maintained on a yeast extract-rabbit serum medium containing 50 units of penicillin (PN) and 50 μg of streptomycin (SM) per ml. Cells, trypsinized with 0.25% trypsin, were suspended in the growth medium to yield 45,000 cells per ml. One ml volumes were pipetted onto coverslips in Leighton tubes and cultures were incubated at 37°C for 48 hours. Two washings with Hanks' Balanced Salt Solution (BSS) were followed with refeeding of yeast extract medium containing the appropriate test serum in 10% concentration. Infected cell cultures were inoculated with 1.2×10^6 to 1.5×10^6 viable units of *M. bovis* (Ravenel);‡ noninfected cultures received 0.05 ml of BSS. The mycobacterial suspension was prepared from a 3- to 4-week-old culture grown on Lowenstein-Jensen medium. Using

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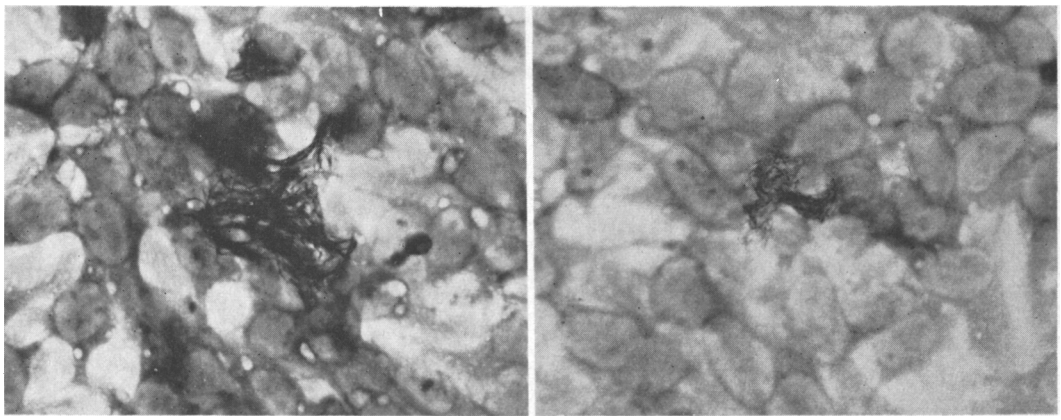

FIG. 1. (left) 8-day *M. bovis*-infected J-111 cell culture in yeast extract-antityphoid rabbit serum medium. Note luxuriant intracellular growth and cording. Aubert's stain. 700 ×.

FIG. 2. (right) 8-day *M. bovis*-infected J-111 cell culture in yeast extract-anti-BCG rabbit serum (inactivated) medium. Extent of growth and cording much less than controls (normal and antityphoid rabbit serum containing medium). Aubert's stain. 700 ×.

BSS, the growth was triturated, washed twice and resuspended. The suspension was then filtered through a single layer of Whatman No. 1 paper and standardized to an optical density of 35 to 45 Klett-Summerson units at 540 mμ wavelength.

Following overnight incubation, infected and uninfected cultures were again washed twice with BSS containing 5 units of PN and 5 μg of SM per ml. Three experimental and control coverslips were then fixed in absolute methanol and stained for acid-fast bacilli employing Aubert's technique(9). A similar number of cell cultures was lysed with 0.5% sodium desoxycholate solution(12) for quantitative colony counts of intracellular bacilli and one-tenth ml aliquots of 1:10, 1:100 and 1:1,000 dilutions were planted onto Lowenstein-Jensen medium. Remaining cell cultures were fed appropriate nutrient (yeast extract medium and 10% test serum) containing 5 units of PN and 5 μg of SM to inhibit extracellular growth(13) and prepared for microscopy 3-4 and 7-8 days postinoculation.

Falcon plastic tissue culture bottles (25 ml) were used to determine cell populations. The cultural method was similar to that employed for Leighton tubes except that 4 ml of medium were used per bottle. Upon completion of the cultural phase of 7-8 days postinfection, both experimental and control cell cultures were trypsinized, fixed with 10% formalin for 3 hours and triplicate counts made in a Neubauer hemocytometer.

Results. A heat labile phagocytosis-promoting substance appears to have been present in anti-BCG hyperimmune serum (Table I). Normal and antityphoid sera did not enhance phagocytosis.

The extent of intracellular growth of *M. bovis* at 3-4 days postinfection was approximately the same in all cell cultures. At 7-8 days, however, there were fewer intracellular mycobacteria in the anti-BCG serum-fed cultures than in the controls. Furthermore, there were only about half as many bacilli in cord-formation in the anti-BCG immune system as in the controls (Fig. 1, 2). This was true even for inactivated anti-BCG immune serum-fed cultures.

In repeated experiments, a decline in cell population was observed in infected cultures

TABLE I. Effect of Hyperimmune Rabbit Serum on Phagocytosis. Colony counts of intracellular *M. bovis* one day postinoculation.

Exp	Mean viable units/ml*		
	Anti-BCG serum	Normal serum	Antityphoid serum
1	4.4×10^5	2.9×10^4	—
2	2.3×10^5	5.1×10^4‡	—
	†2.3×10^4	†5.2×10^4‡	—
3	2.5×10^5	7.0×10^4	4.2×10^4

* Avg of colony counts performed in triplicate.
† Inactivated at 56°C, 30 min.
‡ Commercial rabbit serum.
— Not done.

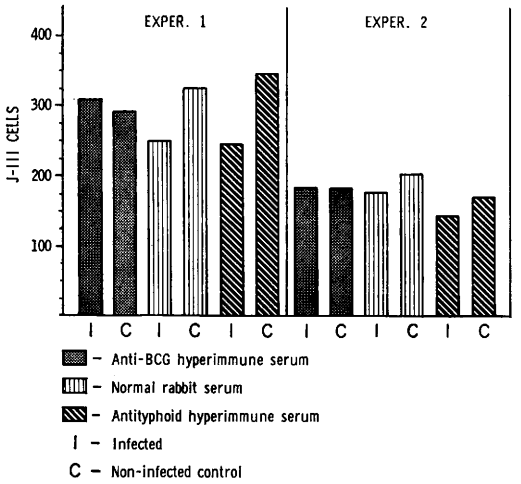

FIG. 3. *M. bovis* in J-111 cells. Mean cell count 7 days postinfection.

maintained on normal or antityphoid serum-containing medium; no appreciable loss of cells occurred in cell cultures fed anti-BCG hyperimmune serum medium (Fig. 3). Employing the F test(14) to analyze the combined results of both experiments, the difference is significant at the level of 0.05 probability in the infected cell population of anti-BCG serum-fed cultures from that of infected normal and antityphoid serum-treated cultures.

Discussion. Youmans(15) and Dubos(16) emphasized that acquired immunity to tuberculosis is the ability of the host to inhibit multiplication of tubercle bacilli. There is considerable evidence that this immune response is primarily cellular(17). However, as suggested by Jenkin and Rowley(18), cell-bound antibody may also play a significant role. Although it is perhaps related to natural rather than acquired immunity, it is worthy of mention that Kochan and Smith(19) found that the ability of macrophages of BCG-vaccinated guinea pigs to inhibit intracellular growth of BCG was serum-conditioned.

Suter(20) showed that cellular destruction may result from an overwhelming number of intracellular growing bacilli. Employing macrophage culture systems, the favorable influence of immune serum in diminishing the cytotoxic effect of intracellular parasitism was observed by Fong(6) and Hsu(7). Hsu emphasized that cellular degeneration was lessened without a corresponding decrease in intracellular multiplication of bacilli.

The *in vitro* culture system used in our experiments provided controlled experimental conditions to study the effect of humoral factors on the interaction between host and parasite. Based on: (a) enhanced phagocytosis, (b) diminished frequency and size of intracellular cords, and (c) prevention of loss of infected cells due to overgrowth of intracellular *M. bovis*, an opsonin-like substance and an antimycobacterial factor were demonstrated in anti-BCG rabbit serum. Serum of normal and typhoid-vaccinated animals did not exhibit these properties.

Summary. The interaction between *M. bovis* and the J-111 cell was influenced by the serum component of a yeast extract-serum nutrient medium. Anti-BCG hyperimmune serum enhanced phagocytosis, suppressed intracellular growth, and prevented loss of infected cells to a greater extent than did normal or antityphoid sera.

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Responses of Xeric-Adapted Rodents to Waterloading.* (30529)

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Several studies have indicated that desert-inhabiting rodents have "difficulty" in eliminating a water load, presumably because of factors associated with their adaptation to conservation of water. Hofmann(1) found that Merriam's kangaroo rat, *Dipodomys merriami*, "excreted the load only with the greatest difficulty." *D. merriami* given 5 ml/100 g of distilled water by stomach tube eliminated 40% of the load only after 367 minutes, while Long-Evans white rats did so in 84 minutes. *D. merriami* loaded intraperitoneally showed a still more pronounced water retention(2). The banner-tailed kangaroo rat, *D. spectabilis*, excreted the same percentage (85-88%) of a water load within 6 hours as did white rats, but only after a diuretic lag of 3 hours(3). A gerbil, *Meriones shawii*, "cannot excrete orally administered tap water by means of a hypotonic diuresis," when kept on a dry diet(4).

We have compared *D. merriami*, *D. agilis* and *M. unguiculatus* in order to check and extended these observations. The genus *Dipodomys* has had its center of evolution in the deserts of southwestern North America. *D. merriami* is one of the most ubiquitous species in these deserts; a few species such as the nimble kangaroo rat, *D. agilis*, have dispersed into semiarid habitats adjacent to deserts. Species of *Meriones* inhabit deserts and semiarid regions of the Old World. The Mongolian gerbil, *M. unguiculatus*, is found in semiarid areas of Mongolia, northern Korea and the Necca Province of China.

Materials and methods. *D. merriami* were live-trapped in the desert near Pearblossom, Calif., and *D. agilis* in chaparral near Etiwanda, Calif. *M. unguiculatus* were bred from stock from Tumblebrook Farms, Brant Lake, N. Y. Body weights of the animals were: *D. merriami*, 32-40 g, *D. agilis*, 60-70 g, *M. unguiculatus*, 63-72 g.

In all cases the water load was 5 ml/100 g of body weight of warm distilled water, given in one dose by stomach tube. Animals were stimulated to empty their bladders before intubation; they were weighed before and after loading to determine the absolute amount received. After loading, animals were placed on wire mesh over mineral oil in tared beakers. Urine excretion was measured by weighing the beakers at half-hour intervals for 6 hours; weights were corrected for any feces present. Urine output was plotted against time, and time for excretion of 40% of the load was estimated from the plot. Rate of excretion was measured after the first water load, and again after a second loading the same day or the next day, or after a third load the next day.

Results. Measurements are summarized in Table I.

Discussion. There are numerous observations of instances in which the abilities of different species of mammals to conserve body water have evolved in direct relationship to the average aridity of their habitats(5). The water conservation of such species often per-

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