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Inhibition by Normal Human Serum of *Mycobacterium avium* Multiplication in Cultured Human Macrophages

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Normal human serum used as a protein supplement in RPMI 1640 medium inhibited growth in blood-derived human macrophages (MP) of virulent *Mycobacterium avium* serovars 4 and 8, derived from patients with acquired immunodeficiency syndrome, but not virulent *Mycobacterium tuberculosis*. A defined serum substitute (SS) promoted the intramacrophage growth of *M. avium* but not of *M. tuberculosis*. The effects of serum or SS were measured by counting viable bacteria in lysates of the MP at 0, 4, and 7 days after their infection by the bacteria. Neither serum nor SS inhibited or enhanced *M. avium* growth in the absence of MP. The results suggest that a nutrient essential for intracellular replication of *M. avium* is made by MP from a pronutrient present in both SS and serum and that something in serum inhibits MP conversion of the pronutrient to nutrient. This inhibition may be an important mechanism of native resistance against *M. avium* in normal people.

Mycobacterium avium is a rare cause of chronic granulomatous infection in people who do not have acquired immunodeficiency syndrome (AIDS) and is a frequent cause of severe disseminated opportunistic infection in patients with AIDS (5, 11). *M. avium* cannot cause disease in most people, so these people must have mechanisms which can suppress growth of the organism in vivo. Conversely, patients with *M. avium* disease must have lost these mechanisms (5). Like *Mycobacterium tuberculosis*, *M. avium* grows in macrophages (MP) (4). While comparing *M. avium* growth in cultured MP from AIDS patients and from normal subjects (A. J. Crowle, D. L. Cohn, and P. Roche, submitted for publication), we discovered, as reported here, that normal human serum itself is a potent inhibitor of *M. avium*, but not of *M. tuberculosis*, via interaction with the MP.

Blood for MP and serum was obtained from several healthy, tuberculin-negative subjects between 20 and 50 years old (informed consent was obtained). Adherent monocytes from the blood were isolated and cultured in Falcon 1008 petri dishes (Becton Dickinson Labware, Oxnard, Calif.) in RPMI 1640 medium (without antibiotics) supplemented with 2 mM L-glutamine and unheated 1% normal serum (autologous or heterologous), as described in detail previously (1–4; Crowle et al., submitted). After 7 days in culture, the adherent MP cells were infected for 30 min with suspensions of *M. avium* or *M. tuberculosis* or were coinfected with both. The ratio of bacilli to MP, usually about 1:2, was adjusted to infect approximately 10% of the MP with single bacilli (1, 2, 4). The bacteria were dispersed ultrasonically for infection and also for CFU counts from samples of lysed MP (1, 6). The sonication as used did not kill the bacilli (1, 4, 6).

The infected MP were incubated after infection for 7 days more in culture medium supplemented with unheated normal serum or with a serum substitute (SS), as described below. SS is an ultrasonically stabilized mixture of human serum albumin, human transferrin, cholesterol, and lecithin in bicarbonate-free Iscove medium (3, 10). Samples of the infected MP were taken at 0, 4, and 7 days after infection for lysing and CFU counts, as previously described (1, 6). CFU counts included viable bacilli from both the lysed MP and their supernatants. The counts are reported as mean CFU per milliliter of MP lysate, where 1 ml of lysate represents an average of 10^5 MP (1). Each CFU value in our results is a

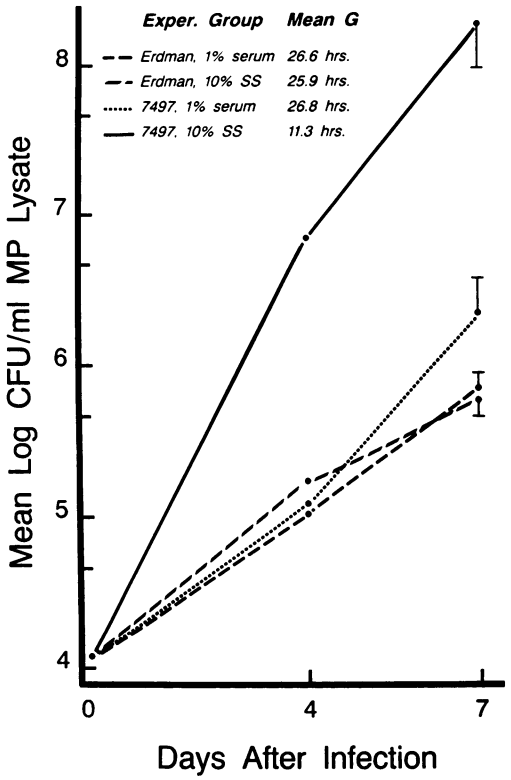

FIG. 1. Growth rates of Erdman and 7497 bacilli in donor 6 MP coinfected with both species of bacteria and cultured in RPMI 1640 medium supplemented with 1% autologous serum or 10% SS. In this and other figures, mean counts of CFU per milliliter of MP lysate (lysate of approximately 10^5 MP) are plotted in $\log_{10}$ values against days after infection. Each datum point is the mean of five values. Bars indicating standard errors of means are shown only for 7-day counts.

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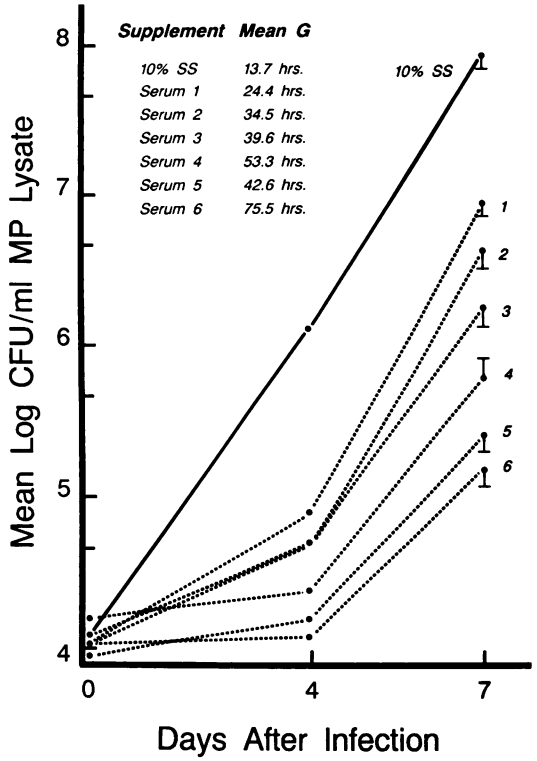


FIG. 2. Comparison of the replication of 7497 in donor 1 MP cultured in medium supplemented with 10% SS, 1% autologous serum, or 1% sera from donors 2 through 6.

mean of five values. For simplicity, standard error of mean bars are shown in the graphs (Fig. 1–3) only for 7-day values; standard errors of the means for 0- and 4-day values were about the same. Some data are presented as mean bacterial generation times (G). G is the doubling time of the bacteria in a MP culture and was calculated from the mean slope of bacterial growth for the $\log_{10}$ CFU counts at 0, 4, and 7 days after infection plotted against time after infection. Microtiter well cultures of the bacteria in 7H9 bacteriologic culture medium were used to test the effects of sera or SS on bacterial growth in the absence of MP (1, 2; Crowle et al., submitted). As reported previously (4, 6), neither *M. avium* nor *M. tuberculosis* grows significantly in the absence of MP in RPMI 1640 medium, whether unsupplemented or supplemented with serum or SS.

Growth rates of *M. tuberculosis* and *M. avium* in MP cultured in medium supplemented with 1% serum were similar. Figure 1 shows the results from one experiment, which are representative of results from several experiments. The results in Fig. 1 are especially significant because they are from MP which were coinfected with *M. tuberculosis* and *M. avium*, and they show that differences in the results between the species are not connected with effects these bacteria have on the MP. The doubling times (mean G) for the two species of bacteria were 26.6 and 26.8 h, respectively. However, *M. avium* grew nonexponentially. In MP cultured in medium supplemented with 10% SS, G for *M. tuberculosis* was essentially unchanged (G = 25.9), but for *M. avium* it was greatly accelerated (G = 11.3), and *M. avium* growth became exponential. These results show that normal serum inhibits growth of *M. avium* but not of *M. tuberculosis* in cultured human MP.

Sera from all healthy donors so far tested (more than 20 in miscellaneous experiments) have inhibited the growth of *M. avium* in MP relative to the rate of growth in SS-supplemented medium, whether the MP were coinfected, as in Fig. 1, or infected with one species of bacteria alone. The degree of inhibition has varied, as can be seen when six sera on MP from donor 1 infected with *M. avium* alone are compared (Fig. 2). Growth was exponential in SS (G = 13.7). In the various sera it was nonexponential, and G ranged from 24.4 to 75.5. The inhibition depends on which combination of MP and serum is used. For instance, when the experiment in Fig. 2 was partly repeated with MP from donor 6 instead of donor 1, the relative abilities of sera from donors 1 and 6 to inhibit were reversed (G = 75.0 and 52.7, respectively), while G in

TABLE 1. Effects of SS and various concentrations of normal serum on growth of *M. avium* in human MP

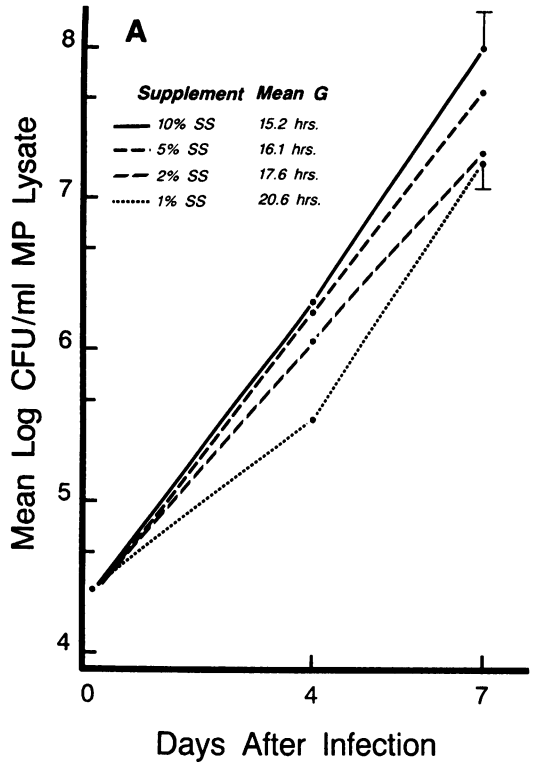
Expt	Bacillus	Serum		SS concn (%)	Mean G (h)[a]
		Donor or serum type	Concn (%)		
P-36	7497	7	1	0	30.0
P-36	7497	7	5	0	Stasis
P-36	7497	7	25	0	Stasis
P-36	7497	7	50	0	Stasis
P-55	Erdman	7	1	0	24.8
P-55	Erdman	7	5	0	24.0
P-55	7497[b]	7	1	0	67.1
P-55	7497	7	5	0	69.4
P-55	T-138	7	1	0	39.6
P-55	T-138	7	5	0	53.1
P-55	7497[b]	AB serum	5	0	300
P-55	T-138	AB serum	5	0	86.9
P-66	7497	8	1[c]	0	13.1
P-66	7497	8	1[c]	0	11.3
P-66	7497	AB serum	1	0	15.6
P-66	7497	8	5	0	71.3
P-80	7497[b]	AB serum	1	0	56.3
P-80	7497	AB serum	5	0	Stasis
P-80	7497	AB serum	10	0	Stasis
P-84	T-138	4	2	0	41.4
P-84	T-138	4	1	0	24.4
P-84	T-138	4	0.2	0	18.8
P-84	T-138	4	0.2	0	13.7
P-84	T-138	4	None	10	11.3
P-85	T-138	4	1	10	36.9
P-85	T-138	4	0.05	0	13.1
P-88	T-138	AB serum	1	0	51.0
P-88	T-138	AB serum	None	10	16.9

[a] Mean generation (doubling) time of intracellular bacilli.
[b] 7497 in experiment P-55 was an unselected, low-virulence batch; in experiment P-80 it was of selected flat-transparent colony morphology but also of low virulence.
[c] The 1% serum in the first of two groups used in P-66 was used throughout the period of incubation; in the second, it replaced 1% AB serum, which was used in the period before infection.

TABLE 2. Effects of serum and SS on growth of *M. avium* in RPMI 1640 medium

Bacterium	Generation time (h) in:			
	7H9	RPMI	RPMI-serum	RPMI-SS
TMC 724	19.9	158	60.4	60.4
7497[a]	13.7	65.3	34.3	43.1
BCG[b]	24.0	123	No growth	69.4
Erdman	20.1	58.5	39.4	58.5

[a] MP-passaged, smooth-transparent virulent batch.
[b] Human MP-passaged Webb-Waring strain; originally Tice strain.



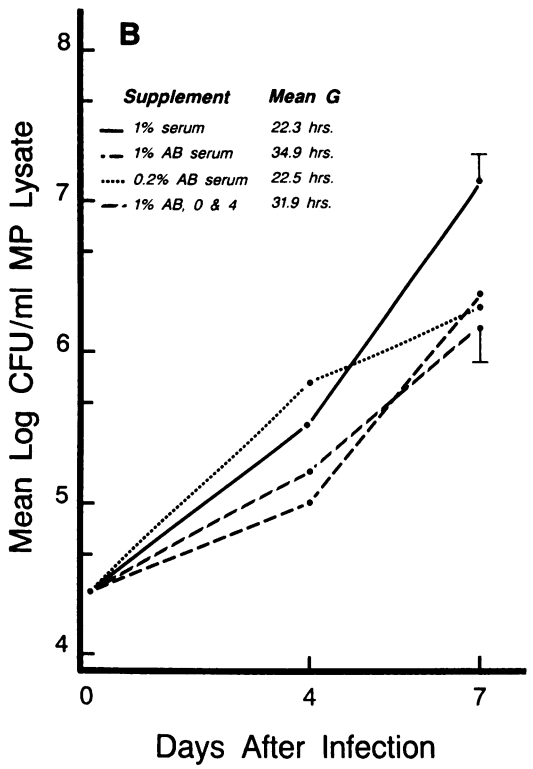
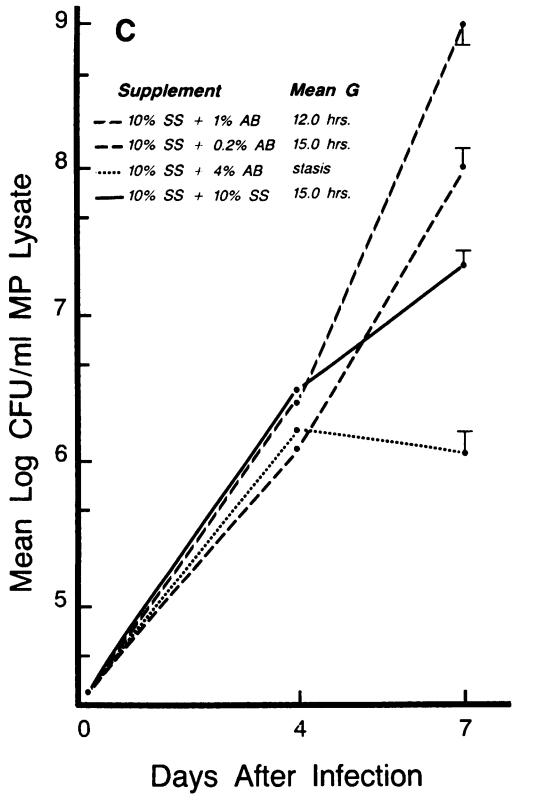


FIG. 3. Growth of T-138 in donor 9 MP in medium supplemented in various ways. (A) Results with SS at different concentrations in medium added to the MP immediately after infection (0 days). (B) Results with control groups that were given 1% autologous or AB serum at 0 days, 0.2% AB serum at 0 days, or 1% AB serum at both 0 and 4 days after infection. (C) Results with cells incubated throughout the 7 days of infection with a combination of 10% SS and 1% AB serum or 10% SS and 0.2% AB serum and with cells incubated for 4 days of the infection with 10% SS and then changed to 4% AB serum or to fresh medium with 10% SS as the supplement still.



SS remained essentially the same. These results show that inhibition is expressed by all normal sera tested but that the degree of inhibition is variable and partly dependent on which donor MP are used.

Inhibition by the serum was concentration dependent and was effective against serovar 8 *M. avium* (T-138) as well as against the serovar 4 (7497) strain most frequently used in these experiments (Table 1). At 5% and higher, serum stopped intra-MP growth of *M. avium* although it did not affect intra-MP growth of *M. tuberculosis* (Table 1) (1). The inhibition was bacteriostatic, not bactericidal, and never caused any significant drop in CFU (Table 1, P-36; Fig. 3C).

Inhibition of *M. avium* growth in MP by serum and relative enhancement of growth by SS were never seen in medium alone (Table 2). Therefore, the promotion of *M. avium* growth by SS in MP is probably due to some product of interaction between SS and MP, and inhibition of the growth by serum is not direct.

Experiments testing various concentrations and mixtures of SS and normal serum (Fig. 3 and 4; Table 3) suggest that inhibition by serum is caused by some rapidly acting inhibitor that is absent from SS. Normal AB serum at 4%, for instance, stopped 10% SS from supporting growth of *M. avium* as soon as the serum was added to the medium (Fig. 3C). By contrast, removing serum from the medium quickly restored support of vigorous growth by SS (Fig. 4). Diluting serum, for instance to 0.2 instead of 1% (Fig. 3B), diminished its inhibitory effect.

The nature of the agent in normal human serum which inhibits growth of *M. avium* but not of *M. tuberculosis* in

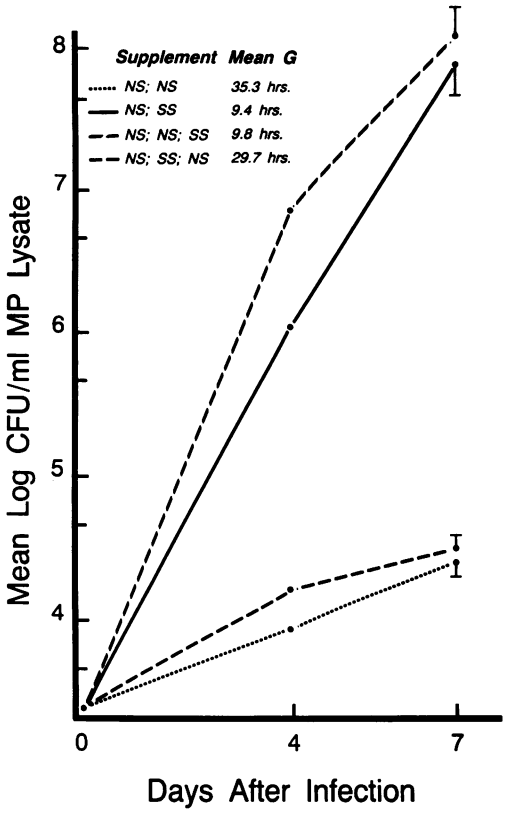


FIG. 4. Growth rate of 7497 bacilli in MP cultured in medium with supplement changed at various times relative to infection. Group 1 (NS; NS) was incubated in 1% normal (autologous) serum for 7 days before infection and for 7 days after infection. Group 2 (NS; SS) was incubated in 1% normal serum before infection and then changed to 10% SS after infection. Group 3 (NS; NS; SS) was incubated in 1% normal serum for 6 days, changed to fresh medium with 1% normal serum at 24 h before infection, and then incubated in 10% SS after infection. Group 4 (NS; SS; NS) was incubated in 1% normal serum for 6 days, 10% SS for 24 h, and then 1% NS after infection.

normal human MP is unknown. Probably it is not antimycobacterial antibody, because antibody-rich sera from patients with *M. avium* infections are unable to inhibit growth (Crowle et al., submitted). Furthermore, the inhibitor is variably effective in MP from different donors (Fig. 1 and 2), which would not be expected of antibody. Most likely, the agent selectively inhibits a MP metabolic function. Both serum and SS appear to contain a pronutrient (e.g., a lipid) which MP can convert to a nutrient (e.g., a fatty acid) needed by intra-MP *M. avium* to divide (7–9). The inhibitor would act by blocking this conversion. The inhibitor is absent from SS, and consequently MP in SS efficiently convert pronutrient to nutrient (7) and support vigorous intracellular growth of *M. avium*. The presence of the inhibitor in serum, however, would block the conversion and starve *M. avium*. In other experiments (Crowle et al., submitted), we have found that serum from patients with AIDS or chronic granulomatous *M. avium* infections acts like SS. These findings suggest that the serum inhibitor is an important native defense against *M. avium*.

TABLE 3. Effects of mixing AB serum and SS in various concentrations on ability of SS to support growth of *M. avium* in MP

Concn (%) of:		Mean G (h)[a]
Serum	Serum SS	
1	None	54.2
None	10	11.8
1	10	12.8
2	10	9.0
4	10	46.5
None	2	13.1
2	2	41.8
4	2	96.9
2	None	134.3
4	None	289.7

[a] Mean generation time.

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LITERATURE CITED

1. **Crowle, A. J.** 1988. The tubercle bacillus—human macrophage relationship studied *in vitro*, p. 99–135. *In* M. Bendinelli and H. Friedman (ed.), *Mycobacterium tuberculosis*: interactions with the immune system. Plenum Publishing Corp., New York.
2. **Crowle, A. J., N. Elkins, and M. H. May.** 1988. Effectiveness of ofloxacin against *Mycobacterium tuberculosis* and *Mycobacterium avium*, and rifampin against *M. tuberculosis* in cultured human macrophages. Am. Rev. Respir. Dis. **137**:1141–1146.
3. **Crowle, A. J., E. J. Ross, and M. H. May.** 1987. Inhibition by $1,25(OH)_2$-vitamin D_3 of the multiplication of virulent tubercle bacilli in cultured human macrophages. Infect. Immun. **55**: 2945–2950.
4. **Crowle, A. J., A. Y. Tsang, A. E. Vatter, and M. H. May.** 1986. Comparison of 15 laboratory and patient-derived strains of *Mycobacterium avium* for ability to infect and multiply in cultured human macrophages. J. Clin. Microbiol. **24**:812–821.
5. **Deo, M. G.** 1988. Immunological approach for control of *Mycobacterium avium-intracellulare* infections in AIDS—an hypothesis. Int. J. Lepr. **56**:455–463.
6. **Douvas, G. S., and A. J. Crowle.** 1985. Macrophages. I. Model for studying antimicrobial immunity *in vitro*, p. 81–90. *In* T. Yoshida (ed.), Investigation of cell-mediated immunity. Churchill Livingstone, Ltd., Edinburgh.
7. **Kottke, B. A.** 1987. Role of macrophages in lipid metabolism. J. Cardiovasc. Pharmacol. **10**(Suppl. 9):S7–S10.
8. **McCarthy, C., and P. Ashbaugh.** 1981. Factors that affect the cell cycle of *Mycobacterium avium*. Rev. Infect. Dis. **3**:914–925.
9. **McCarthy, C. M., M. A. Taylor, and M. W. Dennis.** 1987. Maximum growth rate of *Mycobacterium avium* in continuous culture or chronically infected BALB/c mice. Microbios **52**: 97–103.
10. **Stevenson, H. C., E. Schlick, R. Griffith, M. A. Chirigos, R. Brown, J. Conlon, D. J. Kanapa, R. K. Oldham, and P. Miller.** 1984. Characterization of biological response modifier release by human monocytes cultured in suspension in serum-free medium. J. Immunol. Methods **70**:245–256.
11. **Young, L. S.** 1988. *Mycobacterium avium* complex infection. J. Infect. Dis. **157**:863–868.