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Comparison of the Abilities of *Mycobacterium avium* and *Mycobacterium intracellulare* To Infect and Multiply in Cultured Human Macrophages from Normal and Human Immunodeficiency Virus-Infected Subjects

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Patients with AIDS commonly develop disseminated infections with *Mycobacterium avium* (MA) but not its close relative, *M. intracellulare* (MI). In non-AIDS patients who have these infections, the two species are about equally distributed. The higher incidence of infection with MA than with MI in AIDS patients might be due to the selective susceptibility of these patients to MA. This possibility was tested by comparing the abilities of MA and MI to infect and replicate in cultured macrophages from normal subjects and from patients with AIDS-related complex or AIDS. The macrophages were cultured in medium supplemented with 1 or 5% normal or patient sera or with 1% defined serum substitute. Replication of MA (serovar 4) or MI (serovars 16 and 17) in the macrophages was measured by CFU counts made from lysed samples of the macrophages taken at 0, 4, and 7 days after macrophage infection. MA and MI in infected normal macrophages which were cultured in normal serum replicated in these macrophages at similar rates. MA but not MI multiplied abnormally rapidly in patient macrophages cultured in either normal serum or patient serum. The accelerated growth of MA in patient macrophages was macrophage dependent, because patient sera did not change the rate of MA replication in culture medium lacking macrophages. However, patient sera did increase the permissiveness of normal macrophages to MA but not MI. These results suggest that a selective increased susceptibility to MA compared with a retained normal resistance to MI in human immunodeficiency virus-infected patients as they progress from AIDS-related complex to AIDS accounts for the higher prevalence of MA than MI infection in AIDS patients. The results also indicate that the mechanisms of native resistance in human macrophages to MA and MI are different.

Mycobacterium avium (MA) and *M. intracellulare* (MI) are two closely related but distinct species of mycobacteria commonly referred to as the MAI complex of bacteria (10, 17). MA and MI cause systemic infections with equal frequency (2, 10, 17) in patients who are not infected with human immunodeficiency virus (HIV), but in HIV-infected patients who develop disseminated infections with MAI, MA greatly predominates (8, 10, 12, 14, 18). Among MA, the strain or serovar which predominates is due partly to which serovar the patient is exposed to from water or dust in the environment (2, 8–11, 13). However, there appears to be no direct evidence for differences in environmental exposure accounting for the more frequent infection of AIDS patients by MA than by MI, and obtaining such evidence will be difficult. An alternative explanation for the greater prevalence of MA than of MI in AIDS is that AIDS patients are more susceptible to MA than to MI. When they cause infection, both MA (3) and MI (15) are obligate intracellular parasites which multiply in macrophages. We therefore tested this alternative explanation by investigating the relative susceptibility of macrophages from normal subjects and from patients with AIDS and AIDS-related complex (ARC) to experimental infection in vivo with MA and MI. The results reported here indicate that HIV-infected patients develop a selective susceptibility to MA but not to MI as their virus-caused disease progresses through ARC to AIDS.

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MATERIALS AND METHODS

Bacteria and infections. The materials and procedures used have been described in detail previously (4, 6). The bacteria used were MA serovar 4 (strain 7497) and MA serovars 16 and 17. The MI strains, originally isolated from patients infected with them, were provided and serotyped by Anna Tsang of the National Jewish Center for Immunology and Respiratory Medicine. Serovar 4 was confirmed to be MA and serovars 16 and 17 were confirmed to be MI by Gen-Probe analyses of DNA homology (10, 12, 14, 16). Serovar 4 was 55.7% homologous with reference MA and 2.9% homologous with reference MI, while homology percentages for serovars 16 and 17 were 1.6 and 1.5 for MA and 47.0 and 47.9 for MI, respectively.

Subcultures of smooth transparent colonies of these bacteria on 7H10 agar were made in 7H9 broth in such a manner as to obtain suspensions of the virulent morphotype as previously described (4, 7). These subcultures were frozen in one-use aliquots for storage at −70°C. The macrophages were infected with the thawed and diluted suspensions, as described previously, to obtain infection of approximately 10% of the macrophages with one to two bacteria each (4).

Macrophage isolation and culture. Macrophages were isolated and cultured as previously described (3, 4, 7) and as follows. Briefly, the adherent blood monocytes from normal subjects or from patients were incubated in 35-mm-diameter plastic petri dishes for 7 days to allow them to mature to macrophages. The macrophages were incubated in medium

TABLE 1. Demographic and clinical characteristics of patients with HIV infection

Patient	Age (yr)	Race	HIV transmission category	Concomitant illness	CDC class	$CD4^+$ cell count[a] ($CD4^+$ cells/mm^3)
1	39	White	Homosexual	Oral candidiasis Diarrhea Peripheral neuropathy	IV A	137
2	39	Asian	Homosexual	PCP[b]	IV C-1	70
3	35	White	Homosexual	PCP	IV C-1	9
4	29	Hispanic	Homosexual	Fatigue	IV	149
5	59	White	Homosexual	Oral candidiasis Generalized lymphadenopathy	IV A	198
6	36	Black	Homosexual	PCP	IV C-1	6
7	44	White	Homosexual	Oral candidiasis Ophthalmic zoster Seborrheic dermatitis	IV C-1	64
8	30	White	Homosexual	PCP	IV C-1	11
9	33	White	Homosexual	CMV retinitis Kaposi's sarcoma	IV C-1	35
10	44	White	i.v. drug user	PCP	IV C-1	18
11	42	White	Homosexual	PCP Kaposi's sarcoma	IV D	143
12	33	White	Homosexual	PCP	IV C-1	7

[a] Counted within a mean of 4 months (range, 0 to 17) of venipuncture for serum and macrophages.
[b] PCP, *Pneumocystis carinii* pneumonia.

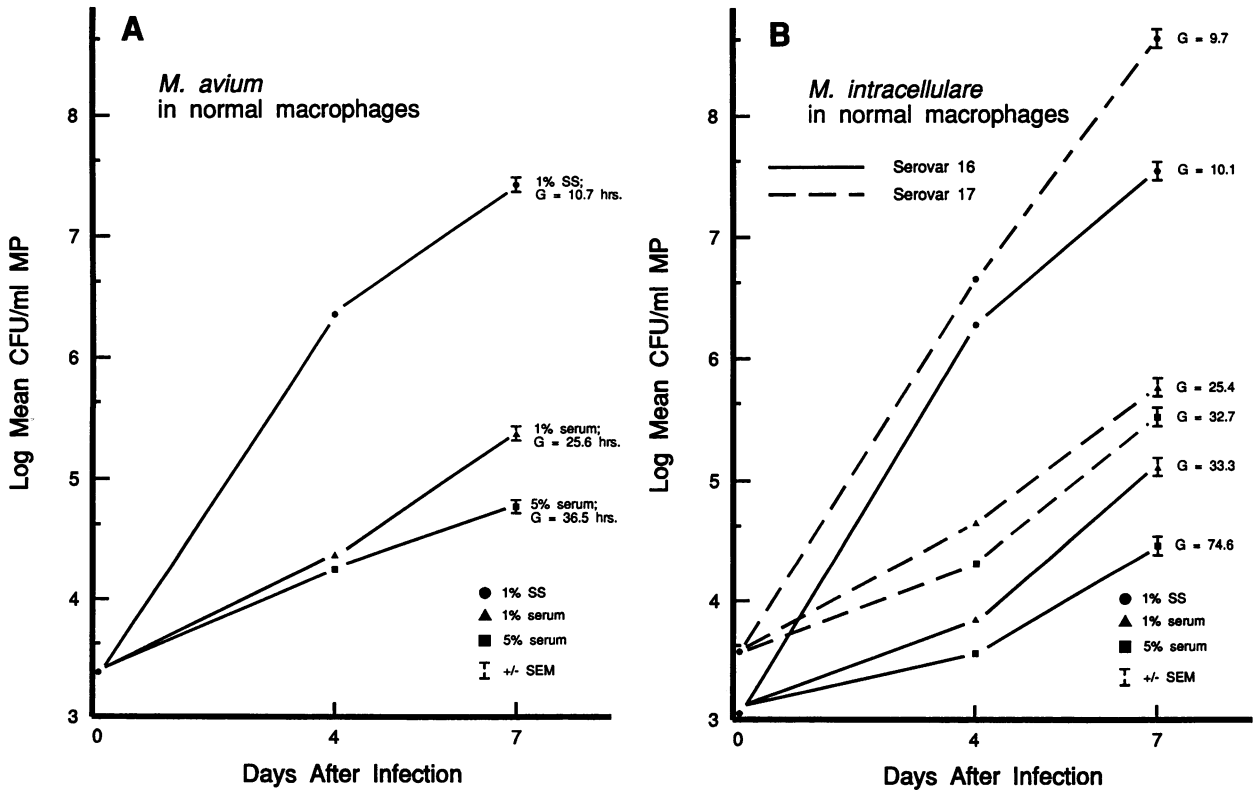

FIG. 1. Growth kinetics of MA (A) and MI (B) in normal human macrophages cultured in medium supplemented with 1 or 5% normal human serum or with 1% SS. The native growth rates of the bacteria are seen in the macrophages incubated in SS, because SS lacks natural inhibitors of their growth in macrophages which are found in serum (6). G, mean generation time (hours; calculated by using 0-, 4-, and 7-day values). Each datum point is the mean of five values. SEMs for the datum points, shown graphically for 7-day values, were less than 10% of the means for all datum points.

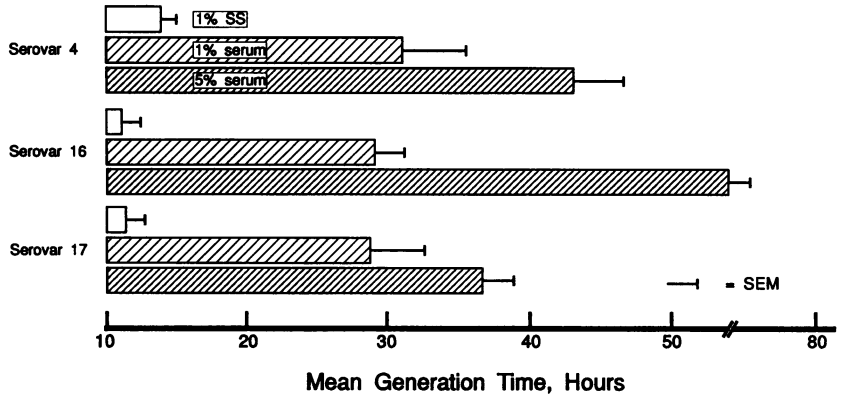

FIG. 2. Mean generation times of MA (serovar 4) and MI (serovars 16 and 17) from results of four separate experiments using macrophages of different normal donors cultured in medium supplemented with 1% SS or 1 or 5% normal AB-type human serum.

supplemented with 1% standardized normal serum (blood type AB serum) until the time that they were infected. After infection, they were incubated in culture medium supplemented with 1 or 5% AB normal serum, 1 or 5% autologous serum, or 1% serum substitute (SS). The composition and effects of SS on MA growth in cultured human macrophages have been described previously (7).

The numbers, morphologic appearance, and relative health of macrophages from the patients were determined by inverted phase microscopy during experiments. Cultures prepared with patient macrophages sometimes consisted of significantly fewer macrophages than did those prepared from normal subjects. However, since rates of bacterial replication were being determined by CFU for each experiment, with internal comparisons derived from succeeding samples over a 7-day culture period, this variable did not affect the results of the experiments.

The macrophage culture medium was RPMI 1640 (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 2 mM L-glutamine and unheated human serum or SS as described above. It contained no antimicrobial drugs. The cell cultures were incubated at 37°C in 7% CO_2 in air. Evidence has been presented (4, 6, 7) that under these experimental conditions, CFU counts (see below) represent principally (by more than 99% [7]) intracellular numbers of the bacteria.

CFU counts. CFU counts were made as previously described (3, 4) from duplicate samples of infected macrophage cultures taken at 0, 4, and 7 days after infection. CFU counts were plotted semilogarithmically for all experiments. Some of the results are presented as intracellular microbial growth in $\log_{10}$ units of the mean CFU per milliliter of lysed macrophages (approximately 10^5) plotted against time (in days) after infection. Most are presented at mean generation times (hours) of the bacteria. In all instances, mean generation times were calculated from CFU values obtained at 0, 4, and 7 days. The values are shown with their standard errors of means (SEMs). The statistical significance of differences between means of generation times, where mentioned, was determined by the *t* test.

Human subjects and patients. Macrophages from four normal subjects (two male and two female) were used. Three of the subjects were white; the fourth was black. This difference is specified where it is significant, because macrophages from black subjects have been reported to be more permissive for mycobacteria than are macrophages from white subjects (5).

Macrophages and serum from 12 HIV-infected patients were studied. The demographic and clinical characteristics of these patients are shown in Table 1. All of the patients were males. None of them had active opportunistic infections, and none were known to be infected with MAI or were receiving antimycobacterial drugs at the time that their blood samples were taken. One of the patients (patient 12) had been diagnosed with disseminated MAI infection 7 months

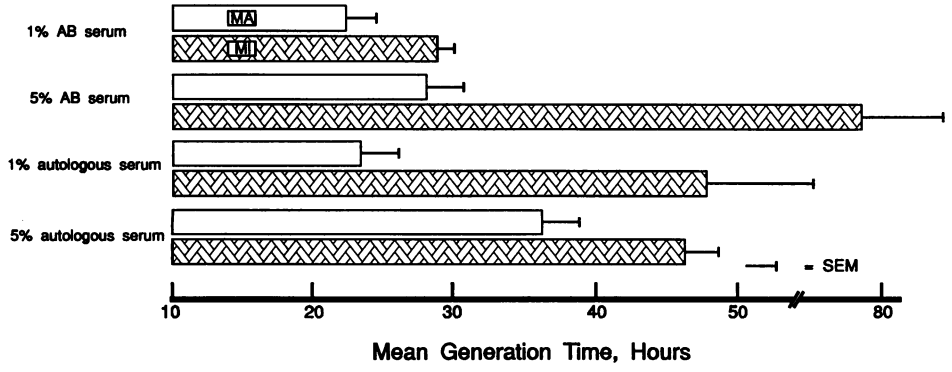

FIG. 3. Mean generation times of MA and MI (serovar 16) in macrophages from the six AIDS patients (six experiments) studied in this investigation. The macrophages were incubated in medium supplemented with 1 or 5% normal AB serum or 1 or 5% autologous (patient) serum.

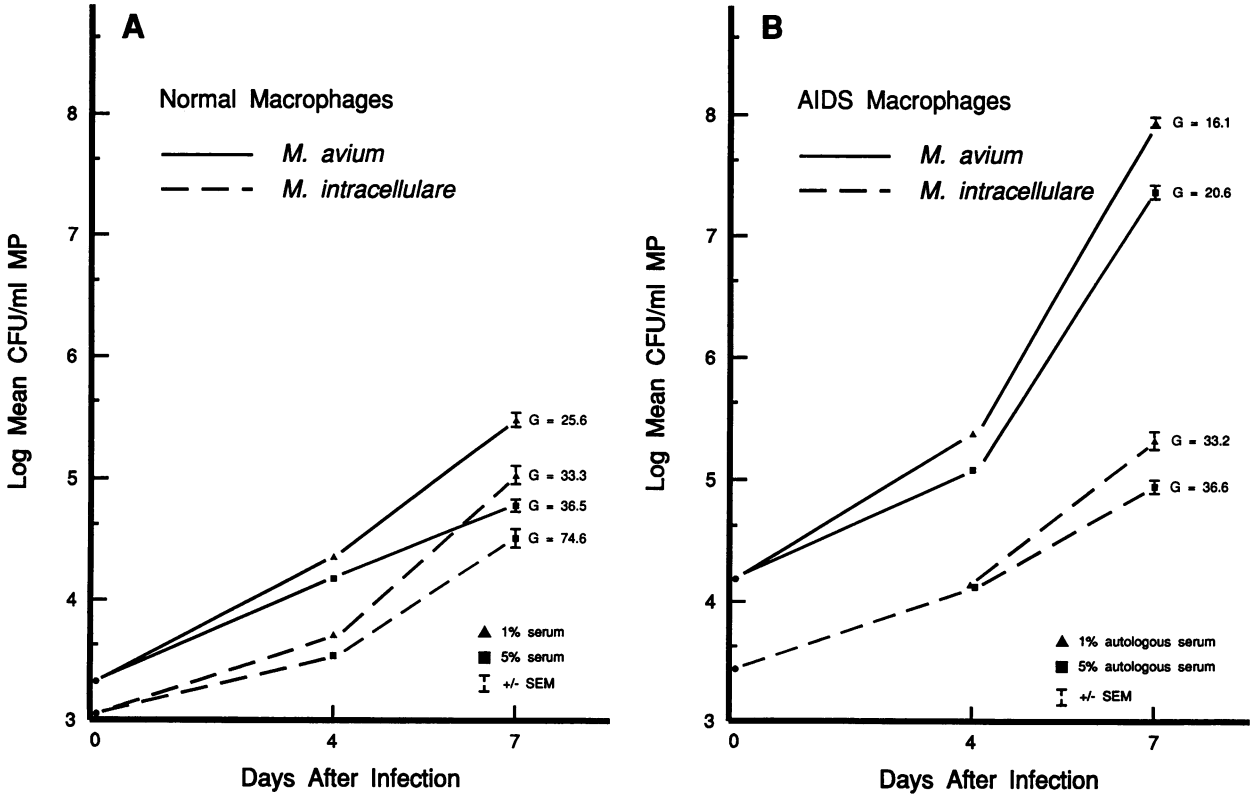

FIG. 4. Contrast of growth rates and kinetics of MA and MI in macrophages from a normal donor (A) and an AIDS patient (B) with macrophages incubated in autologous serum. In these two experiments, the macrophages (MP) were infected with both MA and MI in each experiment, so that their growth could be compared under identical conditions. Each datum point is the mean of five values. SEMs for the datum points, shown graphically for 7-day values, were less than 10% of the means for all datum points. G, generation time in hours.

after the time of venipuncture. The sera of all of these patients were studied. The macrophages from patients 1 to 4, 6, 7, 9, 10, and 12 were successfully studied. Contamination of cultures prevented successful study of the macrophages from patients 5, 8, and 11.

Informed consent was obtained from all donors.

RESULTS

Ability of MA and MI to grow in normal macrophages. The two serovars of MI were compared with the standard serovar 4 MA, regularly used in this laboratory (3, 6), for ability to multiply in macrophages from normal donors in five different experiments. Results from these experiments all agreed in showing that MI infected and multiplied in normal macrophages similarly to MA. The comparative growth kinetics for MA and the two serovars of MI are illustrated by growth curves plotted from one of the experiments in Fig. 1. Figure 2 presents a bar graph summary of results obtained from four of the five experiments. Results from the fifth experiment are not included because it used different batches of the bacteria than did the other four experiments.

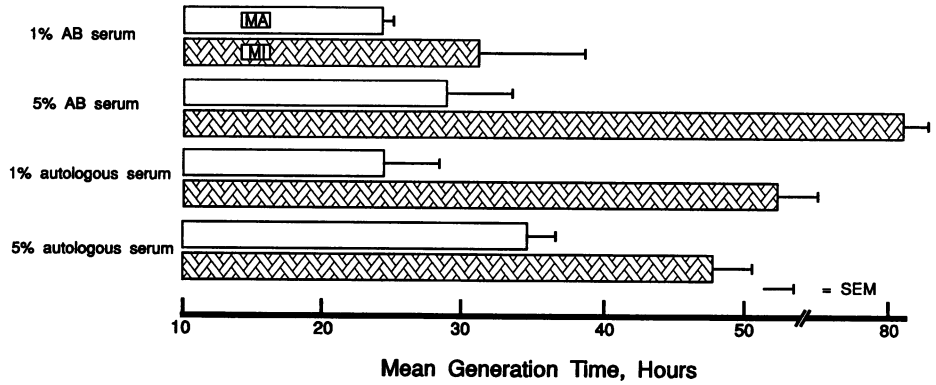

FIG. 5. Mean generation times of MA and MI in macrophages from the five AIDS patients with low (here defined from experimental findings as $<36/mm^3$) counts of $CD4^+$ lymphocytes. Means and SEMs are from values from five separate experiments.

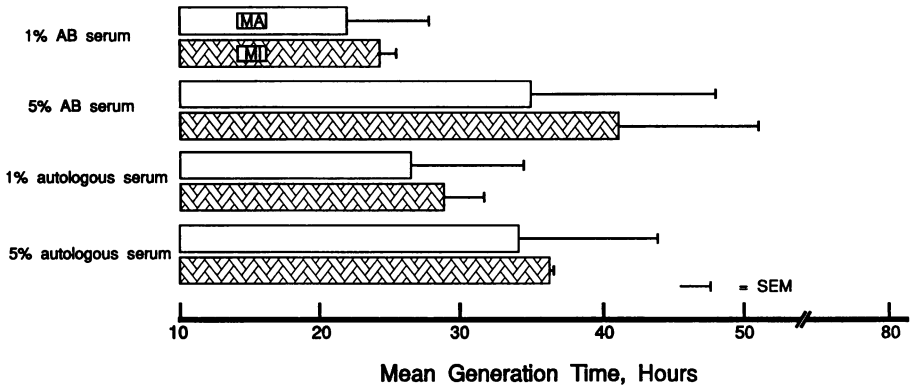

FIG. 6. Mean generation times of MA and MI in macrophages from patients 1 (ARC), 2 (AIDS), 4 (pre-ARC), and 7 (ARC), with moderately depressed (here defined from experimental findings as >63 and <200/mm^3) counts of CD4$^+$ lymphocytes. Means and SEMs are from values of four separate experiments.

Faster replication of MA than of MI in patient macrophages. The comparative rates of growth of MA and MI were successfully measured in six of the seven patients who had AIDS (results from the experiment with cells from patient 11 were lost by bacterial contamination). The results, summarized in Fig. 3 (cf. Fig. 2), showed that MA replicated significantly ($P < 0.01$) more rapidly than did MI in AIDS macrophages cultured in either 5% normal AB serum or 1% autologous patient serum. The rates of replication for MA in these macrophages in 1% normal or 5% autologous serum and for MI in them in either concentration of normal or autologous serum were normal.

The kinetics of the more rapid rate of MA than of MI growth in AIDS patient macrophages suggested by the results in Fig. 3 are illustrated by direct comparison of the bacterial replication in macrophages from a normal donor and cells from patient 1 shown in Fig. 4.

AIDS stage dependency of selectively accelerated MA replication in patient macrophages. MA replicated significantly faster than did MI in AIDS patient macrophages in only two of the four conditions shown in Fig. 3. This result was found to be due to averaging of results from all of the AIDS patients. Thus, when the results were analyzed with respect to the immunologic stage of AIDS in each patient as reflected by CD4$^+$ cell counts, MA was found to replicate abnormally rapidly in all experimental conditions. This finding is shown by plotting the results of these experiments for patients with CD4$^+$ cell counts below 36/ml in Fig. 5 compared with higher CD4$^+$ cells counts in Fig. 6. It can be seen that MA replicated significantly faster than did MI under all experimental conditions in macrophages from the patients with low CD4$^+$ counts (Fig. 5) ($P < 0.001$ for 5% AB serum and 1 and 5% autologous serum and $P < 0.01$ for 1% AB serum). By contrast, in macrophages from the four patients with higher CD4$^+$ lymphocyte counts, three of them clinically classified as ARC patients, there was only a tendency for MA to have replicated faster than MI (Fig. 6). The differences were not statistically significant, and the SEMs were very large. This finding, again, was determined to be due to individual patient characteristics relating to the clinical stage of AIDS and specifically, in these results, to the characteristics of macrophages from patient 4, who had the highest CD4$^+$ count of all patients whose cells were successfully studied. This conclusion is evident from a regraphing of the results by using data from patients 1, 2, and 7 (Fig. 7). Differences between MA and MI replication were of the same order in the macrophages of these patients as for the patients with the low CD4$^+$ counts (Fig. 5). The results in Fig. 5 and 7 therefore show that for 9 of 10 of the patients whose macrophages were studied, MA replicated abnormally rapidly while MI did not, and that the one patient in whose macrophages MA and MI grew at normal rates, and not significantly differently, was distinguished by having only a modestly depressed CD4$^+$ count and in presenting clinically with only the earliest vague symptoms of AIDS-related disease.

Influence of serum on MA and MI growth in normal macrophages. Sera from all of the patients were compared with normal serum for effects on the growth of MA and MI in normal macrophages in two pairs of experiments. One pair used macrophages from a normal black donor, and the other used macrophages from a normal white donor. In one experiment of each pair, the macrophages were infected with MA; in the other, they were infected with MI. The results (Fig. 8) show that black donor macrophages are more permissive than white donor macrophages for both MA and MI. They also show that there was significantly ($P < 0.01$) enhanced growth of MA but not of MI in the normal macrophages cultured in patient sera. The patient sera did not enhance or inhibit growth of MA in RPMI 1640 culture medium in the absence of macrophages (results not shown).

DISCUSSION

This study was prompted by observations that disseminated infections with MA are much more frequent than those with MI in AIDS patients (1, 10), whereas MA and MI infections are equally frequent in non-HIV-infected patients (1, 2, 10). There are two possible explanations. One is the speculation that AIDS patients are selectively infected with MA because they are more likely to be exposed to it from the environment (8, 9, 11), but there is no direct evidence to support this view. The second is that AIDS patients are more susceptible to infection with MA than with MI. This view is supported by the data presented here. MA and MI were found to infect and grow equally in cultured normal human macrophages (Fig. 1 and 2), while MA but not MI was found to grow abnormally rapidly in macrophages from AIDS and ARC patients (Fig. 3 to 5 and 7). This difference was seen for macrophages of 9 of the 10 patients successfully studied. The only exception was for cells from patient 4, who had the

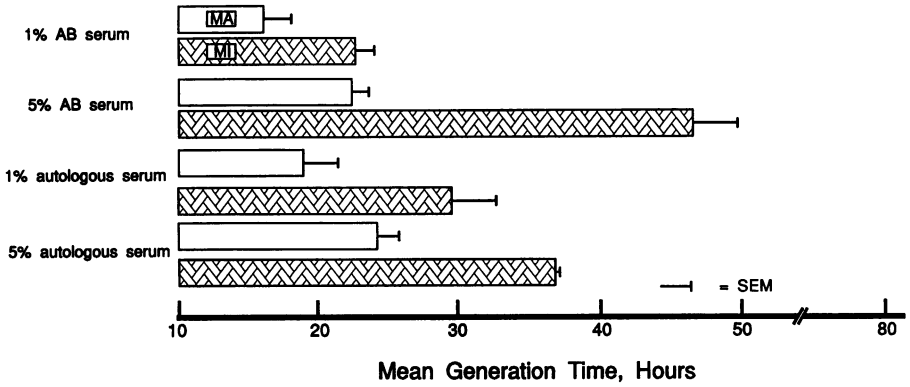

FIG. 7. Mean generation times of MA and MI in macrophages from patients 1, 2, and 7. Means and SEMs are from values from three separate experiments.

highest $CD4^+$ lymphocyte count of all patients whose cells were tested and whose only concomitant illness was fatigue (Table 1).

The selective susceptibility to MA in these AIDS and ARC patients was due to abnormalities in both their macrophages and their sera. Specifically, the patient macrophages (except, as mentioned above, those from patient 4) were abnormally and selectively susceptible to MA when cultured in medium supplemented with 5% normal serum, even though normal serum contains a substance which will strongly inhibit the growth of MA in normal macrophages (6). In contrast, the macrophages from these patients retained the ability to inhibit MI in the presence of either normal or patient serum. Reasons for the greater susceptibility of AIDS patient macrophages to MA than to MI are unknown. There are multiple immunologic abnormalities in AIDS patients which can affect macrophages, ranging from altered hematopoiesis to abnormal production of macrophage-altering cytokines. Which one or more of these factors explain the increased susceptibility of AIDS patient macrophages to MA will be learned only by further experimental analyses.

The contribution of patient serum to the selective susceptibility to MA was seen by the abilities of the patient sera to increase the permissiveness of normal macrophages to MA but not to MI (Fig. 8). These results confirm earlier observations (4) that sera from AIDS and ARC patients either lack a natural inhibitor of MA growth in human macrophages or contain a promoter of it, or both. Work in progress favors both: a deficiency in iron chelation by the serum along with excessive loads of iron, together with abnormally high levels of certain fatty acid precursors. These conditions appear to greatly accelerate replication of MA in cultured human macrophages. Preliminary work suggests that they do not equally well promote such growth of MI and that, in fact, AIDS patient serum may contain an inhibitor of MI intracellular growth.

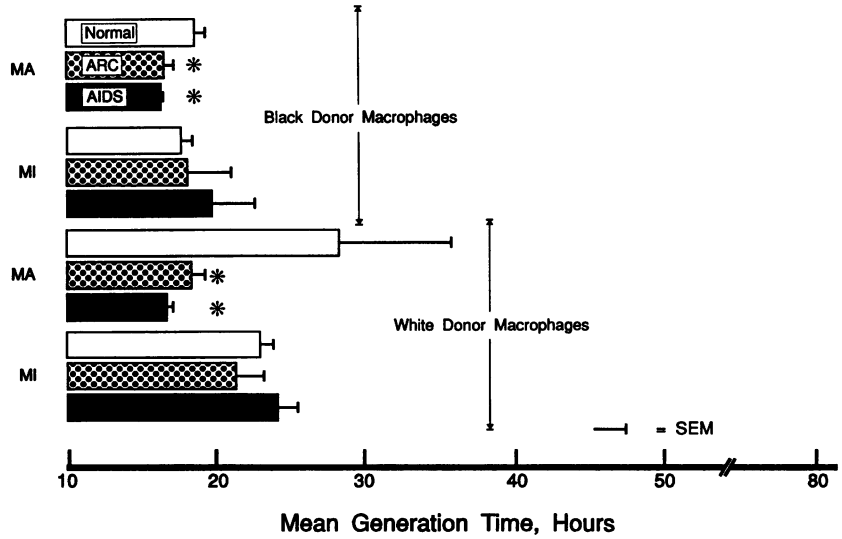

FIG. 8. Results from two pairs of experiments (one with MA infection and one with MI infection in each pair) comparing abilities of ARC and AIDS patient sera to promote growth of MA or MI in normal macrophages. One pair used macrophages from a black donor, and the other used macrophages from a white donor. Asterisks indicate statistically significant ($P < 0.01$, calculated by Student's t test) differences of means for patient sera from normal serum controls. Means and SEMs are for five ARC and six AIDS patient sera and for two normal control sera used in each experiment. Growth promotion of MA was greater in white donor macrophages than in black donor macrophages, because the black donor macrophages are inherently more permissive for MA (unpublished results, and as shown).

The data presented here suggest that the growth of MA and MI in cultured human macrophages is differently regulated by both macrophages and components of human serum and that in HIV-infected persons progressing through ARC to AIDS, concomitant with low (defined in this study by experimental results as $<63/mm^3$) $CD4^+$ lymphocyte counts, both cellular and humoral mechanisms for native resistance to MA are lost while those for MI are retained. This differential shift in microbial susceptibility may explain the much greater incidence of disseminated MA infection than of MI infection in AIDS patients. This possibility should be further investigated by broadening the comparative analyses to a larger variety of MI and MA serotypes and especially to investigating the biochemical reasons for the faster replication of MA than of MI in AIDS patient macrophages and serum.

ACKNOWLEDGMENT

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