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Macrophage-Activating Factors Produced by Murine Leukemia X Fibroblast Hybrid Cells Stimulates Resistance to *Mycobacterium avium* Complex

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A murine leukemia x LM fibroblast hybrid cell line with immune augmenting properties stimulated resistance to *Mycobacterium avium* complex (MAC) in mouse peritoneal macrophages, and in immune deficient beige mice (C57BL/6/bgj/bgj). The proliferation of MAC in mouse peritoneal macrophages was inhibited by medium conditioned by the growth of the hybrid cells (hybrid cell-CM). Under similar circumstances, media conditioned by the growth of LM cells (LM cell-CM), a mouse fibroblast cell line used as one parent in forming the hybrid cell, was exhibited no inhibitory effect. Treatment of mouse peritoneal macrophages with hybrid cell-CM, but not with LM cell-CM, stimulated the expression of each of four previously described macrophage activation antigens, suggesting that the hybrid cells formed immunomodulators in addition to those formed by LM cells. Furthermore, the morphology of the macrophages following treatment with hybrid cell-CM was clearly distinguishable from that following exposure of the cells to LM cell-CM. The therapeutic effects of hybrid cells on the progression of MAC-infection were indicated by the prolonged survival of MAC-infected immune-deficient beige mice. One hundred percent of treated animals survived more than 60 days, while untreated animals died in approximately 22 days.

Key words : Opportunistic infection, AIDS, *Mycobacterium avium*, Macrophage

INTRODUCTION

Organisms of the *Mycobacterium avium* complex (MAC) infect and replicate in the cytoplasm of mononuclear phagocytes. Although infection with MAC is unusual in immunocompetent hosts, the bacteria are a cause of disseminated disease in immunosuppressed patients, including those with the acquired immunodeficiency syndrome (AIDS). Approximately 25% of immunodeficient patients dying from AIDS have evidence of wide-spread infection with this opportunistic microorganism (Young *et al.*, 1986; Pierce *et al.*, 1996). Treatment of MAC-infection with existing chemotherapeutic drugs is usually ineffective because many of the more virulent subtypes are resistant to antibiotic therapy at tolerable doses (Baron & Young, 1986). In contrast, some forms of biological therapy may have a beneficial role in the treatment of MAC-infection in immunodeficient hosts (Yang *et al.*, 1994; Appelberg, 1995; Bermudez *et al.*, 1995).

Immunomodulators are among the newer experimental approaches used to stimulate resistance to infection with MAC. Bermudez and Young (1988) reported that incubation of human peripheral blood monocytes in medium containing recombinant human tumor necrosis factor (TNF) alone or in combination with interleukin-2 (IL-2), could activate the monocytes to inhibit intracellular growth or kill MAC. They obtained similar results by treating human monocytes with recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) (Bermudez and Young, 1990). In contrast, the proliferation of MAC in cells treated with interferon-gamma (IFN-γ) was not suppressed (Douvas *et al.*, 1985). Thus, certain forms of biological therapy may have a beneficial role in the treatment of MAC infection in immunodeficient hosts.

Previously, we found that immunocompetent mice receiving an injection of ASL-1 x LM cells, a murine leukemia x fibroblast hybrid cell line, exhibited augmented immune responses (Cohen and Hagen, 1985). The number and proportion of cells forming antibodies to sheep red blood cells were increased following immunization if the animals were injected with hybrid cells or hybrid cell-CM. The hybrid cells, like LM cells, formed colony stimulating factor-1 (CSF-1;

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M-CSF) constitutively (Chow *et al.*, 1987), and possibly other immunomodulators may have stimulated host immunity.

Here, we report the effect of medium conditioned by the growth of the hybrid cell line on the proliferation of MAC in T cell-depleted cultures of mouse peritoneal macrophages, and on the survival of immune-deficient mice infected with MAC. Both hybrid cells and LM cells secreted IFN-γ and CSF-1. Unlike LM cell-CM, however, hybrid cell-CM stimulated microbicidal pathways in mouse peritoneal macrophages that inhibited the proliferation of MAC, suggesting that the cells formed additional, as yet undefined, macrophage-activating immunomodulators.

MATERIALS AND METHODS

Reagents

Amikacin was obtained from Bristol Laboratories (Evansville, IN). Fluoroscein isothiocyanate (FITC)-conjugated anti-rat IgG sera were obtained by Sigma Chemical Company (St Louis, MO). Middlebrook 7H 10 agar, Bacto Middlebrook OADC enrichment solution and Middlebrook 7H9 broth were purchased from Difco Laboratories (Detroit, MI).

Experimental animals

BALB/c mice, 8-12 weeks old, were obtained from Charles River Breeding Laboratories (Wilmington, MA). C57BL/6/bgj/bg$^+$ and immune-deficient C57BL/6/bgj/bgj (beige) mice were obtained from Jackson Laboratories (Bar Harbor, ME).

Mycobacterium avium complex (MAC)

MAC organisms were kindly provided by Dr. Gangadharam (University of Illinois, Chicago). The virulent strain 101 was used throughout the study. Single cell suspensions from transparent colonies were obtained, as described previously (Bertram *et al.*, 1986), and stored in 1 ml aliquots at -80°C. For each infection, aliquots from frozen stock were thawed quickly and diluted with 7H9 medium, vortexed and sonicated for 30 sec, and then diluted with 7H9 medium to a concentration of approximately 5×10^6 microorganisms/ml. The number of microorganisms was confirmed by colony counting techniques described below.

Infection of peritoneal macrophages with MAC

Macrophages were obtained by lavage from the peritoneal cavities of BALB/c mice immediately after an intraperitoneal (i.p.) injection of 5 ml Dulbecco Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (Grand Island Biologicals, Grand Island, NY) (growth medium). The number of cells in a pooled cell suspension from at least three mice was determined and 1×10^6 cells in 1.0 ml of growth medium were added to individual wells of 24 well plastic cell culture plates (Falcon Plastics, Oxnard, CA). The plates were incubated for 2 hr at 37°C in 7% CO_2/humidified air mixture after which the nonadherent cells were removed. Afterwards, the number of macrophages was determined as described previously (Nakawara & Nathan, 1983). More than 95 percent of the adherent macrophages were viable at the beginning of each experiment. For infection, 5×10^6 MAC in 1.0 ml of growth medium were added to the macrophage monolayers, approximately 5×10^5 cells/well. About 1~2% of infected, or noninfected macrophages were detached from the plates every two days.

Formation and maintenance of ASL-1 x LM fibroblast hybrid cells

ASL-1 cells, a murine leukemia cell line (originally obtained from Dr. Boyse, Memorial Sloan-Kettering, New York, NY), were used as one parent in forming the hybrid cells. They were maintained by serial passage in histocompatible strain A/J mice (H-2^a). A thymidine kinase-deficient subline of LM mouse fibroblasts (LM cells) (C3H/He mouse origin) (H-2^k) (American Type Culture Collection, Rockville, MD) was used as the other parental cell type. These cells were maintained in growth medium under standard cell culture conditions (37°C, 7% CO_2/humidified air mixture). The hybrid cells were prepared, as described previously (Kim *et al.*, 1979). Briefly, approximately 5×10^6 ASL-1 cells from a terminally-ill mouse were mixed with an equivalent number of LM cells from *in vitro* culture in medium containing PEG 6000, used as a fusogen. Selection of fused cells was accomplished in medium containing 100 μM hypoxanthine, 0.4 μM aminopterin, and 16 mM thymidine (HAT). None of the nonfused parental cells (in control cultures) survived *in vitro* in HAT medium. The hybrid cells shared the histocompatibility antigens of both parental cell-types and possessed a hybrid chromosomal number (Garber *et al.*, 1984). The cells were maintained in growth medium under standard cell culture conditions as a cell-line. Their mean generation time was approximately 24 hr and the deliberate addition of growth factors was not required.

Preparation of cell-free conditioned medium from ASL-1 x LM hybrid cells or LM cells

Hybrid cell-CM or LM cell-CM was obtained from 1×10^7 cells of either cell type that had been maintained in 10 ml of growth medium in a 100 mm cell culture dish for 24 hr under standard cell culture conditions, as described previously (Chow *et al.*, 1987).

After incubation, the CMs were decanted from the adherent cells, centrifuged and passed through 0.22 μ nitrocellulose filters. They were stored in aliquots at -80°C before use. Care was taken to ensure that the number of cells and the growth period to generate the CMs were the same for either cell line.

Determination of the number of microorganism from that mouse peritoneal macrophages infected with MAC

The number of microorganism from treated and untreated MAC-infected macrophages was determined, as described previously (Bermudez *et al.*, 1995). Briefly, wells containing infected cells were washed and the infected cells were lysed with ice-chilled sterile distilled water. Afterwards, a lysing solution consisting of 1.1 ml of 7H9 medium and 0.4 ml of 0.25% sodium dodecyl sulfate (SDS) solution in phosphate buffer (pH 7.2) was added to each well for 10 min. The wells were vigorously scraped with a rubber policeman and the cell lysate was resuspended in 20% bovine serum albumin in sterile distilled water. The suspension was vortexed for 2 min and sonicated for 30 sec (power output: 2.5 watt/sec) before serial dilutions were plated on 7H10 agar. As a control for osmotic stability, a suspension of a previously-determined number of organisms (without cells) was subjected to the same procedure before plating. Confirmation of the lysis of all the macrophages was made by microscopic examination after Giemsa staining. After inoculation, the plates were allowed to dry at room temperature for 15 min and then incubated at 37°C in a 7% CO_2/humidified air mixture for 2 weeks. The results are reported as the mean colony forming units (CFUs) per ml of macrophage lysate. Each determination was performed at least in triplicate.

Assay of immunomodulators

Assays for IL-1, IL-2, IL-3, IL-4, and interferon-γ were performed as described previously using the following cytokine-dependent cell lines: D10S cells (IL-1) (Orincote and Dinarello, 1989), CTLL-2 cells (IL-2) (Baker *et al.*, 1979), DA-1 cells (IL-3) (Ihle *et al.*, 1982), CT4S cells (IL-4) (Hu-Li *et al.*, 1989), and WEHI-279 cells (IFN-γ) (Reynolds *et al.*, 1987). In brief, approximately 2×10^4 cytokine-dependent cells were cultured under varying conditions for 3 days in 0.2 ml flat bottom microtiter wells after which 1 μCi of ^{3}H-thymidine was added and incubated for 6 additional hr. Afterwards, the cells were harvested and the radioactivity was determined in a liquid scintillation counter.

Assays for TNF and GM-CSF were carried out by ELISA kits (Genzyme, USA), following the instruction of the supplier.

Antibodies and cytofluorometric assay

Antibodies specific for murine CSF-1 and murine IFN-γ were kindly provided by Dr. Stanley (Albert Einstein College of Medicine, Bronx, NY). Mouse ascites samples containing monoclonal antibodies (mAbs) against each of four macrophage activation antigens (TM-1, TM-2, TM-3 and TM-4) (Paulnock and Lambert, 1990) were kindly supplied by Dr. Paulnock (University of Wisconsin, Madison, WI). Assays for the presence of each of the activation antigens were performed by indirect immunofluorescent staining. Briefly, single cell suspensions from macrophages that had been incubated with hybrid cell-CM (1:1) or LM cell-CM (1:1) were washed with PBS, and then incubated with the mAb-containing hybridoma supernatant fluids for 30 min. After incubation, the cells were washed with PBS containing 0.02% NaN_3 and 0.5% FBS, and then incubated with FITC-conjugated anti-rat IgG for 30 min, followed by analysis in a Coulter EPICS V flow cytofluorograph (Coulter Electronics, Hialeah, FL).

Statistical analyses

Significance of differences between treated *versus* untreated macrophage cultures at identical timepoints was determined by Student's *t* test. A *P* value of <0.05 was considered significant.

RESULTS

Effect of hybrid cell-CM on the progression of MAC-infection in mouse peritoneal macrophages

The effect of hybrid cell-CM on the progression of MAC-infection in T cell-depleted cultures of peritoneal macrophages was determined by treating the cells with varying concentrations of the CM for 24 hr, followed by infecting the cells with predetermined numbers of organisms. As controls, an equivalent number of cells was maintained in growth medium, or medium containing equivalent concentrations of LM cell-CM for the same period, before they were infected.

As indicated (Fig. 1A), over a seven day period, the number of organisms present in cultures treated with hybrid cell-CM (at 1:1 ratio of CM to growth medium) was significantly ($P<0.001$) less than that found in control cultures incubated in growth medium alone. In contrast, the number of organisms in cultures treated according to the same schedule was not affected by LM cell-CM (Fig. 1B). The number was about the same as in the control cultures, at each concentration tested.

Since the above results could be due to nonspecific toxic effects of hybrid cell-CM on the viability of the macrophages, we determined the viability of peritoneal macrophages incubated for up to 7 days in medium containing equivalent concentrations of the CM. Toxicity was not detected and the viability of the cells

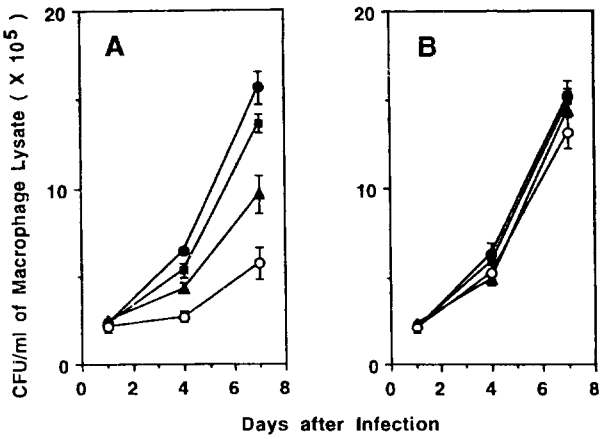

Fig. 1. Effect of hybrid cell-CM and LM cell-CM on the progression of MAC-infection in T cell-depleted cultures of mouse peritoneal macrophages. The CFU per ml of macrophage lysate was determined in cultures containing varying concentrations of hybrid cell-CM (A) or LM-cell-CM (B). The ratios of CM to growth medium were 1:1 (○), 1:2 (▲), and 1:4 (■). As a control, the macrophages were incubated in growth medium alone (●). The numbers are the mean± SD of at least triplicate determinations.

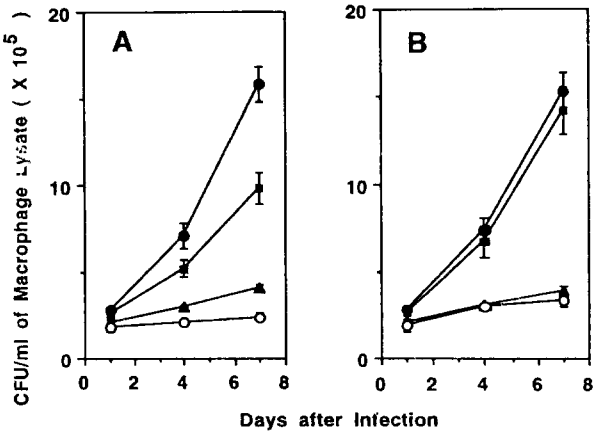

Fig. 2. Combined effect of hybrid cell-CM and amikacin on the proliferation of MAC in mouse peritoneal macrophages. Macrophages were pretreated with hybrid cell-CM (1:2) (A), or with LM cell-CM (1:2) (B) and then infected with MAC. Amikacin (2.5 ug) was added to the infected macrophages. Cell treated with growth medium alone (●), CM (■), amikacin (▲), or with CM and amikacin (○). The numbers are the mean±SD of three different experiments.

was greater than 98%. This was the case for both infected and noninfected cultures treated with hybrid cell-CM. Similarly, hybrid cell-CM did not affect the viability of the microorganisms. In addition, treatment of viable MAC with the lysing buffer had no effect on the microorganisms' viability.

The inhibitory effect of hybrid cell-CM on the proliferation of MAC in infected macrophages could also be explained by a nonspecific inhibition of the uptake of the microorganisms by cells treated with the CM. This possibility was tested by using Kinyoun staining in the presence or absence of hybrid cell-CM. Over a seven day period following the addition of viable MAC, proportion of infected cells was approximately the same in the presence or absence of hybrid cell-CM (data not shown).

To determine if the hybrid cell-CM enhanced the antimycobacterial effects of amikacin, this antibiotic was added to infected mouse peritoneal macrophages treated with hybrid cell-CM, followed by the determination of the number of microorganisms in the infected cells. As shown in Fig. 2, amikacin alone inhibited proliferation of the microorganisms in the infected cells. The addition of hybrid cell-CM led to a small, but significant augmentation of the inhibitory effects of amikacin. After 7 days, the number of organisms present in cells treated with hybrid cell-CM and amikacin was significantly less than that found in cell cultures treated with hybrid cell-CM ($P<0.01$) or amikacin alone ($P<0.05$). LM cell-CM exhibited no effect. The number of bacteria present in cultures treated with amikacin and LM cell-CM, although clearly less than that present in untreated cultures, was not significantly different from that of cultures treated with amikacin alone.

Expression of macrophage activation antigens by mouse peritoneal macrophages treated with hybrid cell-CM or LM cell-CM

Activated macrophages express unique membrane-associated antigens (Paulnock & Lambert, 1990). The antigens, designated TM-1, TM-2, TM-3 and TM-4, were first detected in RAW 264.7 cells, a murine macrophage cell line, during the activation process. Immunofluorescent staining with macrophage activation antigen-specific monoclonal antibodies was used to determine if such activation determinants were expressed by macrophages exposed to hybrid cell-CM or LM cell-CM. The results indicate that mouse peritoneal macrophages exposed to hybrid cell-CM formed each of the four activation antigens. Under similar conditions, however, macrophages exposed to LM cell-CM expressed only three of the four antigens (Fig. 3). Cells stained positively with antibodies for TM-3 were not detected among cells treated with LM cell-CM. Thus, hybrid cell-CM, but not LM cell-CM, stimulated the formation of each of four previously described antigens expressed by activated macrophages.

Morphologic changes in mouse peritoneal macrophages exposed to hybrid cell-CM

During the course of these investigations, we noted morphologic differences between peritoneal macrophages incubated in medium containing hybrid cell-CM and LM cell-CM (Fig. 4). Cells incubated in medium containing hybrid cell-CM were larger and possess-

ed a "ruffled" border, characteristic of activated macrophages. Cells incubated under similar conditions in medium containing LM cell-CM were elongated and fibroblastic. Macrophages incubated in growth medium alone were clearly smaller, rounded and the ruffled border was not present.

Formation of previously described immunomodulators by ASL-1 X LM hybrid cells

Could the results be explained by concentration differences between analogous immunomodulators formed by hybrid cells or LM cells? This point was determined by investigating medium conditioned by the two cell-types for the presence of each of several previously described immunomodulators that might be involved in stimulating resistance to MAC-infection. As shown in Table IA, tests for the presence of IL-1, 2, 3, 4, GM-CSF and TNF were negative in medium conditioned by either cell types. Both CSF-1 and IFN-γ were present in medium conditioned by both hybrid and LM cells. The titers of CSF-1 or IFN-γ in hybrid cell-CM and LM cell-CM were approximately the

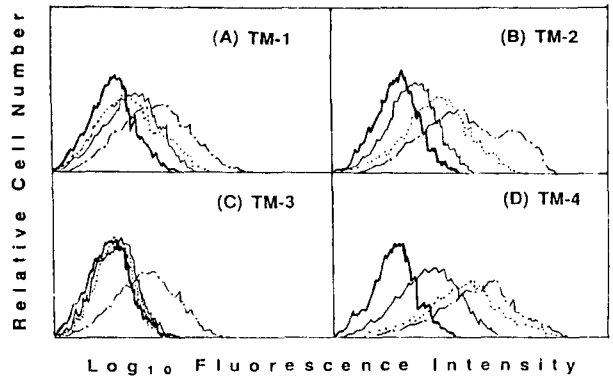

Fig. 3. Flow cytometric analysis of macrophage activation antigens expressed by mouse peritoneal macrophages exposed to hybrid cell-CM. Macrophages were treated with growth medium alone (—), with hybrid cell-CM (1:1) (- - - -), or with LM cell-CM (1:1) (---). After treatment, the cells were incubated with mAbs to each of four macrophage activation antigens (TM-1, TM-2, TM-3, and TM-4), followed by staining with FITC-conjugated rabbit anti-rat IgG. Background levels of staining are represented by the cytofluorometric profile obtained after staining of macrophage monolayers under each condition with FITC-conjugated anti-rat IgG alone (—).

Table I. Effect of immunomodulators on MAC-infection in peritoneal macrophages

A. Formation of immunomodulators

Immunomodulator[a]	Hybrid cell-CM	LM cell-CM
IL-1	-	-
IL-2	-	-
IL-3	-	-
IL-4	-	-
IFN-γ	+	+
TNF	-	-
GM-CSF	-	-
CSF-1	+	+

B. Effect of CSF-1 and IFN-γ on MAC-infection

Treatment[b]	CFU/ml cell lysate[c] ($\times 10^5$)	Relative reduction
Growth medium (GM)	18.4±3.2	1.00
+ anti CSF-1 (1:50)	19.1±1.9	1.03
+ anti IFN-γ (1:25)	18.5±0.5	1.01
+ anti CSF-1+anti IFN-γ	18.5±1.6	1.01
Hybrid cell-CM (1:1)	6.0±1.4	0.33
+ anti CSF-1 (1:50)	11.2±2.0[c]	0.61
+ anti IFN-γ (1:25)	6.8±1.0	0.37
+ anti CSF-1+anti IFN-γ	12.0±1.2	0.65

[a]The assays were performed as described in the Materials and Methods.

[b]Peritoneal macrophages were infected with 5×10^6 MAC and then incubated for 7 days in growth medium alone, or in medium containing hybrid cell-CM (1:1 of CM to growth medium) with or without antibodies.

[c]$P<0.01$, relative to hybrid cell-CM (1:1) and $P<0.01$, relative to growth medium (GM) and CSF-1 antiserum (1:50).

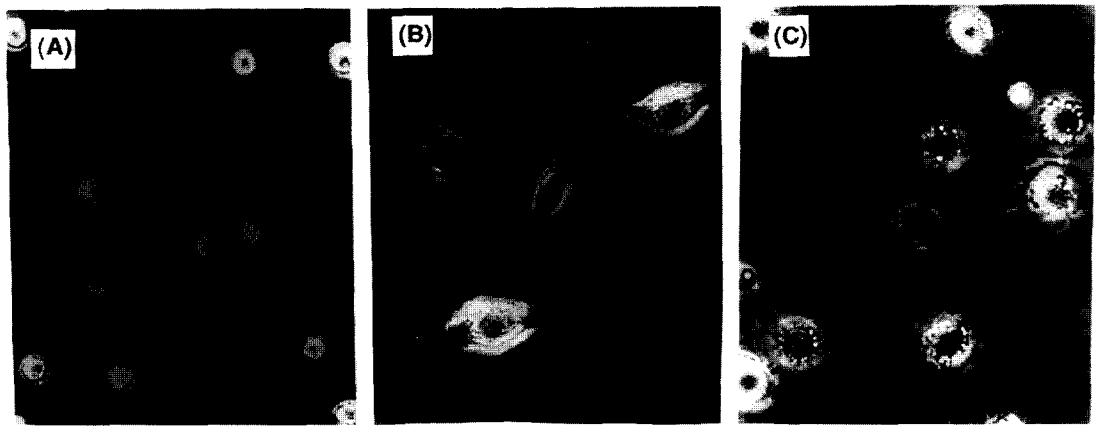

Fig. 4. Morphologic changes in mouse peritoneal macrophages exposed to LM cell-CM or hybrid cell-CM. Peritoneal macrophage monolayers from BALB/c mice were incubated for 5 days in medium containing growth medium alone (A), LM cell-CM (B), or hybrid cell-CM (C). The ratio of CM to growth medium was 1:1. Phase contrast photomicrographs.

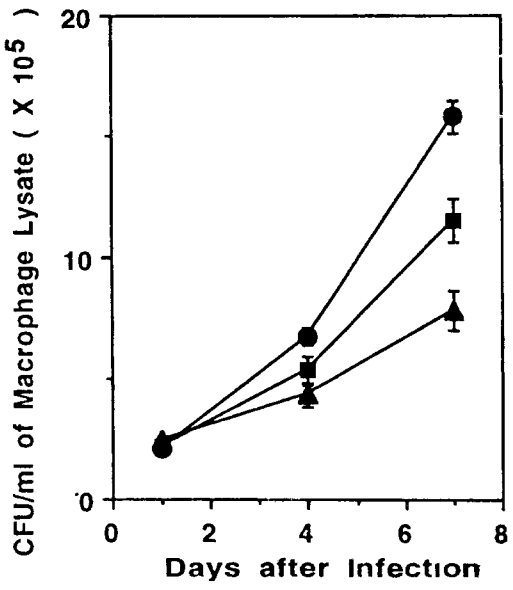

Fig. 5. Treatment of infected mouse peritoneal macrophages with hybrid cell-CM. Peritoneal macrophages were infected with MAC, according to the procedure outlined in the Materials and Method Section. After 24 hr incubation, the cells were treated with varying concentrations of hybrid cell-CM, or with growth medium alone as a control culture (●) . The ratios of CM to growth medium were 1:1 (▲) and 1:2 (■). The numbers are the mean±SD of at least triplicate determinations.

same (data not shown). Whether or not ASL-1 cells form immunomodulators has not been determined because the cells do not grow *in vitro*.

To determine if CSF-1 and/or IFN-γ was responsible for the resistance of stimulating macrophages to MAC-infection, anti CSF-1 and/or anti IFN-γ antibodies were added to hybrid cell-CM before it was added to the cell cultures. As shown in Table IB, the inhibitory effects of hybrid cell-CM were only slightly neutralized by anti-CSF-1 antibodies. Treatment of the CM with higher concentrations of CSF-1 antibodies did not lead to greater neutralizing effects, indicating that CSF-1 was slightly, but not completely responsible for the macrophage activating properties of hybrid cell-CM. Addition of anti IFN-γ was not effective in this respect.

Treatment of infected macrophages with hybrid cell-CM

The treatment of infected cells more closely mimics common clinical needs. To determine if hybrid cell-CM affected the course of MAC-infection, peritoneal macrophages were first infected with MAC and 24 hr later, varying concentrations of hybrid cell-CM were added. Seven days later, the number of microorganisms present in infected cell cultures treated with hybrid cell-CM (at 1:1 ratio of CM to growth medium) was significantly ($P<0.01$) less than that found in untreated cultures (Fig. 5).

Table II. Effect of hybrid cell on the survival of C57BL/6 beige mice infected with MAC

Strain	Treatment[a]	No. of mice	Survival days
C57BL/6/bgj/bgj	MAC alone	6	22±4[b]
C57BL/6/bgj/bgj	MAC+Hybrid cells	12	>60
C57BL/6/bgj/bg$^+$	MAC alone	13	>60
C57BL/6/bgj/bg$^+$	MAC+Hybrid cells	10	>60

[a]Adult homozygous C57BL/6/bgj/bgj (beige) mice or heterozygous C57BL/6/bgj/bg+ mice were injected i.p. with 5×10^6 and s.c. with 2.5×10^6 ASL-1×LM hybrid cells. Five days later, the mice received an i.p. injection of 2.5×10^7 MAC organisms. Other received a single i.p. injection of 2.5×10^7 MAC organisms alone.

[b]$P<0.001$, relative to other groups.

Effect of hybrid cells on the survival of immune-deficient beige mice infected with MAC

Beige mice develop disseminated MAC-infection following inoculation of viable organisms. Can the hybrid cells affect the survival of immune-deficient beige mice infected with MAC? This question was investigated by injecting the adult homozygous mice (C57BL/6/bgj/bgj) with viable hybrid cells before an injection of MAC 5 days later. One hundred percent of the treated animals survived more than 60 days, without evidence of disease. Under similar conditions, mice injected with the organisms alone died in about 22 days. Heterozygous C57BL/6/bgj/bg$^+$ mice, resistant to MAC-infection, survived indefinitely in both circumstances (Table II). There was no evidence of the growth of the allogeneic hybrid cells (H-2$^{a/k}$) in any of the animals. Our prior experience indicated that the cells remained viable in the recipient mice for about 14 days.

DISCUSSION

The mechanism of resistance to MAC, an intracellular parasitic infection of macrophages, is incompletely understood. The organisms rarely cause disseminated infection in immunocompetent hosts, but are associated with a high prevalence of disease in immunosuppressed patients, especially patients with AIDS (Pierce *et al.*, 1996). In experimental animals, depletion of T cells in previously immunocompetent mice enhances the severity of MAC-infection and increases the animals' susceptibility to the disease, pointing to the role of cellular immunity in resistance to the infection. T cells may be required for the secretion of macrophage-activating immunomodulators, leading to a heightened state of intracellular microbicidal activity (Lee *et al.*, 1988).

MAC organisms proliferate less rapidly or may be killed by activated macrophages, indicating that resistance can develop at the cellular level. The cells' phagocytic activity, the total quantity of degradative enzymes, and the release of oxygen-derived products are increased in activated macrophages. Such changes are likely responsible for the more efficient bacterial killing and resistance to MAC-infection (Adams and Hamilton, 1984).

Thus, immunomodulators that are able to activate macrophage-activation may have beneficial effects in stimulating resistance to MAC-infection. GM-CSF, TNF-α, IL-4, and IL-12 have been reported to increase host resistance to the organisms (Hsu *et al.*, 1995; Saunders *et al.*, 1995). However, not all macrophage-activating immunomodulators appear to be beneficial. Interferon-γ activates macrophages, but does not stimulate resistance to MAC (Douvas *et al.*, 1985). Increased resistance to intracellular infectious organisms, including *Chlamydia, Leishmania and Toxoplasma,* has been observed following treatment with interferon-γ, suggesting that different intracellular microbicidal pathways may be present, or that the sensitivities of the organisms to the activation stages of macrophages may differ.

In the studies reported here, medium conditioned by the growth of hybrid cells, but not LM cells, stimulated resistance to MAC in mouse peritoneal macrophages. It is likely, therefore, that the composition of the two CMs might differ. Further evidence was indicated by observation that hybrid cell-CM, but not LM cell-CM, stimulated macrophages to express each of four previously described antigens associated with activated macrophages. One such antigen (TM-3) was not detected in cultures treated with LM cell-CM. It has been reported that TM-3 was expressed on macrophage cell line RAW 264.7 primed by interferon-γ, after subsequent triggering of the primed cells with lipopolysaccharide (LPS), but TM-1, TM-2 and TM-4 were expressed on the cells primed by IFN-γ alone (Paulnock and Lambert, 1990). Therefore, hybrid cell-CM may contain substances that can express one type of activation antigen, TM-3, on macrophages, in combination with interferon-γ which was also detected in the conditioned medium, leading to more fully activated stage of macrophages involved in resistance to MAC. Additional evidence was provided by differences in the morphology induced in macrophages exposed to hybrid cell-CM. The cells were rounded, and formed a "ruffled" border, characteristic of activated macrophages. Under similar conditions, cells exposed to LM cell-CM were fibroblastic in appearance.

Conceivably, the two cell-types formed the same immunomodulators, but at different rates. However, the titer of IFN-γ in LM cell-CM was slightly higher than that present in hybrid cell-CM although LM cell-CM did not stimulate resistance to MAC, indicating that IFN-γ alone was not responsible for stimulating resistance to MAC. Thus, if the difference between hybrid cell-CM and LM cell-CM on the proliferation of MAC in infected macrophages resulted from differences in the titers of analogous immunomodulators, then their rate of formation would have to differ markedly. There is no evidence to support this explanation.

Is CSF-1 formed by the hybrid cells responsible for stimulating the resistance to MAC in mouse peritoneal macrophages? Antibodies to CSF-1 partially reduced the inhibitory effect of hybrid cell-CM on the proliferation of MAC, suggesting that CSF-1 was at least partially involved. But it seems like that CSF-1 alone, or in combination with interferon-γ, cannot stimulate resistance to MAC in mouse peritoneal macrophages because LM cell-CM contained CSF-1 and IFN-γ, but did not stimulate resistance to MAC. We are unaware of prior report indicating that CSF-1 stimulates resistance to MAC although the killing effect of CSF-1 to other microorganisms such as *Trypanosoma cruzi, Leishmania tropica* or *Toxoplasma gondii* has been reported. Since the neutralizing effects of CSF-1 antibodies were incomplete, even at the highest concentrations tested, it is likely that more than one macrophage-activating immunomodulators are present in the hybrid cell-CM.

The beneficial effects of the hybrid cells were also revealed by the cells' effects on the prolonged survival of MAC-infected, immunodeficient beige mice. Control animals infected with MAC alone died in short intervals, about 22 days. Since the hybrid cells expressed histocompatibility antigens that were foreign to beige mice, it is likely that they were rejected. The cells may have provided a continual source of macrophage-activating immunomodulators during the period they remained viable in the immunodeficient hosts.

Differences in the inhibitory effects of hybrid cell-CM and LM cell-CM on the proliferation of MAC in mouse peritoneal macrophages may reflect the effects on gene regulation following fusion of two different cell types. After cell fusion, regulatory controls in each parental cell-type may affect the analogous control in the other, leading to the expression of genes that were previously inactive (Weiss and Chaplain, 1975). Conceivably, fusion of murine leukemia cells with mouse fibroblasts led to the activation of various genes including those encoding possibly unique substances that stimulate resistance to MAC.

This result suggest that treatment with purified immunomodulators used individually is insufficient for a complete effect. Simultaneous treatment with multiple immunomodulators may be required to fully activate the bactericidal properties of mouse macrophages or human monocytes. In addition, MAC organisms in infected macrophages were completely removed when

amikacin and hybrid cell-CM were used in combination, suggesting that combination therapy with antibiotics and multiple immunomodulators may be effective in the treatment of MAC-infection in immunocompromised hosts.

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