Production of Macrophage Activating Factor as Related to Soluable *Listeria monocytogenes* Antigen

by

John K. McNutt

Texas A & M University

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B G Foster

Dr. B. G. Foster, Research Advisor

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INTRODUCTION

Immunology is the study of the host's resistance to disease. This resistance has been traditionally divided into two branches: humoral and cell mediated. Humoral resistance involves the inactivation of invading organisms by soluable antibodies which are produced by B lymphocytes. Another type of lymphocyte, known as T lymphocytes, are derived from the thymus and are responsible for cell mediated immunity. When a lymphocyte binds with a certain foreign antigen, the lymphocyte becomes sensitized, and its mode of action is then directed only against the specific sensitizing antigen. Lately resistance has been shown to also be dependent on macrophages, a type of phagocytic cell which engulfs invading organisms (1). T lymphocytes interact with macrophages in providing resistance against intracellular parasites, such as tuberculosis bacteria and *Listeria monocytogenes*. Much of the nature of this interaction has now been deciphered. Sensitized T lymphocytes, upon stimulation with the sensitizing antigen, release a number of soluable factors known as lymphokines. Some lymphokines such as blastogen are directed towards lymphocytes, causing rapid cellular division and thus an increased production of lymphokines. Other lymphokines act directly on macrophages; it is these lymphokines and their action on macrophages with which this study is concerned.

One of the macrophage-directed lymphokines is Migration Inhibitory Factor, or MIF. MIF inhibits the normal wandering nature of a macrophage. Another important lymphokine is Macrophage Activating Factor, or MAF. MAF causes macrophages to become activated; this activity may be manifested in a number of ways. Activated macrophages demonstrate a higher degree of adherence to glass slides, greater mobility and spreading, an increase in metabolism of glucose, and a higher uptake of particles through non-specific phagocytosis (2). Mackaness has reported that activated macrophages are also endowed with higher numbers of mitochondria and lysosomes (3). Activated macrophages have been shown to have an increased bactericidal effect on phagocytized organisms. This bactericidal property is not directed specifically toward the bacteria which stimulated the T lymphocyte to produce MAF, but rather the bactericidal effect is demonstrated on nearly all types of phagocytized organisms (4). The microphages must be activated to demonstrate these enhanced bactericidal properties; non-activated microphages will be destroyed if infected with intracellular parasites, such as L. monocytogenes (5). Macrophages activated by MAF have even been shown to have a killing effect on syngeneic tumors (6). Thus the cell mediated branch of immunology is non-specific in resistance (7).

Activation of macrophages has been demonstrated by in vitro production of MAF. Suspensions of sensitized T lymphocytes are exposed to the sensitizing antigen for various periods of time. The cell suspen-

sions then may be either directly added to cell cultures of normal macrophages (4, 5), or supernatants prepared from the lymphocyte suspensions are overlayed upon the macrophage monolayers (8, 9). The increased activity of the macrophages is measured by using a variety of techniques and then compared with non-activated control macrophages.

Attempts to determine the chemical nature of the lymphokine MAF have not been very rewarding. Studies have shown MAF is believed to be a globular glycoprotein with a molecular weight of 20,000 to 35,000 daltons (10). It has been reported MAF is not affected by freezing or incubation at 60°C for 30 minutes, but a low pH (2 to 4) tends to inactivate the factor (8). Not much more is known about the lymphokine. Nor is much known about the bacterial antigen which sensitizes lymphocytes and causes them to produce MAF.

When the activity to be measured is bactericidal effects of intracellular parasites, L. monocytogenes is frequently used as a model organism. It has been shown that resistance to Listeria is cell mediated (11), an essential characteristic for a test organism in studies of this type. Coppel, et al. have shown that resistance to L. monocytogenes is incurred only when live organisms are used; various antigen preparations such as heat killed cells or ribosomal fractions apparently do not sensitize mice in vivo (12). However, certain antigen preparations of the bacteria have elicited lymophokine production when added to

sensitized T lymphocytes in vitro. A cell wall preparation has been noted to cause the production of blastogen; this blastogen was noted to affect only B lymphocytes (13). Petit and Unanue have shown that soluable antigens secreted from cultures of L. monocytogens have elicited the production of a similar blastogen. This same preparation was also shown to immunize mice against Listeria and to even activate macrophages in vivo as determined by visual morphological changes in the cells (14). Many studies have shown that killed, whole-cell preparations of Listeria can promote MAF production in vitro; however, these preparations give little insight as to the specific antigen which is responsible for MAF production (15).

It is the purpose of this study to see if a soluable antigen secreted by cultures of Listeria monocytogenes will stimulate MAF production in vitro, as determined by listericidal activity.

METHODS AND MATERIALS

Aseptic technique was maintained throughout all procedures.

Lymphocytes - Lymphocytes were obtained from the spleens of sensitized and normal outbred Hartley strain albino guinea pigs. The animals were sensitized by injecting intravenously 5.8×10^3 virulent Listeria monocytogenes (described below) suspended in 0.85% NaCl solution from an 18 hr culture. Sensitization was demonstrated by resistance to a

challenge of 10 - 100 LD_{50} of Listeria. Eighteen to twenty-three days later the animals were sacrificed by cardiac bleeding and rapid cervical dislocation. The lateral left side of the animal's abdominal region was shaved and saturated with 95% ethanol. An incision was made, the spleen was removed and placed in a beaker containing 15ml of cold Hanks' Balanced Salt Solution (HBSS) (GIBCO) containing 50 units of Sodium heparin per ml, 0.1 units of penicillin per ml, and 100 ug of streptomycin per ml. The spleen was minced into small sections and then forced through a double layer of cotton gauze. The resulting single cell suspension was centrifuged at 100xG for 20 min. The supernatant was discarded and 10 ml of cold 0.9% NH_4Cl were added to the cells for 15 min to lyse any erythrocytes. The cells were then passed through a double layer of cotton gauze and centrifuged at 100xG for 15 min. The supernatant and the cells were washed by centrifugation in 50 ml of cold HBSS as prepared above. The cells were then suspended in 10 ml of cold RPMI 1640 (GIBCO) supplemented with 10% fetal calf serum (FCS) (GIBCO), 0.1 units of penicillin per ml, 100 ug of streptomycin per ml, and adjusted to a pH of 7.2 with 1 M Hepes Buffer. The cells were then counted with a hemocytometer and adjusted to a concentration of approximately $5x10^6$ cells/ml. The cell suspensions were then placed in 125 ml plastic tissue culture flasks.

Antigens - Two types of antigens were prepared: Whole Killed Cell (WKC) and Soluable Secreted (SS). WKC antigen was prepared from

an 18-20 hr Brain Heart Infusion (BHI) broth (Difco) culture of L. monocytogenes (described below) containing 100 ug/ml streptomycin grown at 37°C. The cells were harvested by centrifugation, washed twice by centrifugation with 0.85% NaCl and resuspended in RPMI 1640 or 0.85% NaCl solution. The cells were killed by heating to 50°C for 48 hr. Sterility was checked by a failure of the antigen to grow in BHI broth. SS antigen was prepared as follows: 10x concentrated BHI broth was dialyzed against distilled H_2O for 24 hr at 4°C. The dialysate was dispensed in 50 ml quantities into flasks and autoclaved. Streptomycin was added to give a total concentration of 100 ug/ml. Dialyzed uninoculated media served as a source of control antigen. Two ml aliquats of a 20 hr BHI broth culture of L. monocytogenes (described below) were used to inoculate the flasks. The cultures were grown at 37°C for 96 hr under constant shaking. After growth, the cultures were pooled and centrifuged for 20 min at 10,000xG and the supernatant collected. From this point on, both the control and SS antigens were treated alike. The supernatant was fractionated by adding $(NH_4)SO_4$ to effect a 75% saturated solution at 4°C. Gentle handling of the antigen was maintained to avoid denaturation of the proteins. After 24 hr the proteins were collected by centrifugation and dissolved in 40 ml of sodium phosphate buffer (9.17g NaH_2PO_4, 35.95g $Na_2HPO_4 \cdot 7H_2O$ in 1 l, pH 7.1). The protein solution was dialyzed against the sodium phosphate buffer for 48 hr at 4°C, with three successive changes of the buffer solution. The $(NH_4)_2SO_4$-free protein suspensions were lyophilized for 72 hr. The antigens

were then resuspended in 10 ml of RPMI 1640. The protein concentration was determined by the method of Lowry using a bovine serum albumin standard (17). The final concentrations of the SS and control antigens were adjusted to 500 ug/ml in RPMI 1640. These antigens were sterilized by filtration through a 0.20u Milipore filter.

Generation of MAF Supernatants - WKC antigen was added to the sensitized and control lymphocyte suspensions (prepared above) at the ratio of 10 killed cells to each lymphocyte. SS antigen was added to 15 ml of the sensitized lymphocyte suspensions in amounts of 1000, 500, and 250 ug. Control antigen was added to 20 ml of sensitized lymphocyte suspension in the amount of 650 ug. The lymphocytes with antigens were incubated at 37°C in 5% CO_2 in humidified air for 72 hr. After incubation, the suspensions were centrifuged at 10,000xG for 20 min. The supernatants were collected and supplemented with FCS, penicillin, and streptomycin to achieve a final concentration of 20% FCS, 1.0 units per ml of penicillin, and 89 ug/ml of streptomycin.

Preparation of Peritoneal Exudate Cells - Normal outbred Hartley albino guinea pigs were injected intraperitoneally with 30 ml of sterile mineral oil. After 3 days the animals were sacrificed by cardiac bleeding and rapid cervical dislocation. The ventral portion of the abdominal region was shaved and saturated with 95% ethanol. An incision was made down the center line of the abdomen, and the tissue layers were

clamped back to expose the viscera. The peritoneal exudate was removed by washing the viscera with cold HBSS (prepared as described above) and then withdrawing the liquid from the abdominal cavity with the use of a 50 ml syringe. The viscera were washed with a total of 400 ml of cold HBSS. The HBSS and oil, containing peritoneal exudate cells (determined to be 80-95% macrophages [2]), were transferred to a separatory funnel. The aqueous layer was collected and centrifuged for 20 min at 600xG. The cells were washed twice in 40 ml of cold HBSS, and then resuspended in 10 ml of cold RPMI 1640 (prepared as described above). At this point any cell clumps were broken up by lavage through a 23 guage needle. The cells were counted with a hemacytometer and diluted in cold RPMI 1640 to a concentration of 2.38×10^6 cells/ml. The cell suspensions were then seeded in 0.2 ml quantities into wells of tissue culture plates (Linbro Scientific, Inc., Hamden, Conn.). The cells were incubated overnight at 37°C in 5% CO_2 in air; the following day the cell layers were washed twice with cold RPMI 1640 to remove any non-adherent cells. Each macrophage layer was overlayed with the active and control supernatants to be tested and then reincubated for 72 hr at 37°C in 5% CO_2 in air.

Bacteria - An EGD strain of Listeria monocytogenes was made virulent by serial passage of the organism three times through guinea pigs. The LD_{50} was determined to be 5×10^5 organisms. The bacteria was determined to be completely removed from the animal 18 days

after injection. For culture uses, L. monocytogenes were grown in BHI (Difco) broth with streptomycin added to give a final concentration of 100 ug/ml.

To infect the macrophage layers, a 50 ml stock culture was grown for 18 hr as described above. The cells were washed three times in sterile 0.85% NaCl solution, and then diluted in RPMI 1640 without antibiotics. The cells were then used to infect the macrophage layers at the rate of 10 bacteria/macrophage. The number of bacteria in the infecting dose were determined by standard bacterial pour plate methods.

Infection of Macrophage Monolayers - After incubation the cell layers were washed three times with cold RPMI 1640 containing no antibiotics to remove all of the penicillin which was present in the supernatants. The third wash did not inhibit the growth of Listeria. After washing, the cells which were to be assayed immediately after infection (Time Zero) were counted (as discussed below). All of the tissue culture wells containing the macrophages were infected with 0.2 ml of the bacterial suspension (prepared above). After 30 min the cells were then washed five times in cold RPMI 1640 without antibiotics to remove, as much as possible, all nonphagocytized bacteria. Cells which were to be assayed at times other than immediately after infection were reseeded with their respective supplemented supernatants and incubated at 37^{o} in 5% CO_2 in air.

Lysis of Infected Macrophage Layers - the monolayers were counted to determine the total number of adherent cells, using an ocular with a built-in grid. The cells were washed five times with cold RPMI 1640 without antibiotics to remove all traces of antibiotics from the supernatants. Sterile, distilled water was then added to each tissue culture well in 0.2 ml quantities for 30 min to lyse the adherent cells. After 30 min total lysis was insured by mechanical scraping of the monolayers with a sterile wooden applicator. The lysate was then aspirated out of the wells, and the number of bacteria in the lysates were determined by standard pour plate assay method.

RESULTS

Effect of WKC Antigen - Supernatants derived from sensitized lymphocytes with WKC antigen demonstrated a greater ability in activating normal macrophages when compared with the degree of activation produced by normal lymphocytes and WKC antigen. Figure 1 shows that by 72 hours post infection, there were significantly greater numbers of Listeria per macrophage in the control samples than in the experimental samples, indicating the macrophages were activated and possessed Listericidal properties in the experimental samples. The tests were run in triplicates and the difference is highly significant ($P < 0.001$) using the Students' t test and a F distribution. These results indicate sensitized lymphocytes will produce MAF when the

specific stimulating antigen is present.

Effect of SS Antigen - Table I shows the amount of antigen added to the lymphocyte cultures. Supernatants generated from sensitized T lymphocytes stimulated with SS antigen did not seem to confer a significant increase in activating normal macrophages when compared with the degree of activation conferred upon normal macrophages by the supernatants from sensitized T lymphocytes stimulated with control media antigen. The results are seen in Figure 2; the experiments were run in triplicates. While there are less bacteria per macrophage at 72 hours in the SS antigen samples than in the control media samples, the difference is not significant ($P > 0.1$) using the Students' t test and a F distribution.

TABLE I

Amount of soluable antigen added to sensitized lymphocytes

Antigen	Amount	Concentration of Lymphocytes	log (ug antigen/lymphocyte)
SS	940 ug	6.17×10^6 cells/ml	-3.82
SS	470 ug	6.17×10^6 cells/ml	-4.12
SS	235 ug	4.50×10^6 cells/ml	-4.28
Control	641 ug	4.50×10^6 cells/ml	-3.85

DISCUSSION

As stated before, previous studies have demonstrated that whole killed cell Listeria monocytogenes preparations will stimulate sensi-

WKC ANTIGEN

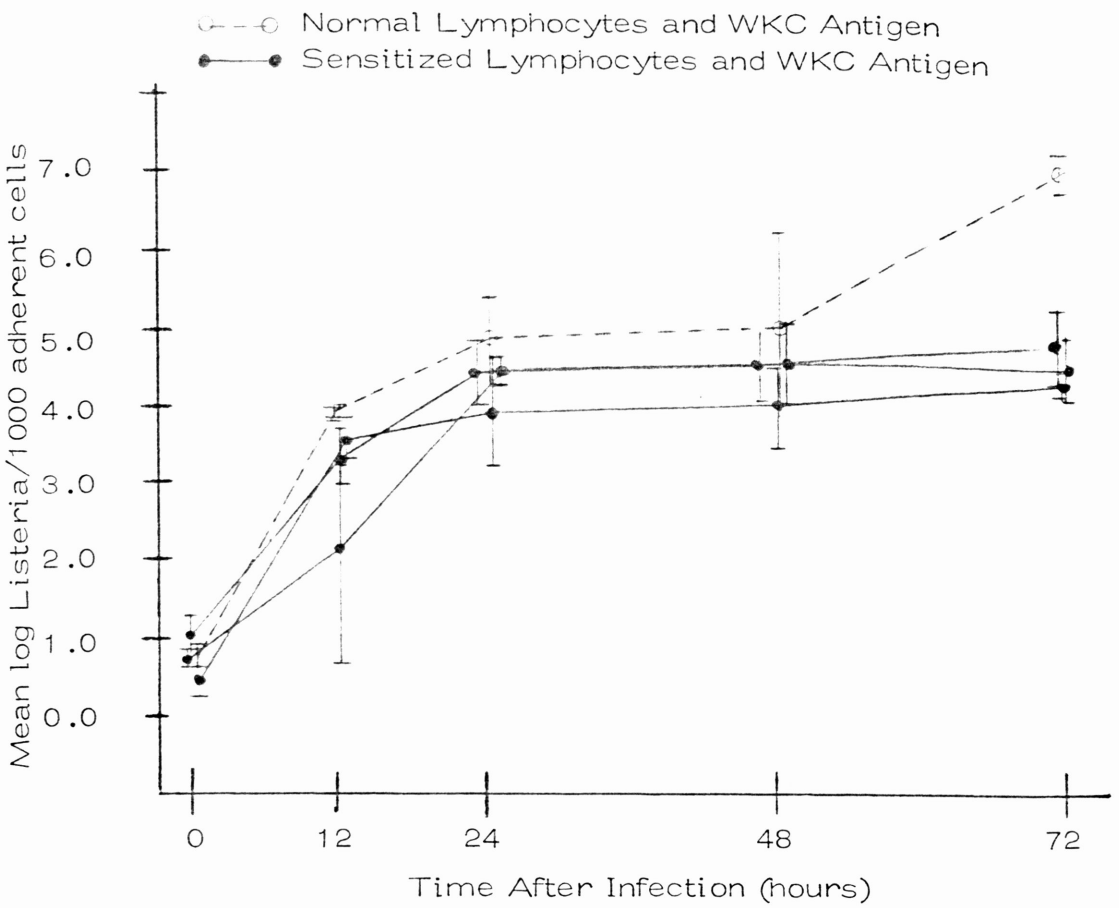

Fig. 1. Macrophage Listericidal activity by incubation with supernatants from normal and sensitized lymphocytes with WKC antigen. Bars indicate standard deviations. Each point represents the mean of three trials.

SS ANTIGEN

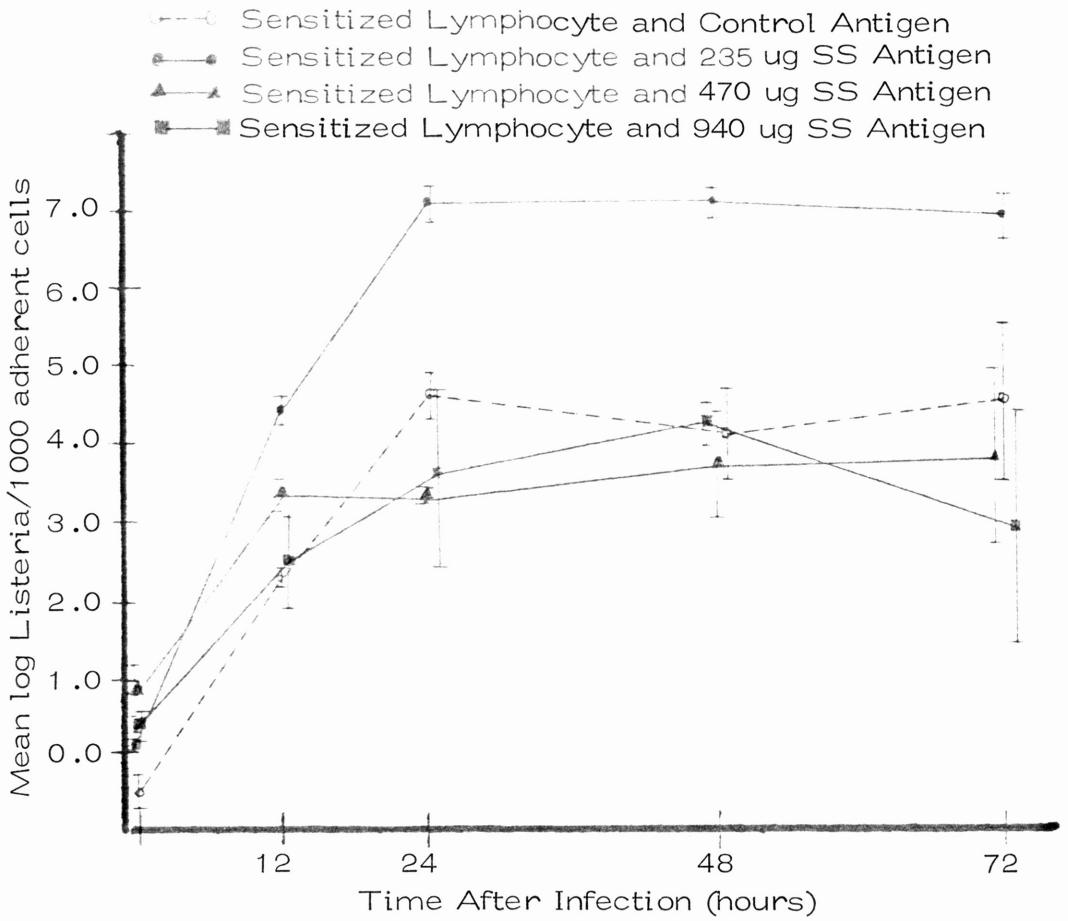

Fig. 2. Macrophage Listericidal activity by incubation with supernatants from sensitized lymphocytes with various amounts of SS antigen and uninoculated media. Bars indicate standard deviations. Each point represents the mean of three trials. Note: The 235 ug supernatant was contaminated, and this contamination contributed significantly to the number of bacteria counted.

tized lymphocytes to produce MAF (15). Therefore, the use of WKC antigen in this study serves as a positive control. The results indicate MAF can be produced in vitro with the given set of conditions under which the experiment was run, as consistant with other studies. The lack of macrophage activation see in the SS antigen fractions when compared to control fractions is therefore taken as a failure of the antigen to stimulate F production of MAF. On the basis of these results, it would appear that the antigen associated with Listeria which is responsible for production of MAF is not secreted as a soluable product during growth.

Some justifications need to be made at this point. The results described above are the averages of triplicate trials. More experimental trials need to be run before it is "proven" that the antigen is not a soluable product. This would include regeneration of SS antigen a number of times. Repeated trials may very well warrant the reverse of the tentative conclusion reached above. An indication of this may be seen by further examination of Figure 2. It will be noticed that at 0 hours the control fraction began with a significantly ($P < 0.001$) less number of Listeria per macrophage than the SS antigen fractions. Calculations show the SS antigen fractions had seven times as many bacteria. However, at the conclusion of the experiment (72 hr) the control fraction had at least the same number of bacteria per macrophage as did the SS antigen fraction. Had both fractions started with an equal number

of bacteria, the slightly higher number of bacteria seen in the control at 72 hours may have been elevated enough so the difference would be significant.

There are also certain justifications of the experimental procedure which need to be made. While sensitization to infections normally are at a peak four to six days after injection, the spleens were not collected for sensitized T cells until the 23rd day. However, Copel, et al. have shown resistance to Listeria to last well over four weeks (12); also, the skin sensitivity tests indicated the animals were still resistant. The spleen was chosen as a source of sensitized T cells because of its convenient accesibility and because it has been shown that T lymphocytes responsible for resistance to Listeria reside in the spleen (16). Penicillin is used to help keep down contamination. However, its use may be questionable, since macrophages can pinocytose penicillin. Is the bactericidal effects observed due to the activation of macrophages or due to the presence of penicillin inside the macrophages? Simon, et al. have shown that while the use of penicillin may give a higher degree of macrophage bactericidal effect, both control and experimental macrophages are affected equally, and penicillin is not responsible for the differences observed between the control and experimental macrophages (5).

SUMMARY

Whole, killed Listeria cells caused the production of MAF as determined by bactericidal activity. Antigens from soluable metabolites of Listeria cultures failed to demonstrate a conclusive production of MAF.

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