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Extracellular Antigens from *Listeria monocytogenes*

II. Cytotoxicity of Hemolytic and Lipolytic Antigens of *Listeria* for Cultured Mouse Macrophages

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Purified preparations of hemolytic and lipolytic antigens were shown to be cytotoxic for tissue cultures of mouse peritoneal macrophage monolayers. The percent reduction in viability was proportional to the concentration of antigen used although, for a brief period, the hemolytic antigen stimulated the growth of cultured macrophages. Hemolytic and lipolytic antigens were synthesized by an hemolysin-producing strain (7973), but were deficient or totally lacking in nonhemolysin-producing strains (9037-7, 4219). The inoculation of strain 7973 onto macrophage monolayers at a bacterium to macrophage ratio of 10:1 resulted in a complete loss of viable phagocytic cells. The decrease in viable phagocytic cells was accompanied by an increase in viable listeria. Pronounced cytopathic activity with proliferation of listeria was also observed when 200 U of lipolytic antigen were added to macrophage monolayers that had been infected with the smooth type nonhemolysin-producing strain (4219) on the previous day. On the other hand, a high percentage of macrophages survived when exposed to nonhemolysin-producing strains only. In this case, listeria were not recovered by routine culture methods. The data presented do not establish that phagocytic cell destruction with accompanying microbial proliferation is the direct result of hemolysin elaboration; however, they suggest that there is need for further exploration of the role of the hemolysin in listeriosis.

A previous report from this laboratory revealed that, although all strains of *Listeria monocytogenes* produce a hemolytic zone on blood agar plates, the production of soluble hemolysin in broth varies depending on the incubation time and temperature, the culture medium, and the particular strain (10). More recently, two distinct antigens were recovered and purified from the soluble hemolysin (3). One antigen designated as a hemolytic antigen because of its ability to lyse erythrocytes from different animal species had an estimated molecular weight of at least 171,000. The other antigen designated as a lipolytic antigen because of its ability to hydrolyze lipoidal substrates had an estimated molecular weight of 52,500. During the purification process, significant quantities of ribonucleoproteins, anthrone-positive carbohydrates, and an erythrocyte-sensitizing substance were removed or drastically reduced. It has become apparent that the soluble hemolysin represents a concentrated culture filtrate from listeria containing a complex of chemical and biologically active materials associated with a hemolytic-active component. The present communication is part of a long-range investigation on mechanisms of pathogenicity and immunity in listeriosis. The primary objective has been concerned with the need for more basic information on the fundamental differences in the total makeup of virulent and avirulent strains of *Listeria*. The present study is an extension of a previous report (3) in which the hemolytic and lipolytic antigens were purified and characterized chemically and serologically. This report characterizes the cytotoxic activities of these antigens for mouse peritoneal macrophage monolayers in tissue culture.

MATERIALS AND METHODS

Preparation of hemolytic and lipolytic antigens. The methods of production and purification and the determination of the specific activity of each antigen have been described in detail (3). The final preparations were frozen in phosphate buffer (0.025, pH 6.8)

and stored in 1-ml amounts at −60 C. The specific activity of various preparations of the hemolytic antigen ranged from 13,000 to 51,000 complete hemolytic U per 0.01 mg of protein. The specific activity of various preparations of the lipolytic antigen ranged from 600 to 1,800 lipolytic U per 0.01 mg of protein. Specific activity will be expressed hereafter in units per milliliter.

Cell culture procedure. Peritoneal exudate cells were harvested from the cavities of CFW mice (Carworth; New City, N.Y.) 3 to 5 days after stimulation by 1 ml of Thioglycollate broth (BBL) for each mouse. The collection medium was diploid growth medium (Gibco) containing 10% inactivated fetal calf serum. Penicillin (200 U per ml), streptomycin (200 μg per ml), and heparin (500 U.S.P. U per ml) were added to the medium. The percent of macrophages in the pooled cell suspension was determined by the neutral red dye uptake method (13), and the viability of the cell population was estimated by the trypan blue exclusion technique (7). The pooled cell suspension was diluted with diploid medium to contain 5×10^5 viable macrophages per ml for seeding Leighton tubes. The following procedure reported by Mackaness (6) was used to select macrophage populations over other cell types. After a 30-min incubation in Leighton tubes at 37 C in an atmosphere of 5% CO_2, 95% air, the macrophages attached to the glass surface. Unattached cells were removed by copious washings with maintenance medium (diploid medium containing 100 U of penicillin per ml and 100 μg of streptomycin per ml). After 24 h, a monolayer composed of 80 to 90% macrophages was observed. Macrophages were operationally defined as phagocytic peritoneal exudate cells capable of neutral red uptake and adherence to glass.

Experimental procedures. In the determination of cytotoxicity, maintenance medium containing antibiotics and supplemented with 10% (vol/vol) of the hemolytic or lipolytic antigen was added to macrophage monolayers. On the day after the addition of antigen and at 24-h intervals thereafter, samples from duplicate or triplicate cell cultures from each experimental condition were removed to determine the percentage of surviving macrophages. Direct visualization of macrophage cultures by phase contrast microscopy was also used in assessing the degree or extent of cell lysis within the culture. The percent of viable macrophages was determined by dividing the number of viable cells by the total number (viable + nonviable) by 100. The final values were expressed as the average of at least three separate counts of replicate cultures from each experimental condition. The range of viability counts varied by 2 to 15% from the average.

The approximate time of antigen association with macrophage monolayers necessary to cause a demonstrable loss in viability was examined indirectly by determining the time necessary to effect the lysis of sheep erythrocytes suspended in buffered saline (with and without 0.01 M cysteine) and diploid medium. The buffered saline solution contained 1.81 g of $NaHPO_4$, 3.17 g of KH_2PO_4, and 7.4 g of NaCl dissolved in water and made up to 1 liter. The pH was adjusted to 6.6. In some experiments, hemolytic and lipolytic antigens were filtered through membrane filters (Millipore Corp.) before their addition to macrophage monolayers. Hemolytic and lipolytic antigens inactivated by heat treatment (60 C for 30 min) or by 0.01 M cysteine, respectively, were diluted to the same extent as the active solutions and served as controls. These experiments were repeated with the antigens neutralized by hemolytic-lipolytic antiserum to exclude the possibility of a nonspecific effect. A predetermined amount of maintenance medium supplemented with either antigen and 10% (vol/vol) of undiluted antiserum (adsorbed and unadsorbed) was incubated at 37 C for 30 min. Undiluted normal rabbit serum was substituted for antiserum in control preparations. One milliliter of the reaction mixture was inoculated onto macrophage monolayers in Leighton tubes and incubated at 37 C.

The antiserum prepared in another study (3) possessed neutralizing titers of 1 to 1,280 and 1 to 160 against the hemolytic and lipolytic antigens, respectively. The antiserum was adsorbed by mixing acetone-dried whole cells of strain 7973 (approximately 50 mg per ml) and incubating the mixture for 2 h at 37 C, followed by an overnight incubation at 4 C.

Listeria. Three strains were selected because of their observed quantitative differences in the production of soluble hemolysin in Trypticase soy broth (BBL). The hemolytic activity of the hemolysin was assayed by a method previously reported (2). Complete hemolytic units (CHU) were defined as the reciprocal of the highest dilution of the hemolysin showing complete lysis of a 1% sheep erythrocyte suspension. The CHU were 4,095 for strain 7973 and less than 20 for strains 4219 and 9037-7. The strains were maintained on Trypticase soy agar slants at 4 C. Preliminary experiments comparing the growth of strains in tissue culture medium with growth in Trypticase soy broth revealed that the medium was not toxic or otherwise inhibitory.

Infection of macrophage cultures. Viable bacterial counts were determined by plating 0.1-ml fractions on Trypticase soy agar. Appropriate dilutions of each strain were washed and suspended in maintenance medium without antibiotics. One tenth milliliter of a suspension of a particular strain was added to the macrophage monolayer (5×10^5 per ml) to give a ratio of 10 listeria for each macrophage in a total volume of 1.0 ml. After a 1-h exposure, extracellular listeria were removed by washing three times with maintenance medium containing antibiotics (1). Finally, this medium was replaced with fresh maintenance medium, and the infected macrophage cultures were incubated statically at 37 C. No attempt was made to quantitate intracellular bacteria; however, the multiplication and proliferation of listeria were followed by microscopic examination of macrophage monolayers grown on cover slips in Leighton tubes. These were then Giemsa stained.

RESULTS

Cytotoxic effect of hemolytic and lipolytic antigens. The cultured macrophages appeared

extremely granular 4 h after exposure to at least 1,000 U of hemolytic antigen or 100 U of lipolytic antigen; however, there was no decrease in the percent viability of macrophages at that time, when compared with uninoculated controls. Repeated experiments revealed that cytotoxicity for macrophage monolayers becomes prominent between 12 and 24 h after initial exposure, although a previous report (4) demonstrated that cytolysis of cultured macrophages occurred within 30 min after exposure to the listerial hemolysin. An inference can be drawn from studies on the minimal contact time necessary for the hemolytic antigen to cause the complete lysis of a 1% suspension of sheep erythrocytes. Table 1 summarizes the hemolytic antigen sensitivities to sheep erythrocytes suspended in buffered saline and diploid growth medium. In this study, cysteine activation was highly effective but not essential in bringing about the complete lysis of sheep erythrocytes when at least 1,000 U or greater of the hemolytic antigen were used. On the other hand, concentrations of less than 500 U were not capable of lysing erythrocytes in the absence of cysteine. In the presence of 10% fetal calf serum or diploid medium (supplemented with 10% fetal calf serum), the lytic activity of the hemolytic antigen was delayed for 1 to 12 h when concentrations greater than 500 U were used. Hemolysis was completely inhibited when less than 500 U of the hemolytic antigen were used. The lipolytic antigen did not possess hemolytic properties. It appears that the cytotoxic activities for both antigens were delayed in the presence of the diploid medium, the serum, or both.

When 100 to 400 U of lipolytic antigen were added to macrophage monolayers, the percent reduction and the time necessary to achieve 100 percent reduction in macrophage viability were proportional to the concentration of antigen (Fig. 1). No cytotoxic effect was observed when less than 70 U of lipolytic antigen were added to macrophage monolayers. When 1,000 to 9,000 U of the hemolytic antigen were used, the percent reduction in viability was proportional to the concentration of antigen used, although 100% reduction in macrophage viability was never achieved. A different pattern of cytotoxicity was observed when the hemolytic antigen was added to macrophage cultures. There was a clearly defined decrease in the percent viability relative to the controls within the first 24-h period. The decrease was followed by an apparent increase in the percent viable cells on the second and third post-inoculation days. This temporary "growth-enhancing effect" was observed routinely when 1,000 U or more of the hemolytic antigen were added to cultured macrophage monolayers. No growth enhancement was seen when less than 500 U of the hemolytic antigen were used. Further characterization of the cytotoxic activities of the hemolytic and lipolytic antigens are summarized in Table 2. In general, the findings are consistent with previous experiences which have indicated that the hemolytic and lipolytic antigens were physically and biologically distinct (3).

Antihemolytic-lipolytic serum consistently neutralized the cell-damaging effects of both

TABLE 1. *Comparison of hemolytic antigen sensitivity in buffered saline and diploid growth medium*

Medium	Incubation time[a]			
	10,000[b]	1,000	400	200
Buffered saline				
Without additives	1	1	–[c]	–[c]
With 10% serum[d]	2	12	–[c]	–[c]
With 0.01 M cysteine	0.5	0.5	0.5	0.5
Diploid growth medium	2	12	–[c]	–[c]

[a] Approximate time (in hours) necessary for complete lysis of a 1% suspension of sheep erythrocytes incubated at 37 C.

[b] Numbers across represent units of hemolytic antigen.

[c] No lysis.

[d] Fetal calf serum.

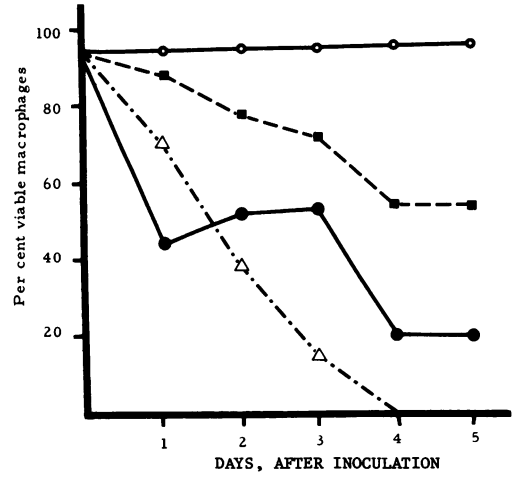

FIG. 1. *Percent decrease in cultured macrophage monolayers after the addition of hemolytic and lipolytic antigens. Each point represents the average of 2 to 3 replicate cultures. Symbols: inactivated hemolytic and lipolytic antigen-control cultures* (○), *200 U of lipolytic antigen* (■), *400 U of lipolytic antigen* (△), *and 9,000 U of hemolytic antigen* (●).

TABLE 2. *Effect of hemolytic and lipolytic antigens on cultured macrophage monolayers after pretreatment by heat, cysteine, or membrane filtration*

Treatment[a]	Viable macrophages remaining 4 days after inoculation (%)	
	Hemolytic antigen (9,000 U)	Lipolytic antigen (400 U)
Heat-treated (60 C/30 min)	98[b]	0
Cysteine-treated (0.01 M)	25	100
Membrane filtered (0.45 μM)	70	0
Membrane filtered (0.22 μM)	100	5

[a] Hemolytic and lipolytic antigens were treated as indicated. Tissue culture medium was supplemented with 10% (vol/vol) of the particular antigen and added to macrophage monolayers.

[b] Percent of control.

TABLE 3. *Effect of hemolytic-lipolytic antiserum on the cytotoxic activity of hemolytic and lipolytic antigens*[a]

Serum	Viable macrophages remaining 4 days after inoculation (%)	
	Hemolytic antigen (9,000 U)	Lipolytic antigen (400 U)
Antihemolytic-lipolytic serum (unadsorbed)	96[b]	92
Antihemolytic-lipolytic serum (absorbed)	20	83
Normal rabbit serum	20	0

[a] Appropriate amounts of hemolytic or lipolytic antigens were mixed with 10% (vol/vol) of undiluted serum and incubated at 37 C for 30 min. One milliliter of the reaction mixture was inoculated onto macrophage monolayers.

[b] Percent of uninoculated controls.

antigens (Table 3). The adsorption of the antiserum by acetone-dried whole listeria (strain 7973) removed antibodies capable of inhibiting lipolytic antigen cytotoxicity but not hemolytic antigen cytotoxicity.

Listeria-macrophage interaction. When strain 7973, a hemolysin-producing strain, was added to macrophage monolayers at a listeria to macrophage ratio of 10:1, there was a complete loss of viable macrophages within 48 h. Direct visualization data derived from Giemsa-stained cover slip cultures implied an increase in numbers of listeria within a few hours after inoculation. This event was accompanied by a decrease in the percent of viable macrophages and a subsequent increase in colony-forming listeria. These organisms most likely represented those that (i) were initially phagocytized, (ii) remained viable, (iii) and multiplied intracellularly. Nonviable listeria (Formalin-inactivated or heat-treated 7973) inoculated onto macrophage monolayers at a ratio of 10:1 or 100:1 did not significantly reduce the percent viability ($<$ 1 when compared with uninoculated control cultures) of macrophage cultures. A higher percentage of macrophages survived, by comparison, when viable nonhemolysin-producing strains were added to monolayers (Fig. 2). These strains were not recovered by cultural means. The three strains, all relatively avirulent, had the following mean lethal dose (LD_{50}) values; strain 7973, 1.5×10^6; strain 9037-7, 5.6×10^9; and strain 4219, 2.4×10^6 organisms when given intraperitoneally to mice. Strain 9037-7 possessed the characteristics of a rough varient, whereas the other two strains were decidedly smooth. A progressive decrease in

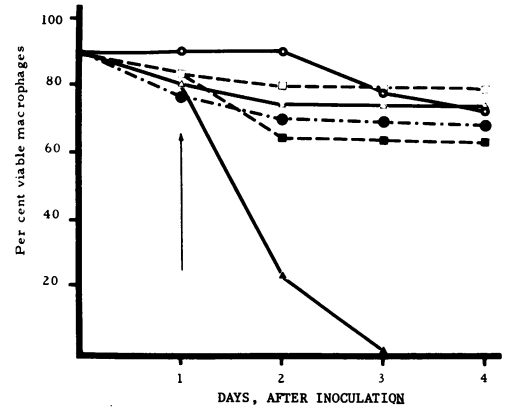

FIG. 2. *Effect of nonhemolytic strains of Listeria and 200 U of lipolytic antigen on the viability of cultured macrophages. In the case of dual inoculations, listeria were added initially (time 0) followed by the lipolytic antigen given 1 day later (indicated by arrow). Symbols: listeria- and listeria antigen-inactivated control cultures (○); lipolytic antigen (●); strain 9037-7 (□); strain 9037-7 + lipolytic antigen (■); strain 4219 (△); and strain 4219 + lipolytic antigen (▲).*

macrophage viability occurred, however, when 200 U of lipolytic antigen were added to macrophage monolayers that had been infected, 24-h earlier, at a bacterium to macrophage ratio of 10:1 (Fig. 2). Since separate additions of strain 4219 and 200 U of lipolytic antigen could have accounted for an approximate reduction in macrophage viability of 15 and 20%, respectively, it is apparent that a synergistic union resulting in a greater destructive effect to mac-

rophage monolayers (100% decrease in viability by the third post-inoculation day) became established. An increase in colony-forming listeria accompanied the loss in macrophage viability. Since no "growth-enhancing effect" was produced, the lipolytic antigen was used instead of the hemolytic antigen, and the cytotoxic response could be predicted with greater accuracy. Under similar conditions of testing another nonhemolysin-producing strain, strain 9037-7 (rough varient), together with 200 U of lipolytic antigen did not cause a progressive decrease in macrophage viability, nor were colonies formed on artificial medium. Undiluted hemolytic-lipolytic antiserum added to cultured macrophages prior to, or with, the hemolysin-producing strain (7973) was ineffective in preventing the progressive destruction of macrophages. On the other hand, the antiserum was highly effective in inhibiting the progressive cytotoxic effect produced by the apparent synergistic union of strain 4219 and 200 U of lipolytic antigen.

DISCUSSION

The *L. monocytogenes* hemolysin, in addition to its lytic activity against erythrocytes from different animal species, possesses cytotoxic activity in tissue culture systems of mouse peritoneal exudate cells (4, 9) and the L-M strain of mouse cells (12). The purification and recovery of two biologically active antigens from the hemolytic-active portion of the hemolysin (3) has led to the conclusion that the hemolysin is, in reality, a concentrated culture filtrate containing, in addition to a hemolytic-active component, a complex of chemical and biologically active materials. For example, an erythrocyte-sensitizing substance associated with hemolysin preparations has been reported to exert a cytotoxic effect on the L-M strain of mouse cells and the Madin-Darby canine kidney cell line in tissue culture (B. B. Watson and E. M. Jenkins, Bacteriol. Proc., p. 77 1967). The erythrocyte-sensitizing substance was removed from hemolytic and lipolytic antigen preparations by batchwise adsorption with diethylaminoethyl cellulose-Sephadex A50 (3).

The present studies revealed that purified preparations of hemolytic and lipolytic antigens are cytotoxic for tissue cultures of mouse peritoneal macrophages. In general, both antigens were capable of causing progressive decreases in macrophage viability and showed many of the characteristics previously ascribed to the hemolysin (9). Kingdon and Sword (4) reported that cytolysis of cultured mouse macrophages occurred 30 min after exposure to listerial hemolysin and that cysteine activation of the hemolysin was essential for lytic activity. Cysteine activation of hemolytic and lipolytic antigens was not required to effect a cytolytic response. At present, it appears that the state or degree of antigen purification decreases the necessity for cysteine activation of cytotoxic activities (Table 1).

The hemolytic antigen produced a temporary "growth-enhancing effect" when added to macrophage monolayers. Waksman and Matoltsy (14) observed that tuberculin had a growth-stimulating effect on peritoneal exudate cells obtained from guinea pigs previously sensitized to tubercle bacilli. An explanation for growth stimulation is unavailable. The macrophage cultures were not recovered from mice previously sensitized to listeria; however, the mice could have been infected and subsequently sensitized by other gram-positive organisms possessing antigens in common with listeria (8, 10). The strains used were relatively avirulent yet the strain capable of hemolysin production was appreciably more lethal for cultured macrophage monolayers than nonhemolysin-producing strains. Additional studies have shown that strains 9-125 (LD_{50} 1.7×10^6; CHU 2,048) and VRL 8418 (LD_{50} 7.6×10^6; CHU 2,048) are as destructive as strain 7973 for macrophage cultures, whereas strain 5248 (LD_{50} 3.2×10^7; CHU <20) behaves similarly to strain 4219 when added to cultured macrophages (B. Watson, unpublished data).

The reactivity pattern of hemolytic-lipolytic antiserum was shown to be highly effective in inhibiting the serological activities of hemolytic and lipolytic antigens (3). Results indicate that the hemolytic-lipolytic antiserum neither prevented cell destruction nor the proliferation of strain 7973 on macrophage monolayers. This observation is in accord with the general finding that antilisteria serums will not enhance the killing of listeria by macrophages (6, 11). It is tempting to suggest that if the hemolysin was the factor responsible for microbial survival in phagocytic cell cultures, the mode of action of the hemolysin must have occurred within phagocytes or in areas unavailable to neutralizing antibody. The antiserum, on the other hand, was effective in inhibiting progressive cytotoxicity produced by the synergistic union of strain 4219 and 200 U of lipolytic antigen on macrophage monolayers. Certain inferences can be drawn from the data presented. The lipolytic antigen caused a reduction in the percent viable macrophages and also the release of viable listeria from intraphagocytic sites. A subsequent increase in extracellular listeria then could destroy the remaining viable macro-

phages by the mass of the bacteria interfering with phagocytic cell function. To insure the validity of this contention, the listeria must be able to survive initially within the phagocytic cells. It is probable that the macrophages grown in a tissue culture system exerted a microbiocidal effect on all phagocytized listeria but with a limited ability to kill certain of the strains. Kingdon and Sword (5) suggested that the hemolysin may promote listeriosis by causing an alteration in the healthy phagocyte-to-bacterium ratio. The significance of our observations and the relationship of hemolysin production to listeric infection have not been established. Studies are underway to critically assess the role of the hemolysin in listeriosis.

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