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Intracellular Hemolysin-Producing *Listeria monocytogenes* Strains Inhibit Macrophage-Mediated Antigen Processing

CHRISTOPHER W. CLUFF,† MARY GARCIA, AND H. KIRK ZIEGLER*

Department of Microbiology and Immunology, Emory University School of Medicine, Atlanta, Georgia 30322

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We found that virulent hemolysin-producing (Hly$^+$) *Listeria monocytogenes* strains inhibit antigen processing and presentation when added to macrophages in vitro. Avirulent Hly$^-$ bacteria caused little or no inhibition. Live Hly$^+$ bacteria inhibited presentation of both heat-killed *L. monocytogenes* and ovalbumin. Several observations indicate that hemolysin produced by intracellular bacteria was responsible for the inhibition. First, inhibition was observed even when extracellular bacteria were removed after a brief 10-min bacterial uptake period. Second, inhibition was not prevented by the addition of cholesterol, a substance which inactivates soluble hemolysin. Third, only very high concentrations of soluble hemolysin were inhibitory. Under conditions which inhibit antigen presentation (10^5 per well), macrophages retained normal levels of Ia, maintained normal morphology, and were not permeable when assayed by chromium release. The uptake and catabolism of ^{35}S-labeled live bacteria by macrophages were similar for both Hly$^+$ and Hly$^-$ bacteria. Only a small decrease in uptake and catabolism of surface-iodinated heat-killed *L. monocytogenes* by macrophages pretreated with inhibitory numbers of live Hly$^+$ bacteria was observed. Additionally, macrophages pretreated with live Hly$^+$ bacteria and fixed 1.5 h later were able to effectively present an ovalbumin peptide (amino acids 323 to 339) to the T-cell hybridoma DO11.10. Hemolysin-producing bacteria inhibited the presentation of antigens that need processing better than they did of antigens that do not require a processing event. Thus, we have demonstrated inhibition of an intracellular antigen processing pathway by hemolysin-producing *L. monocytogenes*, which may contribute to the virulence of this pathogen.

Listeria monocytogenes is a facultative intracellular gram-positive bacterium that is responsible for causing severe infections, usually in newborns and immunocompromised individuals (17, 40). The sulfhydryl-dependent hemolysin secreted by *L. monocytogenes* has been shown to be an important virulence factor. All clinical isolates of *L. monocytogenes* have been shown to be hemolytic (19, 20, 38), and nonhemolytic mutants obtained by transposon mutagenesis have been shown to be avirulent in mice (15, 23). Although hemolysin production does not appear to affect uptake of bacteria by macrophages, production of this toxin has been shown to enhance the survival of *L. monocytogenes* in mouse peritoneal macrophages (29, 37).

The importance of T-cell-mediated immunity in recovery from listeriosis has been established (13, 24, 26, 34, 45, 53). Both CD4$^+$ and CD8$^+$ T cells appear to be involved in a successful anti-*L. monocytogenes* immune response (11, 25, 31). The induction of bactericidal macrophages by gamma interferon (7, 27) and the production of tumor necrosis factor-α (33) have been recognized as crucial events in bacterial clearance.

In attempts to gain insight into the events that take place during infection with virulent strains of *L. monocytogenes*, we have examined the effects of live bacteria on macrophage functions in vitro. Using a modified assay system (8, 35, 44, 46–48, 50–52), we have shown that macrophages treated with hemolytic strains of *L. monocytogenes* present antigens poorly to class II major histocompatibility complex (MHC)-restricted T cells (9). In this study we have investigated the mechanism by which hemolysin inhibits macrophage-mediated antigen presentation. Intracellular interference with an antigen-processing pathway is demonstrated, and a possible mechanism(s) of inhibition is discussed in relation to current models of antigen presentation.

MATERIALS AND METHODS

Media. Peritoneal lavage was performed with Hanks balanced salt solution containing 0.06% bovine serum albumin, 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer, 2 mM L-glutamine, and heparin (10 U/ml). This medium, minus the heparin, was used for cell washing procedures. For cell culture prior to fixation of macrophages, the following medium was used: RPMI 1640 containing 5% fetal calf serum (FCS), 10 mM HEPES, 2 mM L-glutamine, 0.075% sodium bicarbonate, and 0.5 mM sodium pyruvate. T cells were added to the macrophages in this culture medium supplemented with 50 μg of gentamicin per ml. Dulbecco modified Eagle medium (DMEM) containing 10% FCS, penicillin (50 U/ml), streptomycin (50 μg/ml), and 2 mM L-glutamine was used in assays for interleukin-2 (IL-2) production with HT-2 cells. HT-2 cells were maintained in DMEM supplemented with 2 mM L-glutamine, 10% FCS, and 10% Rat-T Monoclone (Collaborative Research Inc., Bedford, Mass.) as a source of IL-2.

***L. monocytogenes* preparation.** *L. monocytogenes* was prepared as described previously (9, 48). Hemolysin-producing (Hly$^+$) *L. monocytogenes* were used for immunization. Bacteria used in vitro were grown in 50 ml of brain heart infusion broth (BHI) for 15 h at 37°C with an inoculum of 10^9 bacteria and then washed twice with cold phosphate-buffered saline (PBS). The concentration of bacteria was determined with a spectrophotometer and confirmed by colony counts on blood-agar (tryptic soy agar with 5% sheep blood).

* Corresponding author.
† Present address: Rocky Mountain Laboratories, Hamilton, MT 59840.

Examination of colonies grown on blood-agar plates confirmed the presence or absence of hemolysin production for all *L. monocytogenes* strains used. Prior to addition to macrophages, bacteria were diluted to proper concentrations in ice-cold culture medium. The Hly^- transposon Tn*1545* mutant and the Hly^+ revertant, designated CNL85/162 and CNL85/163, respectively, were constructed and characterized as described previously (15). Medium used for the growth of the transposon-containing mutant was supplemented with tetracycline (10 μg/ml). The strains used in the experiment shown in Fig. 6 were obtained from the American Type Culture Collection (*L. monocytogenes* strains ATCC 43250, 43251, 43248, and 43249). These strains have been described before (36).

T cells and macrophages. Female BALB/c or C3HeB/FeJ mice 8 to 12 weeks old were purchased from Jackson Laboratories, Bar Harbor, Maine. T cells were purified from the peritoneal exudates of C3HeB/FeJ mice infected intraperitoneally with 1×10^4 to 5×10^4 live *L. monocytogenes*. One week after infection, the mice received an intraperitoneal injection of 10% proteose peptone (1.5 ml) and were killed 3 days later. T-cell enrichment was accomplished by removing adherent cells with culture dishes and nylon wool. Nonadherent cells were then treated with anti-Ia^k serum and complement. Such T cells were "functionally pure" because they would not respond well to antigen unless macrophages were added (48). The T cells active in this system have been characterized as class II MHC restricted, $Ly1^+$, $CD8^-$, Ia^-, $Thy1^+$ cells (44).

The I-A^d-restricted, antiovalbumin T-cell hybridoma (designated DO11.10) has been described previously (41).

L. monocytogenes-immune mice were also used as the macrophage source for most experiments. Peritoneal exudate cells (PEC) were harvested as described above and incubated for 2 h at 37°C in tissue culture vessels to allow macrophage adherence. The nonadherent cells were removed by washing. Macrophages used for the experiments shown in Fig. 4, 5B, 6, and 7 were harvested from the peritoneal cavity of BALB/c mice injected intraperitoneally 3 days before sacrifice with 100 μg of concanavalin A in 1 ml of PBS.

Assay for antigen processing and presentation. The ability of the macrophages to process and present antigen to T cells was measured by the enhanced production of IL-2 after 24 h in culture. Assays were performed in 96-well plates (Costar). In each well, 10^5 T cells were added to macrophages derived from 2×10^5 PEC. Live bacteria or heat-killed *L. monocytogenes* (HKLM) were added as described in the text and figure legends. Macrophages were fixed as described previously (9, 48). T cells were added, and after 18 h, supernatants were collected. Supernatants (80 μl of 1:2 and 1:20 dilutions) were added to IL-2-dependent HT-2 cells (20 μl at 1.5×10^5 cells per ml in 96-well plates). After 24 h, tritiated thymidine (20 μl; 0.025 mCi/ml) was added, and thymidine incorporation was determined 16 h later. Results are expressed as net cpm (cpm from cultures with T cells minus cpm from parallel cultures without T cells).

For the experiment shown in Fig. 5, soluble listerial proteins (SLP) (50 μg/ml) were added along with T cells to macrophages that had been treated with live bacteria and fixed. The preparation of SLP has been described before (48). Ovalbumin was obtained from Sigma Chemical Co. (St. Louis, Mo.), and the ovalbumin peptide was constructed by solid-phase peptide synthesis and purified by reverse-phase high-pressure liquid chromatography in the Microchemistry Department at Emory University. The processing requirements of these antigens has been described previously (41, 48).

Radioimmunoassay for detection of Ia. The radioimmunoassay technique has been described previously in detail (49). Briefly, fixed macrophages were incubated with anti-IA^k (10-2.16) or control immunoglobulin G2b myeloma protein. The next incubation was with rabbit anti-mouse immunoglobulin, and the final incubation was with ^{125}I-*Staphylococcus aureus* protein A. The plate was cut with a hot wire, and radioactivity bound in each well was determined by a gamma counter. Ia is expressed as net cpm (specific minus nonspecific binding).

Assay for antigen uptake and catabolism by macrophages. (i) Intrinsically labeled live bacteria. Hly^+ and Hly^- strains of *L. monocytogenes* were grown overnight in BHI, washed three times with cold PBS, and suspended in 4 ml of cold PBS. A 1-ml amount of each suspension was added to 9 ml of RPMI 1640 (minus methionine) along with 0.1 ml (approx. 1 mCi) of ^{35}S-labeled methionine (ICN Radiochemicals, Irvine, Calif.). The bacteria were incubated while rotating for 6 h, washed three times with cold PBS, and suspended in 5 ml of cold PBS. This labeling procedure did not alter hemolysin production. Bacterial concentrations were determined with a spectrophotometer, and dilutions were made in cold culture medium. Dilutions of live bacteria (100 μl per well) were added to the macrophages. The plates were centrifuged (2,000 rpm, 5 min) and returned to the incubator for 10 min. The unbound bacteria were removed by washing, the plates were incubated at 37°C for various periods of time, and the supernatants and lysates were collected. Trichloroacetic acid (TCA) precipitation was performed on each sample. The amount of radioactivity associated with the soluble and precipitable fractions was determined as described previously (8). In some experiments the number of viable bacteria associated with macrophages was monitored by colony counts (37).

(ii) Extrinsically labeled HKLM. HKLM were surface labeled with ^{125}I by the chloramine T method (18, 52). Macrophages were treated with live Hly^+ and Hly^- *L. monocytogenes* and washed, and radiolabeled HKLM (10^5 cpm per well) were added 30 min later. The plates were centrifuged (2,000 rpm, 5 min) to speed adherence, and, after a 10-min incubation at 37°C, the plates were washed to remove unbound radiolabeled HKLM. The remainder of the assay was carried out according to the catabolism protocol described for the ^{35}S-labeled live bacteria.

Chromium release studies. PEC were plated at 2×10^5 per well (200 μl) in 96-well plates, with 2 μCi of ^{51}Cr added to each well. The plates were incubated at 37°C for 2 h, and the wells were washed. Dilutions of live bacteria were added to the appropriate wells. The plates were centrifuged (2,000 rpm, 5 min) and returned to the incubator. After 10 min the unbound bacteria were removed by washing, and the plates were incubated for a total of 90 min. At this time the supernatants and lysates were collected, and the amount of radioactivity associated with each was determined.

Preparation and quantitation of soluble hemolysin. *L. monocytogenes* were grown for 18 to 24 h at 37°C with constant aeration in BHI supplemented with 1% glucose. The bacteria were removed, and phenylmethylsulfonyl fluoride and EDTA were added to final concentrations of 100 mM and 500 mM, respectively. Proteins were precipitated by addition of solid ammonium sulfate (53 g/100 ml) and stirring for 8 to 12 h at 4°C (20). The precipitate was suspended in PBS (1/100 original volume) and dialyzed

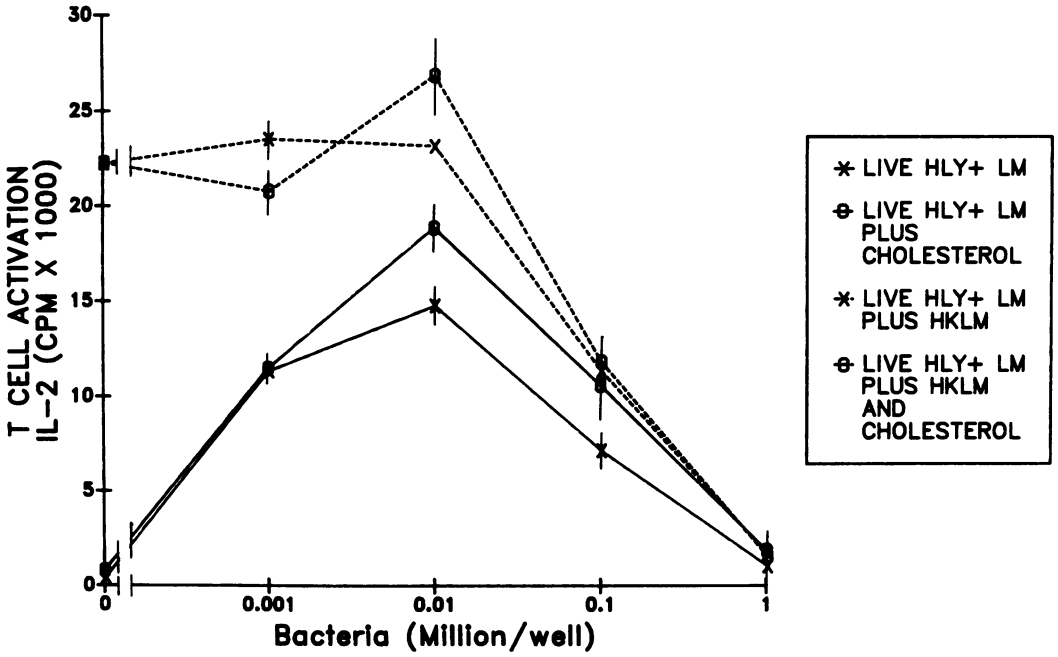


FIG. 1. Addition of cholesterol to macrophages treated with live Hly$^+$ *L. monocytogenes* does not prevent inhibition of antigen processing and presentation. The indicated numbers of Hly$^+$ *L. monocytogenes* (LM) were centrifuged onto macrophages in 100 μl of medium containing diluent or cholesterol (10 μg/ml). After 30 min, HKLM (10 μl of a 10^8/ml solution) (dashed lines) or medium alone (solid lines) was added to the appropriate wells, and the plates were centrifuged. After a 1-h incubation, the macrophages were fixed. T cells were then added, and IL-2 production was measured after 24 h. Values shown are means of duplicate wells ± SD.

against PBS containing 4 mM EDTA. The preparation was assayed for protein concentration and hemolytic activity.

A 90-μl amount of H_2O (100% lysis control) or hemolysin was added to the wells of a microtiter plate (V-bottomed; Flow Laboratories, McLean, Va.) along with 10 μl of 100 mM dithiothreitol, and the assay mixture was incubated (37°C, 10 min). Sheep erythrocytes were added to a final concentration of 1% and incubated at 37°C for 30 min. The erythrocytes were pelleted, and the supernatants were collected and assayed for the presence of hemoglobin by spectrophotometric analysis. One unit of hemolytic activity is defined as the minimum amount of hemolysin required to induce 50% lysis of the erythrocytes (compared with the H_2O control) and is expressed as the reciprocal of the dilution titer.

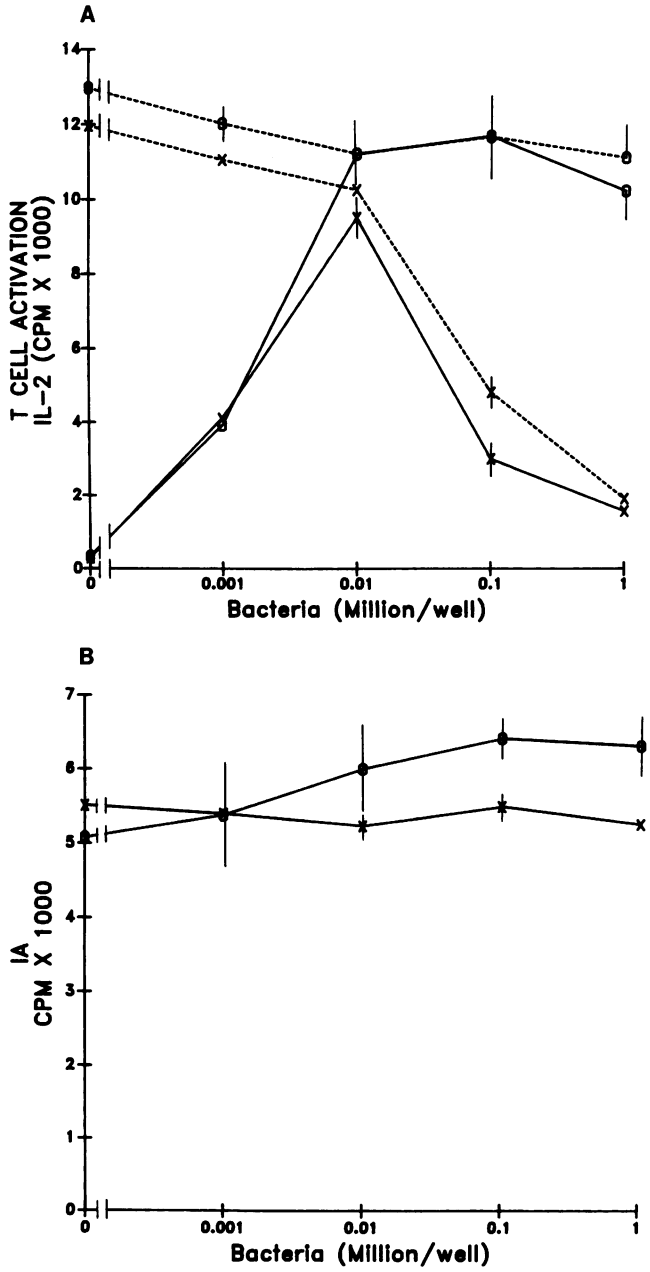
Cholesterol inactivation of hemolysin. Cholesterol (Sigma C-8253) was dissolved in absolute ethanol (2 mg/ml). This solution was diluted in culture medium and added to the appropriate wells (final concentration, 10 μg/ml) to inhibit activity of soluble hemolysin in vitro. An equal amount of ethanol alone in culture medium (diluent) was used as a control. We found that 10 μg of cholesterol per ml completely neutralized 900 U of hemolytic activity per ml.

RESULTS

We found that live *L. monocytogenes* inhibit macrophage-mediated antigen presentation in vitro and have shown that hemolysin production by these bacteria is responsible for the inhibition (9). Experiments were performed to determine whether the inhibition of antigen presentation was due to hemolysin produced by extracellular or intracellular bacteria. To approach this question, we took advantage of the finding that cholesterol irreversibly inactivates soluble hemolysin (6) (Materials and Methods). Cholesterol (10 μg/ml) or diluent (control) was included in the macrophage cultures with various numbers of live Hly$^+$ bacteria, and 30 min later HKLM were added to the appropriate wells. Macrophages were fixed 1 h later, anti-*L. monocytogenes* T cells were added, and IL-2 production was measured after 24 h. As shown in Fig. 1, cholesterol did not prevent the inhibition of antigen presentation caused by bacteria. Thus, the inhibition by hemolysin is expressed in a compartment inaccessible to cholesterol, presumably the intracellular environment of the macrophage.

This conclusion is supported by experiments with partially purified soluble hemolysin. While about 300 U of soluble hemolysin per ml inhibited antigen presentation by 50%, an inhibitory number of live bacteria (10^5 per well) produced <10 U/ml/h whether cultured in the presence or absence of macrophages. Furthermore, in contrast to the inhibition caused by live Hly$^+$ bacteria, the inhibition caused by soluble hemolysin was prevented by addition of 10 μg of cholesterol per ml, as indicated in the following results: macrophages plus HKLM gave 12,371 ± 1,188 cpm; macrophages plus hemolysin (300 U/ml) and HKLM gave 6,506 ± 512 cpm; and macrophages plus hemolysin, HKLM, and cholesterol gave 11,801 ± 1,358 cpm (mean ± standard deviation). We also considered the possibility that hemolysin released from bacterium-treated macrophages inhibited antigen presentation by acting on T cells or by inducing a transmissible suppression. However, the finding that addition of untreated macrophages to cultures of Hly$^+$ *L. monocytogenes*-treated, fixed macrophages relieved the inhibition of antigen presentation (data not shown) makes that unlikely.

Our next goal was to investigate the mechanism by which hemolysin inhibits antigen processing and presentation. One approach was to test the effects of these bacteria on macro-



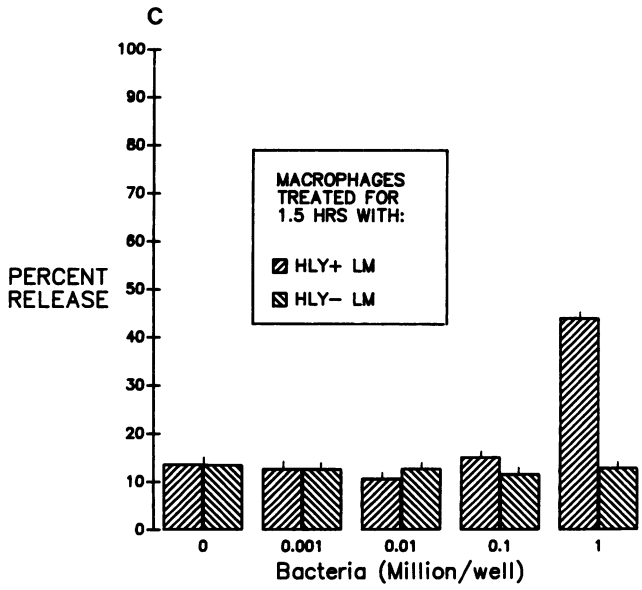


FIG. 2. Effects of live Hly$^+$ *L. monocytogenes* on macrophage functions important for effective antigen presentation. The indicated numbers of Hly$^+$ or Hly$^-$ *L. monocytogenes* (LM) were centrifuged onto macrophages in 100 μl of culture medium. The plates were incubated for 10 min to allow uptake, and the unbound bacteria were removed by washing. (A) Antigen presentation. HKLM (10 μl; 10^8/ml in culture medium) were added to the appropriate wells after 30 min, and the plates were centrifuged. The macrophages were fixed 1 h later, T cells were added, and IL-2 production was measured after 24 h. Symbols: ×, live Hly$^+$ bacteria; ○, live Hly$^-$ bacteria; ——, no HKLM; – – –, plus HKLM. (B) Ia expression. The macrophages were fixed after 90 min and assayed for Ia expression by radioimmunoassay as described in Materials and Methods. Symbols: ×, Hly$^+$; ○, Hly$^-$. (C) Chromium release. During the adherence period (prior to addition of live bacteria), macrophages were incubated with ^{51}Cr (2 μCi per well). The amount of chromium released into the supernatant and the quantity retained by the macrophages were determined 90 min after the live bacteria were centrifuged onto the monolayers. Percent release = (cpm released into the supernatant/total cpm taken up by macrophages) × 100. (D) Catabolism. Live Hly$^+$ and Hly$^-$ *L. monocytogenes* were intrinsically labeled with [^{35}S]methionine. Live bacteria (10^6 per well in 100 μl) were centrifuged onto the macrophages. Catabolism was measured by determining the amount of radioactivity associated with the TCA-soluble (sol) and -precipitable (ppt) fractions of the culture supernatants (supn) and macrophage lysates (lys) after various periods of time. Uptakes of these two strains were found to be similar: 39.8% of added cpm for Hly$^+$ and 38.5% for Hly$^-$ bacteria. For all experiments, values shown are means of duplicate wells ± SD.

phage catabolic activity, membrane permeability, Ia expression, and antigen presentation under identical conditions. Because inhibition was caused by intracellular bacteria and uptake was similar for Hly$^+$ and Hly$^-$ bacteria (Fig. 2D legend and below), this and subsequent experiments were performed by removing unbound live bacteria after a brief 10-min uptake period. Antigen presentation was inhibited by treatment with Hly$^+$ but not Hly$^-$ bacteria (Fig. 2A). Fifty percent inhibition of antigen presentation was noted when 10^5 bacteria were added per well. This dose corresponds to approximately 1 bacterium per macrophage.

The number of viable Hly$^+$ and Hly$^-$ bacteria present during this 1- to 2-h period of antigen processing and presentation was monitored. The number of viable bacteria associated with macrophages immediately after the 10-min uptake period was not significantly different for Hly$^+$ and Hly$^-$ bacteria. After 1.5 h in culture, a small but significant preferential survival and/or replication of Hly$^+$ bacteria was noted; 22% + 8% more viable Hly$^+$ than Hly$^-$ bacteria were observed. These relatively small differences cannot account for the major (10- to 100-fold) differences in antigen presentation when Hly$^+$ and Hly$^-$ bacteria are compared.

Ia expression (Fig. 2B) was not significantly decreased by treatment with Hly$^+$ bacteria. Interestingly, a small yet significant increase in Ia expression was noted with Hly$^-$ but not Hly$^+$ bacteria. Treatment of macrophages with inhibitory doses of Hly$^+$ bacteria (10^5 per well) did not alter macrophage permeability (Fig. 2C). It is clear from both these studies, as well as from microscopic observations of macrophage morphology (9, 37), that gross changes in macrophage membrane structure and viability are not observed

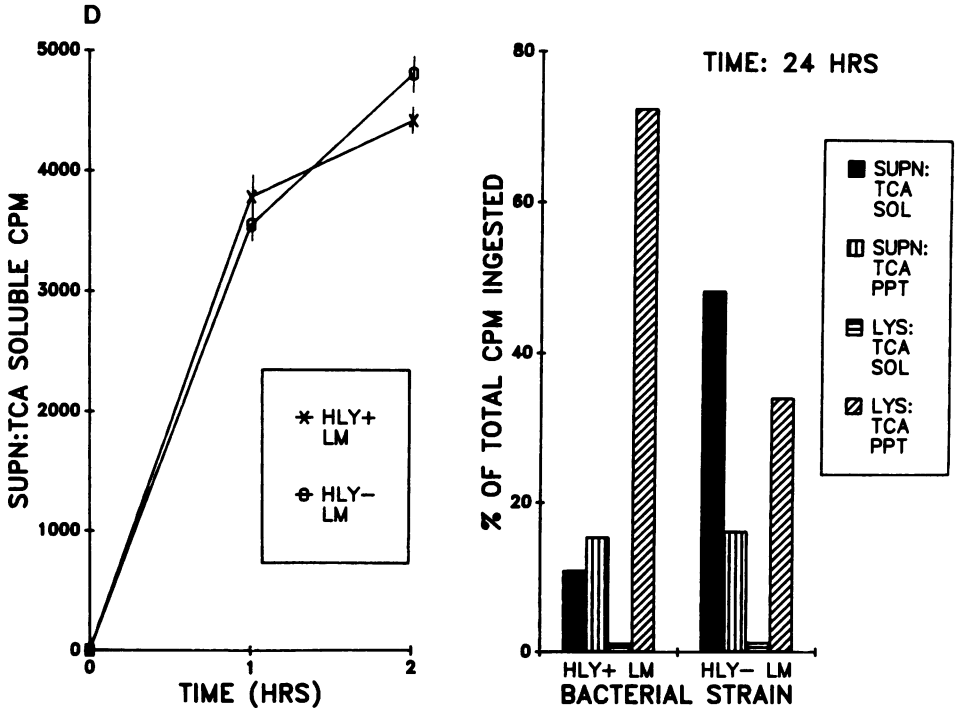


FIG. 2—*Continued*

at a 1:1 bacterium-macrophage ratio. Higher concentrations of *L. monocytogenes* (10^6 per well), however, did cause ^{51}Cr release from labeled macrophages, but it should be noted that the increased ^{51}Cr release (about 30% above background) caused by Hly^+ bacteria was observed only at bacterial numbers 10-fold higher than those that could produce inhibitory effects on antigen presentation.

To measure antigen uptake and catabolism, ^{35}S-labeled live *L. monocytogenes* and surface-iodinated HKLM were used as described in Materials and Methods. Live ^{35}S-labeled Hly^+ and Hly^- *L. monocytogenes* were centrifuged onto macrophages, and unbound bacteria were removed. TCA-soluble and -precipitable fractions of the culture supernatants and lysates were collected, and the radioactivity associated with each fraction was determined. This procedure allowed us to determine the uptake of live bacteria, as well as the amount of protein catabolized and released as TCA-soluble material. Our data indicate that after 2 h, there was no difference in either uptake (39.8% for Hly^+ and 38.5% for Hly^- bacteria) or catabolic activity (Fig. 2D) between macrophages treated with ^{35}S-radiolabeled Hly^+ or Hly^- *L. monocytogenes*. At much later times (24 h), macrophages cultured with Hly^+ bacteria retained about two times more TCA-precipitable material than those cultured with Hly^- bacteria.

Uptake and catabolism also were evaluated with surface-iodinated HKLM. The labeled HKLM were added to macrophages pretreated for 30 min with various numbers of live Hly^+ or Hly^- *L. monocytogenes*. The unbound radiolabeled bacteria were removed, and the fate of the radiolabel was monitored as described above. Only small inhibitory effects were observed. Pretreatment of macrophages with Hly^+ bacteria modestly inhibited uptake of HKLM, by 20% (Fig. 3A). Although both Hly^- (Fig. 3B) and Hly^+ (Fig. 3C) bacteria slightly inhibited the catabolism of ingested ^{125}I-labeled HKLM when added at 10^6 per well, only the Hly^+ strain caused a small inhibition of this activity when added at 10^5 per well.

Another approach to studying the mechanism of inhibition by hemolysin involved the use of antigens that differ in requirements for antigen processing. We used the T-cell hybridoma DO11.10, which recognizes an ovalbumin peptide (amino acids 323 to 339) in association with $I\text{-}A^d$. In contrast to the native protein, which requires processing, the peptide can be effectively presented to DO11.10 by fixed macrophages (Fig. 4). The other antigenic system used was HKLM and SLP; unlike HKLM, SLP can be presented by fixed macrophages as described previously (48).

Macrophages were exposed to various concentrations of live Hly^+ *L. monocytogenes* for 1.5 h, washed, and fixed. In one case, HKLM were present 1 h prior to fixation. In another protocol, T cells were added along with SLP after fixation. Macrophages pretreated with Hly^+ *L. monocytogenes* were inhibited from presenting HKLM, but once fixed, they were able to effectively present the antigens in the SLP preparation to the specific T cells (Fig. 5).

In another series of experiments, we determined whether presentation of the ovalbumin peptide would be inhibited when it was added prior to fixation. Macrophages were treated with live Hly^+ or Hly^- strains of *L. monocytogenes*, followed by addition of ovalbumin or ovalbumin peptide. The macrophages were fixed after 2 h, DO11.10 cells were added, and IL-2 production was measured after 24 h. The Hly^- strains caused only modest inhibition of ovalbumin presentation. The addition of $\geq 10^6$ Hly^+ bacteria per well almost completely inhibited the presentation of ovalbumin (Fig. 6A) yet only partially reduced the presentation of the ovalbumin peptide (Fig. 6B). The results obtained in many independent experiments with 10^5 bacteria per well are presented in Table 1. Hly^+ bacteria caused approximately 50% inhibition of ovalbumin presentation without a significant reduction in the response with the peptide.

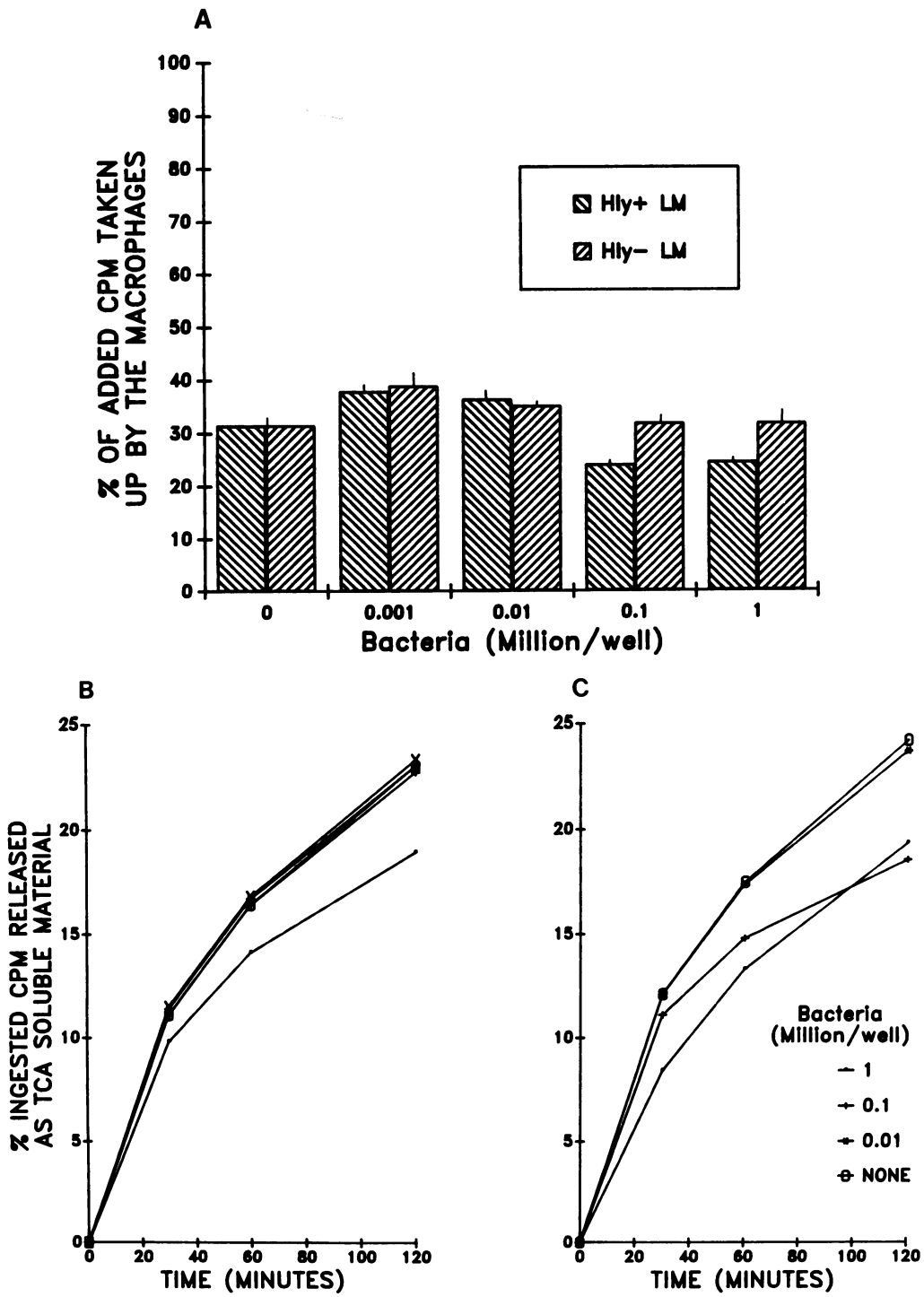


FIG. 3. Effect of live Hly$^+$ *L. monocytogenes* on the uptake (A) and catabolism (B and C) of ^{125}I-surface-labeled HKLM. Various numbers of live Hly$^+$ (A and C) or Hly$^-$ (A and B) *L. monocytogenes* (LM) were added to macrophages. The plates were centrifuged, and after a 10-min incubation, the unbound bacteria were removed. After 30 min, 2×10^5 cpm of labeled HKLM in 10 μl of culture medium was added to the macrophages, and the plates were centrifuged. Unbound labeled bacteria were removed by washing, and catabolic activity was measured as described in the legend to Fig. 2D. For antigen uptake, values shown are means of duplicate wells ± SD. For catabolism, individual values did not deviate more than 5% from any of the means.

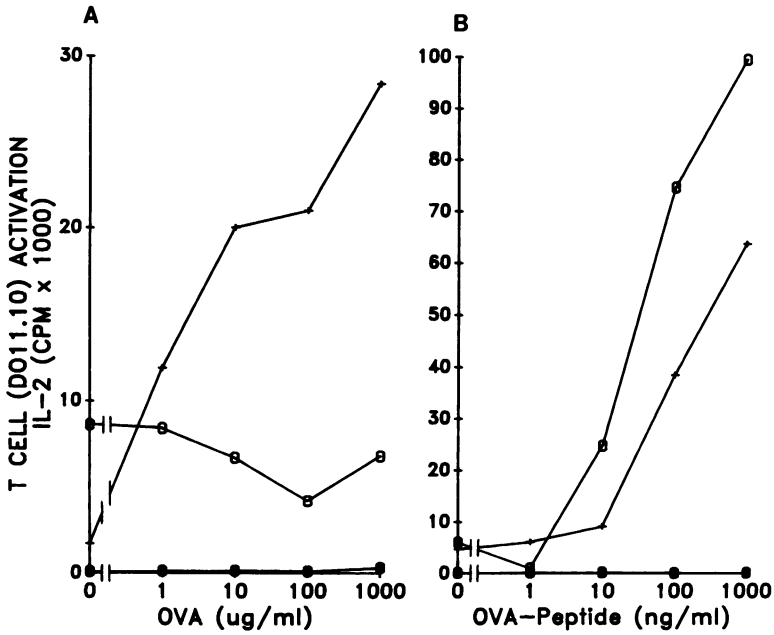


FIG. 4. Processing requirements for ovalbumin and ovalbumin peptide. The indicated concentrations of ovalbumin (OVA, panel A) or ovalbumin peptide (OVA-peptide, panel B) were added to normal (+) or fixed (○) macrophages in the presence of the T-cell hybridoma DO11.10. Cultures without added macrophages are indicated by solid circles. IL-2 activity in the culture supernatants was measured after 24 h. Individual values did not deviate more than 10% from any of the means.

We also obtained similar results with another antigenic system (22), hen egg lysozyme and a corresponding T-cell hybridoma (Hd-1.AC5). With 10^4, 10^5, and 10^6 Hly^+ *L. monocytogenes* bacteria per well, the percentage of control IL-2 values (control without bacteria = 6,820 cpm) were 88, 44, and 7%, respectively. Hly^- bacteria showed corresponding values of 87, 74, and 68%, respectively. Under similar conditions, presentation of a hen egg lysozyme peptide was relatively unaffected (≥76% of the control value).

Using the system involving presentation of ovalbumin, we tested a battery of bacterial strains and species (Fig. 7). Upon examination of different bacterial strains (at 10^6 bacteria per well, as shown in Fig. 7A) within the *Listeria* genus, a consistent correlation between hemolysin production and inhibition of antigen presentation was observed. In this experiment, the effects of 10^5 bacteria per well were also tested; with the Hly^+ *Listeria* strains, the percentages of control IL-2 values (control done with no bacteria) were 54 ± 19 with ovalbumin and 89 ± 10 with the ovalbumin peptide (mean ± standard deviation). One Hly^+ strain of another species, *L. ivanovii*, showed weak but significant effects on presentation, with IL-2 values of 72% of the control with ovalbumin and 103% of the control with the peptide.

From the examination of several bacterial strains not belonging to the *Listeria* genus (Fig. 7B), it was clear that the relationship between hemolysin production and inhibition of antigen presentation was dependent upon the bacterial strain. Notably, hemolysin-positive bacteria such as *Streptococcus pyogenes* and *Staphylococcus aureus* were not greatly inhibitory compared with similar numbers (10^6 per well) of *L. monocytogenes*. Hemolysin-positive *Escherichia coli*, however, were markedly inhibitory. Even at 10^5 bacteria per well, Hly^+ *E. coli* caused marked inhibition, showing IL-2 values of 22% of the control with ovalbumin and 81% of the control with the peptide. Also of note is the finding that a highly virulent smooth *Salmonella typhimurium* strain was not greatly inhibitory.

DISCUSSION

Relationship among virulence, hemolysin production, and inhibition of antigen presentation. The production of hemolysin by pathogenic strains of *L. monocytogenes* has been shown to promote their intracellular survival and replication in cultured macrophages (29, 37). Inhibition of antigen presentation, demonstrable during the first 90 min of macrophage-bacteria interaction, may also contribute significantly to the virulence of these bacteria by preventing T-cell activation. Inhibition of antigen presentation in vitro was associated with virulence in all *Listeria* strains tested (Fig. 7B). The use of antigen presentation assays as an indicator of bacterial virulence was also explored with several other bacterial strains (Fig. 7B). With non-*Listeria* strains, hemolysin production did not correlate precisely with inhibition of antigen presentation, and certain highly virulent strains (e.g., smooth *Salmonella typhimurium*) did not appear to greatly inhibit antigen presentation. The inhibitory effects of Hly^+ strains, however, did extend to gram-negative organisms in that Hly^+ *E. coli* caused dramatic inhibition of presentation. These differences among bacterial strains may relate to the different survival of these bacteria, quantitative differences in hemolysin production, differences in control of bacterial gene expression within the macrophage, and/or different properties of the hemolysins produced. In this latter regard, the hemolysin produced by *Streptococcus pyogenes*, while structurally and functionally similar to listeriolysin in many ways, does not share with listeriolysin the ability to alter membrane permeability in the low pH conditions that are present within the acidic intracellular compartments of the macrophage (16). It is also possible that bacteria differ in their response (e.g., increased hemolysin expression) to signals within the intracellular environment of macrophages. The precise nature of these signals, the mechanisms of regulation, and the role of these parameters in virulence and control of macrophage function remain to be determined.

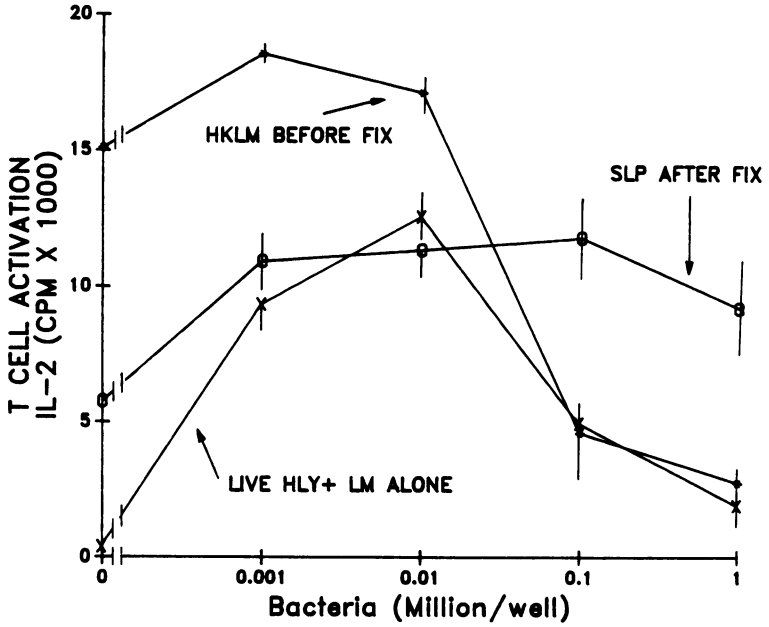


FIG. 5. Macrophages fixed after treatment with Hly$^+$ *L. monocytogenes* are able to present antigens that do not require processing. The indicated numbers of Hly$^+$ *L. monocytogenes* (LM) were centrifuged onto macrophages. After a 10-min uptake period, the unbound bacteria were removed. Thirty minutes after addition of live bacteria, 10 μl of medium alone (control) or medium containing 10^8 HKLM per ml was added to the appropriate wells. The plates were incubated for 90 min, and the macrophages were fixed. T cells alone or T cells plus SLP (50 μg/ml) were added to the appropriate wells, and IL-2 production was measured after 24 h. Values shown are means of duplicate wells ± SD.

Hemolysin production by *L. monocytogenes* has been shown to be important for generating protective immunity (5). This effect might be due to the activity of hemolysin as a principal antigen, an adjuvant, and/or a factor promoting bacterial survival and thus antigen duration (4). Our observations on inhibition of presentation do not conflict with these findings, since low numbers of Hly$^+$ bacteria can be presented effectively (9) (Fig. 1 and 2). As bacterial numbers are increased, a threshold is reached, and inhibition predominates. This phenomenon may mimic the situation in vivo after infection with lethal doses of bacteria or during uncontrolled bacterial growth. In keeping with this threshold effect, our recent studies with recombinant preparations of listeriolysin have revealed that it can serve as an antigen at concentrations almost 100-fold lower than those that cause inhibition of presentation (1a).

Site of inhibition by hemolysin. We used several experimental approaches to determine whether the hemolysin affecting antigen presentation is produced by intracellular or extracellular bacteria. Cholesterol, which inactivates hemolysin, did not prevent the inhibition when added to macrophages treated with live Hly$^+$ bacteria (Fig. 1). In addition, inhibitory numbers of bacteria (10^5 per well) cultured with or without macrophages did not produce enough soluble hemolysin to account for the inhibition observed with viable bacteria. Finally, when extracellular bacteria were removed after a 10-min uptake period, we observed no relief of inhibition (Fig. 2A). Although we cannot formally exclude some contribution by bacteria attached to the macrophage surface, overall the data indicate that intracellular bacteria are responsible for the inhibition. The finding that the hemolysin produced by *L. monocytogenes* is optimally hemolytic at pH 5.5 (16) is compatible with our suggestion that this toxin is delivered from within the acidic microenvironment of the phagosome.

Mechanism of inhibition. We tested the effects of live Hly$^+$ and Hly$^-$ *L. monocytogenes* on macrophage functions that contribute to antigen presentation, including Ia expression, maintenance of membrane integrity, antigen uptake and catabolism, and presentation of antigens that do not require processing. Under conditions that caused about 50% inhibition of antigen presentation (10^5 bacteria per well as in Fig. 1, 2A, and 5, and Table 1), membrane integrity was maintained (Fig. 2C), Ia expression remained normal (Fig. 2B), and macrophages treated with Hly$^+$ bacteria were able to present antigens that do not require processing (Fig. 5 and 6; Table 1). These results suggest that hemolysin produced by intracellular bacteria does not interfere greatly with the minimal membrane events required for effective antigen presentation.

The modest but significant inhibitory effects of $\geq 10^6$ Hly$^+$ bacteria on the presentation of the peptide (Fig. 6) may be due to effects on the proper display of macrophage surface ligands (e.g., Ia molecule display not revealed by anti-Ia antibodies as in Fig. 2B). Since known inhibitors of antigen processing caused slight but significant inhibition of peptide presentation (see legend to Fig. 6), it is also possible that peptide-Ia interactions are partially dependent upon intracellular events and an acidic compartment that is disrupted by Hly$^+$ bacteria. An understanding of the precise nature of this inhibition must await further studies.

The inefficient presentation of antigens associated with live Hly$^+$ bacteria cannot be explained by decreased uptake and/or catabolism by the macrophages, since no differences in these events were noted when live Hly$^+$ and Hly$^-$ bacteria were compared at culture times of <2 h (Fig. 2D). Similar observations of equivalent uptake of Hly$^+$ and Hly$^-$ bacteria were made in previous studies (29, 37). We did find that pretreatment of macrophages with live bacteria caused a modest decrease in both the uptake and catabolism of iodinated HKLM (Fig. 3). We are hesitant to conclude that the effect of live Hly$^+$ bacteria on HKLM uptake is solely

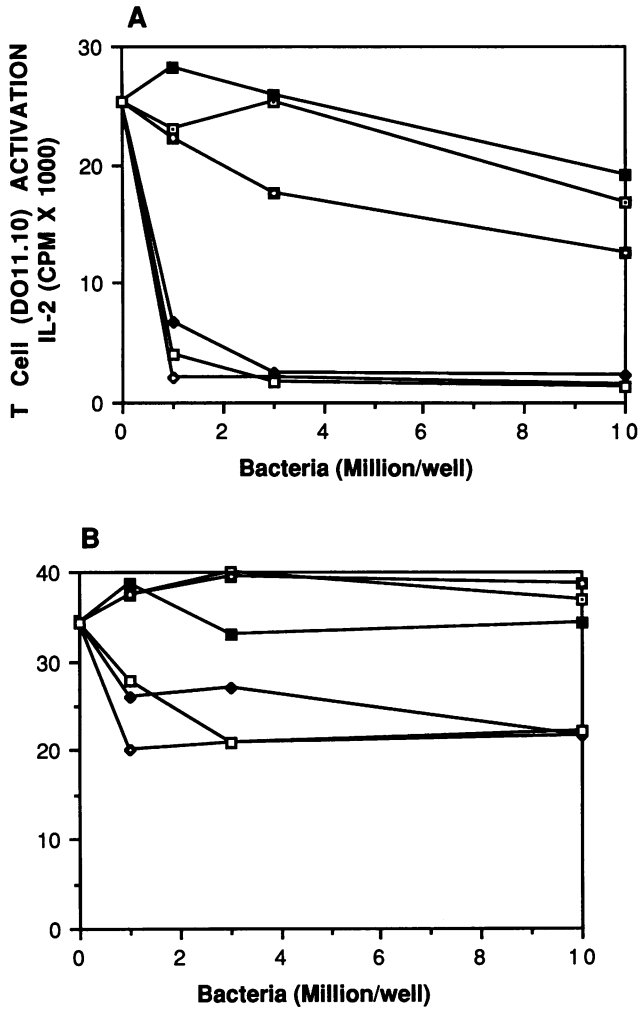


FIG. 6. Presentation of ovalbumin or ovalbumin peptide (amino acids 323 to 339) by macrophages treated with live Hly$^+$ or Hly$^-$ *L. monocytogenes* (LM). The indicated numbers of live bacteria were centrifuged onto macrophages. The unbound bacteria were removed by washing, and 1 mg of ovalbumin (A) or 1 μg of ovalbumin peptide (B) per ml was added in 100 μl of culture medium. The macrophages were fixed after 1.5 h. DO11.10 hybrid cells were added, and IL-2 production was measured after 24 h. Individual values did not deviate more than 10% from any of the means. The following strains of *L. monocytogenes* were used: ⊡, 43250 (Hly$^-$); ◆, 43251 (Hly$^+$); ■, CNL85/162 (Hly$^-$); ◆, CNL85/163 (Hly$^+$); ■, 43248 (Hly$^-$); and □, 43249 (Hly$^+$). In this experiment, chloroquine (10^{-4} M), ammonium chloride (10 mM), and monensin (5 μg/ml) were included as positive controls for inhibitors of antigen processing; these drugs showed IL-2 values of 9.9, 26, and 52% of control with ovalbumin and 75, 94, and 79% of control with the ovalbumin peptide, respectively (control was IL-2 activity with no drug).

responsible for the observed inhibition of antigen presentation, since a 20% decrease in antigen uptake cannot account entirely for the >50% inhibition of antigen presentation function. In addition, increasing the concentration of HKLM did not overcome the observed inhibition (data not shown). Because certain concentrations (10^6 per well) of Hly$^-$ *L. monocytogenes* inhibited catabolism to the same extent as Hly$^+$ bacteria yet did not diminish the ability of macrophages to present antigen (Fig. 2A and 6), it seems unlikely that the inhibition of antigen presentation observed with Hly$^+$ bacteria is due to a decrease in catabolic activity. The possibility exists, however, that Hly$^+$ bacteria differentially inhibit catabolic enzymes necessary for antigen processing and that this inhibition was not detected in our measurement of total protein degradation. It is also possible that the effects of hemolysin on several antigen-handling events can collectively account for the observed inhibition of antigen presentation. Clearly, further analysis is required to definitively identify the mechanism(s) of inhibition.

TABLE 1. Presentation of ovalbumin and ovalbumin peptide in the presence of 10^5 bacteria per well[a]

Condition	*L. monocytogenes* phenotype	Antigen	Presentation (% of control)
1	Hly$^-$	Ovalbumin	94 ± 14
2	Hly$^-$	Ovalbumin peptide	115 ± 11
3	Hly$^+$	Ovalbumin	52 ± 7
4	Hly$^+$	Ovalbumin peptide	99 ± 10

[a] Experimental conditions were as described in the legend to Fig. 7. The values were calculated relative to the IL-2 response observed without added bacteria (control). For conditions 1 and 2, $n = 6$; for conditions 3 and 4, $n = 12$. Values represent the mean ± standard error of the mean. Statistical analysis (Student's t test) of experimental conditions: 1 versus 2, $P = 0.26$; 1 versus 3, $P = 0.01$; 3 versus 4, $P = 0.001$; 2 versus 4, $P = 0.4$.

In a recent study, heat-killed bacteria (HKLM) were shown to inhibit the presentation of lysozyme antigen, with lesser effects on the presentation of a lysozyme peptide (30). With killed bacteria, under the conditions of our assay system, we also observed inhibition of ovalbumin presentation but not peptide presentation, but only at very high concentrations (10^8 per well) of HKLM (data not shown). This effect may be similar to that observed with live Hly$^-$ bacteria, as in Fig. 6, in which a small inhibition of ovalbumin but not of ovalbumin peptide presentation was noted at 10^7 bacteria per well. Thus, while our results are compatible with previous findings (30), it should be noted that the inhibitory effects with live Hly$^+$ *L. monocytogenes* are apparent at concentrations of bacteria 100- to 1,000-fold lower than those that caused the effects we observed with Hly$^-$ live bacteria or HKLM.

Because it is clear that Hly$^+$ bacteria inhibit the presentation of antigens that require processing more strongly than they inhibit that of antigens that do not require processing (Fig. 6, Table 1), a selective effect on an antigen-processing pathway is implied. Although the initial finding that catabolic activity correlated with antigen processing suggested lysosome involvement (52), it is probable that other acidic compartments exist which are used for antigen processing. In fact, our results can be best explained by the existence of separate pathways for antigen degradation and processing and a selective interference with the processing pathway by Hly$^+$ bacteria.

Evidence for at least two pathways for intracellular protein handling comes from the identification of functionally distinct subpopulations of endosomes involved in targeting internalized material to specific intracellular destinations (39). Creswell's findings suggest that early endosomes interact with an Ia-containing compartment within the macrophage (10). An acidic environment may be critical for early endosome function, since it allows the dissociation of receptor-ligand complexes. Perhaps the low pH of these early endosomes denatures ingested antigens and allows these antigens to associate with Ia in the absence of enzymatic fragmentation. We and others have found that nonenzymatic denaturing treatments can create processed antigen (1, 28, 42, 48). Thus, exposure of microbial products to the low pH

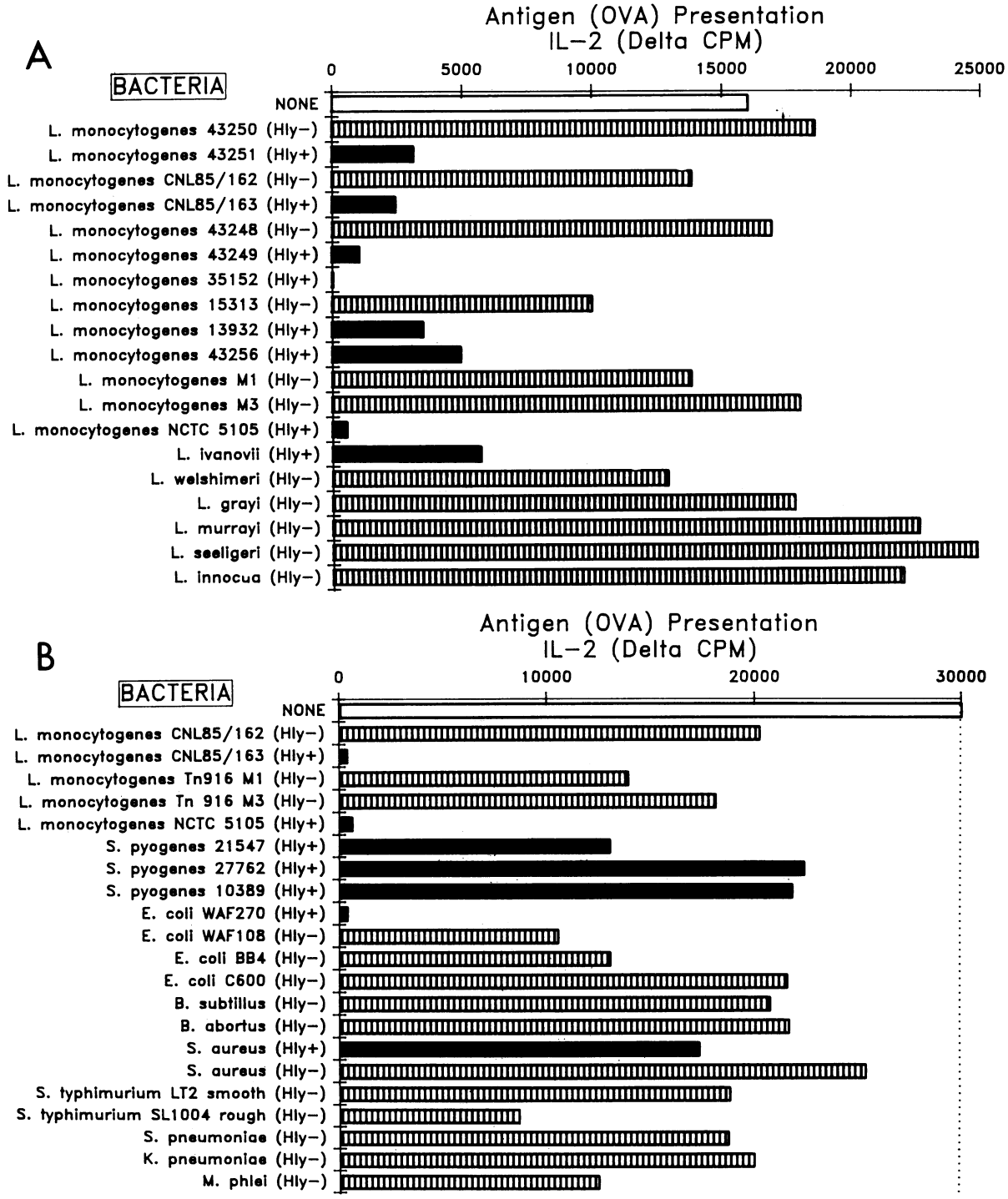


FIG. 7. Inhibition of antigen presentation by live bacteria. Presentation of ovalbumin by macrophages treated with various bacterial strains was tested. Live bacteria (10^6 per well) were centrifuged onto macrophages. The unbound bacteria were removed by washing, and ovalbumin (OVA, 1 mg/ml) was added in 100 μl of culture medium. The macrophages were fixed after 1.5 h. DO11.10 hybrid cells were added, and IL-2 production was measured after 24 h. Two representative experiments are shown in panels A and B. Each strain was judged to be hemolytic on sheep blood-agar plates. Solid bars are Hly^+ strains; hatched bars are Hly^- strains. *Streptococcus pneumoniae*, although judged to be Hly^-, does contain an intracellular pneumolysin. Most strains were obtained from the American Type Culture Collection. The CNL *L. monocytogenes* strains (9, 15) and the M1 and M3 strains (23) have been described previously. The WAF *E. coli* strains were from Rod Welch (43).

of early endosomes may define the processing event for some antigens (12). Additionally, exposure of Ia molecules to low pH may favor the association with processed antigen (22). Conceivably, hemolysins could inhibit processing by acting as an ion channel, thereby neutralizing the pH of this intracellular compartment. In this regard, hemolysin may be analogous to monensin, a known inhibitor of antigen processing. This idea is currently being tested.

The results of others suggest that hemolysin secreted by ingested bacteria can mediate the disruption of the phagosome membrane and promote bacterial replication in the cytoplasm (14, 37). It is possible that the escape of bacteria into the cytoplasm may allow bacterial antigens to bypass a presentation pathway involving class II MHC gene products (Ia molecules). In the cytoplasm, a class I MHC-controlled presentation may predominate (32), which might account for the class I-restricted $CD8^+$ cells noted in the immune response to *L. monocytogenes* (11, 25). Also relevant to the effects on intracellular antigen processing and presentation events is the reorganization of actin filaments that has been shown to occur in infected macrophages (42a). The previous findings that Ia expression increases after antigen uptake (2, 3), that protein synthesis is required for presentation (21), and that newly synthesized Ia and internalized molecules reside in the same intracellular compartment (10) are circumstantial evidence that Ia synthesis, intracellular Ia-antigen binding, and subsequent transport to the cell surface are important for effective antigen presentation. Thus, the possible effects of hemolysin-producing bacteria on these events must also be considered. Indeed, we have found that macrophages treated with Hly^- bacteria increased Ia expression by about 25% (Fig. 2B). In contrast, macrophages treated with Hly^+ bacteria did not show similar increases. Future experiments will be designed to determine whether these bacteria interfere with Ia synthesis, intracellular interactions between Ia and processed antigen, and/or transit of the complex to the cell surface.

In summary, we have provided evidence that hemolysin promotes the virulence of *L. monocytogenes* by inhibiting antigen processing. Our approach not only affords insight into the manner by which *L. monocytogenes* expresses its virulence factors, but also supplies clues regarding the mechanism of antigen processing and presentation.

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