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Parameters That Influence the Efficiency of Processing Antigenic Epitopes Expressed in *Salmonella typhimurium*

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We investigated parameters that affect the efficiency with which antigenic epitopes from *Salmonella typhimurium* are processed for presentation to T lymphocytes. As a model system, the hen egg white lysozyme 52-61 [HEL(52-61)] epitope, which binds the murine major histocompatibility complex class II (MHC-II) molecule I-A^k, was expressed in soluble fusion proteins in *S. typhimurium*. Murine peritoneal macrophages mediated phagocytic processing of viable *S. typhimurium* expressing fusion proteins of the HEL epitope for presentation via I-A^k regardless of the bacterial compartment in which the epitope was contained (i.e., surface exposed, facing the periplasmic space, or in the cytoplasm). Minor differences in processing efficiency observed with different epitope compartmentalizations could be overcome by altering the relative expression level, indicating that epitope abundance is an important factor for efficient processing of epitopes from *S. typhimurium*. This processing pathway required phagocytosis of bacteria followed by passage through an acidic compartment, suggesting a pathway involving phagolysosomal degradation of the bacteria to liberate epitopes that bind MHC-II. HEL(52-61) was processed more efficiently from heat-killed *S. typhimurium* than from viable bacteria, and in addition, the HEL epitope was processed more efficiently from a rough lipopolysaccharide (LPS) strain than from its isogenic smooth LPS counterpart, most likely because of enhanced phagocytosis of the rough LPS strain. These data suggest that the efficiency of epitope processing from *S. typhimurium* for presentation via MHC-II is affected by bacterial viability, epitope abundance, and LPS phenotype, factors which may be important to consider in development of recombinant *S. typhimurium* vaccine strains.

Antigen processing and presentation are central to the cascade of events involved in initiating both a humoral and a cell-mediated immune response to antigenic challenge. The presentation of exogenous antigens involves the uptake of antigen by specialized cells of the immune system, such as macrophages, which degrade the antigen into peptides that bind major histocompatibility complex class II (MHC-II) molecules and are presented at the cell surface for recognition (14, 16). Recognition of peptide-MHC-II complexes activates $CD4^+$ lymphocytes (T helper cells), which then secrete lymphokines that both generate cellular immune reactions and "help" B cells to produce antigen-specific antibody. $CD8^+$ T cells recognize and lyse cells expressing specific peptide-MHC-I complexes, which are generated by distinct pathways of antigen processing (18).

Macrophages are an important first line of defense against bacterial infections because of their phagocytic function, expression of bactericidal mechanisms, and capacity for antigen processing and presentation (16, 33). In addition, macrophages are "professional" antigen-presenting cells and process antigens for presentation via both MHC-I and MHC-II (16, 18, 33). *Salmonella typhimurium* is one example of a facultative intracellular bacterium that is capable of survival within macrophages (3, 6–8, 11, 12), and the survival of *S. typhimurium* within macrophages correlates with virulence in the murine model for typhoid fever pathogenesis (8, 11, 12, 23, 24). Unlike some intracellular bacteria, however, *Salmonella* organisms remain within a vacuolar compartment in the macrophage and do not escape into the cell cytosol (3, 7). *Salmonella* infection occurs by an oral route, and infection elicits both a humoral and cell-mediated immune response (22). Although killed preparations of *S. typhimurium* elicit a strong anti-*Salmonella* antibody response, the ability of such killed preparations to protect against subsequent challenge with virulent organisms is highly variable (reference 22 and references therein). However, viable preparations of *S. typhimurium* induce both humoral and cell-mediated immune responses and consistently confer protection against a subsequent challenge with virulent organisms (22). In light of this, live attenuated *S. typhimurium* strains have been developed and have been shown to elicit protection against a subsequent *S. typhimurium* challenge (21), and recombinant attenuated strains harboring foreign antigenic epitopes have been shown to elicit antigen-specific humoral and cell-mediated responses (1, 13, 26, 30, 32). Thus, there is great interest in understanding the parameters of *S. typhimurium* that are important in generating protective humoral and cell-mediated immune responses in the hopes of developing oral vaccine strains capable of conferring protection to one or more pathogens simultaneously.

The ability of *S. typhimurium* to survive and replicate within macrophages, the very cells responsible for processing and MHC-restricted presentation of epitopes to T lymphocytes, and its ability to modulate the harsh intracellular environment of the macrophage to enhance its survival (3, 7, 8, 11, 23, 24) may combine to diminish the ability of macrophages to effectively process and present *S. typhimurium* antigens. The current studies examine multiple parameters that may influence the processing of defined epitopes expressed in *S. typhimurium* for presentation to T hybridoma cells via MHC-II. Sequences

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from hen egg white lysozyme (HEL) containing the I-A^k-restricted 52-61 epitope (25) were fused to bacterial proteins so that the HEL epitope could be expressed as a soluble protein or as a membrane protein with the epitope exposed in various compartments of *S. typhimurium*. This system was used to examine the effect of epitope abundance and compartmentalization within *S. typhimurium* on the efficiency of epitope processing. In addition, we tested the roles of bacterial viability and lipopolysaccharide (LPS), a virulence factor of *S. typhimurium* (15). Knowledge of the parameters that influence the efficiency of processing and presentation of epitopes expressed in *S. typhimurium* may have important ramifications for development of recombinant *S. typhimurium* vaccine strains.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Standard recombinant DNA techniques were used throughout (31). The plasmids pLamBHEL153, pLamBHEL183, and pJLP-1E have been previously described (29, 34). Either plasmids were transformed into *Salmonella* strains after passage of the DNA isolated from *Escherichia coli* cloning hosts through the $r^- m^+$ *S. typhimurium* SH9312 (9) or plasmids isolated from *E. coli* were electroporated into *S. typhimurium* as described before (10). Growth and induction of *S. typhimurium* expressing the LamBHEL fusion proteins were done as previously described (34). Briefly, strains were grown overnight at 37°C in Luria broth containing carbenicillin (100 μg/ml). These strains were subcultured in the same medium to an optical density at 600 nm (OD_{600}) of 0.1, grown to an OD_{600} of ~0.8, and induced with either 0.4 or 0.8 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 45 min. The OD_{600} of the cultures was determined, and the cells were pelleted and resuspended to 10^9 cells per ml in phosphate-buffered saline (PBS, pH 7.4) containing 0.04% glucose and 10 mM $MgSO_4$. *S. typhimurium* strains expressing the Crl-HEL fusion protein from pJLP-1E were grown on Luria agar plates supplemented with carbenicillin (100 μg/ml). Cells were removed from the plates by washing with PBS (pH 7.4) containing 0.04% glucose and 10 mM $MgSO_4$ and quantitated spectrophotometrically by determining the OD_{600}; cells were resuspended to 10^9/ml. An aliquot of cells was used in the assay for MHC-II-restricted antigen processing and presentation, and another aliquot was used to quantitate the HEL epitope by enzyme-linked immunosorbent assay (ELISA) as previously described (34).

To test the function of LamB fusion proteins expressed in *Salmonella* strains, sensitivity to bacteriophage lambda was determined by a plate plaque assay on *S. typhimurium* SH9312 (9) (rough LPS) harboring pLamBHEL153, pLamBHEL183, or a plasmid encoding wild-type LamB as a control, as previously described (34).

Derivation and characterization of rough LPS *S. typhimurium* strains. A rough LPS derivative of the smooth LPS strain 14028 was made by selecting *S. typhimurium* strains that were resistant to lysis by bacteriophage P22. P22-resistant colonies were passaged several times on Luria agar plates and were then tested for their LPS phenotype by using a series of smooth- or rough-LPS-specific bacteriophage (35). A strain considered to have rough LPS was resistant to the smooth-LPS-specific phage P22c2 and was sensitive to the rough-LPS-specific phage Br60 (35), indicating that the O antigen chains were removed but that the core polysaccharide and lipid A moieties were intact.

Quantitation of the HEL epitope. ELISA was used to quantitate the amount of HEL epitope in cultures induced for expression of the LamBHEL fusion proteins or from cells removed from agar plates as previously described (34). Briefly, cells at 10^9/ml were centrifuged, and the pellet was resuspended in PBS (pH 7.4) and lysed by sonication on ice. Unbroken cells were removed by centrifugation, and the total protein content of the cleared sonicates was determined by the bicinchoninic acid protein determination system (Sigma Chemical Co., St. Louis, Mo.). All samples were standardized to the same total protein concentration, serially diluted in PBS (pH 7.4), and plated in triplicate in 96-well microtiter plates (Nunc Immunosorp). To determine the relative abundance of the HEL epitope in various strains, ELISAs were carried out as described before (20) with primary antiserum raised in rabbits to synthetic HEL(34-61) peptide conjugated to ovalbumin. Prior to use in the ELISA, the primary antiserum was first adsorbed with an acetone powder (20) made from *S. typhimurium* 14028. Secondary antiserum was anti-rabbit immunoglobulin G-peroxidase conjugate (Sigma). The background reactivity of the *S. typhimurium* vector control was subtracted from all test sample values prior to determining the relative abundance of the HEL epitope.

Antigen-processing assay. Phagocytic processing of bacteria expressing a HEL fusion protein for MHC-II-restricted antigen presentation was quantitated as previously described (29, 34). Briefly, peritoneal macrophages were elicited in CBA/J (*H-2^k*) mice (The Jackson Laboratory, Bar Harbor, Maine) by intraperitoneal injection with *Listeria monocytogenes* (19) and collected 7 to 14 days later. Bacteria were added to 2×10^5 adherent macrophages in antibiotic-free medium, and after gentle centrifugation (1,000 × *g* for 5 min), the cultures were incubated at 37°C for the times indicated for individual experiments. After being washed, the cells were fixed in 1% paraformaldehyde for 15 min and extensively washed. A total of 10^5 3A9 T hybridoma cells, which are specific for the HEL(52-61) epitope and secrete interleukin-2 (IL-2) upon specific recognition of the HEL(52-61)/I-A^k complex (2, 25), were added for 24 h; IL-2 production by the 3A9 T hybridoma cells was quantitated by using IL-2-dependent CTLL cells.

Quantitation of macrophage-associated bacteria. Bacteria were grown on agar plates, resuspended at 10^9 cells per ml in PBS (pH 9.1), and labeled with the fluorescent dye 5(6)-carboxyfluorescein-*N*-hydroxysuccimide ester as previously described (29). Labeled bacteria were incubated with adherent macrophages on glass coverslips with the specific conditions indicated in footnote *a* of Table 1 and were then fixed in 2% paraformaldehyde in PBS (pH 9.1). Coverslips were then extensively washed in PBS and mounted on glass slides in 50% glycerol in PBS (pH 9.1) prior to examination under a fluorescence microscope. Macrophage-associated bacteria were quantitated by determining the average number of bacteria associated with 50 individual macrophages per experiment.

RESULTS

Processing of HEL(52-61) from *S. typhimurium* 14028 for presentation to T cells via MHC-II requires phagocytosis and lysosomal acidification. Murine macrophages mediated processing of the soluble Crl-HEL fusion protein expressed in the cytoplasm of *S. typhimurium* 14028, with maximal presentation of the HEL(52-61) epitope occurring after 2 h of coincubation of bacteria and macrophages (Fig. 1A). In addition, incubation of adherent macrophages with cytochalasin D, a compound that interferes with actin polymerization and inhibits the phagocytic capacity of macrophages, abolished processing of Crl-HEL expressed in *S. typhimurium*/pJLP-1E (Fig. 1B), whereas cytochalasin D did not inhibit the processing of soluble HEL (Fig. 1C). These data demonstrate that process-

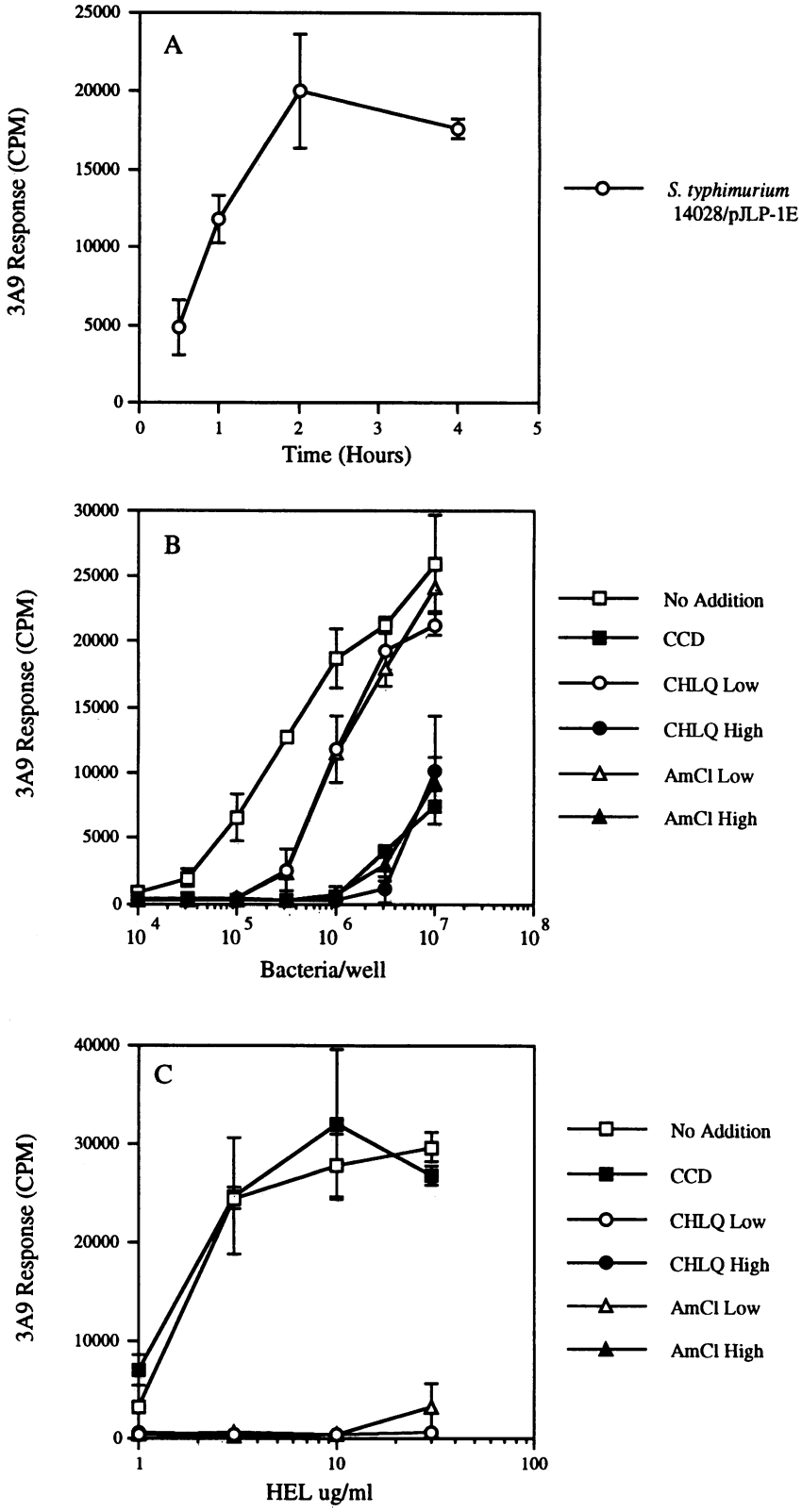

FIG. 1. Processing of HEL(52-61) expressed in *S. typhimurium* 14028 for MHC-II-restricted presentation to 3A9 T hybridoma cells. (A) Kinetics of processing of HEL(52-61) expressed in the cytosol of *S. typhimurium* 14028 from the Crl-HEL fusion protein encoded by pJLP-1E. (B) Effect of inhibitors of phagocytosis or lysosomal acidification on processing of HEL(52-61) from *S. typhimurium* 14028/pJLP-1E. (C) Effect of inhibitors of phagocytosis or lysosomal acidification on processing of HEL(52-61) from soluble lysozyme. For panels B and C, adherent macrophages were incubated with the inhibitors for 30 min prior to addition of bacteria; the inhibitors were then continuously present during the 2-h incubation. The inhibitors used were cytochalasin D (CCD) at 5 μg/ml, chloroquine (CHLQ) at either 390 μM (CHLQ High) or 39 μM (CHLQ Low), and ammonium chloride (AmCl) at either 50 mM (AmCl High) or 10 mM (AmCl Low). Datum points represent the means for triplicate samples. Error bars represent ± 1 standard deviation for triplicate samples.

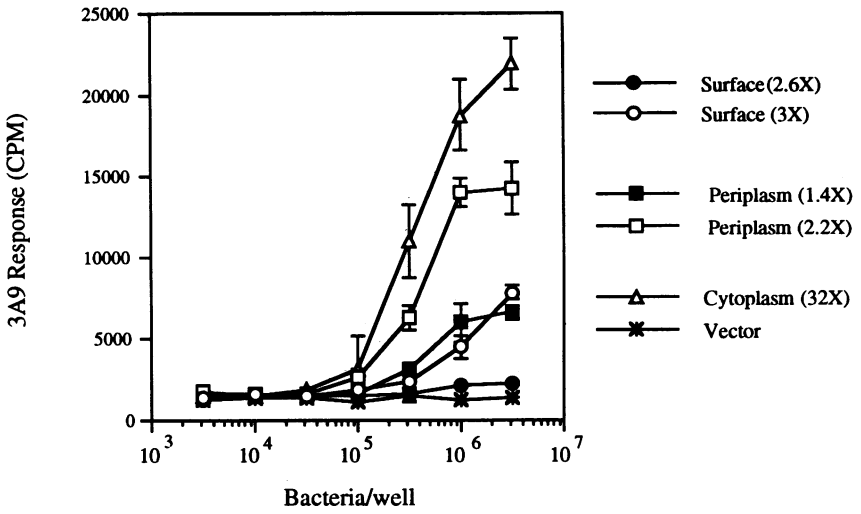

FIG. 2. Effect of epitope compartmentalization on processing of HEL(52-61) from *S. typhimurium* 14028 for presentation via MHC-II. Bacteria carrying either pLamBHEL153 (surface-exposed epitope) or pLamBHEL183 (periplasm-exposed epitope) were grown and induced for LamBHEL expression with either 0.4 mM IPTG (solid circles and squares) or 0.8 mM IPTG (open circles and squares) as described in Materials and Methods; *S. typhimurium* constitutively producing cytoplasmic Crl-HEL from pJLP-1E (open triangles) was grown on agar plates. Bacteria were incubated with macrophages for 2 h before antigen processing was stopped. The 3A9 T hybridoma response to *S. typhimurium* 14028 expressing surface-exposed HEL in LamBHEL153 (circles), periplasmic HEL in LamBHEL183 (squares), or cytoplasmic HEL in Crl-HEL (open triangles) is shown. The response to *S. typhimurium* harboring the vector only is also shown. The relative abundance of the HEL epitope as determined by quantitative ELISA is indicated in parentheses. Datum points represent the means for triplicate samples. Error bars represent ± 1 standard deviation for triplicate samples.

ing of Crl-HEL from *S. typhimurium* requires phagocytosis of intact bacteria and that the observed presentation of the HEL epitope shown in Fig. 1A is not due to the release of soluble Crl-HEL or peptide from extracellular bacteria. To further characterize the processing pathway, the effects of inhibitors of vacuolar acidification on processing of Crl-HEL from *S. typhimurium* were examined. Chloroquine and ammonium chloride both effectively inhibited processing of Crl-HEL from *S. typhimurium*/pJLP-1E (Fig. 1B). Similarly, both compounds inhibited processing of the HEL(52-61) epitope from soluble lysozyme (Fig. 1C) (29). Taken together, these data suggest that processing of epitopes from *S. typhimurium* occurs by phagocytic internalization and subsequent phagolysosomal degradation of the bacteria to release peptides. The peptides generated in this process may then associate with MHC-II molecules either in the phagolysosomal compartment (17) or in other compartments along the endocytic pathway.

Parameters influencing the level of epitope presentation: the role of epitope abundance and compartmentalization. Previous reports suggested that the localization of an epitope within *S. typhimurium* may influence the efficiency of its processing for MHC-II presentation (4). We previously determined that only minor differences in the efficiency of phagocytic processing for T-cell stimulation occurred for the same epitope expressed either in the bacterial cytoplasm, in the periplasm, or on the surface of *E. coli* (34). To test the effect of bacterial compartmentalization of epitopes expressed in *S. typhimurium*, we used fusion proteins to the outer membrane protein LamB, constructed so that the epitope is expressed either at the bacterial surface (LamBHEL153) or facing the periplasmic space (LamBHEL183) (28, 34). The function of the fusion proteins expressed in *Salmonella* strains was assessed by determining the sensitivity of rough LPS strains induced for LamBHEL153 and LamBHEL183 expression to bacteriophage lambda as previously described (27, 34). The sensitivity to lambda was identical to that observed with the same fusion proteins expressed in *E. coli*, suggesting that the LamB fusions function in *Salmonella* strains without gross structural or functional alteration (data not shown) (27, 28, 34).

Figure 2 demonstrates that fusion proteins containing the HEL epitope are processed for presentation via MHC-II regardless of the compartmentalization of the epitope in *S. typhimurium*. However, minor differences in the efficiency of processing were evident with the different constructs. Notably, the epitope facing the periplasmic space was processed more efficiently than the epitope exposed on the surface of the bacterium, given approximately equal expression (Fig. 2). Furthermore, the relationship that approximately two to four times more surface-exposed HEL(52-61) is required to stimulate 3A9 cells to the same level extends over a broad stimulation range of 3A9 cells (34) (Fig. 2). It is unclear, however, if the observed differences in antigen-processing efficiency occurred because of epitope compartmentalization or local protein context of the epitope. Importantly, the minor differences observed in the efficiency of processing the epitope expressed in different bacterial compartments could be overcome by increasing the relative amount of epitope per bacterium. We previously showed that relative amounts of antigen expressed in the cytoplasm of *E. coli* ranging from two to four times is sufficient to give significant 3A9 stimulation (34). In the present study, we chose to use a cytoplasmic construct giving maximal amounts of antigen simply to illustrate the magnitude of the T-cell response under conditions in which maximal antigen abundance is used in our system (Fig. 2). Taken together, these data demonstrate that expressing a defined epitope in a particular compartment of *S. typhimurium* does not necessarily convey an advantage for efficient processing, but rather that epitope abundance seems to be a key factor affecting the efficiency of processing epitopes for MHC-II presentation. Such an observation has relevance for the design and development of recombinant *S. typhimurium* vaccine strains.

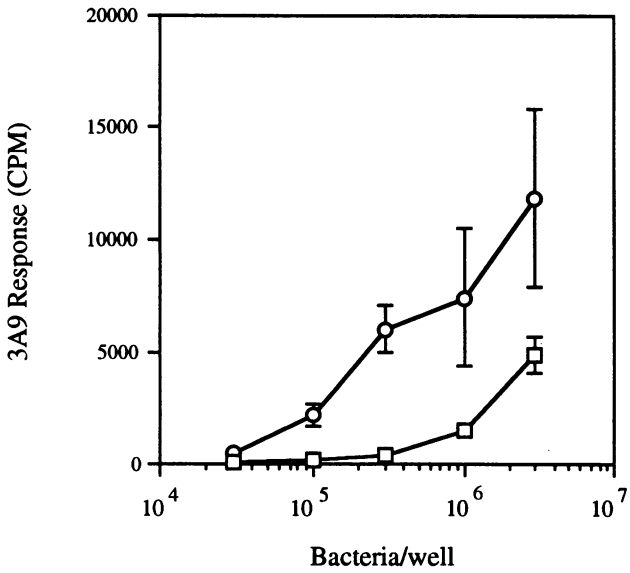

FIG. 3. Effect of LPS phenotype on the efficiency of processing of HEL(52-61) from *S. typhimurium* expressed as cytoplasmic Crl-HEL. The 3A9 T hybridoma response to epitope processed from *S. typhimurium* 14028/pJLP-1E with smooth LPS (open squares) or *S. typhimurium* 14028/pJLP-1E with rough LPS (open circles) is shown; bacteria were incubated with macrophages for 2 h before antigen processing was stopped. Datum points represent the means for triplicate samples. Error bars represent ± 1 standard deviation for triplicate samples.

Role of LPS. The above studies determined that epitope abundance is an important factor influencing the efficiency of processing epitopes expressed in *S. typhimurium* for MHC-II presentation and that epitope expression in a particular bacterial compartment does not necessarily convey an advantage for processing efficiency. We next determined the role of bacterial LPS in antigen-processing efficiency by examining the processing of HEL(52-61) from Crl-HEL expressed in strain 14028 with smooth LPS and its isogenic rough LPS counterpart. As Fig. 3 shows, significantly greater amounts of epitope were processed for presentation via MHC-II from 14028/pJLP-1E with rough LPS than from the strain with smooth LPS despite equivalent amounts of epitope being expressed in these strains, as determined by quantitative ELISA (data not shown). However, approximately 4 to 10 times more bacteria with rough LPS than bacteria of the same strain with smooth LPS were phagocytosed (Table 1). Thus, relatively better phagocytosis of the rough LPS strain than of the smooth strain most likely accounts for the more efficient processing of HEL(52-61) from 14028/pJLP-1E with rough LPS.

Role of bacterial viability. A previous report has demonstrated that, compared with viable bacteria, heat-killed *S. typhimurium* organisms are less able to efficiently buffer pH drops within phagolysosomes during the initial 2 to 3 h after phagocytic uptake by macrophages (3). Since we had already shown that phagolysosomal acidification is required for efficient processing of Crl-HEL expressed in *S. typhimurium* (Fig. 1B), we next examined whether bacterial viability influenced antigen-processing efficiency by comparing the processing of Crl-HEL expressed in live and heat-killed 14028 with rough LPS. Figure 4 demonstrates that Crl-HEL is processed more efficiently from the heat-killed bacteria. This result was not due to differential phagocytosis of the strains, as approximately

TABLE 1. Quantitation of macrophage-associated bacteria[a]

Strain	Condition	LPS phenotype	Mean no. of bacteria/macrophage ± SD	
			Expt 1	Expt 2
14028/pUC18	Live	Smooth	7.5 ± 4.7	2.7 ± 2.8
14028/pJLP-1E	Live	Smooth	8.3 ± 4.9	1.6 ± 1.8
14028/pJLP-1E	Live	Rough	35 ± 10[b]	19 ± 7.1[c]
14028/pJLP-1E	Killed	Rough	43 ± 19[b]	20 ± 11[c]

[a] Bacteria were fluorescently labeled as described in Materials and Methods and incubated with adherent macrophages for 30 min at a bacterium-to-macrophage ratio of either 6:1 (experiment 1) or 10:1 (experiment 2). "Killed" bacteria were heat killed by incubating bacterial suspensions at 65°C for 20 min prior to addition to macrophages. Values are expressed as the mean number of bacteria associated per individual macrophage ± the standard deviation. A minimum of 50 macrophages were counted in each experiment. There was no statistically significant difference between the levels of uptake of the live and killed rough LPS bacteria in either experiment 1 or 2.

[b] $P < 0.005$ compared with 14028/pJLP-1E live, smooth LPS in experiment 1.

[c] $P < 0.005$ compared with 14028/pJLP-1E live, smooth LPS in experiment 2.

equal numbers of live and heat-killed rough LPS bacteria were associated per macrophage (Table 1). Nor was this result due to different levels of epitope abundance, as quantitative ELISA revealed that equivalent amounts of epitope were present in the live and heat-killed preparations (data not shown). In addition, control experiments revealed that the presence of cytochalasin D during the incubation of heat-killed *S. typhimurium* with macrophages completely abolished the antigen-specific response (data not shown), demonstrating that the increased response from the heat-killed bacteria was not due to spontaneous release of antigen which was then processed

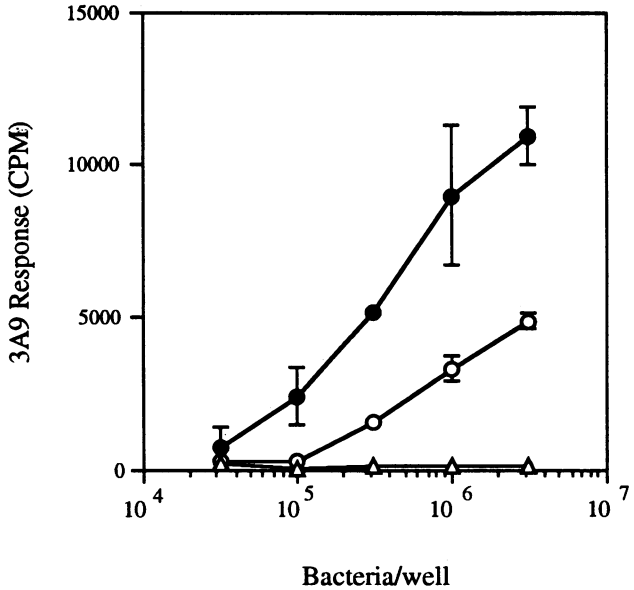

FIG. 4. Effect of bacterial viability on the efficiency of processing of HEL(52-61) from *S. typhimurium* expressed as cytoplasmic Crl-HEL. The 3A9 T hybridoma response to epitope processed from viable *S. typhimurium* 14028/pJLP-1E (open circles) or heat-killed *S. typhimurium* 14028/pJLP-1E (solid circles) is shown; both strains have rough LPS. The response to *S. typhimurium* harboring the vector only is also shown (open triangles). Bacteria were incubated with macrophages for 3 h before antigen processing was stopped. Datum points represent the means for triplicate samples. Error bars represent ± 1 standard deviation for triplicate samples.

through a nonphagocytic route. The better processing observed for the heat-killed bacteria could be due to their inefficient buffering of phagolysosomal pH (3), resulting in liberation of bacterial epitopes from killed bacteria more readily than from live ones. Alternatively, this result could also be due to the enhanced ability of macrophages to degrade previously killed bacteria, independent of any effects that the bacteria might have on phagolysosomal pH.

DISCUSSION

Previous studies on phagocytic processing of *S. typhimurium* revealed that epitopes can be processed by murine macrophages for presentation via MHC-I (28) or by human monocytes for presentation by MHC-II (4). Although processing of epitopes expressed in *S. typhimurium* for presentation by MHC-II has previously been demonstrated (4), these studies were performed with an epitope expressed in an insoluble form in inclusion bodies. Since processing of insoluble proteins can be inefficient (34; unpublished data), many questions remain regarding factors that may influence the processing of an epitope, including its localization within the bacteria and the characteristics of the bacteria. The current study was undertaken to systematically define the roles of epitope abundance and compartmentalization as well as the roles of bacterial viability and LPS phenotype in the efficiency of processing of epitopes expressed in *S. typhimurium* for MHC-II presentation.

Our studies revealed that HEL(52-61) expressed within soluble fusion proteins in any bacterial compartment is efficiently processed from *S. typhimurium* within the first few hours for presentation via MHC-II by a mechanism that requires phagocytosis and passage through an acidic compartment. These results provide an explanation, and indicate a mechanism, for prior observations that high-titer antibody responses can be produced in mice in vivo after inoculation with *S. typhimurium* expressing foreign antigenic epitopes cytoplasmically (5, 30). Similar mechanisms appear to be involved in the processing of HEL(52-61) expressed in the cytoplasm of *E. coli* (29) as well as the processing of an epitope from an insoluble form of influenza virus nucleoprotein expressed in an *aroA* mutant strain of *S. typhimurium* (4). Our model system allows the study of only one aspect of the interaction between the bacteria and peritoneal macrophages and does not address the many other factors that contribute to the eventual immune response in the course of murine infection with *S. typhimurium*. Clearly, in vivo studies are required to directly assess the role of epitope abundance, compartmentalization, and bacterial LPS phenotype on induction of both humoral and cell-mediated immune responses to epitopes expressed in *S. typhimurium*.

We have also demonstrated that epitopes are processed more efficiently from heat-killed bacteria than from viable bacteria (Fig. 4). This in vitro demonstration of efficient processing of epitopes expressed in killed *S. typhimurium* for presentation to $CD4^+$ T lymphocytes is consistent with the in vivo observation that killed preparations of *S. typhimurium* stimulate vigorous antibody responses in mice (reference 22 and references therein). Epitopes present in bacteria killed prior to phagocytosis may be more readily liberated to bind MHC-II than epitopes present in viable *S. typhimurium*, which has some capacity to survive in vacuolar compartments after phagocytosis (6–8, 11, 12, 24), limiting the extent of antigen processing. However, it is also interesting that the result demonstrating the requirement for passage through an acidic compartment for efficient processing of Crl-HEL from *S. typhimurium* is consistent with the result showing more efficient processing from heat-killed than from viable salmonellae, inasmuch as it has been shown that viable *S. typhimurium* cells are capable of buffering pH drops for at least the first 2 to 3 h within the phagolysosome (3). The less efficient processing from the viable bacteria suggests that blocking phagolysosomal acidification may be viewed as a virulence factor on at least two levels. First, a more neutral pH likely contributes directly to bacterial survival, and second, a more neutral pH hinders efficient antigen processing and presentation, thus inhibiting development of a specific immune response.

We investigated the role of LPS phenotype in the efficiency of antigen processing from *S. typhimurium*. Bacterial LPS influenced the efficiency of processing of the HEL epitope, as Crl-HEL was processed more efficiently from *S. typhimurium* with rough LPS than from its smooth LPS counterpart (Fig. 3). The increased processing of the rough LPS strain was probably due to increased phagocytosis by macrophages (Table 1), as viable cell counts of macrophage-associated bacteria paralleled the increased phagocytosis of the rough strain (data not shown), suggesting that intracellular survival within the time frame examined here is similar between the smooth and rough LPS strains. Thus, the increased processing of the rough strain is most likely due to enhanced phagocytosis, although a minor contribution to more efficient processing of the rough strain by altered intracellular survival and intracellular processing efficiency cannot be completely excluded. Likewise, opsonization of *S. typhimurium* enhanced antigen processing (4), although this could also have been due to extracellular lysis of bacteria, as inhibition of antigen processing by cytochalasin D was not demonstrated.

The data presented here reveal that epitope abundance, LPS phenotype, and bacterial viability influence the efficiency of antigen processing from *S. typhimurium* for MHC-II-restricted presentation, whereas localization of epitope expression within *Salmonella* cells has relatively minor effects on antigen processing. Thus, previously identified virulence factors of *S. typhimurium*, such as smooth LPS (15), may have a role in influencing the efficiency with which macrophages process antigenic epitopes from this organism. This may provide a mechanism by which *Salmonella* strains reduce the effectiveness of an immune response and prolong their survival within the host.

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