Infection with *Listeria monocytogenes* Impairs Sialic Acid Addition to Host Cell Glycoproteins

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Summary

Listeria monocytogenes is a facultative intracellular bacterium that causes severe disease in neonates and immunocompromised adults. Although entry, multiplication, and locomotion of Listeria in the cytosol of infected cells are well described, the impact of such infection on the host cell is unknown. In this report, we investigate the effect of *L. monocytogenes* infection on MHC class I synthesis, processing, and intracellular trafficking. We show that *L. monocytogenes* infection interferes with normal processing of N-linked oligosaccharides on the major histocompatibility complex (MHC) class I heavy chain molecule, H-2K^d, resulting in a reduced sialic acid content. The glycosylation defect is more pronounced as the infection progresses and results from interference with the addition of sialic acid rather than its removal by a neuraminidase. The effect is found in two different cell lines and is not limited to MHC class I molecules since CD45, a surface glycoprotein, and LGP120, a lysosomal glycoprotein, are similarly affected by *L. monocytogenes* infection. The glycosylation defect is specific for infection by *L. monocytogenes* since neither *Trypanosoma cruzi* nor *Yersinia enterocolitica*, two other intracellular pathogens, reproduces the effect. The resultant hyposialylation of H-2K^d does not impair its surface expression in infected cells. Diminished sialic acid content of surface glycoproteins may enhance host-defense by increasing susceptibility to lysis and promoting clearance of Listeria-infected cells.

Intracellular pathogens have evolved diverse mechanisms for successful survival in host cells (1). In the few detailed analyses of interactions between the host cell and the intracellular pathogen after cell entry, marked disruptions of host cell functions have been described. These include alterations in host cell cytoskeletal arrangement, organellar distribution, protein metabolism, and trafficking. For example, *Listeria monocytogenes* and *Shigella flexneri*, bacteria that reside in the cytosol of infected cells, induce host actin polymerization to move within and into neighboring cells (2, 3). Another facultative intracellular bacterium, *Salmonella typhimurium*, is transiently surrounded by aggregates of cytoskeletal components such as actin, α-actinin, tropomyosin, and tubulin after invading epithelial cells (4). Organellar redistribution has been described during infection with *Legionella pneumophila* (5) and *Toxoplasma gondii* (6), both of which exist in vacuoles that are associated with host cell mitochondria. The intracellular protozoan, *Trypanosoma cruzi*, recruits host cell lysosomes to the site of entry during infection (7). With respect to host protein metabolism, *Legionella* reportedly causes decreased total host cell protein synthesis (8). *Yersinia pseudotuberculosis* expresses a tyrosine phosphatase that dephosphorylates several host cell proteins (9). Several viruses have been shown to interfere with the trafficking of host cell proteins, especially MHC class I. The adenovirus E3/19K glycoprotein, for example, retains newly synthesized MHC class I molecules in the endoplasmic reticulum (ER)[1] (10). Murine CMV early gene expression also results in selective retention of MHC class I in the ER/*cis*-Golgi compartment (11). Effects of bacterial infection on protein trafficking, however, have not been reported.

L. monocytogenes, an intracellular bacterial pathogen that causes severe systemic disease in newborns and immunocompromised hosts (12), has been well studied both from the standpoint of bacterial pathogenesis and cell-mediated immunity. CTL have been shown to mediate protective immunity to *L. monocytogenes* infection in mice (13). However, at the cellular level, it is unclear to what extent infection with *L. monocytogenes* influences antigen processing and presentation. We investigated the effect of cellular infection with *L. monocytogenes* on antigen processing by examining the synthesis and maturation of MHC class I molecules in infected macrophages. We found that MHC class I molecules synthesized during *L. monocytogenes* infection are of lower molec-

[1] *Abbreviations used in this paper:* BFA, Brefeldin A; CMP, cytosine monophosphate; endo H, endoglycosidase H; ER, endoplasmic reticulum.

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ular weight than those in uninfected cells. This can be attributed to a decrease in sialic acid content and is not limited to MHC class I molecules. This effect is likely due to interference with sialic acid addition to glycoproteins and is specific to infection by virulent *L. monocytogenes*. Altered glycosylation of host cell surface proteins has not been previously described as a response to intracellular infection and may have important implications for host defense.

Materials and Methods

Bacterial and Parasite Strains. *L. monocytogenes* strain 43251 (virulent) and 43250 (avirulent) were obtained from the American Type Culture Collection ([ATCC] Rockville, MD). *Yersinia enterocolitica* and *T. cruzi* (Y strain) were kindly provided by Dr. Norma Andrews (Yale University).

Cell Lines. Macrophage cell lines J774 (BALB/c, H-2^d) and PU5-1R (BALB/c, H-2^d) were obtained from the ATCC and maintained in RPMI 1640 with 10% FCS, penicillin (100 U/ml), streptomycin (100 μg/ml), and gentamicin (50 μg/ml) (RP 10).

Antibodies. The following mAbs were used: SF1-1.1.1 (anti-H-$2K^d$) (HB 159, ATCC); 3-4-20S (anti-H-$2D^d$) (HB 75, ATCC); TIB 122 (anti-CD45) (kindly provided by Dr. Kim Bottomly, Yale University); and ID-4B (anti-LGP 120) (kindly provided by Dr. Norma Andrews).

Cytokines. For the cytokine studies, J774 cells were incubated with IFN-γ (200 U/ml; kindly provided by Dr. Richard Flavell, Yale University) for 44 h and LPS (50 ng/ml; Sigma Chemical Co., St. Louis, MO) for 18 h.

Infection Conditions. For routine immunoprecipitations, tissue culture (25 cm^2) flasks containing 1-2 $\times$ 10^6 J774 cells were infected with *L. monocytogenes* grown overnight in trypticase soy broth (TSB) to an A_{600} of 0.1–0.2. The bacteria were pelleted by centrifugation (3,000 rpm for 10 min) and resuspended in RPMI media without antibiotics (RP-) to an A_{600} of 0.2. Of this, 1 ml/10^6 J774 cells was added to each flask. After 25 min, the media was replaced with RP- containing 5 μg/ml gentamicin to kill extracellular bacteria and infected cells were incubated at 37°C for the indicated times. Under these conditions, we find that all cells are uniformly infected and that, 4 h after infection, there is an average of 20 bacteria/macrophage (data not shown). PU5-1R cells were infected under similar conditions using 1 ml of bacteria resuspended to an A_{600} of either 0.2 or 0.5.

For the infection with *T. cruzi*, 7 $\times$ 10^7 tissue culture–derived trypomastigotes were added to 3 $\times$ 10^6 J774 cells and incubated at 37°C for 24 h. Infection with *Y. enterocolitica* was done with an overnight culture grown in TSB and resuspended in RP- media to an A_{600} of 0.5. A tissue culture flask containing 2 $\times$ 10^6 J774 cells was infected with 2 ml of bacteria for 25 min before replacing the media with RP- containing 5 μg/ml gentamicin. The infected cells were further incubated for 8 h at 37°C, conditions which result in infection of nearly 100% of cells (data not shown).

Metabolic Labeling and Immunoprecipitation. J774 or PU5-1R cells, in flasks, were washed once with PBS and then incubated in 2 ml of methionine-free medium (methionine-free DMEM with 3% dialyzed FCS, 2 mM glutamine, and 20 mM Hepes, pH 7.4) containing gentamicin (5 μg/ml) at 37°C with gentle shaking for 30 min. For infected cells, tetracycline (20 μg/ml) was added to block bacterial protein synthesis. [^{35}S]Methionine in the form of Translabel (1,130 Ci/mmole; ICN Radiochemical, Irvine, CA) was added to a concentration of 50 μCi/ml at 37°C for 30 min. For pulse–chase experiments, cells were washed with PBS and then incubated in 4 ml of RP- media containing gentamicin and tetracycline for the times indicated in the figure legends. After washing with PBS, cells were lysed in 1 ml of ice-cold lysis buffer (1% Triton X-100, 140 mM NaCl, 20 mM Tris-HCl, pH 7.4, 0.5 mM PMSF, and 0.5 mM TLCK). Lysates were cleared by centrifugation at 15,000 rpm for 5 min at 4°C and supernatants were precleared overnight at 4°C with nonspecific mouse IgG (50 μg/ml) and protein A–Sepharose (25 μl of a 50% slurry; all from Sigma Chemical Co.). Specific immunoprecipitation was performed for 1 h at 4°C with 5 μl of the appropriate mAb (1–2 mg/ml stocks) and 25 μl of protein A–Sepharose (50% slurry) or protein G–Sepharose (50% slurry) for rat IgGs. The Sepharose beads were washed three times with wash buffer (0.5% Triton X-100, 140 mM NaCl, and 20 mM Tris-HCl, pH 7.4) and resuspended in 50 μl sample buffer. After boiling for 5 min, the samples were analyzed on 8% SDS-PAGE gels followed by autoradiography.

Endoglycosidase and Neuraminidase Treatment. For endoglycosidase H (endo H) (Boehringer Mannheim, Mannheim, Germany) digestion, immunoprecipitates were washed as described above then eluted by boiling for 2 min in 40 μl of 5$\times$ concentrated endo H buffer (0.1 M sodium phosphate, pH 6.5, 0.5% SDS, and 0.1% sodium azide). After centrifugation, the supernatants were collected, 160 μl of water was added, and the sample was split in two. To one of the samples, 2 μl of endo H (2 mU) was added and then both were incubated for 18 h at 37°C. Digests were analyzed by SDS-PAGE after addition of 10 μl of 10$\times$ concentrated sample buffer (containing 10% β-ME).

For endoglycosidase F/N-glycosidase F (endo F) digestion (Boehringer Mannheim), immune complexes on Sepharose beads were eluted by boiling in 40 μl 0.5% SDS. To the supernatant, 100 μl of 2$\times$ concentrated endo F buffer (40 mM potassium phosphate, pH 7.2, and 100 mM EDTA), 5 μl 20% *n*-octylglucoside, and 55 μl water were added. The sample was split in two and 5 μl of endo F (0.25 U) was added to one. Samples were analyzed by SDS-PAGE after an 18-h incubation at 37°C.

For neuraminidase digestion, immunoprecipitated H-$2K^d$ were eluted from Sepharose beads by boiling in 40 μl 0.5% SDS. To the supernatant were added 100 μl 2$\times$ concentrated neuraminidase buffer (100 mM sodium acetate, pH 5.5, 1.8% NaCl, and 0.2% $CaCl_2$), 5 μl 20% *n*-octylglucoside and 20 μl water. Samples were incubated for 18 h at 37°C in the absence or presence of 40 μl (0.04 U) *Vibrio cholerae* neuraminidase (Boehringer Mannheim) before two-dimensional gel analysis.

Two-dimensional Gel Analysis. For two-dimensional gel analysis, 4 $\times$ 10^6 J774 cells were infected with 3 ml of *L. monocytogenes* culture resuspended to an A_{600} of 0.2. Labeling and immunoprecipitation were carried out as described above. Samples were resuspended in IEF sample buffer (9.5 M urea, 2% NP-40, 1.6% Pharmalyte® 3-10, and 0.4% Pharmalyte 5-8 ampholytes, 5% β-ME) and subjected to IEF and SDS-PAGE according to standard procedures (Hoefer Scientific, Irvine, CA).

Lectin Analysis. Metabolically labeled H-$2K^d$ from *L. monocytogenes*-infected J774 cells on protein A–Sepharose beads was eluted by boiling in 200 μl 0.1% SDS. After centrifugation, the supernatant was split in two and 900 μl wash buffer (0.1% Triton X-100, 140 mM NaCl, and 20 mM Tris-HCl, pH 7.4) were added to each. To one sample, Con A–Sepharose 4B (25 μl of a 50% slurry in a final mixture containing 1 mM each of $CaCl_2$, $MgCl_2$, and $MnCl_2$; Sigma Chemical Co.) was added and to the other sample, lectin from *Triticum vulgaris* (wheat germ) coupled to agarose beads (25 μl of a 50% slurry; Sigma Chemical Co.) was added. After

incubating at 4°C for 60 min, the lectin-coupled Sepharose beads were washed three times in wash buffer, resuspended in sample buffer, and analyzed by SDS-PAGE.

Cell Surface Biotinylation. To study the kinetics of H-2K^d transport to the cell surface, metabolic labeling was coupled with surface biotinylation essentially as described (14). Briefly, after pulsing cells with [^{35}S]Trans-label for 30 min and 2 h of chase, biotinylation was carried out in 130 mM NaCl, 20 mM bicine-HCl, pH 7.5, containing 1.5 mg/ml NHS-SS-Biotin (Pierce, Rockford, IL) for 30 min at 4°C. Free biotin was quenched by adding 50 mM glycine for 15 min at 4°C. After two washes in PBS supplemented with 0.1 mM Ca^{2+} and 1.0 mM Mg^{2+}, the cells were lysed, precleared, and immunoprecipitated as above. The beads were washed with lysis buffer and antigen was eluted by resuspending the beads in 50 μl 2% SDS and boiling for 5 min. To the supernatant we added 1 ml of lysis buffer containing protease inhibitors and 100 μl of a 50% suspension of avidin agarose beads (Calbiochem-Novabiochem Corp., La Jolla, CA) which had been precoated with 1% FCS. After an overnight incubation at 4°C, the beads were washed twice in lysis buffer without protease inhibitors, twice in wash buffer (140 mM NaCl, 20 mM Tris-HCl, pH 7.4, and 0.1% Triton X-100), and twice in 10 mM Tris, pH 7.4. The samples were then analyzed on 8% SDS-PAGE gels.

Brefeldin A (BFA) (5 μg/ml; Epicentre Technologies Corp., Madison, WI) was added to samples 30 min before metabolic labeling and maintained thereafter during the chase and biotinylation procedures.

Results

Infection of Macrophages with L. monocytogenes Results in a Glycosylation Defect of MHC Class I Heavy Chains. MHC class I glycoproteins contain two or three N-linked carbohydrate groups that are processed in the ER and Golgi complex before being transported to the cell surface (15, 16). The time required for complete processing of N-linked oligosaccharides to their complex, endo H resistant forms, is variable for the different alleles of MHC class I but generally is complete within 2 h (17). We first investigated the fate of H-2K^d in uninfected J774 macrophage-like cells by pulse–chase analysis. The H-2K^d heavy chain, immunoprecipitated after a 2-h chase, had an apparent molecular mass of 52 kD and was resistant to endo H digestion, indicating the presence of mature N-linked oligosaccharides (Fig. 1 *A*). J774 cells were then infected for 4 h with a virulent strain of *L. monocytogenes*, pulse-labeled for 30 min with [^{35}S]methionine and chased for 2 h. In contrast to uninfected cells, two forms of H-2K^d were immunoprecipitated from infected cells. In addition to mature 52-kD H-2K^d, an endo H–resistant protein with an approximate molecular mass of 48 kD was observed (Fig. 1 *A*). The 48-kD H-2K^d was not seen when cells were infected with equivalent amounts of the avirulent (listeriolysin minus) strain of *L. monocytogenes* (Fig. 1 *A*).

To determine if the 48-kD H-2K^d seen in infected cells was a proteolytic product of the 52-kD protein or an alternately glycosylated form, H-2K^d immunoprecipitated from infected and uninfected cells was digested with endo F, which removes both high mannose and complex oligosaccharides. A single 44-kD protein was seen in both infected and uninfected cells, indicating that the two forms of H-2K^d seen during *L. monocytogenes* infection share the same protein backbone (Fig. 1 *B*), and thus differ only with respect to their N-linked oligosaccharides.

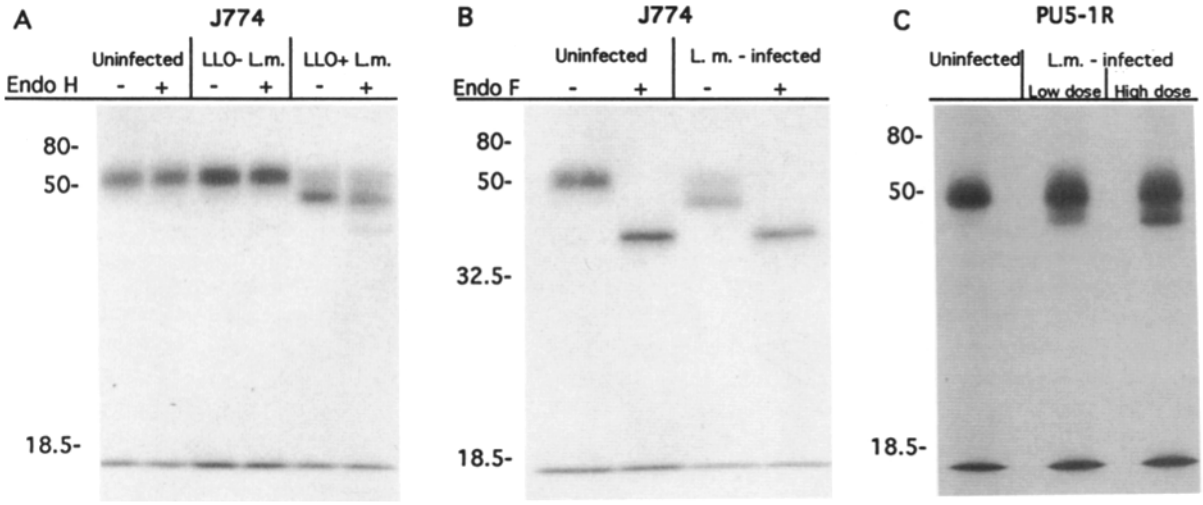

Figure 1. Alterations in the glycosylation pattern of H-2K^d in J774 and PU5-1R macrophages infected with *L. monocytogenes*. J774 cells were infected for 4 h with an avirulent (listeriolysin$^-$ [LLO$^-$]) or virulent (LLO$^+$) strain of *L. monocytogenes*. After a 30-min pulse labeling with [^{35}S]methionine and a 2-h chase, the H-2K^d was immunoprecipitated. Half the sample was digested with the indicated endoglycosidase (+), the other half was mock-digested (−). Another macrophage cell line, PU5-1R, was similarly infected with virulent *L. monocytogenes* using two infecting doses corresponding to A_{600} 0.2 (low dose) and A_{600} 0.5 (high dose). The 12-kD protein present in all lanes is β_2-microglobulin (β_2m). (*A*) Two endo H–resistant forms of H-2K^d (52 and 48 kD) accumulated in J774 cells infected with virulent bacteria. (*B*) Upon endo F digestion, the lower molecular weight protein seen during infection of J774 cells was reduced in size to that seen in uninfected cells. (*C*) The same pattern was seen in PU5-1R cells and was more pronounced at the higher infecting dose.

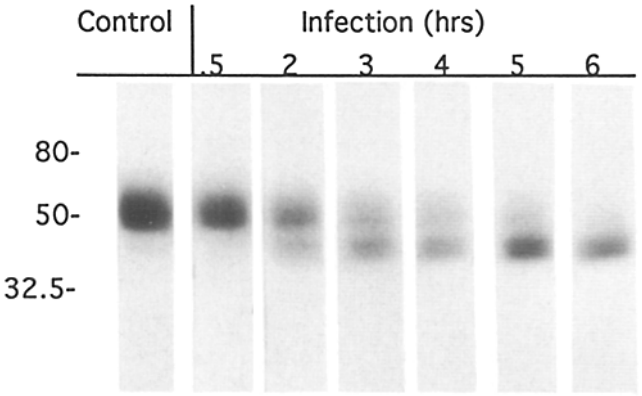

Figure 2. Altered glycosylation of H-2K^d as a function of duration of cellular infection. J774 cells were infected with *L. monocytogenes* for 0.5, 2, 3, 4, 5, and 6 h before 30-min pulse labeling and 2-h chase.

To show that this glycosylation defect was not restricted to one cell line, PU5-1R (also H-2^d), another murine macrophage-like cell line, was investigated. Metabolic labeling and immunoprecipitation of H-2K^d revealed that *L. monocytogenes* infection induced the same glycosylation defect seen with J774 cells and that the effect was enhanced by heavier infection (Fig. 1 *C*).

The Glycosylation Defect Is Related to the Duration of Infection. The degree of cellular infection was varied by allowing intracellular *L. monocytogenes* replication in J774 cells to proceed from a minimum of 30 min to a maximum of 6 h before metabolic labeling. Fig. 2 shows that after 30 min of infection, all newly synthesized H-2K^d was processed to the 52-kD form. However, after a 2-h infection, a small proportion of H-2K^d molecules matured to the 48-kD form. As the time of infection progressed to 3, 4, and 5 h, the relative proportion of H-2K^d molecules that matured to the 48-kD form increased until, after 6 h of infection, all newly synthesized H-2K^d were processed to the 48-kD form. As noted above, the relative amount of the 48-kD form of H-2K^d also increased when a higher dose of *L. monocytogenes* was used to infect PU5-1R macrophages (Fig. 1 *C*). Thus, the proportion of abnormally glycosylated H-2K^d molecules is directly related to the duration and degree of host cell infection by *L. monocytogenes*.

The Glycosylation Defect Is Seen with Other Glycoproteins. To determine if other glycoproteins are similarly affected by infection with *L. monocytogenes*, additional surface glycoproteins were analyzed. The MHC class I molecule, H-2D^d, was found to have the same altered pattern as H-2K^d (Fig. 3 *A*). During infection, CD45, a cell surface glycoprotein common to bone marrow–derived cell lines (18), was also present as a species with an apparent molecular mass less than the normal 120-kD form (Fig. 3 *A*). The glycosylation defect induced by listerial infection is not limited to cell surface glycoproteins since the lysosomal glycoprotein (LGP) 120 was also seen to have lower molecular weight forms in infected cells (Fig. 3 *B*, arrows *a* and *b*). The different lower molecular weight forms of LGP 120 detected in infected cells result from differences in glycosylation since endo F digestion reduced all forms to the same 42-kD protein (Fig. 3 *B*, arrow *c*) (19). These findings indicate that the effect of *L. monocytogenes* infection on glycosylation extends to many, if not all, glycoproteins.

The Glycosylation Defect Is Not a Result of Nonspecific Macrophage Activation. Activation of macrophages in response to cytokine stimulation or infection is accompanied by remodeling of the cell surface. To determine if the glycosylation defect seen during *L. monocytogenes* infection is specific for this organism, the effect on H-2K^d synthesis and processing was investigated in J774 cells exposed to different stimuli. H-2K^d synthesis and maturation were unaffected by

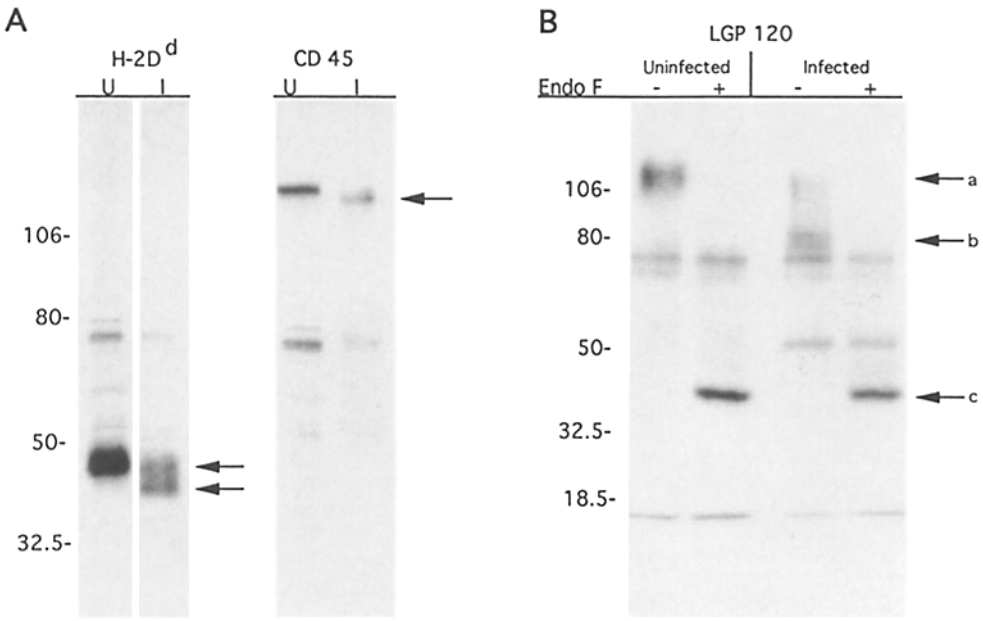

Figure 3. Glycosylation of H-2D^d, CD45, and LGP120 is also affected by *L. monocytogenes* infection. H-2D^d, CD45, and LGP120 were immunoprecipitated from uninfected cells (*U*) or cells infected with *L. monocytogenes* for 4 h (*I*). In all cases, a 30-min pulse labeling was followed by a 2-h chase. (*A*) (*Left, arrows*) Two forms of H-2D^d seen during infection which correspond to the mature form (upper) and an additional lower molecular weight form (lower). (*Right, arrow*) A smaller form of CD45 in infected compared with uninfected cells. (*B*) Altered glycosylation pattern of LGP120 in infected cells. Lower molecular weight form of LGP120 in infected (arrow *b*) as compared to uninfected (arrow *a*) cells. In the presence of endo F (+), LGP120 is reduced to a 42-kD band (arrow *c*).

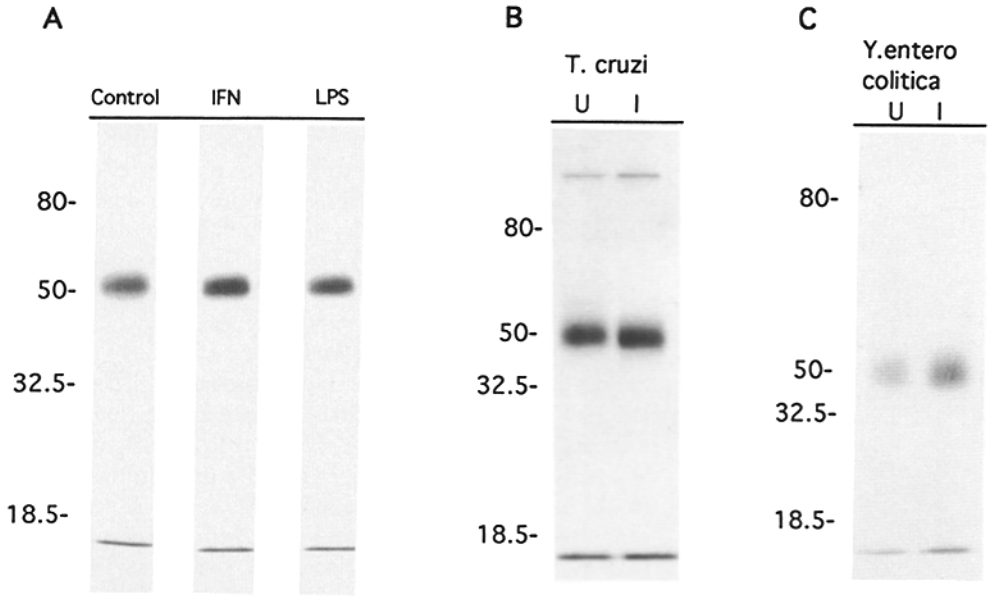

Figure 4. Effects of IFN-γ, LPS, and other intracellular pathogens on the glycosylation of H-2K^d. J774 cells were either pretreated with IFN-γ or LPS (*A*) or infected with *T. cruzi* (*B*) or *Y. enterocolitica* (*C*) as described in Materials and Methods. In both cases, uninfected (*U*) and infected (*I*) cells were compared.

exposure to either IFN-γ or LPS (Fig. 4 *A*). Infection with two other intracellular pathogens, the parasite *T. cruzi*, and another bacterium, *Y. enterocolitica*, also did not reproduce the glycosylation defect (Fig. 4, *B* and *C*).

The Glycosylation Defect Results in Decreased Sialylation. To further define the glycosylation defect in *L. monocytogenes*–infected J774 cells, H-2K^d was analyzed by two-dimensional gel electrophoresis. In uninfected cells, H-2K^d molecules displayed the characteristic isoelectric point heterogeneity which reflects the variation in sialic acid content of the three N-linked glycosylation sites (Fig. 5 *A*). In contrast, H-2K^d isolated from infected J774 cells had a more basic pI (Fig. 5 *B*). This shift towards the cathode was accompanied by a decrease in molecular weight and is consistent with a decreasing sialic

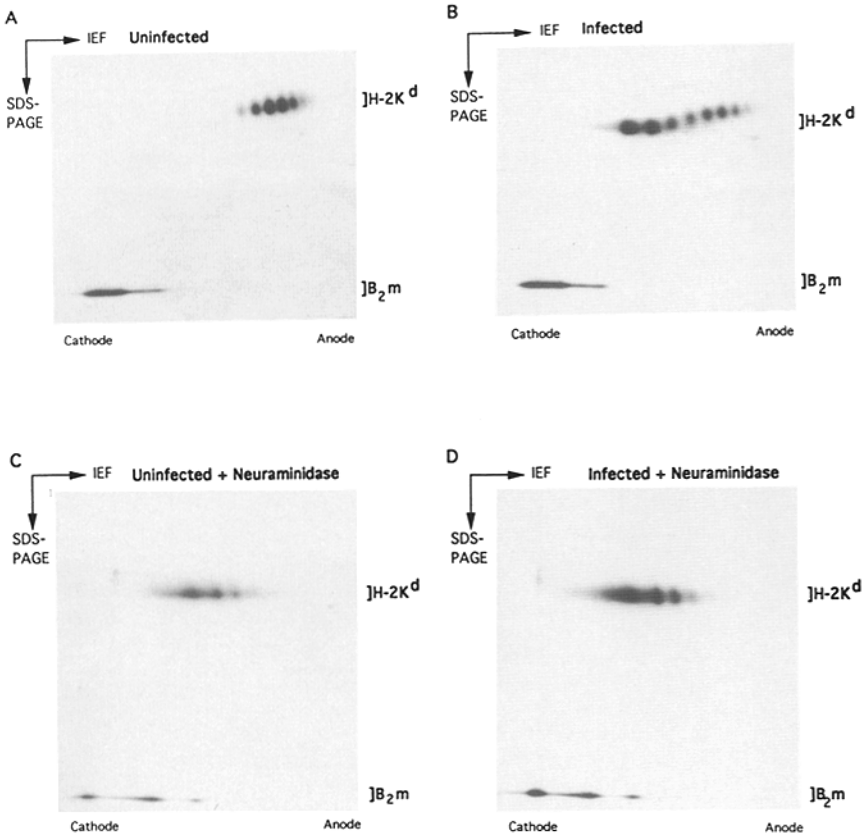

Figure 5. Two-dimensional gel analysis of H-2K^d from uninfected and infected cells. J774 cells were either left uninfected (*A* and *C*) or infected (*B* and *D*) for 4 h with *L. monocytogenes*, metabolically labeled, and chased as in Fig. 1. After immunoprecipitation, radiolabeled H-2K^d were mock-digested (*A* and *B*) or digested with neuraminidase (*C* and *D*), subjected to isoelectric focusing (*IEF*) for the first dimension, then SDS-PAGE analysis on 8% gels for the second dimension. The collection of spots corresponding to the H-2K^d heavy chain and β_2m are indicated.

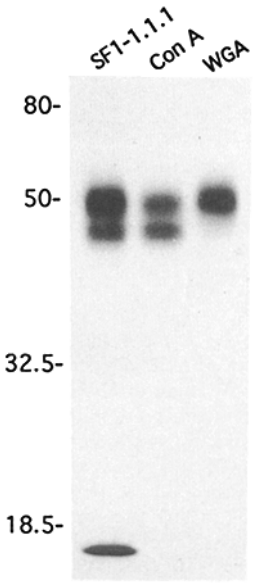

Figure 6. Lectin analysis of H-2K^d in infected cells. J774 cells were infected for 4 h with *L. monocytogenes*, metabolically labeled, and chased for 2 h. Immunoprecipitated, radiolabeled H-2K^d was reprecipitated with Con A or wheat germ agglutinin (*WGA*) immobilized on beads. The first lane (SF1-1.1.1), shows the immunoprecipitated H-2K^d before lectin binding. The 12-kD β_2m is not glycosylated and thus is not lectin bound.

acid content. The differences in charge heterogeneity and molecular weight of MHC class I molecules in uninfected and infected cells were due to different sialic acid contents since they were abolished upon removal of sialic acids with neuraminidase (Fig. 5, *C* and *D*).

Hyposialylation as a cause for the appearance of the aberrantly glycosylated 48-kD form of H-2K^d in *L. monocytogenes*–infected cells was corroborated by lectin analysis. As shown in Fig. 6, wheat germ agglutinin, a lectin specific for sialic acid and outer chain *N*-acetylglucosamine residues of complex oligosaccharides (20, 21) bound the mature 52-kD and not the 48-kD form, indicating that the latter has diminished sialic acid content. As expected, Con A, which binds the core portion of N-linked oligosaccharide chains (22), bound both forms of H-2K^d.

The Altered Form Is not Derived from Mature H-2K^d. To determine if the 48-kD form of H-2K^d is derived from the 52-kD form, J774 cells were metabolically labeled 2 h after infection with *L. monocytogenes* and chased for increasing time intervals. Both 48- and 52-kD forms of H-2K^d were present after 2 h of chase (Fig. 7). As the chase period was lengthened, the relative amounts of both 48- and 52-kD forms of H-2K^d decreased proportionally. This suggests that the 48-kD H-2K^d is not derived from the 52-kD H-2K^d by oligosaccharide trimming due to bacterial or host cell–derived exoglycosidases. *L. monocytogenes* has not been described as having neuraminidase activity and we could not detect such activity in supernatants of *L. monocytogenes* cultures by use of the fluorogenic substrate, 4-methylumbelliferryl-*N*-acetylneuraminic acid (data not shown).

The Hyposialylated Form of MHC Class I Appears on the Cell Surface. Several studies have shown that incomplete glycosylation of MHC class I molecules does not impair their ability to transit to the cell surface (23–25). To investigate if the hyposialylated 48-kD form of H-2K^d is present on the surface of infected cells, ^{35}S-methionine–labeled H-2K^d were biotinylated with NHS-SS-biotin to identify those molecules that were present on the surface. Fig. 8 *A* shows that both the 48- and 52-kD forms of H-2K^d produced during *L. monocytogenes* infection appeared on the cell surface. To insure that intracellular proteins were not inadvertently biotinylated during infection, BFA was added to infected J774 cells before metabolic labeling. Since BFA blocks egress of proteins from the ER, proteins synthesized in the presence of the drug should not be accessible to biotin. Fig. 8 *B* shows that a neglible amount of H-2K^d was biotinylated in infected cells

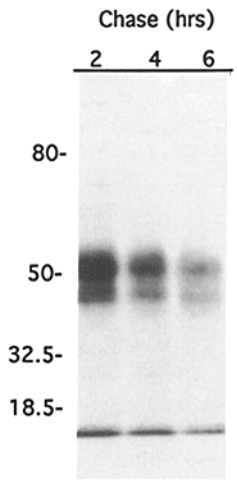

Figure 7. Pulse-chase analysis of H-2K^d in infected cells. After 2 h of *L. monocytogenes* infection, cells were labeled for 30 min and chased for 2, 4, and 6 h.

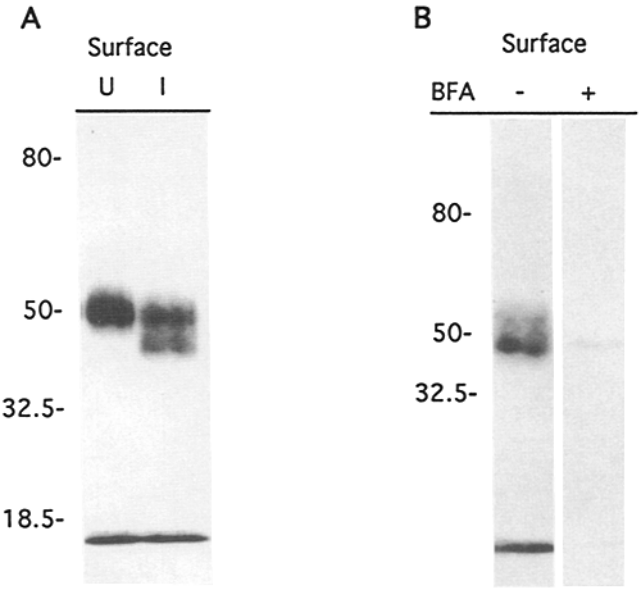

Figure 8. Cell surface appearance of H-2K^d in infected cells. J774 cells were infected with *L. monocytogenes* and subjected to pulse-chase analysis as before. Cell surface biotinylation was performed as in Materials and Methods, prior to immunoprecipitation of H-2K^d. Biotinylated, radiolabeled H-2K^d was purified using avidin–agarose from the immunoprecipitated total H-2K^d. (*A*) Biotinylated, radiolabeled H-2K^d from uninfected (*U*) and infected (*I*) cells. (*B*) As in *A* except Brefeldin A (*BFA*) was added to infected J774 cells before and during the pulse and chase to prevent trafficking of H-2K^d to the surface. The control lane was done in the absence of BFA.

Table 1. *Relative Amounts of Surface H-2K^d in Infected Cells*

Chase	Normal H-2K^d (N)	Hyposialylated H-2K^d (H)	H/N Ratio
h	*cpm*		
0.5	871	412	0.47
1.0	1,782	828	0.47
2.0	2,342	1,086	0.46
4.0	1,039	462	0.45

Metabolic labeling, surface biotinylation, and immunoprecipitation were done as described in Materials and Methods. Bands on the gel corresponding to the 52- (N) and 48-kD (H) forms of H-2K^d were cut out and ^{35}S-incorporation was determined using a TR liquid scintillation counter (model 2500 TR; Packard Instruments, Meriden, CT).

in the presence of BFA. The lower molecular weight form of H-2K^d synthesized in the presence of BFA is characteristic of proteins retained in the ER. Thus, biotinylation accurately reflects surface localization of proteins in infected cells.

The kinetics of surface appearance of the hyposialylated form parallels that of the normal H-2K^d as assessed by pulse–chase analysis. Specifically, when the relative amounts of surface 48- and 52-kD forms of H-2K^d were quantitated, the ratio between the two forms remained constant (Table 1). This suggests that despite altered glycosylation, there is no inherent abnormality in trafficking to the cell surface in infected cells.

Discussion

In this report, we describe the effect of infection by the intracellular bacterium, *L. monocytogenes*, on processing and transport of host cell glycoproteins. *L. monocytogenes* infection of macrophage cell lines results in an alteration of the processing of N-linked oligosaccharides so that surface and intracellular glycoproteins have reduced sialic acid content. This effect is seen within 1 h of infection and its magnitude is directly proportional to the duration and the severity of infection. The effect appears to be specific to infection by *L. monocytogenes* since it is not reproduced by either infection with other intracellular organisms or activation of cells with IFN-γ or LPS. The glycosylation defect results in diminished sialic acid addition and does not result from removal of sialic acids by a neuraminidase. In the case of MHC class I molecules, the resultant hyposialylation does not impair surface expression.

Interference with this aspect of host cell metabolism by an intracellular pathogen has not previously been described. Sialic acid is normally synthesized in the cytosol and coupled to cytosine monophosphate (CMP). After translocation across Golgi membranes, CMP-sialic acid serves as the sialic acid donor for Golgi sialyltransferase in the terminal step in oligosaccharide processing (26). Although the exact mechanism by which *L. monocytogenes* impairs sialic acid addition cannot be defined from our analysis, several mechanisms can be proposed. *L. monocytogenes* may inhibit the synthesis of CMP-sialic acid or metabolize the activated sugar or its precursors. Alternatively, the bacterium may interfere with the activity of the CMP-sialic acid transporter or the sialyltransferase in the Golgi complex. Another possibility is that earlier steps of oligosaccharide processing required for sialic acid addition may be impaired. Inhibition of Golgi galactosyltransferases and/or *N*-acetylglucosaminyltransferases, for example, could prevent sialic acid addition and produce endo H–resistant forms such as we have observed. Alternatively, infection may lead to decreased availability in the Golgi complex of UDP-*N*-acetylglucosamine or UDP-galactose, either by depletion of intracellular pools or impairment of transport. This also could inhibit the late steps of oligosaccharide processing and result in hyposialylation. Another mechanism that has been demonstrated in melanoma cells is the induction of α1,3 fucosyltransferase which prevents sialic acid addition by α2,3 sialyltransferase in the Golgi (27). A similar mechanism could be occurring in *Listeria*-infected cells.

There are several potential functional consequences of hyposialylation of cell surface glycoproteins on host–defense mechanisms. With respect to MHC class I, manipulating the glycosylation state with specific inhibitors or site-specific mutations, does not impair surface expression (23–25, 28, 29) and usually does not affect recognition by CTL (28–30). However, a specific role for sialic acid on MHC class I in antigen presentation has been suggested (31, 32). In one case, mouse strains with mutations in H-2D^b were unable to present certain antigens to specific CTL (32). The block in antigen presentation could be released by decreasing the number of sialic acid residues associated with the APCs by treatment with neuraminidase or glycosylation inhibitors such as deoxymannojirimycin and swainsonine (31). Use of dendritic cells which contain MHC class I molecules that are relatively hyposialylated compared with those in macrophages or B cells, as CTL targets, could also overcome the specific immunologic unresponsiveness found in the H-2D^b mutant mice (32). It was proposed that absence of sialic acids on N-linked sites which normally lie at the extremities of the α_1-α_2 helices outside the peptide binding groove, improved interaction with CTL by virtue of a reduction in electrostatic repulsion. Thus, hyposialylation of MHC class I molecules induced by *L. monocytogenes* infection may augment CTL recognition of infected cells, thereby enhancing host defense.

The consequences of glycoprotein hyposialylation in infected cells, with its resultant net decrease in surface negative charge, may extend beyond the possible role played in MHC class I antigen presentation. It has long been known that there are receptors on liver, spleen, and peritoneal macrophages that specifically bind to desialylated oligosaccharide ligands on cells (33, 34). Sialidase treatment of erythrocytes and lymphocytes, for example, results in clearance from the circulation after binding to appropriate macrophage lectins (35, 36). Two macrophage lectins specific for galactose residues exposed by desialylation have been characterized from mouse tumoricidal (37) and rat peritoneal macrophages (38). It is possible that hyposialylation of surface molecules during cellular infection

with *L. monocytogenes* plays a role in host defense by sequestering infected cells in the reticuloendothelial system, thereby containing and eventually eliminating the infection.

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