Diet-Induced Obese Mice Have Increased Mortality and Altered Immune Responses When Infected with Influenza Virus$^{1,2}$

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Abstract

Obesity is associated with an impaired immune response, an increased susceptibility to bacterial infection, and a chronic increase in proinflammatory cytokines such as IL-6 and TNFα. However, few studies have examined the effect of obesity on the immune response to viral infections. Because infection with influenza is a leading cause of morbidity and mortality worldwide, we investigated the effect of obesity on early immune responses to influenza virus exposure. Diet-induced obese and lean control C57BL/6 mice were infected with influenza A/PR8/34, and lung pathology and immune responses were examined at d 0 (uninfected), 3, and 6, postinfection. Following infection, diet-induced obese mice had a significantly higher mortality rate than the lean controls and elevated lung pathology. Antiviral and proinflammatory cytokine mRNA production in the lungs of the infected mice was markedly different between obese and lean mice. IFNα and β were only minimally expressed in the infected lungs of obese mice and there was a notable delay in expression of the proinflammatory cytokines IL-6 and TNFα. Additionally, obese mice had a substantial reduction in NK cell cytotoxicity. These data indicate that obesity inhibits the ability of the immune system to appropriately respond to influenza infection and suggests that obesity may lead to increased morbidity and mortality from viral infections. J. Nutr. 137: 1236–1243, 2007.

Introduction

Obesity has been associated with immune dysfunction (1–3). In humans, responses of peripheral lymphocytes to mitogen stimulation are reduced (4). Some studies have reported higher T cell counts (5), others report no change (6) or lower T cell counts (7), whereas still others demonstrate changes in the T cell population frequency (8). In addition, obese individuals have a low level of circulating TNF-α and IL-6, suggesting a state of chronic inflammation. Studies using animal models of obesity, both genetic and diet induced, also report immune dysfunction (9–11). In vitro response to mitogens is diminished in obese animals (12) and secretion of IL-4 and IFN-γ is altered (13). Because obesity is associated with immune dysfunction, it is plausible that obese mice infected with a viral pathogen would be unable to mount an effective immune response. To test this hypothesis, we infected lean and diet-induced obese mice with influenza virus, a serious human pathogen. Studies examining the effect of obesity on infection are minimal, and, to our knowledge, this is the first report on the effect of obesity on an influenza virus infection.

Influenza infection is a major cause of morbidity and mortality worldwide. Once infected, an appropriate coordination of both innate and adaptive immune responses is necessary for elimination and recovery from the virus. Groups at high risk for increased influenza virus mortality include the elderly, the very young, and individuals with chronic pulmonary and/or cardiovascular conditions (14). Interestingly, obesity is also associated with chronic pulmonary and cardiovascular diseases (15,16). Upon infection with influenza virus, dendritic cells, macrophages, and lung epithelial cells upregulate the expression of cytokines and chemokines. These molecules play essential roles in the early inhibition of viral replication (17), the stimulation of an inflammatory response (18), and recruitment of immune cells to the site of infection (19). Additionally, cytokines activate natural killer (NK) cells, which are among the first cell types to become mobilized during an influenza infection. NK cells assist in eliminating infected cells and help limit viral spread until a specific cell-mediated response can be assembled (20). The expression of cytokines and chemokines during an influenza virus infection occurs in a coordinated and specific cascade. Antiviral and proinflammatory cytokines are induced first, followed by IL-6 expression, and finally chemokines, such as monocyte chemotactic protein (MCP)-1 and macrophage inflammatory protein (MIP)-1α are expressed (21). Changes in the expression of any of these molecules can alter subsequent immune responses (22).

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$^{1}$ Abbreviations used: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MCP, monocyte chemotactic protein; NK, natural killer cells; p.i., postinfection; RANTES, regulated upon activation, normal T-cell expressed, and secreted; SOCS, suppressor of cytokine signaling; STAT, signal transducer and activator of transcription.
In this study, we investigated whether obesity affects the early immune response to influenza virus infection, and therefore alters viral pathogenesis.

Methods

Animals. Weanling C57BL/6J mice were obtained from Jackson Laboratories. All mice were housed at the University of North Carolina Animal Facility, which is fully accredited by the American Association for Accreditation of Laboratory Animal Care. Animals were maintained under protocols approved by the Institutional Animal Use and Care Committee. Mice were randomized to receive either a low-fat/no-sucrose diet or a high-fat/high-sucrose diet for 22 wk. Mice were housed 4/cage with free access to food and water, with the exception of an 8-h food deprivation period prior to blood draws for glucose and insulin measurements as well as for d-3 splenic NK cell cytotoxicity.

Diets. The diets, which have been previously described (23), were obtained from Research Diets.

Virus and infection. The mouse adapted strain of Influenza A/Puerto Rico/8/34 (A/PR8) (American Type Culture Collection) was propagated in the allantoic fluid of fertilized chicken eggs and the viral titer was determined by hemaggulination assay (24). After 22 wk on the diets, mice were anesthetized with an intramuscular injection of a ketamine (0.6 mg/kg)/xylazine (0.35 mg/kg) solution and infected intranasally with 0.05 mL of 2 hemaggulinating units of A/PR8 virus diluted in PBS. Preliminary studies from our laboratory determined that this dose of virus is sufficient to effectively elicit an immune response while causing little mortality in infected control mice.

Measurement of blood glucose and serum insulin and leptin. To determine whether circulating glucose and insulin concentrations were affected by infection, mice were food deprived for 8 h and blood samples were collected prior to infection (after 22 wk on the diet) and at d 3 and 7 postinfection (p.i.). Blood glucose concentrations were measured with a Freestyle blood glucose monitor (Abbott Laboratories). Serum insulin was measured by ELISA (LINCO Research). Serum leptin was measured by ELISA (R & D Systems) in fed mice prior to infection and at d 3 and d 6 p.i.

Histopathology of lungs. Lungs were removed at d 0, 3, and 6 p.i. and perfused with 4% paraformaldehyde, paraffin embedded, cut in 6 μm sections, and stained with hemotoxylin and eosin. Pathology was graded semiquantitatively, as previously described (25).

Quantitation of viral titers. Viral titers were determined by a modified tissue culture infections dose 50 (TCID50) using hemaggulination as an endpoint, as previously described (26). Briefly, half of the right lobe of the lung was removed, weighed, and ground in 150 μL minimal essential medium. Samples were centrifuged at 9000 × g for 20 min and the supernatant was serially diluted starting at 1:5 in MEM containing 20 mg/L trypsin. Each diluted supernatant (100 μL) was added, in duplicate, to 80% confluent MDCK cells and incubated at 37°C for 72 h. A 0.5% suspension of human O RBC (50 μL) was added to each well and incubated at room temperature for 2 h. Viral titer was expressed as the reciprocal of the highest dilution at which the RBC agglutinated. This value was then normalized to total protein in the sample. Total protein was determined by bichinchonic acid assay, as previously described (27).

Quantitation of lung mRNA cytokine levels. Half of the right lobe of the lung was removed at d 0 (uninfected), 3, and 6 p.i. Total RNA was isolated using the TRIzol method (Invitrogen). Reverse transcription was carried out using Superscript II First Strand Synthesis kit (Invitrogen) using oligo (dT) primers. Concentrations of mRNA levels for murine IFNα, IFNβ, IFNγ, IL-1α, IL-6, IL-10, IL-12, TNFα, MCP-1, MIP-1α, regulated upon activation, normal T-cell expressed, and secreted (RANTES), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were determined using quantitative RT-PCR. Fluorescent reporters were detected using a Bio-Rad iCycler PCR machine, and primers and probes were designed using Primer Express 1.5 (Applied Biosystems). The levels of mRNA for GAPDH were determined for all samples and were used to normalize gene expression. All data are expressed as fold induction over lean, uninfected controls. There were no significant differences between lean and obese uninfected controls.

Enumeration of NK cell populations in the lung and spleen. Lungs were removed at d 0 and 3 p.i. and incubated in a collagenase solution (1500 units/set of lungs) for 1 h. Both spleen and lung tissue were processed into single cell suspensions by mechanical agitation of a Stomacher and stained through a 40 μm nylon filter. Cells were stained with fluorescein (FITC)-labeled anti-DX5 and Phycoerythrin-labeled anti-CD3 (BD Pharmingen) and analyzed by FACScaliber. The lymphocyte population was gated and NK cells were identified as CD3‘‘DX5‘‘ within that gate.

Determination of natural killer cell cytotoxicity. To determine whether obesity had an effect on the ability of NK cells to lyse a target, isolated lung and spleen cells were analyzed in a standard NK cell cytotoxicity assay (28), using 51Cr-labeled YAC-1 tumor cells (ATCC) as targets. Briefly, serial dilutions of 0.1 mL lung or spleen effector cells, starting at 1 × 107 cells/L, were plated with 1 × 104 cells 51Cr-labeled YAC-1 target cells in a 96-well V-bottom microplate. The resulting effector to target ratios were 100:1, 50:1, and 25:1. All samples were prepared in triplicate. To determine maximum lysis, 0.1 mL of 10% sodiumdodecylsulfate was added to labeled YAC-1 cells. To determine spontaneous release, 0.1 mL of the culture medium was added to radioactive YAC-1 cells. Release of 51Cr content was analyzed using a gamma counter (Cobra II, Hewlett Packard) and percentage of specific lysis was calculated by the following equation:

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\text{% specific lysis} = \frac{\text{cpm (sample)} - \text{cpm (spontaneous release)}}{\text{cpm (maximum release)} - \text{cpm (spontaneous release)}} \times 100
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Statistical analysis. Statistical analyses were performed using JMP Statistical Software (SAS Institute). Normally distributed data were analyzed by 2-way ANOVA with diet and day postinfection as main effects. Student’s t test was used for post-hoc comparison between the dietary groups and Tukey’s HSD was used for post-hoc comparisons among the days p.i. Nonparametric data were analyzed using Kruskal Wallis test. Differences were considered significant at P < 0.05.

Results

Elevated serum insulin concentrations in obese mice during influenza infection. Because diabetes is a risk factor for complications of an influenza infection (29), we wanted to determine whether mice fed the high fat/high sucrose diet had elevated glucose and insulin concentrations both before and during infection. At baseline, serum insulin concentrations did not differ between lean and obese mice, but increased during infection in the obese mice (Fig. 1A). Blood glucose concentrations were significantly greater than lean mice at baseline and at d 3 p.i. in the obese mice, but had decreased by d 7 p.i. to a concentration that did not differ from lean mice (Fig. 1B). Conversely, blood glucose concentrations in lean mice were unaffected by influenza infection.

Obesity reduces serum leptin concentrations during influenza infection. To determine the effect of influenza infection on serum leptin concentrations and whether this was altered by obesity, we analyzed serum leptin both prior to and during influenza infection. As expected, baseline leptin concentrations were higher in obese mice than in lean mice (Fig. 1C).
Interestingly, leptin responses during infection differed between the 2 groups. In lean mice, circulating serum leptin levels tended to increase transiently following infection; the concentration at d 3 p.i. tended to be greater than before infection ($P = 0.1$) whereas concentration at d 6 p.i. was not different from before infection but was significantly lower than on d 3 p.i. On the other hand, obese mice had a transient decrease in serum leptin during infection, with levels at d 3 p.i. being significantly lower than at d 0 or d 6 p.i. (Fig. 1C).

**Obesity results in high mortality during influenza infection.** During influenza infection, the morality rate of the obese mice (42%) was 6.6-fold greater than that of lean mice (5.5%). The obese mice died at d 8 p.i. (Fig. 2).

**Viral titers and lung pathology are similar between lean and obese mice.** The high mortality rate in the obese mice indicated that an influenza virus infection is significantly altered by obesity. To determine whether increased viral load was associated with an increased viral load in the obese mice, we measured lung viral titers at d 3 and 6 p.i. Viral titers did not differ between lean (14,625 ± 4,642 viral titer/mg protein) and obese (15,818 ± 6,044 viral titer/mg protein) mice at d 3 p.i., although by d 6 p.i. the lung virus titer was significantly decreased in the obese (9,222 ± 3722 viral titer/mg protein) mice compared with lean (15,818 ± 6044 viral titer/mg protein; $P < 0.05$). However, viral titers were still high in both groups.

Lung inflammation contributes to increased mortality in influenza-infected mice. To determine whether obese mice had significantly elevated lung pathology compared with lean mice, we examined lung tissue following infection. Although lung pathology increased significantly in both groups after influenza infection, the obese mice tended to have greater lung pathology (2.65 ± 0.59) compared with lean mice (1.7 ± 0.18; $P < 0.1$).

Obese mice have reduced expression of antiviral cytokines. The expression of IFNa and IFNb is induced early during infection and these cytokines function to control viral replication and activate NK cell cytotoxicity (30). At d 3 p.i., lean mice had robust increases in mRNA expression of IFNa (Fig. 3A) and IFNb (Fig. 3B) unlike obese mice whose antiviral cytokine expression remained low throughout the infection. It is important to point out that lung viral titers did not differ between lean and obese mice at d 3 p.i., suggesting that the differences in IFNa and IFNb expressions were not due to different amounts of virus.

**Reduced NK cytotoxicity in influenza-infected obese mice.** Because IFNa and β were greatly reduced in obese mice, we reasoned that NK cell cytotoxicity may be impaired in these mice. To determine whether NK cell activity was affected by obesity, we measured lung and splenic NK cell cytotoxicity both prior to and 3 d after infection. NK cell cytotoxicity prior to infection was low in both lean and obese mice, with cytotoxicity in the spleen being <10%, and in the lungs, cytotoxicity was below the detection limit of the assay (data not shown). However, during infection, obese mice had significantly less NK cell-mediated killing than lean mice. At d 3 p.i. lung NK cell cytotoxicity was reduced by >50% in obese mice (Fig. 4A). This reduction may have been due, in part, to the decreased proportion of NK cells in the lungs of obese mice; at d 3 p.i., the proportion of NK cells in the lungs of obese mice was 22%, whereas NK cells made up 30% of the lung lymphocyte population in lean mice (Fig. 4B).
Analogous to the lungs, NK cell killing in the spleens of obese mice was also dramatically reduced (Fig. 4C). However, unlike the lung, this was not due to a difference in NK cell frequency, because the percentage of splenic DX5^+ CD3^− cells at d 3 p.i. did not differ between lean and obese mice (Fig. 4D).

Because NK cell cytotoxicity can be affected by other cytokines, we also measured the mRNA expression of IL-12 and IL-18, which are known to enhance NK cell cytotoxicity (31,32). Although IL-12 mRNA levels did not differ between lean and obese mice, IL-18 mRNA levels were lower in obese mice following infection (fold of lean, uninfected mice was 3.73 ± 0.058 and 0.27 ± 0.02 in obese mice, P < 0.005), suggesting a role for IL-18 in the decreased cytotoxicity of NK cells in obese mice.

**Lung mRNA expression of proinflammatory cytokines and the anti-inflammatory cytokine IL-10.** In addition to antiviral cytokine responses and NK cells, inflammatory responses are a key part of the innate immune response to influenza infection. We examined gene expression of the proinflammatory cytokines IL-6 (Fig. 5A), TNFα (Fig. 5B), and IL-1β (Fig. 5C) in the lungs of obese and lean mice. Lung mRNA expression at d 3 p.i. for all 3 cytokines was significantly lower in obese than in lean mice. Interestingly, expressions of TNFα and IL-6 were downregulated by d 6 p.i. in lean mice, whereas obesity led to enhanced expression at this time point.

To determine whether the differences in proinflammatory cytokine expression at d 3 p.i. could be attributed to upregulation of IL-10, a cytokine that counteracts the proinflammatory effect, we measured its expression in the lungs of obese and lean mice. Similar to the proinflammatory cytokines, IL-10 mRNA levels were significantly lower in the obese mice at d 3 p.i. compared with the lean mice (Fig. 5D). Unlike IL-6, however, the expression of IL-10 at d 6 p.i. was not greater in obese than in lean mice, indicating that the lungs of obese mice were in a proinflammatory state 6 d after the initial infection.

**Decreased lung MCP-1 and RANTES mRNA expression in influenza-infected obese mice.** The induction of chemokines is an important component of infection with influenza virus because they function as attractants for immune cells at the site of infection (22). Lung mRNA expressions of MCP-1 (Fig. 6A) and RANTES (Fig. 6B) were significantly lower in obese mice 3 d p.i. than in lean mice. However, there was no difference in the expression of MIP-1α (data not shown) between lean and obese mice, indicating that obesity may cause selective recruitment of immune cells to the site of infection.
Although there are reports of genetically obese mice (ob/ob or db/db) having impaired immune function (9,33), few studies have examined the effect of diet-induced obesity on immune function. Diet-induced obesity is a more physiologically relevant model of human obesity, as only a small number of individuals are obese due to mutations in the leptin gene (34). Furthermore, to our knowledge, there are no reports on the effect of diet-induced obesity on infection with influenza virus. With 30% of the population in the U.S. obese, and influenza virus infecting 5–20% of the entire U.S. population each year, it is important to understand how obesity may impact the host’s ability to respond to infection with influenza virus.

In our study, diet-induced obese mice had a 6.6-fold higher mortality rate, postinfection. Along with the increase in mortality was an altered immune response, including diminished NK cell cytotoxicity and delayed proinflammatory cytokine expression. To our knowledge, this is the first report of obesity interfering with normal host responses to influenza infection.

It is well established that leptin concentrations in diet-induced obese mice are higher compared with mice of normal weight (35). This chronic elevation, however, appears to cause a state of leptin resistance (36,37), which may be disadvantageous given that numerous studies have demonstrated a significant role for leptin in the immune response (9,38,39). For example, when wild type mice were infected with Klebsiella pneumoniae, leptin concentrations increased in the blood and lung homogenate and these mice had a normal survival time following infection. Conversely, when mice lacking leptin were infected, there was no rise in leptin concentrations following infection, and these mice had reduced macrophage function as well as high mortality rate (9). Therefore, we measured circulating leptin concentrations in influenza-infected lean and obese mice. We found that
leptin concentrations in lean mice transiently increased during influenza infection. Surprisingly, in obese mice serum leptin decreased significantly during infection. The cause for this reduction is unknown, but given that the infection was more detrimental to obese mice than lean, the obese group may have stopped eating. Limited food intake will result in lower serum leptin concentrations (38,40). This decrease in food intake may also have occurred in the lean mice given the high levels of IL-1β and TNFα. However, in addition to their anorectic effects, these cytokines increase leptin production (41,42); therefore, leptin concentrations in the lean mice may have been appropriately balanced.

Given leptin’s influence on immune function, the reduction in leptin may have impaired the innate immune responses in the obese mice. However, it should be noted that although the obese mice had a drop in leptin at d 3 p.i., the concentration of serum leptin at this time was similar to lean mice. Therefore, if obese mice were still sensitive to leptin, this level should be sufficient to generate a response. Usually during an infection, leptin’s effects on the innate immune system increase the inflammatory response. Leptin acts on macrophages and dendritic cells and augments their production of the proinflammatory cytokines IL-1β, TNFα, and IL-6 (43). We found that obesity resulted in a notable delay in lung proinflammatory cytokine expression. Given the lack of an early proinflammatory response in obese mice, it is plausible that leukocytes from the obese mice no longer respond to leptin. Evidence for this comes from a recent report by Papanathanassoglou et al. (36) who demonstrated that signal transducer and activator of transcription 3 (STAT3) nuclear translocation is attenuated in T cells from obese mice following leptin stimulation. A leptin concentration of 6.25 nmol/L caused a 5-fold change in STAT3 DNA binding in T cells from lean mice, whereas no change was observed from cells taken from obese mice. This study clearly shows obesity can cause T cells to become insensitive to leptin and suggests that other cells of the immune system would be similarly affected.

An alternative explanation for the reduced early cytokine expression in obese mice is a reduction in number and/or maturation of macrophages in the lungs during infection. Work by Krishnan et al. (2) demonstrated that obese humans have similar numbers of circulating monocytes, but the number of monocytes that matured into macrophages was almost 3 times less in these individuals. Moreover, the ability of a mature macrophage to elicit an antimicrobial and cytotoxic response may be inhibited (44). Because macrophages are a major contributor to proinflammatory cytokine production, fewer macrophages in the lungs, as well as a decrease in their functional capacity, could explain the reduction in cytokine levels. It is of note, however, that infiltration of monocytes may also be reduced in the obese mice as they expressed significantly lower levels of MCP-1 at d 3 p.i.

Despite the early lack of induction of inflammatory genes in obese mice, expression of IL-6 and TNFα did increase by d 6 p.i. to levels comparable to what lean mice expressed at d 3 p.i. This late response may be due to increased numbers of infected lung cells and infiltration of cytokine producing T cells into the lung. Importantly, the late rise in inflammatory gene expression was not balanced by a concomitant rise in IL-10 expression, indicating that the lungs of obese mice were in a heightened proinflammatory state. Indeed, lung pathology tended to be more severe in obese mice, and it is likely that the upsurge in cellular infiltrate was due to this high level of inflammatory gene expression. Furthermore, because T cells contribute substantially to lung pathology and peak T cell responses do not occur until 8–10 d after the initial influenza infection (45), it is possible that lung pathology continued to escalate after d 6 p.i. and resulted in the death of the obese mice.

In addition to dysregulation of proinflammatory mediators, obesity resulted in the impairment of other important components of the early immune response. The induction of the antiviral cytokine response, induced primarily by viral factors, was severely blunted in obese mice despite equivalent lung viral titers. This may be due to impaired janus kinases/STAT signaling caused by an elevation in suppressor of cytokine signaling (SOCS) proteins in obese mice. SOCS proteins are activated by cytokines signaling through janus kinases/STAT pathways and function as negative regulators of the pathways. However in obese animals, these suppressor proteins are persistently elevated (46,47). Specifically, SOCS1 and 3 are amplified in obese animals and these proteins inhibit signaling by type I interferons (48,49). Because IFNα/β upregulate their own expression (17), it is possible the lack of induction of these cytokines is due to impediment of the signal.

Because IFNα/β are required for NK cell proliferation and cytotoxicity (30), a deficiency in NK activity may have resulted as a consequence of reduced expression of these cytokines. NK cells, together with type I interferons (IFNα/β), provide an innate defense against infection with peak activity occurring within 3–4 d p.i. (50). In the current study, obese mice had >50% reduction in lung and splenic NK cell activity at d 3 p.i. The reduction of NK activity in the obese mice is in agreement with previous studies showing that NK cell function is decreased in splenocytes from rats fed a high fat diet (51,52). This reduction in activity, however, was thought to be caused by a decrease in cellular arachidonic acid, as splenic lymphocytes from rats fed diets high in saturated fat showed reduced levels of arachidonic acid (51). Thus, the reduced cytotoxicity of NK cells in obese mice may be a result of a change in splenocyte fatty acid composition, leading to an impaired immune response.

The importance of the current findings is underscored by the fact that millions of people worldwide are affected by influenza infection every year and the universal prevalence of obesity has reached epidemic proportions. In this study, we found that obesity led to dysregulated innate immune responses to influenza infection and increased mortality. Because the innate immune response also activates and polarizes the appropriate cell mediated response, these data indicate that overall immune function may be affected by obesity. Furthermore, these data suggest that, in addition to influenza infection, obesity may increase susceptibility to other viral infections by way of immune system dysregulation.

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Literature Cited


