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Influenza Virus Infection of Mice Induces Anorexia: Comparison with Endotoxin and Interleukin-1 and the Effects of Indomethacin

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SWIERGIEL, A. H., G. N. SMAGIN AND A. J. DUNN. *Influenza virus infection of mice induces anorexia: Comparison with endotoxin and interleukin-1 and the effects of indomethacin*. PHARMACOL BIOCHEM BEHAV **57**(1/2) 389–396, 1997.—The effects of infection of mice with influenza virus on ingestive behavior were assessed by both 22-h intake of food pellets, and intake of sweetened milk in a 30-minute access period. Infection with a lethal dose of virus resulted in losses in body weight as well as a reduction in food pellet intake and milk intake. By contrast, infection with a sublethal dose of virus decreased body weight and food pellet intake to a lesser extent, but did not alter milk intake. Acute intraperitoneal injection of endotoxin (LPS, 0.3-5 μg), interleukin-1α (IL-1α, 50–100 ng) or IL-1β (100 ng) reduced milk intake, suggesting that the reduction of ingestive behavior may be associated with immune activation in general, and IL-1 in particular. Pretreatment of the mice with the cyclooxygenase inhibitor, indomethacin (10 mg/kg SC) substantially attenuated, but did not completely reverse, the reduction in milk intake by LPS and IL-1. However, chronic treatment with indomethacin failed to alter the body weight or the intake of sweetened milk in influenza-infected mice, although there was some attenuation of the reduction in food intake. These results suggest that although IL-1 may play a role in the anorexia caused by influenza virus infection, it is not the only factor involved. © 1997 Elsevier Science Inc.

Influenza virus Food intake Interleukin-1 Endotoxin Indomethacin Cyclooxygenase

INFECTIONS in animals are known to be associated with a variety of behavioral changes, including decreased locomotor activity, increased sleep time, anorexia, decreased libido and decreased interest in exploring the environment, frequently accompanied by elevations in body temperature. This pattern of responses has been termed ''sickness behavior'' (18). Hart suggested that the behavior of a sick individual is not necessarily a maladaptive and undesirable effect of illness, but rather a highly organized strategy that is at times critical to the survival of the individual (9). The behavioral changes drive the animal to retreat into hiding to conserve energy and to avoid predators that may take advantage of its weakened state.

Many studies have indicated that substances that stimulate the immune system, such as endotoxin (lipopolysaccharide, LPS), and cytokines such as interleukin-1 (IL-1) and tumor necrosis factor α (TNFα) can mimic the behavioral responses to sickness (2,19,31). These results suggest that these, and possibly other cytokines, may be mediators of the sickness-related behavioral changes.

To study the potential role of cytokines during an infection, we have used infection of mice with influenza virus and studied their ingestive behavior. Because the reduced food intake during sickness may reflect factors other than decreased demand for food, we measured not only the intake of food pellets, but also the intake of a more palatable food, sweetened condensed milk, to assess the potential for motivational factors to overcome the anorexia. The results were compared with the acute effects of IL-1 and LPS on sweetened milk intake in mice. We also tested the effects of treatment with indomethacin, a selective cyclooxygenase inhibitor, known to inhibit many actions of cytokines.

MATERIALS AND METHODS

Animals

Six-week old male mice of the CD-1 outbred variety were purchased from Charles River (VAF Plus Colony R16 from

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the Raleigh-Durham facility). They were housed in groups of five in plastic cages provided with wood shaving bedding under a 12 h light-dark cycle with lights on at 6 AM. Mice were given free access to water and food pellets (Purina®). Three days before experimental treatment the mice were housed in individual cages.

Milk Consumption

One part of sweetened condensed milk (Carnation®) was diluted with three parts of water. Pilot experiments showed that this was preferred to water and that mice would consume significant quantities in a short time period. Twenty-five ml glass bottles with standard spouts were filled with diluted milk. The bottles were weighed and placed in the cage hoppers along with larger water bottles. Milk consumption was recorded as the difference between initial and final bottle weights. In this double bottle situation, we observed that mice drank significantly more milk (3.06 ± 0.25 g) than water (0.07 ± 0.01 g), and that the availability of water did not decrease milk intake. When milk was available for one hour, most was consumed in the first 15 min. Careful handling of the bottles minimized spillage. On the three days preceding virus inoculation, the mice were given 30 min access to the milk bottles to acquire a taste for the milk and to habituate to a short-term drinking session. Mice in all groups showed a learning effect on the first three days of access to the milk. The very few individual mice that did not reach the criterion drinking of 1.5 g of milk in the 30 min session were discarded. Every morning the food pellets were removed and weighed, the mice were weighed and at 10 AM the weighed milk bottles were placed in the cages for 30 min. The bottles were then reweighed and a weighed amount of food placed in the hopper.

Locomotor Activity

Spontaneous exploratory locomotor activity was quantified in an open field, at the beginning of the light phase and before presenting the milk bottles. The open field was a box measuring 43 by 43 cm with its floor divided into four squares. The field was illuminated by a red light. Locomotor activity was observed in five minute sessions and line crossings and rears were recorded. Line crossing was scored when a mouse left one square (all four paws) and entered another. Rears were scored when a mouse raised its front paws from the floor. The field was cleaned with 1% acetic acid to mask the odor of preceding animals.

Inoculation with Influenza Virus

The influenza virus (A/PR8/34) was grown in our laboratory in chick embryos. Amniotic fluid was diluted with saline to the required concentration. The mice were anesthetized with halothane and inoculated intranasally at the beginning of the light phase. One group was inoculated with a lethal dose of the virus, $10^{5.5}EID_{50}$ in 50 μl (EID_{50} = embryonal infectious dose, i.e., the dose that would infect 50% of chick embryos). A second group was inoculated with a 100-fold lower dose, $10^{3.5}EID_{50}$. The control group received sterile isotonic saline. In preliminary experiments, it was established that the lethal dose resulted in over 70% mortality within 7 days, with the first deaths occurring on the fifth or sixth day after inoculation. If postmortem analysis revealed no changes in the lungs, these mice were considered to have received unsuccessful inoculations and the related data were removed from the final analysis.

IL-1 and LPS

Recombinant human IL-1α was donated by Hoffmann-La Roche and recombinant mouse IL-1β was obtained from R&D Systems (Minneapolis, MN). *E. coli* LPS was obtained from Sigma (L3755). IL-1α IL-1β and LPS were dissolved in sterile pyrogen-free isotonic saline such that the total dose for each mouse was contained in 0.1 ml. Mice were allowed access to sweetened milk for 30 min each day for two or three days, until their intake reached 1.5 g. On the third or fourth day, IL-1 or LPS was injected intraperitoneally in doses of 20, 50 and 100 ng (IL-1α), 50 or 100 ng (IL-1β) or 0.1–5 μg (LPS). Two hours later, the milk bottle was inserted into the top of the cage for 30 min as described above.

Indomethacin Treatment

Indomethacin was suspended in sterile isotonic saline and injected subcutaneously at a dose of 10 mg/kg. Previous experiments have shown that this treatment with indomethacin maintained a therapeutic concentration of indomethacin in plasma for more than 12 hours (unpublished observations). For the IL-1 and LPS experiments, indomethacin was injected 10 or 30 min before IL-1 or LPS, and access to milk given 2 h after IL-1 or LPS. In the influenza virus-infected mice, indomethacin was injected twice daily at 12 h intervals. The first injection occurred immediately after inoculation with influenza virus.

Experimental Procedure

Three separate experiments were performed with influenza virus-infected animals. In the first experiment, mice received control, sublethal and lethal doses of the influenza virus. Body weight, daily food intake and 30 min milk consumption were observed. The second experiment was performed like the first, except that locomotor activity was quantified on the 2nd, 4th, 6th and 12th days after the inoculations. In the third experiment, the mice received either control or lethal dose of the virus and were treated six times daily with saline or indomethacin. Four groups were thus created: control-saline; control-indomethacin; influenza-saline and influenza-indomethacin. Body weight, food and milk intake were recorded daily, and locomotor activity was observed on the 2nd, 4th, 6th and 10th days after the inoculation.

Data Analysis

One or two-factor analysis of variance was performed using SuperAnova (Abacus Concepts, Inc.). If analysis of variance indicated significant effects, Student's or Dunnett's *t*-tests were used post-hoc. In the experiment with indomethacin and influenza virus infection (Figures 8 and 9), we first performed a two-way ANOVA for the data from Days 1 through 5 following inoculation using a repeated measures design. Subsequently, two-way ANOVA was performed on each experimental day to determine drug-infection interactions. All data are reported as mean ± the standard error of the mean.

RESULTS

Effects of Infection with Lethal and Sublethal Doses of Influenza Virus on Ingestive Behavior

The first deaths in the group of mice administered the lethal dose of virus occurred on the sixth day, and by the eighth day all the mice in this group were dead. All the mice in the control

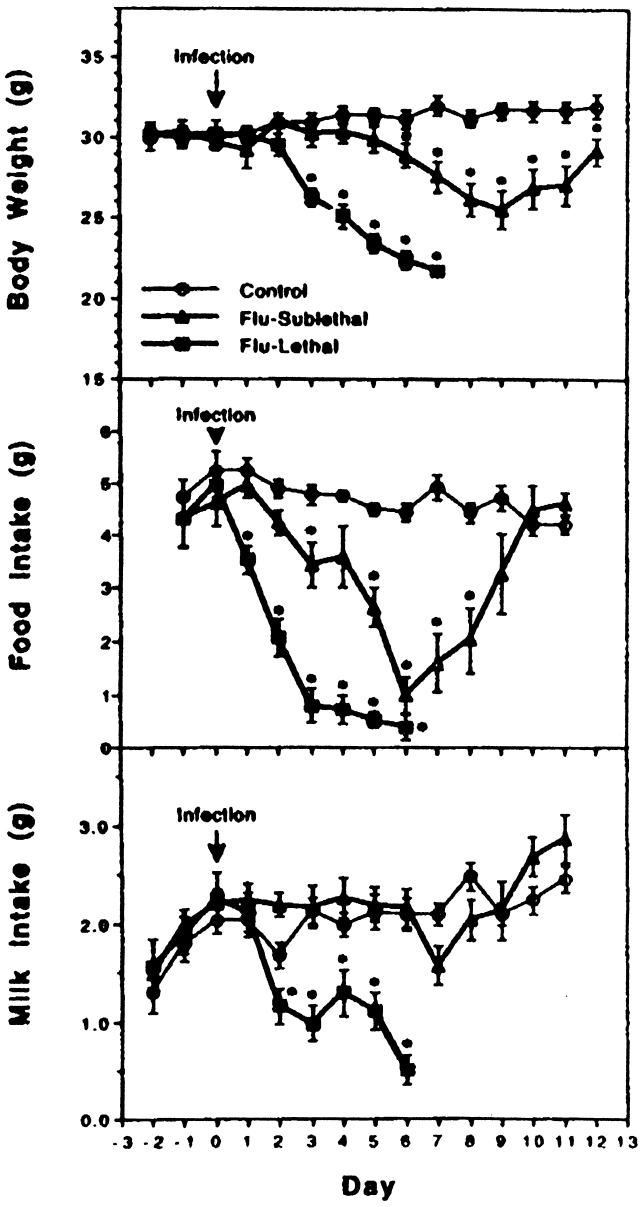

FIG. 1. Body weight, food intake and milk intake in mice inoculated with influenza virus. Mice (n = 10) were inoculated intranasally with vehicle or lethal or sublethal doses of influenza virus immediately after the third exposure to milk. Each day, the mice were weighed and their food pellet intake for the previous 22 h measured, as well as the amount of sweetened milk consumed in a 30 min period. *Significantly different from the control vehicle-injected group ($p < 0.05$).

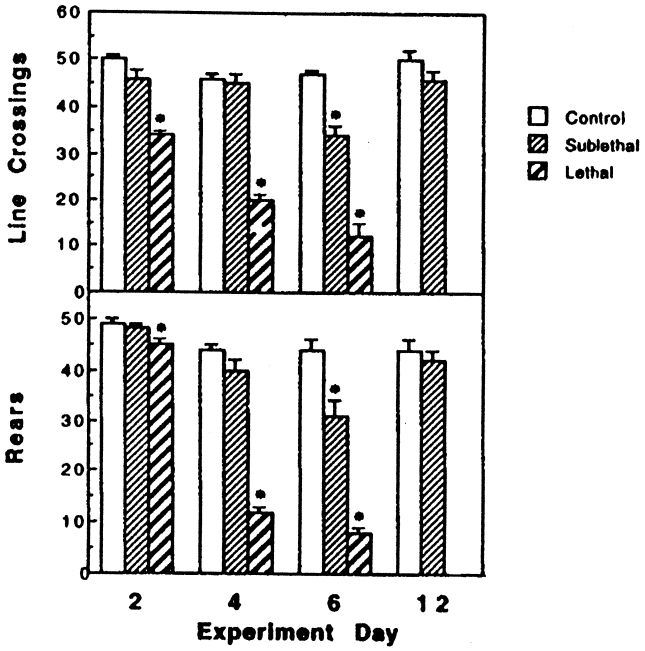

FIG. 2. Locomotor activity of mice inoculated with influenza virus. Mice (n = 10) were tested for 5 min in an open field, scoring line crossings and rears, on the 2nd, 4th, 6th and 12th days following inoculation with influenza virus. *Significantly different from the vehicle-injected group ($p < 0.05$).

and sublethal groups survived. Mice infected with the virus lost weight [$F(2, 347) = 176$, $p < 0.001$: Figure 1 top graph], and the effect was much more marked in the group that received the lethal dose. Significant depression in body weight was observed 72 h after the inoculation in the lethal group, and after 144 h in the sublethal group. The animals from the latter group were recovering body weight by the end of the experiment. The intake of food pellets was also reduced in both groups of mice infected with virus [$F(2, 301) = 129$, $p < 0.001$: Figure 1 middle graph]. Food intake was already depressed in the second 24 h period after inoculation in the lethal group and in the fourth 24 h period in the sublethal group. In the latter group, food intake returned to normal by the ninth or tenth day after virus inoculation. Milk intake was depressed 48 h after a lethal dose of virus, but was not affected in the control and sublethal groups [$F(2, 326) = 41.2$, $p < 0.001$: Fig 1 bottom graph].

Figure 2 shows the results for locomotor activity scored on Days 2, 4, 6 and 12 after inoculation. Virus infection clearly affected both line-crossings and rears. On Days 2, 4 and 6, the animals from the lethal group displayed significantly less locomotor activity than the animals from the control and sublethal groups (Line Crossings, $F(2, 93) = 163$, $p < 0.001$; Rears, $F(2, 93) = 105$, $p < 0.001$). The animals from the sublethal group were significantly less active than the control animals only on Day 6 after the inoculation.

The Effects of LPS on Ingestive Behavior

Mice were allowed access to the sweetened milk for 30 minutes for two consecutive days. On the third day, they received an intraperitoneal injection of saline or 0.1, 0.3, 1 or 3 μg of LPS 2 h before the milk bottle was placed in the cage hopper. The results are shown in Fig. 3. LPS treatment consistently decreased the milk intake on the day of injection [$F(4, 35) = 3.02$, $p < 0.05$], but this had recovered by the next day [$F(4, 35) = 0.9$]. The effect was statistically significant at the 0.3, 1 and 3 μg doses of LPS. Food pellet intake in the 24 h following the LPS injection was also significantly decreased by all doses of LPS tested [$F(4, 34) = 10.7$, $p < 0.05$; Fig. 3]. Similar results were found consistently in several other experiments, although the magnitude of the inhibition varied significantly. We also tested the time course of the response

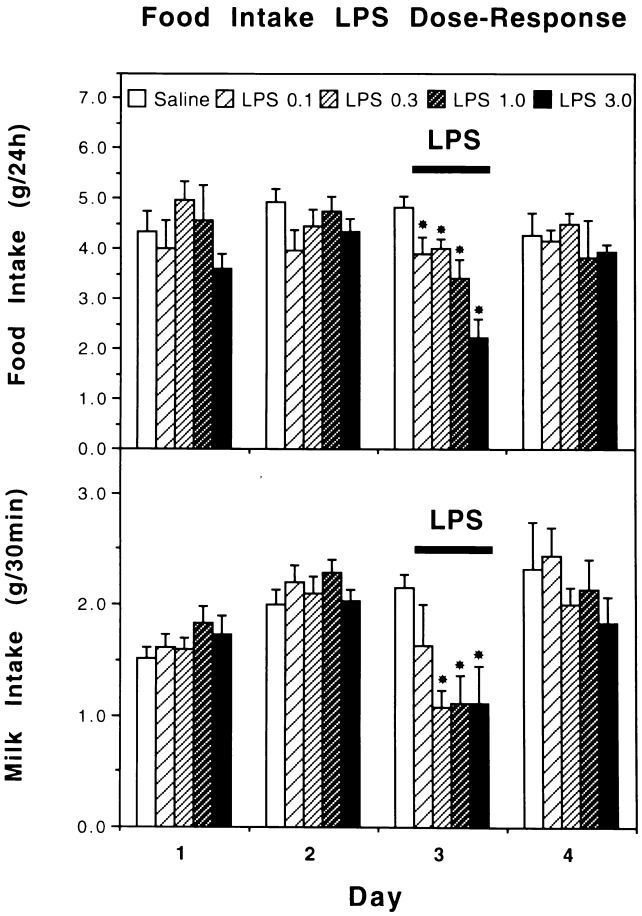

FIG. 3. Milk intake in mice treated with different doses of LPS. Mice ($n = 7$) were habituated for two days to drink sweetened milk for 30 min. Their 24-h food pellet intake, and the amount of sweetened milk consumed in a 30 minute period was monitored for four consecutive days. On the third day, LPS was injected IP at doses of 0.1, 0.3, 1.0 and 3.0 μg 2 h before they were allowed access to sweetened milk. On the fourth day, access to milk was again allowed, but with no prior injections. *Significantly different from the saline-injected group ($p < 0.05$).

to a 1 μg dose of LPS. The results (Fig. 4) indicate that LPS affected milk intake [$F(4, 39) = 6.33$, $p < 0.001$]. Milk intake was already depressed when mice were allowed access to it one hour after LPS ($t_{14} = 2.46$ $p < 0.05$). The maximum response appeared at 2 h ($t_{14} = 5.6$, $p < 0.001$), and some recovery had occurred by 4 h ($t_{14} = 2.14$, $p > 0.05$). In most experiments, recovery was complete within 24 h (see, for example, Fig. 3).

The Effects of IL-1 on Ingestive Behavior

IL-1α and IL-1β were tested in a similar paradigm. Figure 5 shows that IL-1α treatment significantly decreased milk intake at doses of 50 and 100 ng 2 h after injection [$F(3, 20) = 5.52$, $p < 0.01$]. Similarly, IL-1β significantly decreased milk intake at a dose of 100 ng [$F(2, 16) = 5.66$, $p < 0.05$]. Heat inactivated samples of the IL-1 had no effect on milk intake.

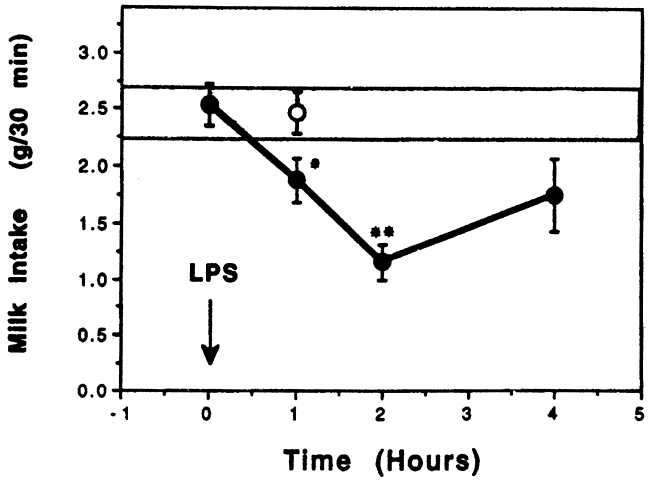

FIG. 4. Milk intake in mice treated with LPS. Mice ($n = 8$) were tested for their intake of sweetened milk at 0, 1, 2 and 4 h after an injection of 1 μg of LPS. Open circle: the milk intake of all of the mice tested 1 h following a saline injection on the previous day; closed circles: milk intake of mice injected with LPS. *Significantly different from the group tested immediately after LPS (*$p < 0.05$, **$p < 0.01$).

The Effects of Indomethacin on the Responses to IL-1, LPS and Influenza Virus

We then tested the effect of a single subcutaneous dose of indomethacin (10 mg/kg) on the responses to IL-1. Indomethacin pretreatment largely prevented the reductions in milk intake in response to 100 ng IL-1α or IL-1β (Fig. 6). ANOVA indicated a statistically significant interaction between the IL-1 and indomethacin treatments [IL-1α: $F(1, 18) = 4.56$, $p < 0.05$; IL-1β: $F(1, 17) = 5.45$, $p < 0.05$]. Very similar results were observed in a separate experiment with LPS (Fig. 7). ANOVA did not indicate statistical significance for the interaction between LPS and indomethacin pretreatments [$F(1, 28) = 1.82$, $p = 0.19$], but it is clear that the indomethacin pretreatment attenuated the usual reduction of milk intake by LPS which was statistically significant in saline-treated mice, but not in indomethacin-treated ones. These results suggest that the anorexic actions of IL-1β and LPS are at least partially dependent on cyclooxygenase activity.

We then tested the effects of chronic treatment with indomethacin in mice infected with a lethal dose of influenza virus. Three (out of 18) animals that were successfully inoculated with influenza virus died by the end of the fifth day after virus inoculation and all the remaining mice were terminally sick or dead by the eighth day after inoculation. There were no statistically significant effects of indomethacin on the mortality of influenza-infected mice.

Body weight was significantly depressed by the influenza virus infection (Fig. 8 top graph); ANOVA for the first five days after inoculation indicated no significant interactions, but a significant effect of the virus inoculation [$F(1,182) = 67.7$; $p < 0.0001$]. Two-way ANOVA on individual days indicated significant effects of the virus on Days 3, 4, 5 and 6 after infection, with no significant effects of indomethacin or any significant interaction between virus and indomethacin. Food pellet intake was depressed by the virus (Fig. 8 middle graph);

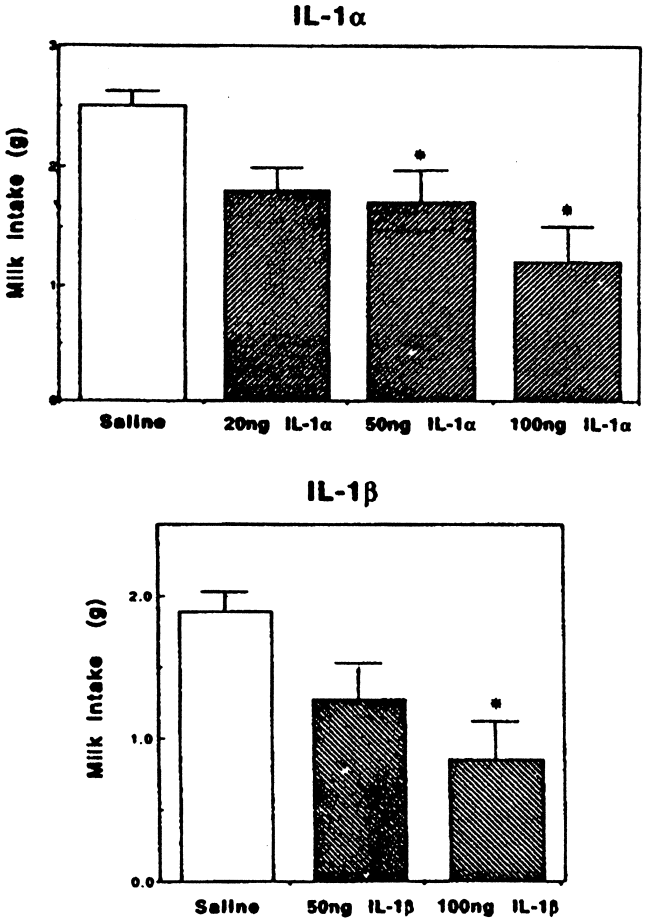

FIG. 5. Milk intake in mice treated with IL-1. Mice were exposed for 30 min to sweetened milk, 2 h after injection with recombinant human IL-1α at doses of 20, 50 or 100 ng (n = 8, upper figure) or recombinant mouse IL-1β at doses of 50 or 100 ng (n = 6, lower figure). *Significantly different from the saline-injected group ($p < 0.05$).

ANOVA for the first five days after inoculation indicated a significant interaction between the virus inoculation and indomethacin [$F(1, 175) = 13.0$; $p < 0.001$], and a significant effect of the virus inoculation [$F(1, 175) = 196$; $p < 0.0001$). Two-way ANOVA on individual days indicated a significant interaction between influenza and indomethacin on Days 1, 4 and 5, and significant effects of the virus on Days 1, 2, 3, 4, 5 and 6 after infection. Milk intake was decreased in inoculated mice (Fig. 8 bottom graph); ANOVA for the first five days after inoculation indicated no significant interactions, but a significant effect of the virus inoculation [$F(1, 177) = 16.7$; $p < 0.0001$). Two-way ANOVA on individual days indicated significant effects of the virus on Days 2, 3, 4, 5 and 6 after infection, with no significant effects of indomethacin or any significant interaction between virus and indomethacin. These results suggest only a small effect of the indomethacin on food pellet intake in the influenza virus-infected mice, and that only on three days after inoculation.

Motor activity was significantly suppressed on all observation days in both infected groups (Fig. 9). On Day 2, ANOVA indicated significant main effects for influenza virus infection

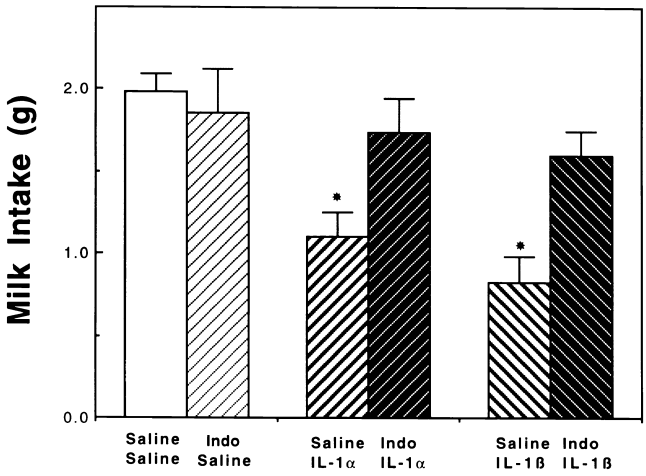

FIG. 6. Milk intake in mice pretreated with indomethacin followed by IL-1. Mice were habituated for at least two days to drink sweetened milk for 30 min. On the next day, they were injected with 10 mg/kg indomethacin SC followed 10 min later by 100 ng hIL-1α or mIL-1β (100 ng IP). Two hours later they were allowed access to the sweetened milk. n = 6, except in the saline groups, where n = 5. *Significantly different from the control saline-injected group ($p < 0.05$).

on line crossings [$F(1, 33) = 36.9$, $p < 0.001$] and rears [$F(1, 33) = 28.3$, $p < 0.001$]. On Days 4 and 6, ANOVA indicated a significant interaction between influenza virus infection and indomethacin on line crossings [Day 4: $F(1, 33) = 5.1$, $p = 0.03$; Day 6: $F(1, 33) = 6.7$, $p = 0.015$] and a significant main effect of influenza virus for rears [Day 4: $F(1,33) = 10.1$, $p < 0.01$; Day 6: $F(1, 33) = 54.4$, $p < 0.001$]. Thus indomethacin significantly attenuated the effects of the influenza virus infection on line-crossings on Days 4 and 6.

DISCUSSION

The time courses for the mortality and the changes in body weight and food intake following influenza virus infection were very similar to those observed in our previous experiments

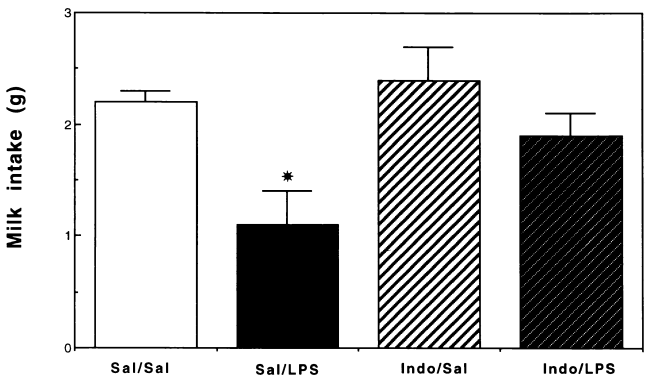

FIG. 7. Milk intake in mice pretreated with indomethacin followed by LPS. Mice (n = 8) were habituated for at least two days to drink sweetened milk for 30 min. On the next day, they were injected with indomethacin (10 mg/kg SC) followed 30 min later by LPS (1 μg IP). Two hours later they were allowed access to the sweetened milk. *Significantly different from the saline-injected group ($p < 0.05$).

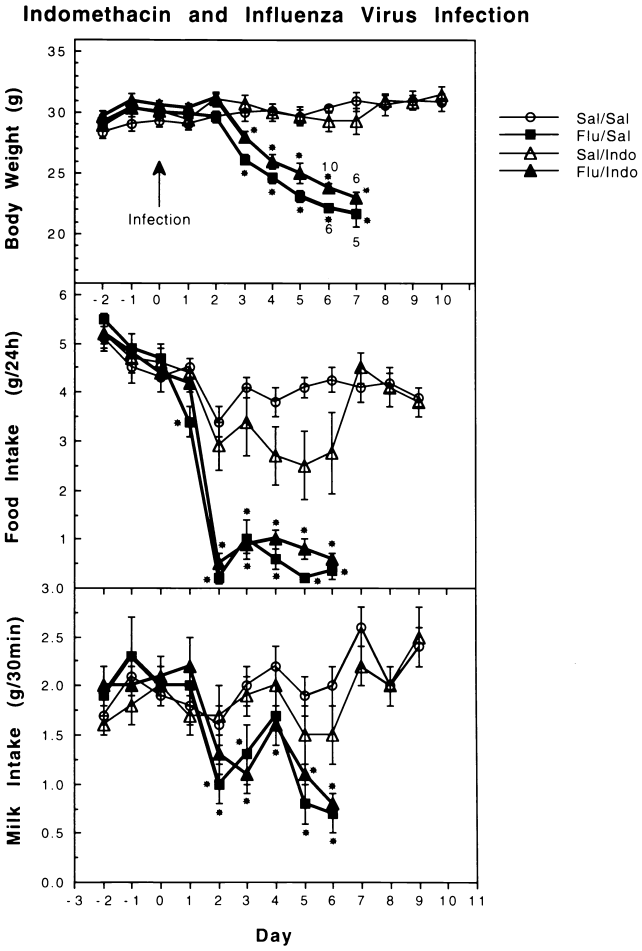

FIG. 8. Changes in body weight, food intake and milk intake in mice pretreated with indomethacin and inoculated with influenza virus. Mice (n = 10) were inoculated intranasally with a lethal dose of influenza virus immediately after the third exposure to milk. The experiment was performed like that of Figure 1 except that only a lethal doses of influenza virus was used and two groups of mice were injected with indomethacin (10 mg/kg SC) every 12 h starting immediately after virus inoculation. *Significantly different from the respective groups injected intranasally with saline ($p < 0.05$). The numbers on Days 6 and 7 indicate the numbers of the surviving mice in the influenza-virus-infected groups.

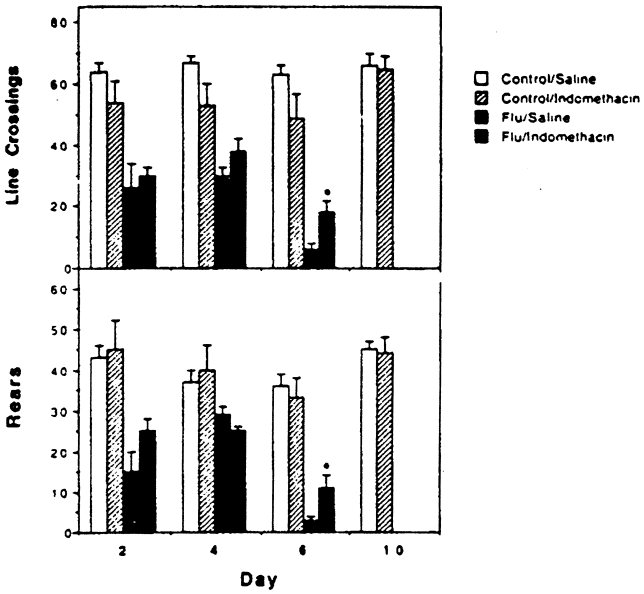

FIG. 9. Locomotor activity of mice pretreated with indomethacin and inoculated with influenza virus. The same experiment as Fig. 8. Mice were tested for 5 min in an open field on the 2nd, 4th, 6th and 10th days following inoculation with influenza virus. *Significantly different from the influenza virus-injected group ($p < 0.05$).

(6). As expected, the infection with influenza virus decreased the intake of food pellets and the effect of a lethal dose of virus was greater than that of a sublethal dose. The intake of sweetened milk was also decreased, but only with the lethal dose of virus, not with a sublethal one. This suggests that the mice were more motivated to drink milk than to eat food pellets, perhaps because the milk was more palatable or easier to consume. The experiments with LPS and IL-1α and IL-1β indicated that each also inhibited milk intake, but that the effects were relatively short-lived. A single dose of LPS early in the light period, also decreased 22-h food pellet intake.

In common with the human experience, infections in animal experiments have long been known to depress food intake (9,18,24). Moreover, LPS has been shown to inhibit food intake in rats (21,24,25,35,36) and chickens (14). Although the behavioral effects of LPS may be attributable to a learned aversion (34), Bauer et al. found that this aspect of the response to LPS was of limited importance in its anorexic effects (1).

Anorexic effects have also been demonstrated for cytokines. IL-1 has been shown to have anorexic effects in rats injected with single doses (10,24,27) or after continuous infusion with IL-1α (28,30). Tumor necrosis factor α (TNFα) can also induce anorexia in rats (2,7,17) and man (37). A decade ago, this effect of IL-1 led McCarthy et al. to suggest that IL-1 might be involved in the anorexia associated with infection (24). Likewise, Calapai et al. have suggested a role for TNFα in the LPS-induced changes in ingestive behavior (4).

Because LPS is known to result in synthesis and secretion of IL-1β shortly after IP injection (38), it is possible that the anorexic effects of LPS are mediated by IL-1. Langhans et al. compared the anorexic responses to IL-1 and LPS in rats, and concluded that ''IL-1β and LPS do not affect feeding through exactly the same mechanism'' (22). Moreover, Kent et al. reported that the IL-1-receptor antagonist (IL-1ra) did not alter the LPS-induced effects on food-motivated behavior in the rat (20).

Infection with influenza virus is also associated with cytokine production. Early work focused on interferon-α (IFNα) (26) which was shown to play a significant role in combatting the infection (8,12). In the lungs, Hennet et al. (11) noted early increases in IL-1α, IL-1β, IL-6, TNFα, granulocyte/macrophage-colony-stimulating factor and interferonγ. Conn et al. (5) have emphasized the role of IL-6 in the acute phase response to influenza virus infection, and failed to demonstrate elevations of circulating interferons in mice using the same strain of virus used here. The latter result conflicts with an earlier report which found elevation of IFN on Days 3–10 after infection, with a peak on Day 7 (13).

Recent results indicate that the LPS and IL-1-related reductions in ingestive behavior may involve the vagus, because

subdiaphragmatic vagotomy prevents the reductions in ingestive behavior (3). Whether or not this is a factor in influenza virus infection will require further experimentation.

Our results indicated that indomethacin treatment was able to significantly attenuate the effects of IL-1α, IL-1β and LPS on milk intake, but not to block them. The effects on the anorexia induced by IL-1 were more clear cut than those of LPS. These results are largely consistent with the existing literature on the effects of cyclooxygenase inhibitors. The LPS-induced reduction in food intake was attenuated by pretreatment of rats with indomethacin (21) or paracetamol (22), by indomethacin in chickens (15), and blocked by indomethacin in pigs (16) and ibuprofen in man (32). Indomethacin blocked the IL-1-induced anorexia in rats (35) and similar results were reported for ibuprofen (10). Ibuprofen and a lipooxygenase inhibitor, AA861, partially prevented the anorexic response to IL-1β in rats (33). Langhans et al. noted that paracetamol was more effective in reversing the anorexia due to IL-1 than to LPS, leading them to suggest that LPS did not act exclusively through IL-1 (22).

Our results did not indicate any effect of chronic indomethacin on sweetened milk intake in influenza virus-infected mice, and a very small but statistically significant effect on food pellet intake. This is consistent with the failure of indomethacin and ibuprofen to alter the food intake of rats bearing tumors (23). Chronic infusion of IL-1 may be a more appropriate model for infection than acute injection, and it is significant that the anorexic effects of IL-1α habituated with such infusions (28, 30). Our results are also consistent with the observation that the anorexic effects of chronic IL-1α were insensitive to the cyclooxygenase inhibitor, piroxicam (29). Taken together these results suggest that although IL-1 might be involved in the influenza virus infection-induced anorexia, it cannot be the only factor.

ACKNOWLEDGEMENTS

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