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Glycyrrhizin, an Active Component of Licorice Roots, Reduces Morbidity and Mortality of Mice Infected with Lethal Doses of Influenza Virus

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The antiviral effect of glycyrrhizin (GR), an active component of licorice roots, was investigated in mice infected with influenza virus A_2 (H_2N_2). When mice that had been exposed to 10 50% lethal doses of the virus were treated intraperitoneally with 10 mg of GR per kg of body weight 1 day before infection and 1 and 4 days postinfection, all of the mice survived over the 21-day experimental period. At the end of this period, the mean survival time (in days) for control mice treated with saline was 10.5 days, and there were no survivors. The grade of pulmonary consolidations and the virus titers in the lung tissues of infected mice treated with GR were significantly lower than those in the lung tissues of infected mice treated with saline. GR did not show any effects on the viability or replication of influenza virus A_2 in vitro. When splenic T cells from GR-treated mice were adoptively transferred to mice exposed to influenza virus, 100% of the recipients survived, compared to 0% survival for recipient mice inoculated with naive T cells or splenic B cells and macrophages from GR-treated mice. In addition, the antiviral activities of GR on influenza virus infection in mice were not demonstrated when it was administered to infected mice in combination with anti-gamma interferon (anti-IFN-γ) monoclonal antibody. These results suggest that GR may protect mice exposed to a lethal amount of influenza virus through the stimulation of IFN-γ production by T cells, because T cells have been shown to be producer cells of IFN-γ stimulated with the compound.

Each year many people contract influenza virus. Although it is not life threatening for most people, it is debilitating and recovery requires 2 to 4 days of bed rest (13). In addition, influenza virus infection causes substantial morbidity among school-age children and excess mortality among elderly people during each influenza season (13). Recent reports (17) described an increase in the number of patients who have been hospitalized or who have died from acute pneumonia or chronic cardiopulmonary diseases or other conditions that can be induced by influenza virus infection. Although vaccination is a good method for providing individuals with improved resistance to influenza virus infection (13), it is only effective against a particular strain of the virus and is dependent upon the type of viral antigen (13). The type of influenza virus which will be prevalent in any coming year is unpredictable. Although therapeutic agents such as amantadine (5) and ribavirin (14) seem to reduce the intensity of the infection, they have toxicities (4, 6). Therefore, new antiviral strategies against influenza virus are necessary to control the infection.

Glycyrrhizin (GR), which is extracted from licorice roots (*Glycyrrhizae radix*) (15) and which has a structure of 20β-carboxy-11-oxo-30-norolean-12-en-3β-yl-2-*O*-β-D-glucopyranuronosyl-α-D-glucopyranosiduronic acid (34), has been used clinically in Japan to treat patients with active chronic hepatitis (34). GR has been shown to express anti-inflammatory activities (7). GR also augments natural killer cell activity (12) and induces interferon production by T cells (1). GR has been described as an antiviral agent against human cytomegalovirus, herpes simplex virus type 2, and human immunodeficiency virus in vitro and in vivo (8, 16, 18). Therefore, the antiviral effect of GR was examined in a mouse model of influenza virus infection. The results of the study found that mice exposed to lethal amounts of the virus survived when they were treated with GR. Also, pulmonary consolidations associated with the infection and the growth of influenza virus in lung tissues were reduced in mice treated with GR. The antiviral functions of the host, including gamma interferon (IFN-γ) production by T cells, were suggested as being part of the protective mechanism of GR against the influenza virus infection in mice.

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MATERIALS AND METHODS

Mice, cells, and viruses. Eight-week-old BALB/c mice (Jackson Laboratories, Bar Harbor, Maine) were used throughout the experiment. Madin-Darby canine kidney (MDCK) cells were maintained in Eagle's modified minimum essential medium (EMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and antibiotics. For the titration of the virus in vitro, MDCK cells which were cultured in EMEM supplemented with 2% FBS and 2 μg of trypsin per ml and which were exposed to influenza virus were used (35). The mouse-adapted Kumamoto strain of influenza virus A_2 (H_2N_2) was maintained in a mouse-to-mouse system as described previously (27). Before being used for the infection, the virus was propagated once in the allantoic cavity of embryonated eggs and was stored at −70°C. The titer of the allantoic fluid in a 50% egg infectious dose (EID_{50}) was 2.6×10^8/ml; this corresponded to 10^4 50% lethal doses (LD_{50}s) for mice when it was inhaled by our standard infection procedures (27).

GR. GR was supplied by Minophagen Pharmaceutical Co., Tokyo, Japan. This compound consists of one molecule of glycyrrhetinic acid and two molecules of glucuronic acid (34). GR was dissolved in saline at appropriate concentrations, and 0.2 ml of the solution was administered intraperitoneally (i.p.) to mice infected with influenza virus or to healthy mice.

Virus inoculation procedures. Mice were infected with the influenza virus by inhalation with a glass vaporephirine type nebulizer that sprayed 10 ml of diluted allantoic fluid for 30 min (10). The mice were in a caged rotating container in which the nebulizer was inserted. By this procedure, each mouse received 20 μl of the virus solution (10).

Evaluation of the antiviral effect of GR in mice. Mice exposed to the influenza virus at doses of 1 to 100 LD_{50}s were treated i.p. with various doses (1.25 to 80 mg/kg of body weight) of GR 1 day before infection and 1 and 4 days postinfection. Infected mice treated with saline (0.2 ml/mouse) served as controls. The antiviral effects of GR were evaluated on the basis of (i) survival rate, (ii) mean survival time in days (MSD), (iii) virus growth in lung tissues, and (iv) lung consolidation scores. The results obtained for the groups treated with GR were compared with those obtained for the groups treated with saline. The survival rate was calculated 21 days after the infection. The mortality rate for all control mice infected with 10 to 100 LD_{50}s of influenza virus was 100%. Within 21 days of infection, the MSD, the average number of days that the mice in each group survived, was calculated. For the titration of pulmonary viruses, lungs of infected mice were disrupted with a glass homogenizer (Wheaton) to make a 10% suspension in 1/100 M phosphate-buffered saline (pH 7.2). After centrifugation at $1,580 \times g$ for 20 min, supernatants were serially diluted 10-fold and were inoculated into groups of four embryonated chicken eggs. The allantoic fluids were tested for hemagglutinin activity after incubation for 72 h at 36°C (22). The EID_{50} was calculated as described by Reed and Muench (19). For calculation of the lung consolidation score, lungs were removed from mice various days after the infection, after removing erythrocytes by irrigation by administration of 4 ml of phosphate-buffered saline to the heart. The consolidation scores were an average of the scores obtained for each mouse. The five possible scores that each mouse could be rated were as follows: 0, less than 25% consolidations; 1, 25 to 49% consolidations; 2, 50 to 74% consolidations; 3, 75 to 99% consolidations; and 4, 100% consolidations or death. The grade of consolidations was evaluated microscopically (24). Ribavirin, an antiviral agent known to have activity against influenza viruses, was used as a positive control (4, 6). Mice exposed to various doses of the virus were treated i.p. with 50 mg of ribavirin (ICN Pharmaceutical Inc., Costa Mesa, Calif.) per kg 24, 3, and 1 h before infection, 1 and 3 h after infection, and then twice daily for 4 days (32).

Virucidal and virustatic tests. In order to determine the effect of GR on the viability of influenza virus, 10 to 1,000 μg of GR per ml in 1 ml of EMEM supplemented with 2% FBS, 2 mM L-glutamine, and antibiotics (medium) was mixed with 1 ml of solution containing virus (2.6×10^6 EID_{50}s/ml). After an incubation period of 1 h at 37°C, the GR-treated virus solution was subjected to plaque titration in MDCK cells after it was serially diluted 10-fold. As controls, the same virus solution was mixed with medium or 10% formalin, and the mixture was incubated for 1 h at 37°C. The growth of MDCK cells cultured with diluted GR (10 to 1,000 μg/ml) was determined by a trypan blue dye exclusion test. From this experiment 10 to 100 μg of GR per ml was shown to have no cytotoxic activity on the growth of MDCK cells. To test the virustatic activity of GR, MDCK cells adsorbed with 2.6×10^4 50% tissue culture infective doses of influenza virus (37°C for 1 h) were treated with nontoxic doses of GR. Forty-eight hours after the treatment, the titer of virus harvested from the cultures was determined in MDCK cells by a plaque method (35).

Preparation of various splenic cells. As described previously (33), spleens were removed aseptically from healthy mice treated or not treated with GR (at 10 mg/kg i.p. at 1, 3, and 5 days before harvest). Single-cell suspensions were prepared from these spleens with a wire screen, and they were designated whole spleen cells (WSCs) (33). In some experiments, plastic surface-adherent cells (macrophages) were prepared from WSCs by using FBS-coated petri dishes (33). Thus, WSCs (2×10^7 to 5×10^7 cells/10 ml) were placed into petri dishes coated with FBS for 90 min. The dishes were then kept for 15 min at 37°C to allow the macrophages to attach. At the end of the incubation period, floating cells were removed with warm RPMI 1640 medium supplemented with 25 mM HEPES buffer. Macrophages were harvested by scraping them from the dishes with a rubber scraper after the dish was kept for 10 min at 4°C. To obtain B cells or T cells, WSCs (10^8 cells/ml) were passed through a B-cell or a T-cell enrichment column (R&D Systems, Minneapolis, Minn.), respectively. When the B-cell fraction obtained was treated with anti-immunoglobulin antiserum and complement, a 97% reduction in the number of viable cells was demonstrated, whereas treatment of these cells with anti-CD3 monoclonal antibody (MAb) followed by treatment with complement caused only a 4% reduction in the number of viable cells. When the T-cell fraction obtained was treated with anti-immunoglobulin antiserum and complement, only a 3% reduction in the number of viable cells was demonstrated, whereas treatment of these cells with anti-CD3 MAb followed by treatment with complement caused a 98% reduction in the number of viable cells. This indicates that B cells and T cells obtained by this procedure have a purity of more than 97%.

Adoptive transfer of splenic effector cells. In order to examine the mechanism of the antiviral activity of GR in vivo, mice exposed to a lethal amount of influenza virus were inoculated with various splenic cell fractions from mice treated with GR. The following cells were adoptively transferred and administered intravenously to the infected mice at an amount of 5×10^6 cells/mouse: WSCs or macrophages and T cells and B cells purified from WSCs. As a control, mice were inoculated with the same amounts of WSCs from mice treated with saline. Two hours after the adoptive transfer, recipient mice (10 mice each) were exposed to 10 LD_{50}s of influenza virus. Survival rates and the MSDs for these mice were compared with those for control mice treated with saline (0.2 ml/mouse).

Anti-IFN-γ MAb treatment. To test the effect of IFN-γ on the antiviral activity of GR in vivo, four groups of 10 mice each were infected with 20 LD_{50}s of the virus. The first group was a control group treated with saline. The second group was the positive control group treated with 10 mg of GR per kg. The third group was treated with GR in combination with anti-IFN-γ MAb (5,000 neutralizing U/kg administered 1 day before infection and 1 day postinfection; Pharmingen, San Diego, Calif.). The last group was treated with anti-IFN-γ MAb only. The survival rates and MSDs were observed for a 21-day experimental period.

Statistical analysis. All of the following data were analyzed statistically: percent survival by χ^2 analysis and MSD, viral growth, and lung consolidations by Student's *t* test. If the *P* value that was obtained was below 0.05, the results were considered significant.

TABLE 1. Effect of GR on the survival of mice infected with various doses of influenza virus A_2

Virus dose (LD_{50})	Treatment[a]	No. of mice	MSD[b]	% Survival[c]
100	Saline	15	6.7	0
	Ribavirin	10	8.7	0
	GR	20	8.4	0
20	Saline	15	10.1	0
	Ribavirin	10	>15.3	20
	GR	10	>19.4[d]	70**
10	Saline	15	10.5	0
	Ribavirin	20	>16.9[d]	30*
	GR	20	>21.0[d]	100**
1	Saline	15	>14.5	50
	Ribavirin	15	>21.0[d]	100**
	GR	20	>21.0[d]	100**

[a] Mice infected with 1 to 100 LD_{50}s of influenza virus A_2 were treated i.p. with saline (0.2 ml/mouse) or GR at a dose of 10 mg/kg 1 day before infection and 1 and 4 days postinfection. As a positive control, ribavirin was administered i.p. at a dose of 50 mg/kg to mice 24, 3, and 1 h before infection, 1 and 3 h postinfection, and then twice daily for 4 days.

[b] MSD during the 21-day experimental period.

[c] Percentage of mice surviving 21 days after the infection. (*, $P < 0.01$; **, $P < 0.001$, χ^2 analysis).

[d] Student's *t* test, $P < 0.001$.

RESULTS

Protective effect of GR against influenza virus infection in mice. In the first experiments, mice were exposed to 1 to 100 LD_{50}s of influenza virus and treated i.p. with GR at a dose of 10 mg/kg 1 day before infection and 1 and 4 days postinfection. When mice infected with 100 LD_{50}s of the virus were treated with GR or ribavirin, no protective effect of the reagents was demonstrated. When mice infected with 20 and 10 LD_{50}s of influenza virus were treated with GR, 100 and 70% of the mice, respectively, survived over 21 days ($P < 0.001$), while control mice treated with saline had MSDs of 10.1 (20 LD_{50}s) or 10.5 (10 LD_{50}s) days and 0% survival (Table 1). The positive controls treated with ribavirin had MSDs of 15.3 days (20 LD_{50}s) and 16.9 days (10 LD_{50}s) ($P < 0.01$), and 20% (20 LD_{50}s) to 30% (10 LD_{50}s) survival rates ($P < 0.01$) were obtained for mice infected with the virus. In the next experiments, seven groups of 10 mice were each infected with 10 LD_{50}s of virus and were treated with GR at a dose of 0 (saline) to 40 mg/kg according to the schedule described above. The protective effect of GR was demonstrated when infected mice were treated with greater than 2.5 mg of the compound per kg (Fig. 1). The observed effect was dose dependent, and the effect was observed with doses ranging from 2.5 to 10 mg/kg. The maximum protection of mice exposed to the virus was produced when doses of GR of 10 to 40 mg/kg were administered.

Virus titers in lung tissues of GR-treated mice. The effect of GR on the replication of influenza virus in mouse lungs was

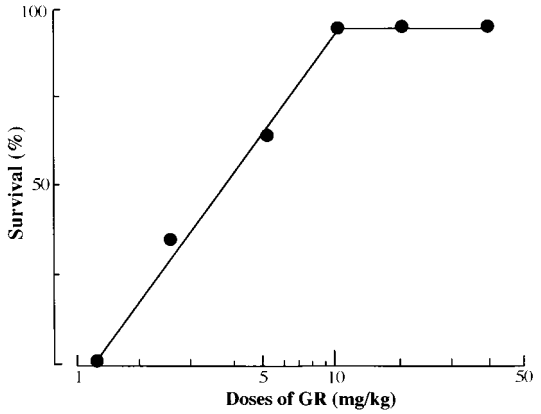

FIG. 1. Effects of various doses of GR on influenza virus infection in mice. A group of 20 mice infected with 10 LD_{50}s of influenza virus A_2 was treated i.p. with GR (1.25 to 40 mg/kg) 1 day before infection and 1 and 4 days after infection. The percentages of mice which survived more than 21 days after infection are plotted.

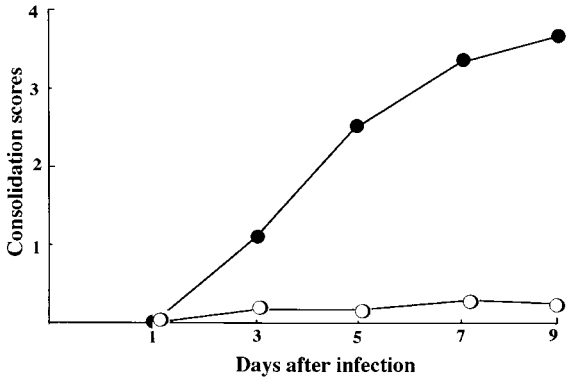

FIG. 3. Effect of GR on the development of pulmonary consolidations in mice infected with influenza virus A_2. The lung consolidation scores for infected mice treated with GR (○) or saline (●) were determined as described in the text. All mice were exposed to 10 LD_{50}s of the virus.

examined. Two groups of mice (30 mice each) exposed to 10 LD_{50}s of the virus were used in this experiment. One of them was treated with GR and the other was treated with saline, which was used as a control. As shown in Fig. 2, the virus titers in the lungs of the treated group of mice 2 to 6 days after the infection were 10 or more times lower than those in the lungs of the control mice. On day 6, viral activity was not detected in the lungs of the treated mice, while the lungs of the control group of mice had titers that remained at 5×10^7 EID_{50}s/ml (Fig. 2).

Effect of GR on the development of lung consolidations. The effect of GR on the development of lung consolidations in mice exposed to the virus was examined. Forty mice exposed to 10 LD_{50}s of influenza virus were divided into two groups. One group was treated i.p. with GR, and the other group, which was used as a control, was treated with saline. For the evaluation of lung consolidations, five mice in each of these groups were sacrificed consecutively 3, 5, 7, and 9 days after the infection. Over a 9-day observation period, the treated group never had a mean score greater than 1, while the scores for the control group reached the maximum (score of 4) by day 9 (Fig. 3).

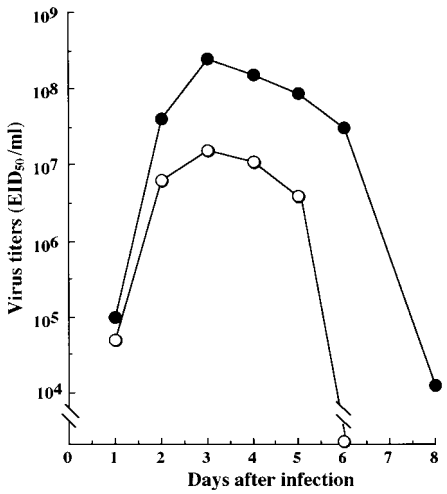

FIG. 2. Effect of GR on the growth of influenza virus in lung tissues. The virus titer in the lungs of GR-treated mice (○) infected with 10 LD_{50}s of influenza virus A_2 was assayed and compared with that in the lungs of controls treated with saline (●). The lung tissue samples were prepared as described in the text.

Mechanisms of antiviral activity of GR. The virucidal activity of GR was not demonstrated when 1 ml of 2.6×10^6 EID_{50}s of influenza virus A_2 was incubated with 1 ml of medium containing 10 to 1,000 μg of GR per ml for 1 h at 37°C. However, under the same conditions, 100% of the virus activity was inactivated by 10% formalin (data not shown). In the virustatic test, MDCK cells were adsorbed with 2.6×10^4 50% tissue culture infective doses of influenza virus, and 1 h later these cells were treated with noncytotoxic concentrations (10 or 100 μg/ml) of GR and were cultured for 48 h at 37°C. The replication of influenza virus was not inhibited by GR for a period of up to 48 h postinfection (data not shown). These results suggest that the observed antiviral effect of GR is neither virucidal nor virustatic, but may be induced through antiviral functions of the host.

In the next set of experiments, various cell fractions prepared from GR-treated mice were adoptively transferred to mice exposed to 10 LD_{50}s of influenza virus. As shown in Fig. 4, when infected mice (recipients) were inoculated with WSCs from GR-treated mice, 100% of the recipients survived, whereas 0% recipient survival was achieved for those mice which received WSCs from saline-treated mice ($P < 0.001$). These results suggested that the protective effect of GR in mice infected with influenza virus A_2 is expressed through the functions of lymphocytes contained in spleens of GR-treated mice. When infected mice were inoculated with T cells prepared from WSCs of GR-treated mice, 100% of recipients survived (Fig. 4) ($P < 0.001$), while all mice inoculated with B cells or macrophages prepared from WSCs of GR-treated mice died within 15 days of the infection (Fig. 4). These results suggest that GR may protect mice infected with the virus through functions of T cells, but not through those of B cells and macrophages.

Since it has previously been reported that GR has an ability to induce IFN (1), we confirmed the IFN-inducing activity of the compound in mice. IFN titers in serum specimens from mice treated i.p. with various doses of GR were determined by a sandwich enzyme-linked immunosorbent assay technique. When mice were treated with a 10-mg/kg dose of GR, IFN-γ was induced in their circulation. The IFN activity reached its peak (35 ng/ml) 24 h after the stimulation, and it then gradually decreased. Therefore, we examined whether IFN-γ plays a role in the antiviral activity of GR in mice exposed to the virus. In this experiment mice exposed to 20 LD_{50}s of the virus were

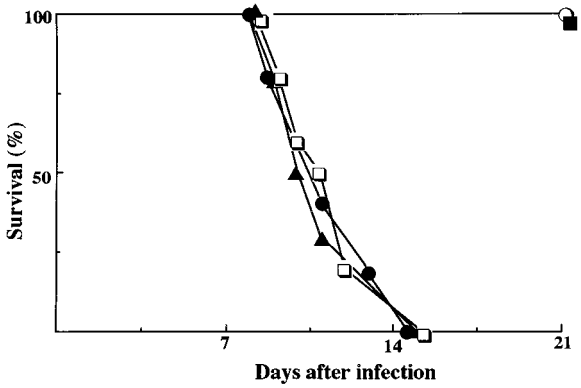

FIG. 4. Effect of adoptive transfer of WSCs and their cellular components from GR-treated mice on the survival of mice infected with influenza virus A_2. WSCs (5×10^6 cells/mouse; ■), T cells (5×10^6 cells/mouse; ○), B cells (5×10^6 cells/mouse; □), or macrophages (5×10^6 cells/mouse; ▲) prepared from GR-treated mice were adoptively transferred to recipient mice (10 mice each) infected with 10 LD_{50}s of virus. As a control, 10 mice were inoculated with the same amount of WSCs from mice treated with saline (0.2 ml/mouse; ●).

treated with GR in combination with anti-IFN-γ MAb (5,000 neutralizing U/kg). When all infected mice treated with saline died, 80% ($P < 0.001$) of mice treated with GR survived more than 21 days after the infection (Fig. 5). However, GR did not protect infected mice (100% mortality) when it was administered to mice in combination with anti-IFN-γ MAb (Fig. 5). Anti-IFN-γ MAb itself had no effect on the mortality of mice infected with the virus (Fig. 5). Although the participation of other factors cannot be ruled out, these results suggest that IFN-γ induced by GR appears to play a key role in the resistance of mice treated with GR.

DISCUSSION

In the present study GR was shown to have antiviral activity in mice exposed to lethal doses of influenza virus A_2 (H_2N_2). The protective effect of GR on the influenza virus infection in mice was demonstrated by (i) an increase in survival rates, (ii) prolongation of the MSD, (iii) inhibition of virus growth in lung tissues, and (iv) reductions in pulmonary consolidations. The protective effect of GR was dependent upon the severity of infection in the mice. The compound showed protective effects in mice infected with 1 to 20 LD_{50}s of the virus (Table 1); however, GR did not increase the survival rates for any of the mice infected with 100 LD_{50}s of the virus (Table 1). We have also previously examined the effects of various schedules of GR administration on the survival of mice infected with influenza virus (36). Prophylactic treatment with GR (1 and 2 days before the infection) or therapeutic treatment with GR (1 and 4 days after the infection) produced 80 and 70% survival rates, respectively. However, five consecutive GR treatments once a day starting 2 days after the infection did not affect the mortality of the infected mice. These results suggest that the treatment schedule is a very important factor in the protection of infected mice by GR. The protection of mice by GR was dose dependent (Fig. 1), and the replication of influenza virus in lung tissues was reduced in infected mice treated with GR (Fig. 2). In addition, the development of lung consolidations associated with viral replication was clearly reduced in GR-treated mice compared to that in the controls (Fig. 3). We have previously reported (24, 27) that the consolidations in the lungs of infected mice are caused by the infiltration of lymphocytes. Our previous study (36) has shown that the number of lymphocytes infiltrated into the lungs of infected mice is reduced after the administration of GR.

Since the compound did not exhibit any virucidal or virustatic activities against the viability of the virus or the growth of influenza virus in vitro, the participation of host antiviral immunity was suggested as the mechanism by which GR protected mice infected with influenza virus. When WSCs and their T-cell fractions from GR-treated mice were adoptively transferred to mice exposed to the virus, marked increases in the resistance of recipients to the infection was demonstrated (Fig. 4). However, mortality rates stayed at 100% for infected recipients when they were inoculated with macrophages or whole B cells from WSCs of GR-treated mice (Fig. 4). These results suggest that the antiviral activity of GR may be expressed through the functions of T cells.

It has also been reported that GR has an ability to induce IFN-γ in mice (1). Therefore, a role of IFN-γ on the antiviral activity of GR was also examined. The results indicated (Fig. 5) that 80% of the mice exposed to 20 LD_{50}s of the virus survived after GR treatment, but there were no survivors among infected mice treated with GR in combination with anti-IFN-γ MAb. These data suggest that GR exerts antiviral effects in mice infected with influenza virus A_2 through the induction of IFN-γ. In a previous study by Hoshino et al. (9), the anti-IFN-γ MAb treatment caused an increased mortality among mice infected with a low dose of influenza virus. This result may support our results presented herein that GR may protect infected mice through its IFN-γ-inducing activity. In our previous study, IFN was not produced in the serum of T-cell-deficient nude mice stimulated with GR (1). Also, GR does not stimulate the IFN production in healthy mice previously irradiated with X rays or treated with hydrocortisone acetate (1). These facts suggest that T cells are the major producer cells for the IFN produced in serum by GR stimulation. Taken together, this study indicates that GR protects mice infected with influenza virus through the stimulation of IFN-γ production by T cells (1). Although it is not directly proven, the activation of natural killer cells and the generation of activated macrophages, which may play a role in the protection of mice infected with the virus, have been shown in mice treated with GR-induced IFN.

In this report, we described the protective effect of GR on influenza virus infection in mice and how the IFN response stimulated by GR is involved in the protection of mice exposed to the virus. Some reports have described the effect of exogenous IFN against influenza virus infection (21, 30). Usually, in

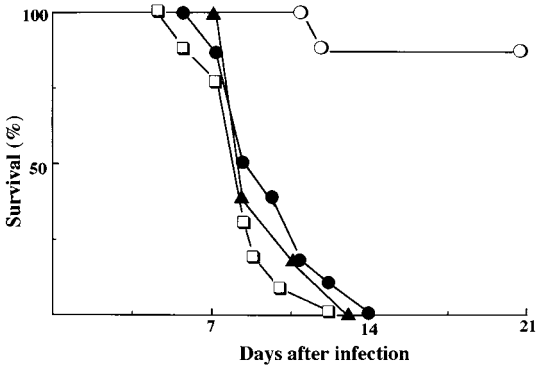

FIG. 5. Effect of anti-IFN-γ MAb on the antiviral activity of GR in mice infected with influenza virus. Mice infected with 20 LD_{50}s of influenza virus were treated i.p. with GR (10 mg/kg; ○), anti-IFN-γ (5,000 IFN-γ neutralizing U/kg; ▲), GR and anti-IFN-γ in combination (□), or saline (0.2 ml/mouse; ●).

those studies relatively high doses of exogenous IFN were required for the protection of individuals infected with the virus. In addition, many reports have described the antiviral effects of IFN inducers on influenza virus infection in mice (20, 26, 28–32). It was proven that these IFN inducers express their protective activities against the virus infection through the induction of IFN (2, 28, 29, 31). However, the titer of IFN in the sera of mice treated with these IFN inducers such as 9-methylstreptimidone and dextran phosphate was relatively low compared with the titer of exogenous IFN required for the protection of infected mice. This suggests that the protective mechanism of IFN against the influenza virus infection in mice may be different when it was administered exogenously or induced endogenously (self-induced IFN). At this time, descriptions of these phenomena are insufficient. Therefore, this study did not address IFN-γ treatment of influenza virus infection.

We have previously demonstrated that local IFN, particularly in lungs, is very important to the survival of mice exposed to influenza virus (21, 30, 31). When 5×10^5 U of IFN per kg was given to mice intravenously, no protective effect was obtained and only 4×10^2 U of IFN per lung was detected in lung homogenates 15 min after administration. In contrast, IFN showed clear protective effects when it was administered intranasally at the same dose to infected mice. By this method of administration, 4×10^4 U of IFN per lung persisted in the lungs up to 90 min after administration. This suggests that the maintenance of a higher titer of IFN in the lung tissues produces the higher protective effect of IFN against the influenza virus infection. Similar results against influenza virus infections have been described in experimental animals treated with certain IFN inducers (28, 29, 31). Dextran phosphate and 9-methylstreptimidone are weak inducers of IFN in serum. However, these compounds effectively protect mice against influenza virus infection when they are given lethal amounts of virus (20, 30). A higher titer of IFN was induced in the lung tissues of mice treated with the inducers 9-methylstreptimidone at 760 U/ml and dextran phosphate at 980 U/ml, whereas a relatively low titer of IFN was produced in the sera of the same mice when they were treated with 9-methylstreptimidone at 120 U/ml and dextran phosphate at 82 U/ml (29). Since influenza virus replicates in a limited area, such as the respiratory tract and lungs, this suggests that the effectiveness of IFN in the protection of mice exposed to lethal amounts of influenza virus is associated with the IFN titer in the lungs.

In our preliminary study, IFN-γ was detected in the lungs of mice 20 h after GR treatment (10 mg/kg). In addition, it has been reported (25) that IFN-γ was produced in cultures of human peripheral blood lymphocytes stimulated with GR. Furthermore, this IFN response was stimulated with T-cell mitogens (25). This suggests that GR may stimulate IFN-γ production by T cells. In this study mice exposed to a lethal dose of influenza virus were protected by adoptive transfer of splenic T cells from GR-treated mice. In our preliminary study, the hemagglutination inhibition titer was measured in the sera of mice 6 days after the infection (5 LD_{50}). A fourfold decrease in the titer was found in the sera of infected mice inoculated with WSCs from GR-treated mice compared with that in the sera of infected mice inoculated with WSCs from saline-treated mice. These results suggest that the total amount of the viral antigen may be reduced in mice inoculated with WSCs from GR-treated mice.

We have described previously that the mortality of mice exposed to lethal doses of influenza virus was clearly reduced when they were treated with anti-T-lymphocyte antibodies (11). This suggests that T cells play an important role in the pathogenesis of influenza virus infection. The T cells that infiltrated into the lung tissues of infected mice produced lymphotoxins and skin-reactive factors. These cytokines may be involved in the progression of lung consolidations directly related to the mortality of infected mice. GR has been shown to be an inducer of anti-type 2 T cells which are able to counteract type 2 cytokine production by type 2 T cells (37). From this, there is a possibility that the anti-type 2 T cells induced by GR may have a protective role against influenza virus infection in mice if type 2 T cells are involved in the pathogenesis of this infectious disease. The generation of type 2 T cells or the production of type 2 cytokines in mice exposed to influenza virus has been described previously, although the production of type 1 cytokines has also been demonstrated in some cases (3, 23). Further experiments are required to clarify the answers to these questions.

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