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Effect of Antilymphocyte Serum on Influenza Virus Infection in Mice (38047)

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(Introduced by S. Baron)

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Several studies suggest that the course of certain viral infections is determined at least in part, by the immunologic response of the host, rather than as a direct effect of virus multiplication (1–4). A good example is the infection of mice with LCM virus (5, 6). Mice inoculated neonatally are protected from the lethal effects of LCM virus. Furthermore, the morbidity and mortality of adult mice are reduced or delayed by various immunosuppressive methods, including X-ray irradiation, neonatal thymectomy and administration of antilymphocyte serum (ALS). A similar experience with ALS was noted by Hirsch with yellow fever virus infection in mice (7). He concluded that cellular immunity was of utmost importance in such murine infections. However, in his review (8) Hirsch reported that pretreatment of mice with ALS did not alter the clinical signs or mortality of influenza virus infections and concluded that cellular immune responses were not important to host defenses under these conditions.

In performing a similar study with influenza virus, we have found that the administration of ALS to mice infected with the Kumamoto strain of A2 virus not only increased survival rates but also reduced the development of lung consolidation without any apparent effect on lung virus titers or humoral antibody responses. Although the mechanisms by which ALS influences the course of infections are not certain, our results suggest the probable participation of cellular immunity in the pathogenesis of influenza infections in mice.

Materials and Methods. Mice. White mice of the dd strain weighing 14–16 g were used for the infection. They were obtained from the Central Farm of Tohoku University where care was taken to prevent infections with Sendai virus.

Virus. The Kumamoto strain of Influenza A2 virus (H_2N_2) was used. For the purpose of inoculation, a mouse adapted virus strain was propagated once in the allantoic cavity of embryonated eggs. The egg infectious titer (EID_{50}) of the fluid was $10^{9.0}$/ml, which corresponded to 10^5LD_{50} in mice when inoculated intranasally by our standard inhalation procedure (9).

Virus inoculation procedures. Virus infection was made by inhalation of the influenza A2 virus. A vaponephrine type nebulizer was connected to the compressor to spray about 10 ml of diluted allantoic fluid in 30 min. This procedure resulted in the inoculation of 1/500 ml of the fluid per mouse (9). The administered dose per mouse is specified for each experiment.

Preparation of antilymphocyte serum (ALS) and antilymphocyte globulin (ALG). ALS was prepared by immunizing rabbits by a slight modification of the method described by Levey and Medawar (10). Briefly, 4 albino rabbits (2–3 kg of body weight) were given 2 iv injections, 7 days

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apart, of single-celled suspensions of 3.2 × 10^8 living thymocytes in Hanks' balanced salt solution obtained from the dd strain of three weeks old mice (10). The rabbits were bled 7 days after the final injection of thymocytes, and the pooled serum was inactivated at 56° for 30 min. The hemolysin was removed from this serum by adsorption with mouse red blood cells. This antiserum and normal rabbit serum used as a control were stored at −20° until use.

ALG was obtained by salting out 40 ml of ALS with ⅓ saturated $(NH_4)_2SO_4$, followed by dialysis against $M/100$ phosphate buffered saline of pH 7.2 (PBS) and fractionation through Sephadex G-200 column (11). The active fraction eluted in 7S globulin fraction was lyophilized and had a minimum leucocyte agglutination activity of 15 μg/ml. The same globulin fraction purified from normal rabbit serum was used as a control. Antihamster thymocyte rabbit serum (AHLS) and its globulin fraction (AHLG) were obtained by the same procedure.

Assays of ALS or ALG. The effectiveness of each preparation was assayed by its ability to (a) diminish peripheral blood lymphocyte counts after intraperitoneal injection *in vivo*, and (b) agglutinate thymocytes *in vitro* when incubated at 37° for 2 hr (10). Both ALS and ALG were shown to be free of antiinfluenza virus activities as assayed by neutralization test. This was done by injecting a 1:1 mixture of 10^3 EID_{50} Kumamoto strain with the serum diluted 1:5, incubating for 1 hr at 37° before injection into the chorioallantoic cavity of 10 embryonated eggs and testing the hemagglutinin production after 48 hr at 36°. Antilymphatic activities of ALG obtained by the above procedure *in vivo* are illustrated in Fig. 1. Peripheral blood lymphocytes of mice were clearly reduced after a single administration of 1.5 mg/kg to 25 mg/kg of ALG.

Determination of the amount of virus in mouse lung by EID_{50} and hemagglutinin (HA) titrations. Infected mouse lungs were disrupted in sterile sea sand with mortar and pestle and made up to a 10% suspension with nutrient broth. The suspension was

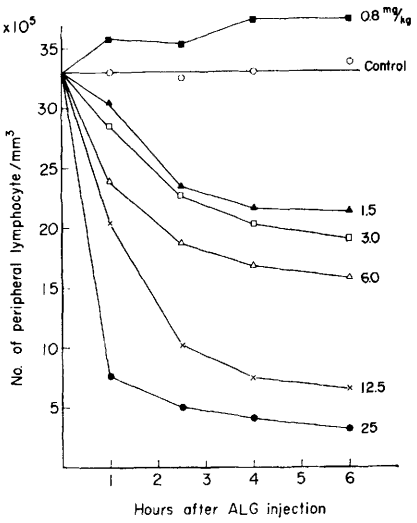

FIG. 1. Effect of ALG on the peripheral lymphocyte count in mice. ALG, in doses indicated in the figures, was injected intravenously into three mice. After injection, leucocyte counts were made of blood samples taken at hourly intervals. Each point represents the average count from 3 mice.

then clarified by centrifugation at 3,000 rpm for 10 min. The supernatants were serially diluted 10-fold with broth and 0.2 ml of each dilution was inoculated into the allantoic cavity of three embryonated eggs. After incubation for 72 hr at 36°, the allantoic fluid was removed from each egg and tested for HA. The EID_{50} was calculated by the method of Reed and Meunch. Salk's pattern method was used for HA titration.

HA inhibition (HAI) test. The technique recommended by the committee on Standard Serological Procedures in Influenza Studies was followed to determine the antibody response in infected mice (9).

Calculation of lung consolidation score. Before autopsy, the lungs of infected mice were irrigated by injecting into the heart $M/100$ phosphate buffer (pH 7.2) to remove trace amounts of red blood cells. The amount of consolidation was scored according to the method of Horsfall (12).

Results. Effect of ALS on influenza infection in mice. Employing the same administration schedule, 3 groups of 10 mice were treated with ALS, normal rabbit serum (NRS) and amantadine hydro-

chloride, respectively. Another group of 20 mice receiving saline served as a control. All of the mice were challenged with 5 LD_{50} of virus intranasally. The ALS used in the experiment had a titer of 320 units when tested for thymocyte agglutination activity and hemolysin titer of less than 5 units. Each group of 10 mice received 0.2 ml of either a 1:10 dilution of ALS (equivalent to 7.5 mg/kg ALG), or NRS, or 8 mg/kg of amantadine intraperitoneally. These doses were administered twice before infection (−24 hr and −4 hr), twice after infection (+1 hr and +3 hr) and once daily for 4 consecutive days after infection. As shown in Table I-a, all of the 20 mice receiving either saline or NRS died within 13 days. The survival rate of mice treated with ALS was 50%, which was far better than that obtained after amantadine treatment (10%) and the difference of survival rate between the ALS treated and 3 control groups was significant at the 1% level.

However, when the challenge dose of virus was increased to 1,000 LD_{50}, both the ALS and amantadine hydrochloride treated animals exhibited no protection against influenza virus infection (Table I-b).

Effect of purified ALG on influenza infection in mice. Different concentrations of lyophilized ALG were examined for their effect on influenza infection (5 LD_{50}) in mice. Twenty mice were used for each concentration of ALG and for the control group. Different concentrations of ALG were given intraperitoneally at 24 hr and 1

TABLE I. Effect of ALS on Influenza A2 (H_2N_2) Infection[a] in Mice.

Materials	Dose[b]	No. of mice	Survival days	Mean survival days	% survivors
a. When challenged with 5 LD_{50}:					
ALS[c]	0.02 ml	10	10, 10, 10, 12, 12, 14, S[e], S[e], S[e], S[e], S[e]	>15.6	50
NRS[d]	0.02 ml	10	9, 9, 10, 10, 10, 11, 11, 11, 12, 13	10.1	0
Amantadine	8 mg/kg	10	9, 10, 10, 10, 11, 11, 11, 11, 12, S[e]	>10.1	10
Saline	0.5 ml	20	9, 9, 9, 9, 9, 10, 10, 10, 10, 11, 11, 11, 11, 11, 11, 11, 11, 13, 13	11.0	0
b. When challenged with 1000 LD_{50}:					
ALS	0.02 ml	15	9, 10, 10, 10, 10, 11, 11, 11, 11, 11, 11, 11, 12, 13, 13	10.9	0
NRS	0.02 ml	10	9, 9, 9, 10, 10, 10, 10, 10, 11, 11	9.9	0
Amantadine	8 mg/kg	10	9, 10, 10, 10, 10, 10, 10, 10, 10, 11	10.0	0
Saline	0.5 ml	20	9, 10, 10, 10, 10, 10, 10, 10, 10, 10, 10, 10, 10, 10, 10, 10, 11, 11, 11	10.1	0

[a] Virus was given by inhalation.

[b] Treatment intraperitoneal 24 hr and 4 hr before virus infection, 1 hr and 3 hr after virus infection, and it was continued once daily for 4 consecutive days.

[c] Rabbit antimouse thymocyte serum.

[d] Normal rabbit serum as a control.

[e] Survived more than 15 days after virus infection.

hr before, 1 hr and 3 hr after virus infection and once daily for 4 consecutive days. It may be noted that ALG at a dose of 25 mg/kg, 12.5 mg/kg and 6.2 mg/kg was toxic as evidenced by 50, 60 and 20% deaths respectively at 5–6 days after virus infection, when all of control mice died during 10–12 days of influenzal disease (Fig. 2). At the nontoxic dose range of 3.1 mg/kg to 0.8 mg/kg, an apparent protective effect on the life span of the animals was observed with more than 50% of the mice surviving beyond 15 days (Fig. 2). Moreover, ALG-treated groups at the doses of 6.2 mg/kg and 3.1 mg/kg showed survival rates of 75 and 90% respectively, whereas that of control mice which received 5 mg/kg of normal rabbit globulin (NRG) or saline was 0%.

Virus and antibody titrations in mice treated with ALG. One hundred twenty mice were infected intranasally with 5 LD_{50} of A2 virus and divided into two groups. In this experiment, the administration of ALG was started immediately after infection. One half of animals received intraperitoneal injections of 5 mg/kg dose of ALG at 1 hr, 3 hr after infection, and once daily for 4 consecutive days. The other half serving as controls, received NRG at the same dose schedule. Five mice from each group were sacrificed at daily intervals from day 1 to day 9 and virus titrations of the lung extracts and HAI antibody titrations of the serum were performed. Ten mice from each group were left for observation and calculation of the survival period.

The results obtained show no difference in virus titers up to 5 days after infection (Fig. 3). Thereafter the virus titers in the surviving animals of the treated group remained constant through day 7 while that in the untreated survivors decreased very significantly. Antibody responses were first detected on day 4 and were equal. The HAI titers on days 5, 6 and 7 were higher in the control group than in the treated group. The difference was ten-fold by day 7.

The ALG-treated group exhibited a 50% survival rate as opposed to the 0% survival rate of the control group, as in the former experiments.

Difference in lung lesion between the

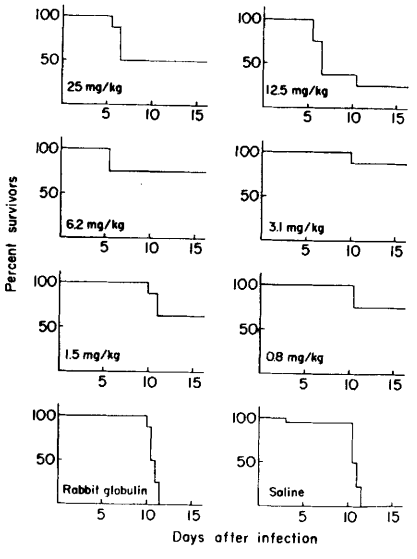

FIG. 2. Effect of ALG on influenza infection in mice. Each concentration of lyophilized ALG or 5 mg/kg of NRG dissolved in saline was injected intraperitoneally. The administration schedule is described in the text. Each mouse received 5 LD_{50} of influenza A2 (H_2N_2) virus intranasally.

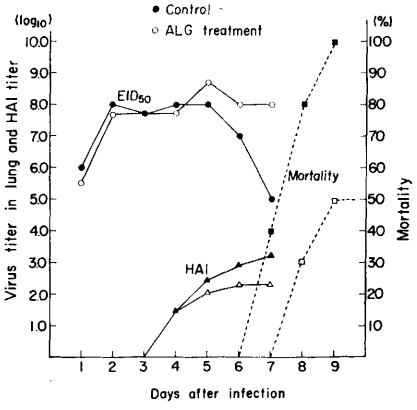

FIG. 3. Effect of ALG on virus growth and humoral antibody response in influenza infected mice. Two groups of mice were infected intranasally with 5 LD_{50} of influenza virus. Each group then received either 5 mg/kg of ALG or NRG intraperitoneally according to the following schedule: twice on the day of infection and once daily for 4 consecutive days (see text). ALG treated group: ○ = EID_{50}/0.2 ml; △ = HAI titer/ml; and □ = mortality. Control group: ● = EID_{50}/0.2 ml; ▲ = HAI titer/ml; and ■ = mortality.

treated and untreated groups. Although signs of lung consolidation were found in all mice of the control group on day 4, no lesions were observed in the corresponding treated group at this time. (Fig. 4). On day 6 the lungs of the control group showed almost complete consolidation. In contrast, the consolidation score in the treated group on day 7 was comparable to that of the control on day 4. Thus, almost a 3-day delay in consolidation score was evident in the treated group and full lung consolidation was never obtained even when the surviving mice in the treated group were sacrificed.

When the effect of 125 mg/kg and 50 mg/kg of antihamster thymocyte globulin (AHLG) was examined in comparison with ALG in the mouse-influenza system, the former did not reveal any effect either on the consolidation score, and thus indicated the species specificity of anti-lymphocyte globulin. The AHLG agglutinated hamster thymocytes at a concentration of 30 μg/ml, but required 240 μg/ml to agglutinate mouse thymocytes.

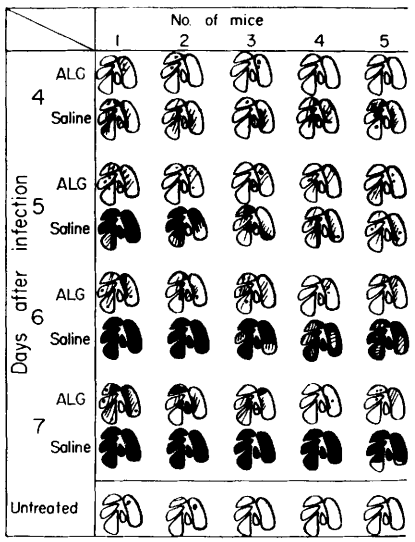

FIG. 4. Effect of ALG administration on lung consolidation. Mice were infected intranasally with 5 LD_{50} of influenza virus and received 5 mg/kg of ALG twice on the day of infection and once for 4 consecutive days. On the designated days after infection, the mice were killed, bled and irrigated with hypotonic solution to remove blood from the vascular system. The evaluation and illustration of the consolidation rate are made as previously described and darkened areas indicate portions of lung consolidated.

Discussion. A central question concerning the pathogenesis of experimental influenza in the mouse is what are the roles of antibody and cellular immunity in this pathogenesis? Previous studies have established the protective role of preexisting antibody especially when the antibody is located at the surface of the respiratory tract (16). However, antibody administered after the establishment of influenza infection is generally nonprotective and also does not enhance disease (16). These findings suggest that antibody serves to prevent reinfection but does not participate in the immunopathogenesis of the influenza disease.

The present and previous studies employed various types of immunosuppression during this model infection to further define the roles of the immune responses. The results of the present study demonstrated that immunosuppression with ALS or ALG protected against morbidity and mortality while enhancing virus titers late in infection. Assuming that ALS specifically suppresses immune and normal *T* lymphocytes it can be inferred that some of these lymphocytes participate in the influenza disease manifestations. Actually the ALS also affected the humoral antibody response as has been observed by others (17), since the peak antibody response was decreased in the treated mice. However, this effect on antibody was not correlated with retardation of disease since early antibody appeared normally on day 4 in treated mice but disease was inhibited at that time. This lack of correlation is consistent with the previously mentioned failure of passive antibody to enhance influenzal disease (16). Thus, by exclusion, it appears that the immunologically sensitized or normal lymphocyte adds to the disease manifestations of influenza.

A role for lymphocytes in the pathogenesis of the disease also comes from other findings. Immunofluorescent studies of mouse lungs infected intranasally with influenza virus revealed the initial site of virus multiplication to be the epithelial cells of the bronchial and alveolar lining (13).

However, the histological background for lung consolidation is the conspicuous infiltration of small round lymphocytes in the interstitial area and these cells do not contain any viral antigen. Thus, the interstitial pneumonitis of mice following influenza infection has been interpreted as a secondary effect of influenza multiplication. Immunopathological considerations of such histological descriptions stimulated the present work to examine the effect of ALS on influenza virus infection in mice as was done in the case of yellow fever virus infection (7). The repeated intraperitoneal injections of ALS or ALG at the time of infection clearly reduced the consolidation score of the lung not only macroscopically but also by histological criteria (to be reported elsewhere). These findings are consistent with those of Singer (18) who used cyclophosphamide immunosuppression and those of Berlin (19) who used X-ray immunosuppression but they are different from those of Hirsch (8) who used ALS. In the latter case the absence of protection against disease may be due to differences in experimental conditions such as virus challenge dose, virus strain and type of ALS since similar differences have been noted with different model poxvirus infections (17, 20, 21).

A marked amplification of morbidity and mortality of mice by pretreatment with ALS has been noted in mice infected with vaccinia (1), herpes simplex (2), reovirus (3), murine leukemias (4, 14), adenovirus type 12 (15) and polyoma (4). In contrast, ALS is reported to reduce or delay mortality in mice following intracerebral administration of LCM (5) and yellow fever viruses (7). On the basis of the experimental results obtained here, the intranasal infection of ALG-treated mice with influenza virus should fall in the latter category. It would appear that in such infections the cellular immunological response to the virus is detrimental to the host.

Additional studies of the influenza model infection are indicated to determine whether immune or nonimmune lymphocytes participate in the disease manifestations of influenza virus infection. Such studies should include (a) reconstitution experiments of immunosuppressed mice with immune or normal lymphocytes and (b) additional use of specific types of immunosuppression such as neonated thymectomy and nude mice. Finally, studies in man are indicated eventually, to determine whether similar mechanisms govern human type of influenza which is more of an upper respiratory disease rather than a pneumonia.

Summary. Repeated injections of rabbit antiserum against mouse thymocytes (ALS) were found to increase the survival rates of mice infected with low doses of influenza A2 virus. Purified immunoglobulin of such rabbit antiserum (ALG) produced the same survival effect without affecting virus growth in lung or serum antibody responses. Lung consolidations of virus infected mice were clearly reduced by ALG administration.

The study supports the concept that the cellular inflammatory reaction to influenza virus in mice plays a potentiating part in the development of morbidity and mortality after intranasal virus inoculation.

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