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Protective Mechanisms against Pulmonary Infection with Influenza Virus. I. Relative Contribution of Polymorphonuclear Leukocytes and of Alveolar Macrophages to Protection during the Early Phase of Intranasal Infection

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SUMMARY

The relative contribution of polymorphonuclear leukocytes and macrophages in the early protection against intranasal infection of mice with influenza virus was investigated. Virus multiplication in the lung in the early phase of infection with less than 1.5×10^3 plaque-forming units was enhanced by X-ray irradiation. The intranasal administration of carrageenan did not influence the titre of virus. However, when mice were infected with 1.5×10^4 plaque-forming units, the virus titre was elevated by intranasal administration of carrageenan as well as by X-ray irradiation, but not by intraperitoneal administration of carrageenan. The intranasal administration of carrageenan not only inhibited the phagocytic activity of alveolar macrophages but also enhanced susceptibility to the virus. On the other hand, polymorphonuclear leukocytes were capable of phagocytosing the virus *in vitro* and were non-permissive for virus infection. Neutralizing antibody and interferon were not detectable in the early stage of the infection. These results suggested that polymorphonuclear leukocytes (X-ray-sensitive, carrageenan-resistant) were the cells primarily responsible for early protection in influenza virus infection and that after infection with a high dose of the virus alveolar macrophages (X-ray-resistant, carrageenan-sensitive) also played a protective role in the early phase.

INTRODUCTION

Polymorphonuclear leukocytes (PMN) are sensitive to X-irradiation, but their functions are not affected by carrageenan (Catanzaro *et al.*, 1971; Tatsukawa *et al.*, 1979). Both blood monocytes and tissue macrophages are selectively sensitive to carrageenan, yet tissue macrophages, except for monocytes, are resistant to X-irradiation (Benacerraf *et al.*, 1959; Kornfeld & Greenman, 1966; Volkman & Collins, 1971; Gillette & Lance, 1973). The relative contribution of the two series of phagocytes, PMN and macrophages, to early protection against bacterial infection has been investigated using their differential susceptibility to X-irradiation and carrageenan (Mitsuyama *et al.*, 1978; Tatsukawa *et al.*, 1979; Tsuru *et al.*, 1981). In contrast, little is known of the protective role of such phagocytes in the early phase of generalized virus infection prior to the development of specific immune responses. In a previous study, we noted a significant role of macrophages in early protection against ectromelia virus infection in mice (Tsuru *et al.*, 1983). However, there has been little study of the potential contribution of PMN to protection in the early phase against virus infections *in vivo*. In the case of influenza virus, contradictory results have been reported. There is negative evidence that PMN are damaged and lose chemotactic and phagocytic activities in the presence of influenza virus (Schlesinger *et al.*, 1976; Ruutu *et al.*, 1977) and positive evidence that PMN might participate in the early response to virus infection and inactivate the virus *in vitro* (Sweet & Smith, 1980). Our preceding

study clearly showed that PMN were responsible for protection in the early stage against infection resulting from intravenous inoculation with influenza virus (Tsuru *et al.*, 1987). Recent studies suggested that macrophages and natural killer (NK) cells might participate in non-specific resistance to influenza virus infection (Mak *et al.*, 1983; Gangemi *et al.*, 1983). We analysed the relative contribution of the two series of phagocytes to the early protection against intranasal infection and our findings are reported herein.

METHODS

Mice. Female BALB/c mice, 8 to 12 weeks of age, were obtained from Charles River Japan, Inc. (Kanagawa, Japan).

Virus. The A/PR/8/34 (H1N1) strain of influenza virus was obtained from the Japan National Institute of Health (Tokyo, Japan) and virus pools were prepared by two passages of allantoic fluid from fertile hen's eggs inoculated 48 h before.

Intranasal infection and virus titration. Mice were anaesthetized with diethyl ether (Virelizer, 1975) and inoculated intranasally with 50 μ l of virus suspension diluted in cold Eagle's MEM (Nissui Pharmaceutical Co., Tokyo, Japan). After bleeding from the axillary artery and vein, lungs were aseptically excised and manually homogenized in phosphate-buffered saline (PBS) to give a 10% extract. The homogenate was dispersed by sonication (200 W, 2 A for 10 min; Insonator model 200M, Kubota K.K., Japan) and centrifuged (10000 *g*) at 4 °C for 1 min and then stored at -70 °C until titration. Tenfold dilutions of the supernatant were prepared in PBS supplemented with 0.25% bovine serum albumin (BSA). The virus infectivity in the supernatant was titrated by plaque assay on Madin-Darby canine kidney (MDCK) cells (Tsuru *et al.*, 1987).

Preparation of PMN, peritoneal and alveolar macrophages. PMN were prepared from the peritoneal cavity of mice given 1 ml of casein (1% in PBS) or glycogen solution (0.1% in saline) 3 to 6 h previously. Peritoneal cells were harvested in Hanks' solution (Nissui) supplemented with 0.5 units of heparin, washed with RPMI 1640 medium (Gibco) supplemented with 10 mM-HEPES (Sigma), 2 mM-glutamine, 100 μ g/ml of streptomycin, 100 units/ml penicillin and 10% foetal calf serum (FCS; Gibco), and PMN were purified by centrifugation (300 *g*, 30 min) after layering onto Mono-Poly Resolving Medium (Flow Laboratories). The PMN-enriched fraction was collected and washed three times to remove the medium. Peritoneal macrophages were prepared as adherent cells from the peritoneal cavity, after stimulation with 1% tryptose peptone (Difco) 3 days previously. Cells were incubated at 37 °C for 1 h in a 5% CO₂ atmosphere and non-adherent cells were removed by washing with MEM. Alveolar macrophages were obtained by a modification of the method of Sone *et al.* (1980). Mice were anaesthetized with sodium pentobarbital (Somnopenyl; Pitman-More, N.J., U.S.A.) given intraperitoneally and exsanguinated by cutting the renal artery to reduce the amount of blood trapped in the lungs. After opening of the chest cavity, the trachea was cannulated with a needle and anchored by suturing. The lung was lavaged ten times with a total of 10 ml of PBS prewarmed at 37 °C and the cells were collected by centrifugation. PMN, peritoneal and alveolar macrophages were resuspended in supplemented RPMI 1640 medium and used in the following experiments. The purities of PMN, peritoneal and alveolar macrophages were over 97%, 99% and 98%, respectively, determined by histological observation after Giemsa staining.

X-ray irradiation. Mice were exposed to 800 or 1000 rad of whole body irradiation 2 days before the virus infection. Radiation was delivered from a Softex 50 kV machine (Softex, Tokyo, Japan) operating at 50 kV, 10 mA with 0.1 mm Cu filtration, 30 cm from the target focus.

Carrageenan treatment. Carrageenan type II (Sigma) was dissolved in distilled water (10 mg/ml). Mice were injected intraperitoneally with carrageenan (200 mg/kg) to deplete mainly tissue macrophages in the liver and spleen, and blood monocytes (Catanzaro *et al.*, 1971) as well as to increase PMN in the blood (Tatsukawa *et al.*, 1979; Tsuru *et al.*, 1983). To impair the function of alveolar macrophages, 50 μ l of carrageenan (1 mg/ml) diluted in MEM was administered intranasally to mice (2 mg/kg) 2 days before the virus infection.

Serological testing. Measurements of neutralizing antibody and interferon (IFN) were carried out as described (Tsuru *et al.*, 1987). Sera obtained from eight mice in each group were treated with receptor-destroying enzyme (Takeda Pharmaceutical, Osaka, Japan) at 37 °C for 18 h and incubated at 56 °C for 30 min. After incubating virus (1000 to 2000 p.f.u./ml) with an equal volume of test serum, diluted in twofold steps, at 37 °C for 1 h, the virus titre in the mixture was measured by MDCK cell plaque assay. The reciprocal of the dilution showing 50% reduction of plaque number (ND₅₀) was calculated from each titration point.

Serum IFN was measured according to Neumann & Sorg (1977). After incubating L cells (2 \times 10³ cells/10 μ l) in a microplate (Falcon 3034) at 37 °C for 6 h, medium was discarded and a twofold dilution of serum obtained from eight mice of each group (15 μ l) was added to the wells and the plate was further incubated at 37 °C for 20 h. Fifty TCID₅₀ of vesicular stomatitis virus (New Jersey) (10 μ l) was added to wells and after adsorption, wells were washed and the plate was incubated for 2 days. The IFN titre was determined in International Units by reference to standard mouse interferon- α/β (Enzo Biochemicals, New York, N.Y., U.S.A.).

Measurement of phagocytosis. Phagocytic activity of alveolar macrophages was measured by a modification of

the method of Jakab *et al.* (1980). Alveolar macrophages (2×10^5 cells/0.1 ml) were allowed to settle on a round glass coverslip at 37 °C for 30 min in a 5% CO₂ atmosphere. After washing, a yeast suspension (5×10^6 cells/0.1 ml) in supplemented RPMI 1640 medium was added to the coverslip. The macrophage monolayer was incubated for 1 h at 37 °C and washed four times with PBS to remove the excess yeast cells. The monolayers were fixed with 100% methanol, stained with Giemsa solution, and the stained preparations were examined under the light microscope at 1000-fold magnification. More than 500 cells were scored and the percentage of cells containing at least one yeast cell was recorded.

Measurement of chemiluminescent response. The chemiluminescent response of PMN was measured using a commercial reagent kit (Laboscience, Tokyo, Japan). PMN (3×10^5 cells/100 µl) were mixed with luminol solution (100 µl, Laboscience) in a counting tube, and the tubes were incubated for 3 min at 37 °C. After the incubation, influenza virus suspension, zymosan (ZAP; Packard) or formylmethionyl leucylphenylalanine (FMLP; Laboscience) were added. Chemiluminescent emission was measured immediately at 37 °C with a lumiphotometer (TD-4000; Laboscience).

Virus infection in vitro. PMN, peritoneal and alveolar macrophages (1×10^6 cells) were infected with influenza virus at a m.o.i. of 1 or 10 in a volume of 0.2 ml at 25 °C for 2 h. Cells were washed five times with MEM to remove virus, suspended in supplemented RPMI 1640 medium and cultured at 37 °C for 24 h and 48 h in a 5% CO₂ atmosphere. At the end of the experiment, infected cells were washed four times with MEM, resuspended in 1 ml of PBS supplemented with 0.25% BSA and disrupted by sonication (200 W, 2 A, for 5 min; Insonator model 200M, Kubota). The virus titre in the infected cells was determined by plaque assay and the results expressed as the mean p.f.u. \pm S.D./ 10^6 cells of triplicate cultures.

Counting of PMN and macrophages. Blood specimens were collected by puncture of the retro-orbital venous plexus. Total numbers of leukocytes in the blood, peritoneal exudate and lung lavage buffer were counted after staining with Turk's solution and differential counts were carried out after staining the smears with Giemsa.

RESULTS

Pulmonary virus titre after intranasal infection with a low dose of influenza virus

Following intranasal infection of untreated mice with 1.5×10^3 p.f.u., the virus titre in the lung increased progressively to day 3 and declined gradually from day 7. When X-irradiated mice were infected with the virus, the virus titre in the lung increased rapidly from day 1 to reach a level higher than that in untreated mice and gradually increased to day 9 (Fig. 1). The virus titre was not influenced by intranasal administration of carrageenan 2 days before the virus infection, although the phagocytic activity of alveolar macrophages *in vitro* was reduced to less than 25% and the number of macrophages to approx. 30% of the corresponding values for untreated mice (Table 1). All X-irradiated mice died within 10 days, although the dose of virus given was not lethal for untreated mice or for those treated with carrageenan (data not shown).

Pulmonary virus titre after intranasal infection with a high dose of influenza virus

When untreated mice were infected with 1.5×10^4 p.f.u., the pulmonary virus titre was markedly increased on day 1, reached a plateau by day 5, and then decreased gradually from day 7. The pulmonary virus titre in the X-irradiated mice rapidly increased to a level higher than that in untreated mice from day 1 after the virus infection. The titre in the lungs of mice which had been given carrageenan intranasally 2 days before the virus infection increased from day 1 to reach a level similar to that seen in the X-irradiated mice. Intraperitoneal injection of carrageenan 2 days before the virus infection did not increase the virus titre in the lung by day 5 (Fig. 2). The pulmonary virus titre in X-irradiated mice increased up to day 9 by which time all mice in this group had died, but a similar sequential increase was not seen in mice pretreated intranasally with carrageenan. The titre in the latter group of mice declined gradually from day 5 (Fig. 2).

Production of neutralizing antibody and of IFN

Neutralizing antibody in untreated mice was detected from day 7 after intranasal inoculation with either 1.5×10^3 or 1.5×10^4 p.f.u. of the virus. The antibody titres in mice given carrageenan intranasally or intraperitoneally increased from day 5. The production of antibody in X-irradiated mice was not detected by day 9 after infection (Fig. 3a, b). No IFN was detected in serum from any group until 3 days after the infection (data not shown).

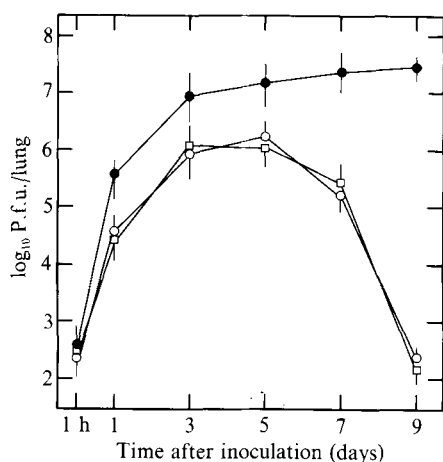


Fig. 1

Fig. 1. Effects of carrageenan treatment and X-irradiation on virus titres in mouse lung after intranasal infection with 1.5×10^3 p.f.u. of influenza virus. \circ , Untreated mice; \square , mice given carrageenan intranasally 2 days before infection; \bullet , mice X-irradiated 2 days before virus infection. Each point and bar indicates the mean value \pm s.d. for eight mice.

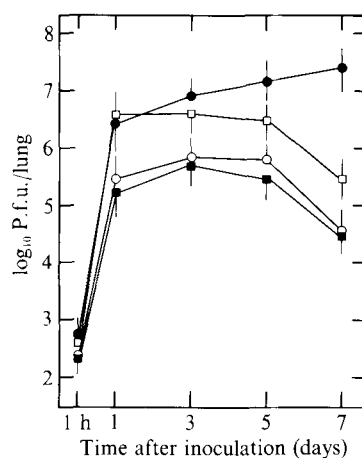


Fig. 2

Fig. 2. Effects of carrageenan treatment and X-irradiation on virus titres in the lung after intranasal infection with 1.5×10^4 p.f.u. of influenza virus. \circ , Untreated mice; \square , mice given carrageenan intranasally 2 days before infection; \blacksquare , mice given carrageenan intraperitoneally 2 days before infection; \bullet , mice X-irradiated 2 days before virus infection. Each point and bar indicates the mean value \pm s.d. for eight mice.

Table 1. *Effects of carrageenan treatment and of X-ray irradiation on the number of PMN in peripheral blood and of alveolar macrophages, and on phagocytosis by alveolar macrophages*

Treatment*	Number of PMN ($\times 10^3/\text{mm}^3$)†	Alveolar macrophages	
		Phagocytic cells (%)‡	Cell number $\times 10^5/\text{lung}$ †
None	0.73 ± 0.12	68.4	1.8 ± 0.4
Carrageenan i.p.	3.4 ± 0.8	55.1	1.5 ± 0.2
Carrageenan i.n.	1.1 ± 0.3	14.6	0.52 ± 0.08
Irradiation	0.05 ± 0.02	53.4	1.7 ± 0.3

* Mice were given carrageenan intraperitoneally (i.p.) (200 mg/kg) or intranasally (i.n.) (2 mg/kg), or exposed to 1000 rad of whole body irradiation 2 days previously.

† The number of PMN in the peripheral blood and of alveolar macrophages in the lung lavage. Results indicate the mean value \pm s.d. for eight mice.

‡ Percentage of alveolar macrophages phagocytosing one or more yeast cells.

Virus susceptibility of phagocytes

The susceptibilities of PMN, peritoneal and alveolar macrophages to influenza virus were examined *in vitro*. PMN did not permit the replication of the virus and virus replication was almost completely inhibited 48 h after infection, whereas both peritoneal and alveolar macrophages were moderately susceptible to the virus infection, as compared to MDCK cells. Replication of virus in alveolar macrophages was enhanced by intranasal pretreatment with carrageenan but not by X-ray irradiation (Table 2). Intraperitoneal pretreatment with carrageenan did not augment the susceptibility of alveolar macrophages (data not shown).

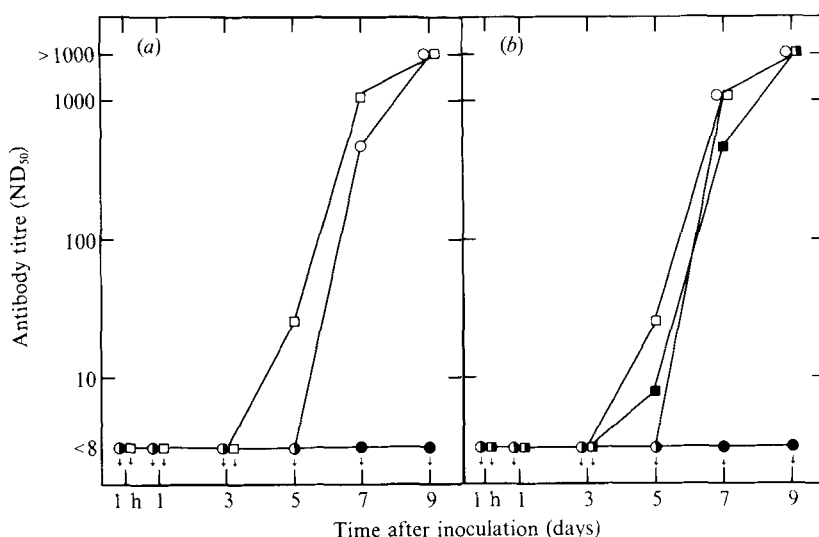


Fig 3. Production of neutralizing antibodies in mice infected intranasally with 1.5×10^3 (a) and 1.5×10^4 (b) p.f.u. of influenza virus. \circ , Untreated mice; \square , mice given carrageenan intranasally 2 days before infection; \blacksquare , mice given carrageenan intraperitoneally 2 days before infection; \bullet , mice X-irradiated 2 days before infection. Each point indicates ND_{50} calculated from the mean p.f.u. in triplicate cultures.

Table 2. Susceptibility of phagocytes to influenza virus

Treatment*	Infected cells†	M.o.i.	P.f.u. $\times 10^{-3}/10^6$ cells‡	
			24 h	48 h
None	Alveolar M ϕ	1	9.0 ± 0.3	2.8 ± 0.6
Irradiation	Alveolar M ϕ		6.8 ± 0.6	2.5 ± 0.8
Carrageenan	Alveolar M ϕ		36.0 ± 5.6	47.4 ± 6.5
None	PMN		0.99 ± 0.02	0.06 ± 0.01
—	MDCK		32.4 ± 4.2	36.2 ± 5.1
None	Alveolar M ϕ	10	130 ± 20	45 ± 8
Irradiation	Alveolar M ϕ		86 ± 37	34 ± 7
Carrageenan	Alveolar M ϕ		254 ± 40	162 ± 21
None	Peritoneal M ϕ		147 ± 11	69 ± 18
None	PMN		22 ± 2.3	3.0 ± 1.3

* Mice were exposed to 800 rad of whole body irradiation, or were administered carrageenan intranasally (2 mg/kg).

† PMN, alveolar and peritoneal macrophages (M ϕ) were prepared 2 days after the treatments and 2.5×10^5 cells were infected with influenza virus at m.o.i. 1 or 10.

‡ Virus titres in the cells were measured by MDCK cell plaque assay 24 h and 48 h after the infection. Results are expressed as the mean value \pm s.d. in triplicate cultures.

Chemiluminescent response of PMN to influenza virus

To investigate whether PMN were able to phagocytose influenza virus, the chemiluminescent response of PMN to the virus was measured and compared with the response to zymosan or FMLP. The addition of FMLP to the suspension of PMN produced a rapid increase in chemiluminescence; the response reached a maximum level in 3 min and then declined less rapidly in 10 min. In contrast, the peak response to zymosan appeared in 5 min and a high level of response was maintained even after 10 min. The addition of influenza virus to give a m.o.i. of 1 induced a chemiluminescent response similar to the pattern with zymosan (Fig. 4).

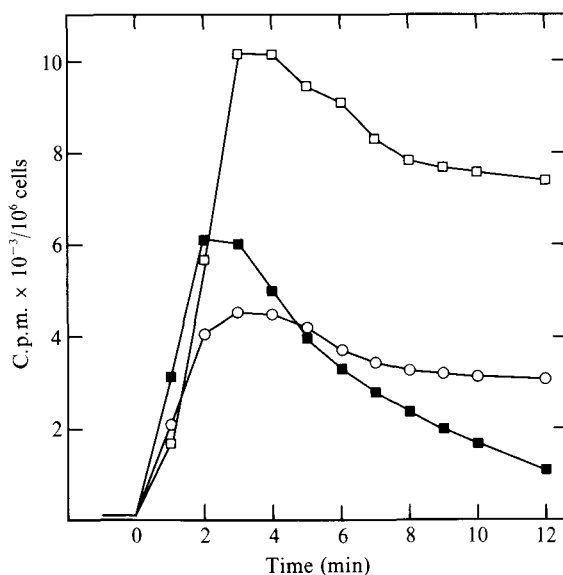


Fig. 4. Time course of chemiluminescence in PMN. Peritoneal PMN (3×10^5 cells) were mixed with influenza virus (3×10^5 p.f.u.) (○), zymosan (□) or FMLP (■) in the presence of luminol and the light emission was measured at 37°C .

DISCUSSION

Our study showed that PMN and macrophages contribute to early protection against intranasal infection with influenza virus. When mice were infected with a low dose of virus (1.5×10^3 p.f.u.), the pulmonary virus titre was increased after X-ray irradiation, whereas the titre was not influenced by intranasal administration of carrageenan before the virus infection (Fig. 1). After intranasal infection with 1.5×10^4 p.f.u. of the virus, the titre was increased by both X-irradiation and intranasal administration of carrageenan but not by intraperitoneal administration of carrageenan before the infection (Fig. 2). The number of peripheral PMN was diminished by X-irradiation but increased by intraperitoneal treatment with carrageenan; both the phagocytic activity and the number of alveolar macrophages were markedly reduced by intranasal administration of carrageenan but not by X-irradiation nor by intraperitoneal treatment with carrageenan (Table 1). These observations indicate that PMN (X-ray-sensitive and carrageenan-resistant) have an important role in the early phase of protection against intranasal infection with influenza virus, and suggest that alveolar macrophages (X-ray-resistant and carrageenan-sensitive) also contribute to protection against pulmonary infection with a comparatively high dose of the virus. This interpretation is also supported by the finding that the PMN were non-permissive for virus replication (Table 2) and were capable of phagocytosing the virus (Fig. 4), and that the susceptibility of alveolar macrophages to virus was markedly enhanced by administration of carrageenan (Table 2). Toms *et al.* (1977) reported that the inflammatory response in the upper respiratory tract after intranasal infection of ferrets with the virus consisted of 90% PMN and 10% mononuclear cells 1 day after infection. The accompanying study also indicated that PMN contributed to the early phase of protection against intravenous infection with influenza virus (Tsuru *et al.*, 1987). An increase in the number of peripheral PMN after intraperitoneal treatment with carrageenan led to a decrease in virus titre in the lung and enhanced the pulmonary inflammatory response due to PMN after infection with the low dose of virus (unpublished data). This evidence strongly suggests that PMN are the cells mainly responsible for the protection in the early phase against influenza virus infection. It has been reported that NK cells are also sensitive to carrageenan but resistant to X-irradiation (Kiessling *et al.*, 1977; Cudkowicz & Hochman, 1979) and that NK cells participate in non-specific resistance in several virus infections (Bukowski *et al.*, 1983; Habu *et*

al., 1984). In our studies, however, intraperitoneal administration of carrageenan before intranasal infection with the high dose of influenza virus did not increase virus titre in the lung at the early phase of infection (Fig. 2). Furthermore, intravenous injections of anti-asialo GM1 antiserum before or after the virus inoculation did not result in the increase of the pulmonary virus titre (unpublished data). Thus, it seems unlikely that NK cells contribute greatly to early protection against intranasal infection with influenza virus. Rodgers & Mims (1981, 1982) reported that murine alveolar macrophages might be capable of spreading the infection *in vivo* since such infected macrophages were able to act as infectious centres for tissue culture cells. However, the present study indicated that both PMN and alveolar macrophages participated in early protection after intranasal infection with a high dose of the virus. After infection with a low dose, no contribution of alveolar macrophages to early protection could be demonstrated. These events require further analysis.

The production of neutralizing antibody was not evident up to day 3 but was present from day 5 after the virus infection, in both untreated and carrageenan-treated mice (Fig. 3). Iwasaki & Nojima (1977) reported that antibody contributed to protection in the transient plateau phase but not in the early phase of primary infection in mice. Raut *et al.* (1975), however, noted that local antibody might be important early in intranasal infection, as antibody-producing cells were detected on day 1 after the virus infection. In addition, there is evidence that protection by PMN and macrophages against bacteria is enhanced in cooperation with antibody to the bacterial antigen (Fukutome *et al.*, 1980; Akeda *et al.*, 1981). Further studies are underway to clarify the protective role of antibody plus PMN against influenza virus infection.

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