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Respiratory Diseases in Cyclophosphamide-Treated Mice

II. Decreased Virulence of PR8 Influenza Virus

S. H. SINGER, P. NOGUCHI, AND R. L. KIRSCHSTEIN

Laboratory of Pathology, Division of Biologics Standards, National Institutes of Health, Bethesda, Maryland 20014

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Mice infected intranasally with the PR8 strain of influenza virus were treated with cyclophosphamide, a potent immunologic suppressor. During the first week of infection, mortality in the unmodified influenza infection averaged 65%, whereas in those animals also treated with cyclophosphamide it averaged 22.5%. After the first week, the mortality rate in the infected cyclophosphamide-treated animals rose to that seen during the first week in the animals only infected. This decreased mortality in the first week was found despite the fact that the cyclophosphamide-treated and infected animals had higher virus titers which persisted longer, decreased circulating antibody, and a decreased interferon response. This delayed mortality appeared to be related to the finding of decreased cellular infiltration in the lungs of infected cyclophosphamide-treated animals.

We have reported on the increased virulence of a mycoplasma respiratory pathogen in cyclophosphamide-treated mice (11). In a continuing effort to investigate what role circulating and cellular immunity has on respiratory diseases, we looked at influenza infection in cyclophosphamide-treated mice. Previous reports have indicated that treatment with cyclophosphamide increased the mortality caused after various virus infections (3, 8, 13-15), and we expected to find similar results. However, instead we found a delayed mortality in these animals during the first week postinfection.

MATERIALS AND METHODS

The animals and routes and times of inoculation were the same as previously described (11). Briefly, 20-g female Swiss general purpose mice, obtained from the Rodent and Rabbit Production Section, Laboratory Aids Branch, National Institutes of Health (NIH) were inoculated intranasally with infectious material. Simultaneously, animals were inoculated intraperitoneally with 0.1 ml of either pyrogen-free saline or cyclophosphamide at a dose of 80 μg per g of body weight. Intraperitoneal inoculations were given once daily for a total of 4 days. At least 10 animals per group were used in these experiments. Based on previous effects seen on peripheral blood counts (11), observations were limited to 7 days (168 hr) postinitiation of infection and the first cyclophosphamide injection.

Inoculum. The PR8 strain of influenza virus was kindly supplied by S. Baron, NIH, Bethesda, Md., and a pool was prepared in the allantoic fluid of 10-day-old embryonated chick eggs. The pool titered 1:128 to 1:256 by the hemagglutination (HA) test and contained $10^{8.25}$ $\log_{10}$ egg infectious dose. It was diluted 100-fold prior to the inoculation of one minum (0.06 ml) intranasally.

Virus and antibody assays. Assay for virus from selected organs was performed by preparing a 10% suspension in Eagle minimal essential medium and then performing a standard HA test with chicken red blood cells. Similarly, a standard hemagglutination-inhibition (HAI) test was used for detection of antibody.

Interferon assay. Materials to be assayed for interferon were first acidified to *p*H 2 and stored at 40 C for 72 hr to eliminate infectious virus. After this, the *p*H was adjusted to 7, and the materials then were assayed for antiviral action on primary mouse fibroblast cultures by using a yield reduction method with vesicular stomatitis virus as the challenge virus. The interferon titer was taken as the last dilution resulting in a 0.5 $\log_{10}$ or greater reduction of virus yield (2). The inhibitor was characterized as interferon by its species specificity, broad antiviral action, and its stability at *p*H 2.

RESULTS

The effect of cyclophosphamide treatment on mortality due to PR8 influenza virus can be seen in Table 1. The PR8 strain of influenza virus at the dose given caused substantial mortality itself. In a series of four experiments, the average was 65% (range 50 to 80%). Death typically began 4 to 5

days postinfection. However, in infected animals which also received cyclophosphamide, the mortality was reduced to 22.5% (range 10 to 30%). There were fewer deaths, and the time to death was delayed in the infected animals treated with cyclophosphamide. Cyclophosphamide itself caused a 5% mortality rate in this series.

Virus was isolated from the lungs and livers of infected mice, but not from brain or spleen, the two other organs which were assayed. The geometric mean titers (GMT) of HA values are shown in Table 2. The lungs of cyclophosphamide-treated mice yielded significantly more virus (GMT of 7.9) at 48 hr postinfection than non-treated mice (GMT of 1.2). In addition, there was persistence of infectious virus in the cyclophosphamide-treated animals, and a GMT of 7.7 was present in the lungs of the cyclophosphamide-treated mice at 7 days postinfection, at a time when virus was no longer recoverable from the lungs of non-cyclophosphamide-treated mice. Pooled serum from non-cyclophosphamide-treated mice 7 days postinfection contained circulating HAI antibody (titer 1:8). No antibody could be found in the cyclophosphamide-treated group. Thus, cyclophosphamide treatment resulted in delayed appearance of circulating antibody and, consequently, a prolonged period during which virus was isolated from the lungs of mice infected with influenza virus.

The production of interferon during the course of this infection was investigated. It has been reported that cyclophosphamide treatment of animals has resulted in decreased interferon production during a viral infection (7, 10). On the other hand, cyclophosphamide treatment has not been reported to have an adverse effect on interferon production after exposure to a synthetic polynucleotide (16). Since interferon titers have been reported to peak at an average of 3 days post-influenza infection (6), lung suspensions from animals infected 72 hr previously were tested for interferon content. The results (Table 3) indicate that there is some interferon in the lungs of cyclophosphamide-treated animals, but it is present at lower levels (average titer of 34 units) than in the non-cyclophosphamide-treated group (average titer of 220 units). Because higher virus titers appeared earlier in the cyclophosphamide-treated group and because it was possible, therefore, that interferon production occurred earlier, experiments were repeated using lung suspensions from animals infected only 48 hr previously. Increased levels of interferon were not found in the cyclophosphamide-treated group at this time.

Histological studies were undertaken to try to explain the differences in mortality seen between the cyclophosphamide-treated and untreated groups. In infected animals without cyclophosphamide, a patchy pneumonia was seen approximately 2 days postinfection and was well developed by 4 days postinfection. In cyclophosphamide-treated animals, there was much less cellular infiltration of the lungs at this time. Although some pneumonia did develop, the cellular infiltration was less dense and less widespread. This was most marked in the lungs of animals infected 4 days previously. It also appeared that there was a direct correlation between the peripheral white

TABLE 1. *Cumulative mortality[a] of influenza virus in cyclophosphamide-treated and untreated mice*

Expt	Influenza alone (%)	Influenza and cyclophosphamide (%)	Cyclophosphamide alone (%)
1	70	20	20
2	50	30	0
3	60	10	0
4	80	30	0
Avg	65	22.5	5

[a] Cumulative mortality 7 days (168 hr) post-intranasal infection with influenza virus. Each experiment represents a minimum of 10 animals per group.

TABLE 2. *Influenza virus titers from cyclophosphamide-treated and untreated mice*

Time (hr)	Lung		Liver	
	Control	Cyclophosphamide	Control	Cyclophosphamide
48	1.2[a]	7.9	0.3	0.6
96	8.7	5.4	0	0.3
168	0	7.7	0	0

[a] Titers expressed as geometric mean titer of hemagglutination values from six test animals per determination.

TABLE 3. *Interferon titers from lungs of cyclophosphamide-treated and untreated mice 72 hr postinfection*

Expt	Control	Cyclophosphamide
1	32,[a] 128, 512	8, 16, 64
2	32, 256, 256	16, 32, 64
Avg	220	34

[a] Expressed as units of interferon per 2 ml of a 10% lung suspension. Three different lungs per experimental group were assayed.

blood count (WBC) and the amount of cellular infiltration of the lungs. At 7 days after the first cyclophosphamide injection (4 days after the last injection) when the peripheral WBC was rising, the cellular infiltrate in the lungs of the cyclophosphamide-treated animals began to approach that of the non-cyclophosphamide-treated animals. Simultaneously, the mortality rate in this group began rising and during the second week postinfection reached that of the non-cyclophosphamide-treated animals.

DISCUSSION

Cyclophosphamide treatment of influenza virus-infected mice resulted in decreased mortality during the first week as contrasted to non-cyclophosphamide-treated animals. This is so despite the fact that, in the cyclophosphamide-treated animals, the virus titers in the lungs were higher and persisted longer, the circulating antibody response was lower, and the interferon level was lower. Thus, the outcome of the disease cannot be predicted solely by the levels of virus, neutralizing antibody, and interferon. Although these are of undoubted importance, the role of the cellular inflammatory response of the host must also be considered. Suppression of virus replication by interferon and antibody may be of no avail if the alveoli of the lungs become so congested with cells that respiratory exchange becomes difficult or impossible. The decreased cellular response to the viral infection seen in the cyclophosphamide-treated group might be a result of decreased cellular immunity, or perhaps a reflection of the nonspecific decreased inflammatory response secondary to cyclophosphamide which is independent of effects on cellular immunity (1). This has yet to be determined. Another possibility is that the simultaneous administration of cyclophosphamide and the influenza virus resulted in a state of tolerance to the virus. The mortality seen after 1 week in the infected cyclophosphamide-treated group may have been due to an escape from the tolerant state with the emergence of antibody-producing cells. This possibility should be considered in view of a recent report (12) that demonstrated that simultaneous administration of cyclophosphamide and a soluble antigen could induce tolerance.

Earlier reports from 1952 (5) and 1955 (4) describe an acid-precipitable bacterial product from *Achromobacter* sp. no. 134 initially designated APM and then xerosin, which was reportedly capable of decreasing the inflammatory response and, thus, mortality in the mouse lung after influenza infection. However, it was also noted in one report (4) that corticosteroids, which significantly reduced the inflammation in the lung after exposure to chemical irritants, had no effect on the inflammation caused by the influenza virus. Thus, xerosin may have suppressed cellular immunity as well as suppressed inflammation.

The effects on cellular infiltrate in the lung in cyclophosphamide-treated animals which have been infected with mycoplasma and influenza differ. In the former, no differences in the lung infiltrates were noted between animals treated or not treated with cyclophosphamide, whereas in the latter there was much less infiltration in the cyclophosphamide-treated group. The signal for invoking the cellular response may be different in these two infections, and indeed the source of the cells contributing to the inflammatory response may be different. The reason for this may be somehow related to the cellular level of infection. Mycoplasma infection is thought to primarily involve the outer cell membranes of the cell (9), whereas viral replication is clearly an intracellular event.

In experiments where the cyclophosphamide-treated animals were observed beyond 1 week, the mortality began increasing in this group and ultimately reached the level of that in the non-cyclophosphamide-treated group. It may be postulated that, as escape from the pharmacological effects of the immunosuppressant cyclophosphamide occurs, the cellular inflammatory response approaches the normal level and the animals begin to succumb. We have not yet attempted to repeat a course of cyclophosphamide therapy in these animals, but, under proper conditions and with an optimal dosage range, it might be possible with cyclophosphamide to establish a chronic nonlethal infection in place of a rapidly lethal one.

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