Effect of Chlorite-Oxidized Oxyamylose on Influenza Virus Infection in Mice

A. BILLIAU, J. J. MUYEMBE, AND P. DE SOMER

Rega Institute for Medical Research, University of Leuven, 3000 Leuven, Belgium

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Intraperitoneally administered chlorite-oxidized oxyamylose (COAM) provided protection of mice against intranasal infection with several influenza virus strains. Treated animals invariably showed a reduced consolidation of the lungs and, in the case of infection with lethal strains of virus, also a delay in mortality. With a small dose of influenza A/PR8 virus, an increase in final survival rate could be observed. The effect of COAM on influenza virus infection lasted for at least 4 to 8 days. Inhibition of lung consolidation was not paralleled by a decrease in virus multiplication in the lung. The significance of this finding in relation to the mechanism of the antiviral action of COAM is discussed.

In previous papers, we described the antiviral effect of polyacetal-carboxylic acids, of which chlorite-oxidized oxyamylose (COAM) is a representative (1, 2). Intraperitoneal administration of COAM can protect mice against several systemic experimental viral infections. It also acts against intranasal infection with influenza virus (A/PR8-strain). For several reasons, we decided to explore further the latter effect. Firstly, the inhibition of myxovirus pathology by systemic administration of polyanions has only sporadically and irregularly been observed. Specifically, it was found that polyacrylic acid could delay mortality of mice infected with influenza A/PR8 (A. Billiau and E. De Clercq, unpublished data) but did not increase the survival rate. The very potent interferon inducer polyriboinosinic-ribocytidylic acid (In.Cn) was found to give weak protection in mice against one (B/Maryland) out of four tested influenza virus strains (4). Therefore, we wanted to know whether COAM had a systematic effect against more than one type of influenza virus and whether such an effect would be of long duration. Secondly, the pathogenesis and pathology of influenza virus infection in mice are limited to a single organ. Therefore, we thought that this system, rather than any other one, might help to clarify the mechanism whereby the polymer inhibits viral disease. Finally, we thought that the system presented a certain interest for its possible implications as to the practical applicability of this type of compound in myxovirus infections in their natural host.

MATERIALS AND METHODS

COAM was prepared by P. Claes, Rega Institute for Medical Research, as described earlier (2).

Experimental animals used in this study were weaned female NMRI mice obtained from the Centrum voor Selectie en Kweek van Laboratoriumdieren of the University of Leuven. The animals were infected by the intranasal route by administering 0.05 ml of diluted allantoic fluid in each nostril under light anesthesia.

The following influenza virus strains were used: A/PR8, A/NWS, A2/Hong Kong (A2/HK), and B/Netherlands (B/Neth). Virus stocks were prepared by inoculating 11-day-old chick embryos.

The potency of influenza virus stocks was expressed in 50% mouse lethal dose (LD50) or in 50% mouse-infecting dose (MID50). For LD50 determinations, groups of 12 mice were infected intranasally with serial 10-fold dilutions of the virus and observed for lethal effect for 3 weeks; LD50 values were calculated by Reed and Muench interpolations (5). For MID50 determinations, groups of six mice were similarly infected and sacrificed after 6 days, at which time the lungs were removed and homogenized individually as described above. The homogenate was inoculated in embryonated eggs to detect virus growth. A mouse was considered infected when its lung homogenate yielded a hemagglutination-positive egg test. Again interpolations were carried out following Reed and Muench (5).

To determine the virus content of the lungs, each pair of lungs was homogenized in 10 volumes of saline. The homogenate was clarified by centrifugation at 2,000 × g for 15 min and stored at −70°C until titration. Virus titers were determined by inoculating 10-fold serial dilutions in duplicate embryonated eggs, which were incubated at 37°C for 48 hr, harvested, and screened for hemagglutinin content. Titers in 50% egg-infecting dose (EID50) were calculated by Reed and Muench (5) interpolation.

Note: "Bevoegdverklaard navorser" of the Belgian N.F.W.O. Assistant of the University of Lovanium, Kinshasa, Democratic Republic of Congo, and fellow of the Belgian O.C.D.
The antiviral effect of COAM was measured by drawing mortality-time graphs for both treated and control groups of animals and by graphically determining the time when 50% of the animals had died. Unless stated otherwise, the animals were observed for 3 weeks post infection, and the excess survival percentage of treated over control groups was taken as a second criterion. In addition, the degree of lung consolidation in mice sacrificed at different times after challenge with influenza virus was evaluated by the method of Horsfall (3). The animals were anesthetized and killed by section of the abdominal aorta to avoid aspiration of blood in the lungs. One-fourth of the lung consolidated was rated as 1, one-half as 2, three-fourths as 3, and complete consolidation as 4. The consolidation ratio of a group, e.g., of five mice with consolidation degrees of 2, 3, 3, 4, and 4, respectively, was calculated as 0.80 (80%) according to the following formula: \((2 + 3 + 3 + 4 + 4)/(5 \times 4) = 0.80\).

RESULTS

Influence of COAM on the mortality curves of mice infected with influenza viruses. Four strains of influenza virus were used to test the antiviral effect of COAM. Only three of them, namely A/PR8, A/NWS, and B/Neth, caused reproducible mortality in mice.

Groups of 12 mice were given 2 mg of COAM intraperitoneally 24 hr before intranasal administration of different doses of influenza virus. The death score was recorded daily. Figure 1 shows results obtained with influenza A/PR8 strain. It can be seen that the effect was most pronounced with a dose of virus around 2.5 LD\(_{50}\). Similar but less convincing results were recorded for influenza strain B/Neth. When a dose of 1.6 LD\(_{50}\) was given, administration of COAM caused a delay of 24 hr in 50% mortality time but no excess in final survival percentage. With A/NWS, mortality curves for treated and control mice were identical.

The antiviral effect of a single dose of COAM against mengovirus lasts for several weeks (1, 2). On the other hand, no effect was noted when the drug was given 1 or 2 days after the virus (1). To determine whether similar time-effect relationships would exist for respiratory myxovirus infections, groups of 11 to 12 mice were given 2 mg of COAM intraperitoneally. A dose of 2.5 LD\(_{50}\) of influenza A/PR8 virus was given intranasally at different times before or after the drug. The pooled results of two experiments are summarized in Fig. 2. They show that COAM was not active when given simultaneously with virus or 1 day afterwards. Conversely, the mice were partially protected when COAM was given 1, 2, 4, or 8 days before influenza virus.

Effect of COAM on lung consolidation and on the replication of influenza virus in the lungs of mice infected with different influenza virus strains. To investigate the effect of COAM on pathology induced by influenza virus strains which were not lethal or were slightly lethal for mice, groups of 12 mice were injected intraperitoneally with 2 mg of COAM. One day later they were given influenza virus of different strains intranasally in different doses as indicated in Table 1. Six days after virus inoculation, the mice were autopsied and lungs lesions were scored. For each group, consolidation ratios were calculated. The data show that for each strain of influenza virus tested, i.e., A/PR8, A/NWS, A/\(\text{HK}\), and B/Neth, an inhibition of the consolidation ratio could be observed. These data corroborate the observations on the delay of mortality.

Next we determined whether COAM had an effect on the replication of influenza virus in the
lungs. We expected this system to allow us to work with still smaller inocula of virus and thus to increase the sensitivity of our tests. Moreover, the conditions of such a test would more resemble infection by natural transmission. Finally, the outcome of this test was expected to provide useful information on the mechanism of inhibition of influenza pathology by COAM.

The experiment was carried out with influenza A/PR8 virus. In a preliminary test, we determined the MID$_{50}$ of the influenza A/PR8 stock used for the experiment. It was found that 1 LD$_{50}$ corresponded to 200 MID$_{50}$. Subsequently four groups of 12 mice, half of them pretreated 24 hr before with 2 mg of COAM intraperitoneally, were infected intranasally with 5 and 50 MID$_{50}$ of influenza A/PR8 virus (i.e., 0.25 and 0.025 LD$_{50}$, respectively). After 3 and 6 days, half of the mice were sacrificed and the lungs were removed for determination of virus content. Figure 3 shows that COAM caused only a suggestive decrease in virus titer in mice infected with a very small inoculum (5 MID$_{50}$) but not in mice infected with 10 times this amount. Apparently, determination of virus multiplication did not increase the sensitivity of the test system. Also decreasing the virus dose to the point at which only 5 MID$_{50}$ was administered, thus imitating what happens in natural infection, did not lead to more clear-cut results. Indeed 5 MID$_{50}$ corresponded to 0.025 LD$_{50}$. With this dose, the difference in consolidation ratio was not more demonstrative than with higher doses (Table 1). Finally, if the interferon mechanism would have been responsible for the observed inhibitory effect on lesion development, one would have ex-

![Survival curves of mice injected intraperitoneally with COAM (2 mg/mouse) at different time intervals from intranasal infection with 2.5 LD$_{50}$ of influenza A/PR8 virus. The numbers in parentheses indicate the number of animals per group.](image-url)
EFFECT OF COAM IN INFLUENZA VIRUS INFECTION

FIG. 3. Influence of COAM (2 mg/mouse intraperitoneally) on multiplication of influenza A/PR8 virus in the lungs of mice.

TABLE 1. Influence of COAM on the development of lung consolidation in mice infected with different strains and doses of influenza virus

| Virus strain | Dose of virus | Consolidation ratio |
|--------------|---------------|---------------------|
|              | Dilution of allantoic fluid | Calculated LD50 | Control groups | COAM-treated groups |
| A/PR8        | 10^-2         | 25                 | 0.55           | 0.35               |
|              | 10^-3         | 2.5                | 0.58           | 0.33               |
|              | 10^-4         | 0.25               | 0.53           | 0.27               |
|              | 10^-5         | 0.025              | 0.32           | 0.17               |
| A/NWS        | 10^-1         | 0.32               | 0.53           | 0.34               |
|              | 10^-2         | 0.032              | 0.36           | 0.27               |
| A/Hong Kong  | 10^-1         | <0.1               | 0.47           | 0.30               |
|              | 10^-2         | <0.01              | 0.35           | 0.25               |
| B/Netherlands| 10^-1         | 1.6                | 0.52           | 0.32               |
|              | 10^-2         | 0.16               | 0.38           | 0.20               |

- A 2-mg amount of COAM per mouse given intraperitoneally 24 hr before virus.
- Consolidation ratio determined on 6 of 12 surviving mice.
- Consolidation ratio determined on 12 of 13 surviving mice.

Intraperitoneally administered COAM, a polyacetal carboxylic acid (1, 2), had an antiviral effect against influenza virus infection in mice. Treated mice invariably showed a reduced consolidation of the lungs and, in the case of infection with a lethal strain of virus, also a delay in mortality. With a small dose of influenza A/PR8 virus, a reproducible increase of final survival rate could be noted. As is the case for other viral infections, e.g., mengo virus or vaccinia virus, the antiviral effect of a single dose of COAM lasted for several days, but no effect could be noticed when the drug was given after virus inoculation (1, 2).

Inhibition of lung consolidation was not paralleled by a significant inhibition of virus replication in the lungs. Even with a very low infecting dose (5 MID50 or 0.025 LD50), only a suggestive decrease in virus titer in the lungs was noted. With a 10 times higher dose, no effect at all was observed, although the consolidation ratio in these groups was clearly reduced. These results plead against the interferon mechanism being the mediator of the antiviral effect of COAM. Indeed, interferon acts through inhibition of intracellular virus replication, and this inhibition is independent from the input multiplicity. It may be argued that a decrease in virus replication, too small to be measured by our technique, may cause a measurable decrease in lesion score. However, decreasing the dose of virus with a factor 10 times caused a clearly measurable decrease in virus titer at days 3 and 6. This decrease corresponded to an inhibition in lesion score, comparable to that obtained by COAM treatment. It seems probable, therefore, that a mechanism other than mere suppression of viral replication by interferon must be responsible for the inhibition of lung consolidation. Whatever the mechanism may be, it must be triggered early during viral infection, as indicated by the fact that COAM must be injected before the virus inoculation to cause a measurable effect.

In a separate study (Billiau, Muyembe, and De Somer, in preparation), we found that intraperitoneal injection of various polycarboxylates, including COAM, protects mice against experimental infection with mengovirus by mobilizing some host defense mechanism. Although the polyanions did induce circulating interferon, the experimental evidence again showed that the larger part of resistance to mengovirus infection was not
interferon-mediated but originated in the retention of challenge virus within the peritoneal cavity. It may be speculated that such trapping of virus may result from increased phagocytosis of virus, or killing, or both, by peritoneal macrophages. A similar activation of reticuloendothelial cells of the lungs might account for the observed effects on influenza virus infection.

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