Inactivation of Influenza and Other Viruses by a Mixture of Virucidal Compounds

J. S. OXFORD,1 C. W. POTTER, C. McLAREN, AND W. HARDY

University of Sheffield Virus Research Laboratory, Lodge Moor Hospital, and Department of Human Biology and Anatomy, Sheffield University, Sheffield, England

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A mixture of benzalkonium chloride, Triton X100, and citric acid (Resiguard F) had a marked virucidal effect on lipid-containing deoxyribonucleic and ribonucleic acid viruses, such as vaccinia virus, herpesvirus, and influenza virus. Adenoviruses and picornaviruses were more resistant to inactivation. Electron microscopy showed that influenza particles became aggregated in the presence of Resiguard F and that the outer fringe of hemagglutinin and neuraminidase spikes seen in control virus preparations became indistinct. The mixture had no detectable antiviral activity in mice infected with influenza AO/PR/8/34 virus, and this was attributed to the reduced virucidal effect of Resiguard F in the presence of serum proteins.

The majority of antiviral compounds at present being tested in man, such as 5-iodo-2-deoxyuridine (13), 1-methylisatin, 3-thiosemicarbazone (4), and 1-adamantanamine hydrochloride (7) have no direct virucidal action, but act by interference with some stage of virus replication. However, it may be possible to destroy viruses extracellularly if sufficiently active and specific compounds can be synthesized, and a series of isoquinolines have been tested recently which may act in this manner (5, 12). We describe the antiviral activity of a mixture of virucidal compounds, Resiguard F, which contains quaternary ammonium, detergent, and citric acid. Although some of the constituents of Resiguard F would be expected to have individual antiviral activity (1, 2, 9), we were particularly interested in any synergistic effect which could result in a more potent virucidal activity. In particular, it was considered that such a mixture of compounds might exhibit a greater virucidal effect in vivo than that of its individual constituents.

MATERIALS AND METHODS

Chemicals. Resiguard F, kindly supplied by J. M. Weston, Aspro Nicholas Research Laboratories, Slough, England, was stored at 4 C. The constituents were 3.0% benzalkonium chloride, 2.75% octylphenoxy polyethoxethanol (Triton X100), and 0.25% picloxoyde digluconate in 10.0% citric acid. The pH of the mixture when diluted 1:80 in deionized water was 2.8.

Cell cultures. Rhesus monkey kidney cells were grown in Eagle’s basal medium containing 10% inactivated calf serum and were maintained in medium 199 containing 4.4 g of sodium bicarbonate per liter.

Chick kidney, HEP-2, and W138 cells were grown in Eagle’s basal medium with 10% fetal bovine serum (Flow Laboratories, Irvine, Scotland) and were maintained in the same medium modified to contain 2% fetal bovine serum.

Viruses. Influenza strains AO/PR/8/34 and A2/Hong Kong/1/68 were propagated allantioically in 10-day-old embryonated hens’ eggs (14). Herpesvirus hominis and vaccinia virus were cultured and titrated for infectivity in BHK-21 cells. Chick embryo lethal orphan (CELO) virus was propagated allantioically in fertile hens’ eggs and was titrated for infectivity in chick kidney cells. Semliki Forest virus was grown and titrated in mouse L cells. Rhinovirus HGP was grown and titrated in W138 cells incubated in a roller machine at 33 C.

Virucidal activity of chemicals. For use in virucidal tests, 9.0 ml of a dilution in deionized water of Resiguard F, or its individual constituents, was incubated for 20 min at 22 C with 1.0 ml of test virus. The mixture of virus and Resiguard F was then diluted rapidly in Eagle’s minimal essential medium containing 10% calf serum and was titrated for residual infective virus. Control virus preparations were incubated in water under identical conditions.

Electron microscopy. Influenza AO/PR/8/34 was concentrated by centrifugation at 25,000 X g for 30 min in a Spinco L preparative ultracentrifuge with the use of an SW-50L swinging-bucket rotor. The virus was resuspended in phosphate-buffered saline (pH 7.2) and centrifuged in a preformed 10 to 40% linear sucrose gradient at 35,000 X g for 45 min; the banded virus was removed by bottom puncture. Purified virus (10,000 hemagglutinating units/ml) was mixed with different dilutions of Resiguard F and incubated for 20 min at room temperature. The mix.
**Table 1. Virucidal activity of Resiguard F**

| Virus                        | Titer of virus (log_{10} TCD_{50}/ml) after incubation with | Reduction in virus titer (log_{10}) |
|------------------------------|-------------------------------------------------------------|-------------------------------------|
|                              | Water | Resiguard pH |                             |                                    |
| Influenza AO/PR/8/34         | 7.7   | 0.2          | >4.7                         | 3.25                               |
| Influenza A2/Hong Kong/1/68  | 5.0   | <2.5         | >2.0                         | 2.5                                |
| Semliki Forest virus         | 4.2   | <2.2         | >2.0                         | 2.0                                |
| Vacinia virus                | 6.2   | <1.2         | >5.0                         | 5.0                                |
| Herpesvirus hominis          | 7.4   | 5.6          | 1.8                          | 1.4                                |
| Chick embryo lethal orphan virus (CELO) | 4.8 | 3.4          |                               |                                    |
| Rhinovirus HGP               | 5.0   | 0.2          | >4.7                         | 3.25                               |

*Resiguard F diluted 1:80 in deionized water.

**Results**

In vitro virucidal activity of Resiguard F. Influenza virus strains were particularly susceptible to the virucidal activity of Resiguard F (Table 1). Thus, 9 log_{10} EID_{50} of influenza AO/PR/8/34 were inactivated after 20 min of incubation with Resiguard F. Other lipid-containing viruses, Semliki Forest virus, vaccinia virus, and herpesvirus hominis, were also inactivated. In contrast, the infectivity titers of CELO virus and rhinovirus HGP were reduced only slightly after incubation with Resiguard F.

High protein concentrations inhibited the virucidal activity of Resiguard F. Resiguard F was diluted 1:200 in deionized water or in water containing 20% calf serum. Influenza virus A2/Hong Kong/1/68 was added, and the mixture was incubated for 20 min at room temperature. The Resiguard F diluted in deionized water inactivated 3.5 log_{10} TCD_{50} of influenza virus. In contrast, no virucidal activity of Resiguard F for this influenza virus strain was detected in the presence of 20% calf serum.

Mode of action of Resiguard F. Table 2 compares the virucidal activity of the different constituents of Resiguard F against influenza A2/Hong Kong/1/68 virus and Semliki Forest virus. Picloxydine had no detectable virucidal activity for these viruses. The degree of virucidal activity of benzalkonium chloride was similar to that of the Resiguard F mixture. Thus, 1:800 dilutions of Resiguard F and benzalkonium chloride reduced the infectivity titer of Semliki Forest virus 1.8 and 1.5 log_{10} TCD_{50} respectively. The detergent Triton X100, and also citric acid, had no detectable virucidal effect on influenza A2/Hong Kong/1/68 at a dilution of 1:800 or greater.

The effect of Resiguard F on the hemagglutinin...
The activity of Resiguard F on influenza virus AO/PR/8/34 hemagglutinin (HA) and neuraminidase antigens

| Dilution of Resiguard F or constituent | Log reciprocal HA titer<sup>a</sup> | Neuraminidase activity<sup>b</sup> |
|----------------------------------------|----------------------------------|----------------------------------|
|                                       | Resiguard F | Triton X100 | Citric acid | Benzalkonium chloride | Resiguard F | Triton X100 | Citric acid | Benzalkonium chloride |
| 1:80                                   | <0.6        | 2.41        | <0.6        | <0.6                  | 1.3         | 1.8         | 0.77        | 1.35                  |
| 1:160                                  | <0.6        | 3.31        | <0.6        | 0.6                   | 1.5         | 1.8         | 1.7         | 1.7                   |
| 1:320                                  | 0.6         | 3.31        | <0.6        | 0.6                   | 1.5         | 1.8         | 1.7         | 1.7                   |
| 1:640                                  | 1.51        | 3.31        | <0.6        | 1.51                  | NT          | NT          | NT          | NT                    |
| 1:1280                                 | 1.51        | 3.01        | 2.11        | 1.81                  | NT          | NT          | NT          | NT                    |
| 1:2560                                 | 1.20        | 3.31        | 2.41        | 2.41                  | NT          | NT          | NT          | NT                    |
| 1:5120                                 | 2.11        | 3.31        | 2.41        | 2.71                  | NT          | NT          | NT          | NT                    |

<sup>a</sup> After 20 min of incubation with each compound. The log HA titer of the control virus preparation was 3.09.

<sup>b</sup> Expressed as optical density at 549 nm after incubation with each compound for 20 min. The value for the control virus preparation was 1.8. NT = not tested.

The activity of Resiguard F on influenza virus AO/PR/8/34 was determined. Unpurified influenza AO/PR/8/34 was diluted 1:10 in either deionized water or in dilutions of Resiguard F. The mixtures were incubated at room temperature for 20 min and then immediately dialyzed against phosphate-buffered saline (pH 7.2) overnight at 4°C. The dialyzed virus was then tested for residual hemagglutinin and neuraminidase antigens (15). The results are shown in Table 3. The hemagglutinin titer of influenza AO/PR/8/34 virus was reduced after incubation with Resiguard F, citric acid, and benzalkonium chloride, at dilutions of <1:1,280. The experiment does not indicate, however, whether the hemagglutinin was denatured or, alternatively, released as nonhemagglutinating monomers (16). In contrast, Triton X100 appeared to increase the hemagglutinin titer, perhaps by disaggregation of virus particles or by splitting off free hemagglutinin from the virus particle. Triton X100 had no detectable inhibitory effect on the virus neuraminidase antigen (Table 3). In contrast, citric acid at a dilution of 1:80 was the most active constituent against the neuraminidase and caused a 57.2% inhibition of enzyme activity; benzalkonium chloride caused a 25.0% inhibition and Resiguard F caused a 27.8% inhibition.

The mode of action of Resiguard F was further studied by electron microscopy. In control preparations of influenza virus incubated with deionized water only, the virus appeared as pleomorphic particles with an outer fringe. After 20 min of incubation with a 1:80 dilution of Resiguard F, the outer fringe had become indistinct in the majority of virus particles, and disintegrated and clumped influenza virus particles were seen in the preparations (Fig. 1 and 2).

**In vivo activity of Resiguard F**. Groups of Swiss white mice were treated with an aerosol, or alternatively with drops of Resiguard F immediately before aerosol infection with influenza AO/PR/8/34 virus. Resiguard F diluted 1:10 and administered intranasally to mice as an aerosol immediately before infection with virus resulted in a decreased mortality at 10 days post-infection (7 of 20 treated mice surviving; 0 of 20 controls surviving). However, by 15 days postinfection in this experiment, there were no mice surviving in either the control or the Resiguard F-treated groups. Administration of Resiguard F aerosols diluted 1:40 and 1:80 had no effect on mortality. Similarly, no in vivo activity for Resiguard F was detected in mice treated intranasally with the compound under ether anesthesia and then infected with influenza virus.

**DISCUSSION**

Resiguard F has been shown in the present study to have the wide spectrum of virucidal activity expected of a mixture containing benzalkonium chloride (2). The mixture was most active against influenza, vaccinia, and herpes viruses, which contain lipid in their outercoat. Further investigations are required on the inactivation of rhinoviruses with Resiguard F, because the present study detected only a marginal virucidal effect on the HGP strain of rhinovirus.

Particularly interesting was the low activity of Resiguard F in vivo compared to its marked virucidal action in vitro against influenza viruses. However, the inhibition of the virucidal action of Resiguard F in vitro by high concentrations of protein may provide an explanation for the differing results. In a previous study, virucidal ac-
tivity was detected in 7 of 18 detergents tested in vitro against influenza AO/PR/8/34 virus (8). However, none of the seven detergents active in vitro protected mice against AO/PR/8/34 virus when instilled intranasally. These results suggest that the usefulness of Resiguard F as a virucidal agent would be limited. However, field trials are required to determine whether the compound could have an application in reducing viral contamination, particularly in veterinary situations.

Highly specific virucidal agents may have a use in topical application against certain viruses. Virucidal properties have been described for sodium magnesium-chlorophyllin (11), and electron microscope studies suggested that this compound acted by fragmenting the virion membrane and destroying the nucleic acid of vaccinia and influenza A2 viruses. The results of electron microscopy in the present study suggested that Resiguard F may affect the lipids constituting the virion membrane. Mild treatment of influenza virus with a nonionic detergent, Triton N101, leads initially to the removal of the outer coat of the virion (6).

No evidence was detected of any synergistic activity of the constituents of Resiguard F. Early studies by Bauer (3) indicated possible synergistic activity between isatin thiosemicarbazone and certain phenoxypyrimidines in vaccinia infection in mice. In addition, an additive effect has been described for interferon and amantadine (10). Studies are in progress on the antiviral activity of combinations of virucidal compounds and also of mixtures of virucidal and virustatic compounds. Such mixtures could extend the spectrum of antiviral activity of the chemoprophylactic agents at present available.

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