The Antiviral Effect of a Triazinoindole (SK&F 40491) in Rhinovirus Infected Gibbons (36800)

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A triazinoindole, 4-((8-amino-7-chloro-5-methyl-5*H*-*as*-triazino (5,6-b) indole-3-yl)amino)-2-methyl-2-butanol (SK&F 40491), is a potent inhibitor of a broad spectrum of picornaviruses *in vitro* (1). Since its *in vitro* activity extends to human rhinoviruses, it was of interest to evaluate the compound for activity against a human rhinovirus infection in experimental animals as a prelude to clinical trials. Asymptomatic infections in chimpanzees (2, 3) and gibbons (4) with human rhinoviruses have been described. The latter system was chosen for study due to an available supply of gibbons, the relative ease of handling this species, and our background of experience in using the gibbon for human rhinovirus infections. This report describes the effect of orally and intranasally administered SK&F 40491 on the yield of virus in nasal secretion and on antibody response following infection.

Materials and Methods. Animals. Male and female gibbons (*Hylobates lar*), ranging in weight from 1.8 to 7.7 kg, were employed. These animals, acquired from a variety of sources, have been maintained in our laboratory for 1 to 4 yr. In this environment they are caged individually in common rooms, and have remained in overt good health.

Viruses. Rhinovirus 1A/2060, obtained from G. Douglas, was passaged 3 times in WI-38 cell cultures in our laboratory. Rhinovirus 2/HGP was obtained from D. A. J. Tyrrell, and was subsequently passed 5 times in WI-26 and once in WI-38 cells.

Compound formulation. SK&F 40491 was prepared for oral administration at 16 mg/kg in a 5 ml dose by grinding in a mortar with an aqueous solution of 8.5% lactic acid and 0.5% gum tragacanth. A 2.5% suspension for intranasal use was prepared with tross-milled compound in water containing 1% carboxymethyl cellulose (type 7 MF) and 0.1% Tween 80 at pH 6.3. A 0.02% solution for intranasal use was obtained in water with 10% ethyl alcohol and 20% polyethylene glycol-200 at pH 4.6. No additional compound could be solubilized under these conditions. Comparable formulations lacking SK&F 40491 were used for placebo.

Virus titration. Virus was assayed either by cytopathic effect (CPE) in WI-38 cell cultures or by plaquing in HeLa cell monolayers; the sensitivity of the 2 methods was comparable. For CPE titration each of 2 to 4 WI-38 tube cultures with confluent monolayers was inoculated with 1 ml of serial 10-fold dilutions of virus in maintenance medium: Eagle's minimum essential medium with Earle's balanced salt solution (MEM), 5% heated fetal calf serum (FCS), with 100 units/ml of the sodium salt of penicillin G and 100 μg/ml each of streptomycin sulfate and neomycin. The cultures were incubated at 34° on a roller drum. The tissue culture infectious dose ($TCID_{50}$) of virus was determined on the basis of viral CPE 7 to 10 days after inoculation. For plaque assay 0.2 ml aliquots of serial 0.5 $\log_{10}$ virus dilutions in maintenance medium were added to duplicate HeLa cell monolayers in 60 $\times$ 15 mm Linbro multidish "Disposo Trays." After 60 min at 22°, residual medium was removed, the cultures were washed 3 times with 3 ml of phosphate buffered saline (PBS), and 6 ml of an agar-containing medium were added: MEM with 2% FCS, 30 m*M* $MgCl_2$, 400 μg/ml DEAE-dextran, the above antibiotics,

and 1% Noble agar. The cultures were incubated for 4 days at 34° in an atmosphere of 5% CO_2. Plaques were counted after staining with 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride for 1 hr at 37°. The number of plaque forming units (PFU) in the inoculum was calculated.

Antibody titration. Serum neutralizing antibody assays were carried out as follows. Serial 4-fold dilutions (0.2 ml) of heated serum (56° for 30 min) were mixed with an equal volume of virus containing 50 to 100 $TCID_{50}$ in maintenance medium. After 1 hr at 22°, 0.4 ml of the mixtures were added to duplicate WI-38 tube cultures containing 0.6 ml of maintenance medium. After incubation for 2 to 6 days at 34° on a roller drum, 50% neutralization endpoints were determined by inhibition of viral CPE. Titers were expressed as reciprocal $\log_2$ geometric means/0.2 ml. Neutralizing activity of nasal wash was also determined. Nasal washings were heated at 56° for 30 min. Serial 2-fold dilutions of the nasal wash in PBS with 0.1% bovine serum albumin (PBS-BSA) were then incubated with equal volumes of PBS-BSA containing 50 PFU of virus for 1 hr at 22°. Each mixture (0.4 ml) was assayed for PFU in duplicate HeLa cell monolayers as previously described. Fifty percent plaque reduction titers per 0.2 ml of nasal wash were calculated. Selected serum specimens were similarly assayed, showing that this assay procedure had comparable sensitivity to the CPE method using WI-38 cells for determining neutralizing antibody titer.

Gibbon infection. The animals were anesthetized (4) by intramuscular injection of Ketalar (ketamine hydrochloride; Parke, Davis & Co.). They were then infected by aerosol with a DeVilbiss No. 82 atomizer having a modified nozzle for more efficient delivery. Each animal received 0.5 ml of inoculum in each naris. Virus was diluted for inoculation in maintenance medium.

Compound administration. Anesthetized animals were dosed orally by intubation. Intranasal dosing was accomplished as described for virus inoculation.

Specimens. In initial experiments (oral treatment) nasal secretion was collected for qualitative assay of virus. The nares of anesthetized animals were swabbed with a sterile, cotton-tipped applicator which had been wet with maintenance medium. The specimen was eluted into 1.5 ml of the medium to which 50 μg/ml of Mycostatin had been added. The eluate was assayed for virus by CPE in WI-38 tube cultures at 1:5 and 1:25 final dilutions in maintenance medium. The proportion of cultures showing CPE, without regard to the inoculated dilution, was used to express the virus content of a secretion. Attempts to recover virus by blind passage from cultures showing no CPE were uniformly unsuccessful.

In later experiments nasal wash was collected for quantitation of virus, and determination of viral neutralizing activity. Five milliliters of PBS-BSA were flushed into one naris of an anesthetized animal using a syringe with a modified fistual tip and the fluid was collected from the second naris by gravity. The nasal wash was recycled through the nares in reverse order. It was then homogenized by grinding in a mortar with sterile Alundum, and subsequently clarified by centrifugation. Virus titers and viral neutralizing activity were assayed by the plaquing methods described above.

Blood was drawn from the femoral vein of anesthetized animals. Serum was separated from the clotted blood by centrifugation, and was assayed for neutralizing antibody in WI-38 cell cultures (see above).

Results. Oral treatment. The effect of orally administered SK&F 40491 was evaluated in a single experiment against an infection with 32 $TCID_{50}$ of rhinovirus 1A. The compound was given at 16 mg/kg/dose 3 times daily at 3 hr intervals for 5 days. The virus was inoculated between the second and third doses. Six animals were given SK&F 40491 and two were dosed with placebo. Data from a control bank of 20 additional gibbons infected on other occasions with the same quantity of virus were also used for comparison. Values obtained from the 2 placebo-treated gibbons were within the limits of the control data bank as determined by covariance analysis. Two of the 6 compound-treated gibbons were found to have preexisting serum

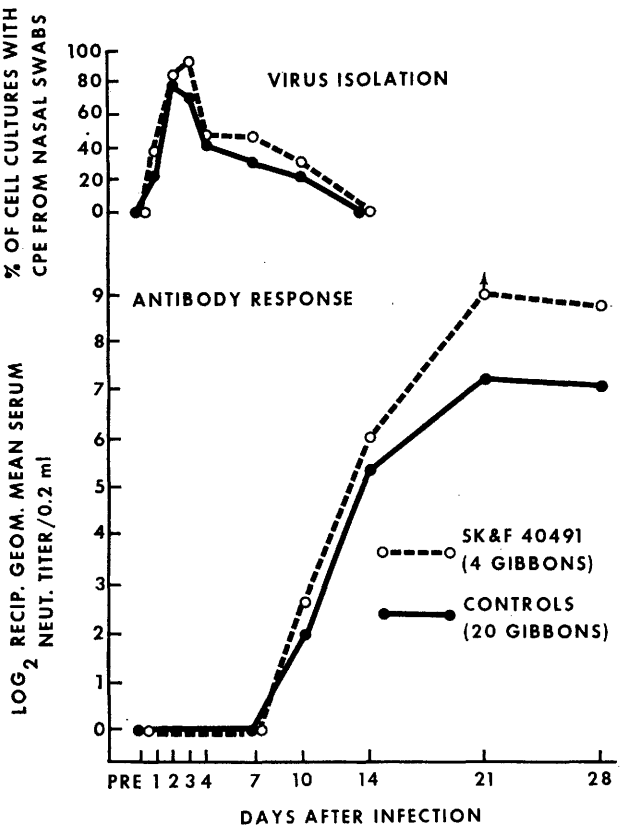

FIG. 1. Effect of SK&F 40491 in rhinovirus 1A infected gibbons—oral administration.

antibody to rhinovirus 1A (titers of 1:4), although they had not been experimentally infected with this virus previously. Similarly, 5 control animals from the data bank, and 1 of the placebo-treated group from this experiment, had preinfection titers ranging from 1:4 to 1:16. The data from all animals with preexisting antibody were analyzed separately. For comparison of antibody responses within this group, all titers were adjusted to a common base line ($< 1:2$).

Figure 1 compares the incidence of viral isolation from the nasopharynx, and serum antibody response of compound-treated and control animals having no preexisting serum antibody to rhinovirus 1A. Covariance analysis showed no significant difference between the values obtained for these 2 groups. Maximum virus recovery was obtained 2 to 3 days after infection. Serum antibody was first detected on day 10; peak titer was observed on day 21.

Figure 2 presents similar data from the animals with preexisting serum antibody titers. Less virus was recovered from the nasopharynges of these animals than from those which were originally seronegative, suggesting less viral replication and earlier resolution of the infection. There was no significant difference in this respect between the compound-treated and placebo groups. Though serum antibody response in compound-treated animals was somewhat less than in the control group on days 10, 14, and 21, this difference was not statistically significant.

Intranasal treatment. Two internally controlled experiments were carried out to evaluate the efficacy of a 2.5% suspension and a 0.02% solution of SK&F 40491 when applied intranasally against a gibbon infection produced with 32 to 100 $TCID_{50}$ of rhinovirus 2. In both experiments treatment was initiated 3 hr before virus inoculation, and con-

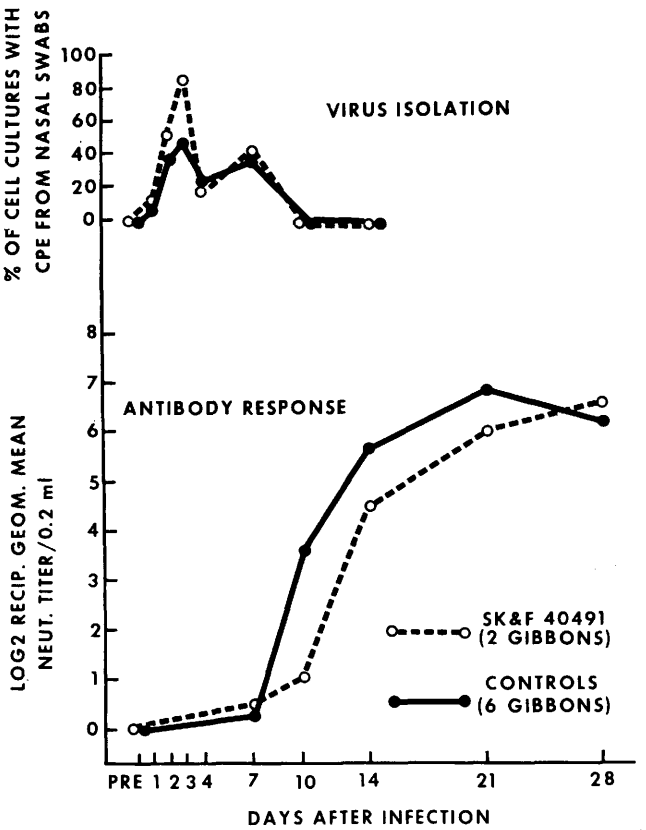

FIG. 2. Effect of SK&F 40491 in rhinovirus 1A infected gibbons with existing antibody—oral administration.

tinued around-the-clock at 4 hr intervals for 24 hr. Thereafter, on days 2, 3, 4, and 5, the last daily dose was eliminated.

Twelve gibbons which had been infected 11 to 20 mo previously with the same virus were used in the first experiment. The animals were ranked on the basis of peak serum antibody response to the initial infection (1:8 to > 1:1024, geom. mean 1:120). When this provided no discrimination between animals, the residual titer at the time of use was employed (< 1:2 to 1:64, geom. mean 1:3.5). Three gibbons were placed in each of 4 blocks by sequential rank. Within each block a gibbon was randomly assigned treatment with placebo, compound suspension, or compound solution.

In the second experiment 8 seronegative gibbons, not used previously for rhinovirus 2 infections, were considered part of the same block and randomly assigned to each treatment group. Three were dosed with compound suspension, 3 with compound solution, and 1 was treated with each placebo.

All gibbons became infected except for 1 placebo-treated animal in the first experiment. This animal had a preexisting neutralization titer of $\geq$ 1:32 in a nasal wash, whereas the remaining animals were negative in this respect. The patterns of viral isolation from nasal wash, and viral neutralization responses in serum and nasal wash were comparable for a given formulation in both experiments. Similar results were obtained with each placebo except for the uninfected animal. Consequently, all data obtained, except that from the uninfected animal, were pooled.

Figure 3 shows the virus titers and incidence of virus isolation from nasal wash specimens. Virus titers in the placebo-treated controls rose to a maximum of 3.1 $\log_{10}$

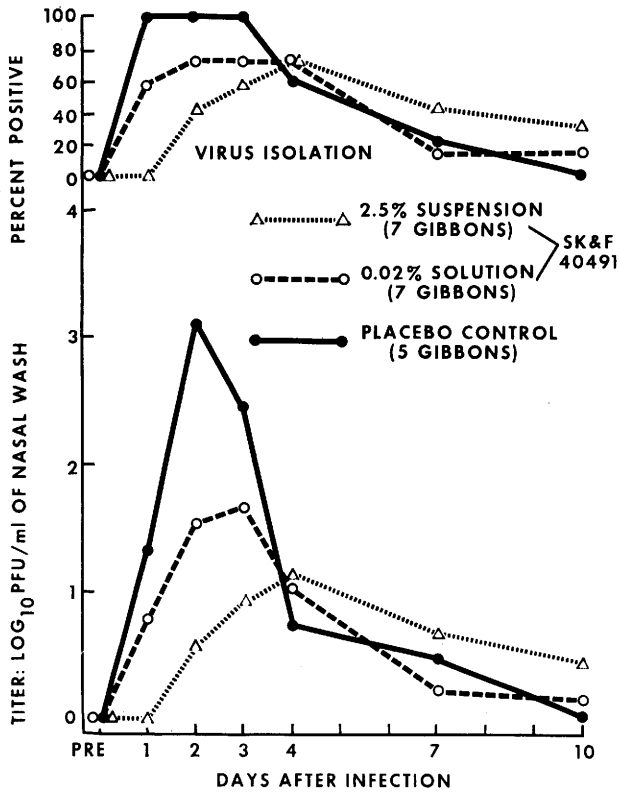

FIG. 3. Antiviral effect of SK&F 40491 in rhinovirus 2 infected gibbons—nasal administration (differences are significant on days 1, 2, 3; $p < .05$).

PFU/ml by the second day after infection. At this time the nasal washes from the 2 compound-treated groups contained 40- and 320-fold less virus. Greater virus yields were obtained from the nasal washes of compound-treated animals later in the infection (3 and 4 days), but the maximum titers obtained remained less than the control maximum by 32- and 100-fold. All nasal wash virus titers diminished through day 10. At that time no virus could be detected in control specimens, but low levels were still evident in both compound-treated groups. Adjusted averages were developed using a mathematical model for a randomized block design. Analysis of these averages showed a significant difference ($p < .05$) between values for control and both compound formulations on days 1, 2, and 3. On day 1 there was a significant difference between solution and suspension.

The lower nasal wash virus titers in compound-treated groups, attributed to compound action in the animal, could result from residual compound carried over into the *in vitro* assay. To preclude this possibility, residual nasal wash was removed from the cell monolayers after virus adsorption, and the cells were washed prior to addition of the overlay medium. Under these conditions nasal washes collected during treatment periods, but containing no detectable virus, did not reduce the titers of either rhinovirus 2 or rhinovirus 1A added to them in known concentrations. Rhinovirus 1A was used since it is more sensitive than rhinovirus 2 to SK&F 40491 *in vitro*.

Serum neutralizing antibody response and incidence of seroconversion are shown in Fig. 4. Values for gibbons having preexisting antibody titers were reduced in proportion to the initial titer to provide a common base line ($< 1:2$). Antibody rise was first observed in the placebo group 7 days after infection. Titers rose rapidly to maximum levels by day

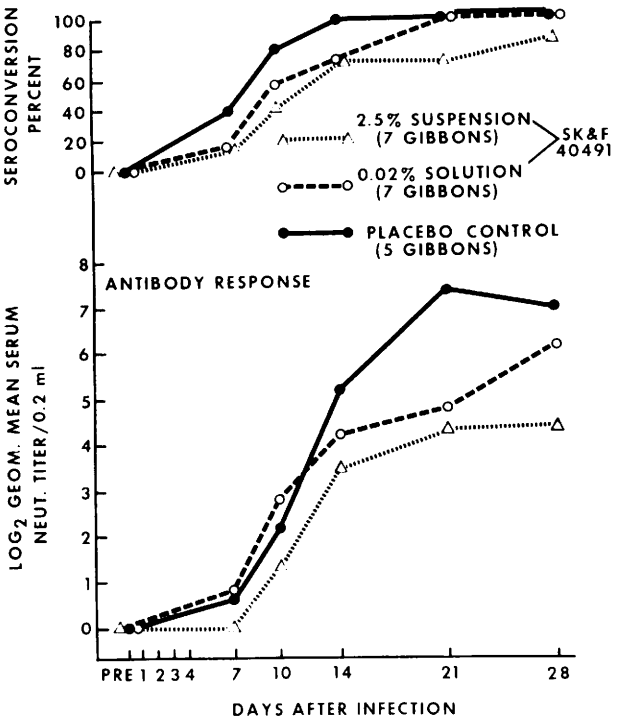

FIG. 4. Antiviral effect of SK&F 40491 in rhinovirus 2 infected gibbons—nasal administration (differences are significant on days 14, 21, 28; $p < .05$).

21. Antibody response in compound-treated groups was somewhat delayed, and mean maximum titers were slightly lower than those of the control group. Statistical analyses of the individual experiments and the combined data separates compound-treated groups from the control. Differences are significant ($p < .05$) on days 14, 21, and 28. There is no significant difference between responses to compound solution and suspension.

Figure 5 illustrates the effect of treatment on the rise in virus neutralizing activity in nasal wash. This response is similar to that of serum antibody, but differences between compound-treated and control groups are greater. Only 2 of 7 gibbons in each compound-treatment group showed a rise in titer by 21 days after infection, whereas all placebo-treated animals responded within this time. The low geometric mean titers which were obtained reflect dilution of nasal mucus in wash fluid; no effort was made to concentrate the specimens or to represent values on the basis of protein content. The neutralizing activity is apparently specific for rhinovirus 2; no response was obtained to rhinovirus 1A. Presumably, these titers are a measure of nasal antibody, although no attempt was made to characterize the material as IgA.

Discussion. SK&F 40491, when applied intranasally, is an effective inhibitor of rhinovirus 2 replication in the nasopharynx of gibbons. These data support and extend the observation that SK&F 40491 suppresses the replication of rhinovirus 2 in cultured cells (1). The delayed and reduced antibody response in treated animals is probably an indirect measure of this effect; less antigen is produced than in controls.

Despite the greater apparent activity observed with the 2.5% suspension than with the 0.02% solution, the difference in activity between these formulations is statistically significant only on the first day after infection. With no further data for comparison, it must be concluded that the 2 formulations have not been shown to differ in their activity.

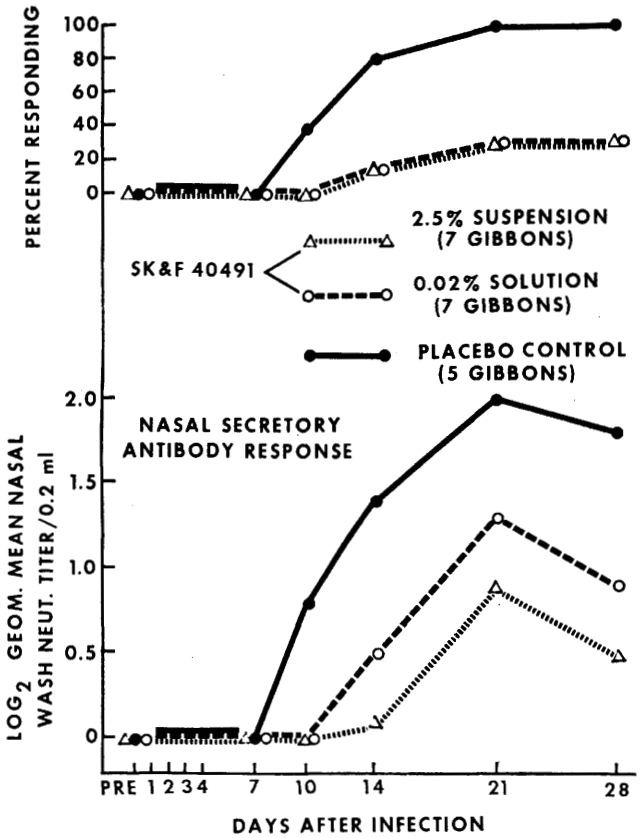

FIG. 5. Antiviral effect of SK&F 40491 in rhinovirus 2 infected gibbons—nasal administration (differences are significant on days 14, 21, 28; $p < .05$).

This is a reasonable contention, since potency is probably related to the amount of compound in solution. Although the amount of SK&F 40491 which is soluble in nasal secretion is unknown, the 2.5% suspension introduces the compound at a soluble concentration of approximately 20 μg/ml compared with 200 μg/ml for the solution formulation. Both values are well above the inhibitory concentration of SK&F 40491 for rhinovirus 2 *in vitro* (1). Neither formulation produces overt irritation of the nasopharynx or systemic toxicity. However, the solution is probably the preferred dosage form due to its physical characteristics and lesser compound content. The effect of dosing frequency on activity was not determined. It is likely, however, that fewer doses than employed here could provide a comparable effect. It has been observed that SK&F 40491 is not readily removed from cultured cells by washing (1). If the compound is similarly bound to cells of the nasal mucosa, its removal by the flow of mucus would be impeded.

The clinical value of an antiviral effect of the magnitude shown here is open to speculation, since the gibbon infection is asymptomatic. Efforts to produce illness in gibbons with other rhinoviruses having multiple cell culture pasage, or contained in nasopharyngeal washings from persons with colds, were unsuccessful. However, it has been observed that the incidence and severity of illness in human volunteers infected with a rhinovirus are commonly related to the pattern of viral replication. Those individuals with early appearance and high yields of virus in nasal wash are more prone to symptomatic infection than those in whom virus is first isolated later and at a lower titer (5). The pattern of

virus isolation in the gibbon control group resembles the former, whereas that of the gibbon groups treated with SK&F 40491 is similar to the latter.

Although the antiviral activity of SK&F 40491 in the gibbon was demonstrated only against rhinovirus 2, probably other rhinovirus infections would be similarly inhibited on the basis of projections from the spectrum of compound activity *in vitro* (1). Studies with other rhinovirus serotypes could be carried out in the gibbon, should it be desirable to do so. Gibbons can be routinely infected with rhinoviruses 1A and 2 as shown. Less regularly we have infected gibbons with rhinoviruses 14, 30, and 45. On the other hand, no infection could be produced with types 16, 17, 19, 21, 25, and 30 in single experiments involving only several animals. On the basis of this experience, it appears that infectivity of rhinoviruses for the gibbon is unrelated to the extent of their cell culture passage, or to their potential for isolation in monkey or human cells (M and H strains). It is unlikely that a short-lived heterotypic resistance of the type described for human rhinovirus infections (6) was responsible for the refractoriness of gibbons to certain strains since sequential infections were spaced a minimum of 11 wk apart.

The lack of activity with orally administered SK&F 40491 against rhinovirus 1A infection in the gibbon reflects our experience using the compound systemically against various other animal infections (1). These results are not surprising in light of its poor absorption from the gut, short plasma half-life, and conjugation to inactive forms in the rhesus monkey (1). Fragmentary data suggest that the gibbon handles the compound similarly.

For practical reasons, the antiviral activity of SK&F 40491 cannot be extensively evaluated in the gibbon. Also, its clinical efficacy against rhinovirus infections cannot be determined in this system. Consequently, it is more convenient and pertinent to carry out further studies in man. Plans have been made to do so.

Summary. SK&F 40491, a triazinoindole, provided antiviral activity when administered intranasally to gibbons infected with human rhinovirus type 2. The compound was effective in a multidose regimen either as a 0.02% solution or a 2.5% suspension. The effect of drug treatment was demonstrated by a decrease in the quantity of virus recovered from the nose, and by a decrease and delay in serum and nasal antibody response when compared to placebo-treated animals ($p < .05$).

Oral treatment with SK&F 40491 at 16 mg/kg/dose in a multidose regimen did not significantly inhibit viral replication or alter the characteristic antibody response in gibbons infected with rhinovirus 1A when compared to a pooled bank of placebo controls.

The authors thank Dr. S. M. Free, Jr., for biostatistical analyses, and Messrs. B. Zabko-Potapovich, P. Ferry, and P. Sereni for excellent technical assistance.

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Received May 25, 1972. P.S.E.B.M., 1972, Vol. 141.