AN INHIBITOR OF REOVIRUS IN SHEEP SERUM

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Reoviruses do not multiply well in cultured cells if serum is present in the medium, and cytopathic changes are delayed. Both virus yields and cytopathic effects are enhanced when the serum is replaced by foetal serum (Gomatos et al., 1962) or by serum substitutes (Wallis, Melnick and Bianchi, 1962). The inhibitory effect of calf and sheep serum seemed likely to be due to the presence of specific antibody since reoviruses of all three types appear to be ubiquitous (see review by Stanley, 1967). In the experiments to be described this hypothesis was examined.

MATERIALS AND METHODS

Virus strain. Reovirus type 3 was obtained from the Central Public Health Laboratory, Colindale, as 15th monkey kidney passage of the Dearing strain. The strain was passaged subsequently in mouse fibroblast L cells and cloned by three consecutive isolations from single plaques.

Infectivity assay. Virus yields during the course of a single multiplication cycle of the virus in L cells were studied by withdrawing the culture fluids at various times after infection to provide duplicate samples for the assay of extracellular virus. An equivalent volume of fresh medium was added and the cells scraped into suspension and rapidly frozen for a subsequent assay of cell-associated virus. Infectivity was assayed by the procedure described by Gomatos et al., except that serum was omitted from all media used for virus propagation and assay. Cell monolayers in 5cm diameter plastic petri dishes were washed free of serum before inoculation. The volume inoculated per dish was 0.2 ml, and 1 hr was allowed for adsorption. The monolayers were washed again with serum-free medium and overlaid with 10 ml of a solid serum-free medium containing 0.6 per cent. Difco Noble agar. It had been observed previously that the size of plaques was enhanced by reducing the concentration of agar in the overlay. The infected monolayers were incubated for 6–7 days at 37°C and then fixed and stained with a saturated solution of methylene blue in 4 per cent. formaldehyde. Plaque counts obtained by this method were identical with those obtained with solid overlays containing up to 10 per cent. foetal calf serum.

Serum. Calf serum was obtained from Burroughs, Wellcome & Co., foetal calf serum from Flow Laboratories, and sheep serum locally. Foetal sheep serum was obtained from foetuses immediately on removal from the uterus.

DEAE-cellulose chromatography. Sheep serum was fractionated on a DEAE-cellulose column according to the method of Levy and Sober (1960). The γG globulin fraction was concentrated by passage through a CM-cellulose column and the remaining serum protein by dialysis under vacuum.

Sephadex G-200 chromatography. Calf and sheep sera were fractionated on Sephadex G-200 according to the method of Flodin and Killander (1962); 5 ml of serum was applied

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to a column 50 x 3.5 cm. The column effluent was monitored at 254 nm and five fractions corresponding to the γM (19S) globulin, γG (7S) globulin and albumin peaks, and the two intermediate zones, were collected. These fractions were reduced to approximately 5 ml by dialysis under vacuum. The purity of the fractions was checked by centrifugation in a Spinco model E analytical ultracentrifuge.

Mercaptoethanol treatment. Undiluted serum was mixed with an equal volume of 0.2 M-mercaptoethanol in 0.04M phosphate buffer at pH 7.6 and held at 22°C for 12 hr. The mercaptoethanol was removed by dialysis.

Neutralisation test. Dilutions of whole serum and serum fractions were added to convenient volumes of a standard virus preparation giving approximately 80 plaque-forming units per 0.2 ml inoculum. Duplicated serum-virus mixtures were held at +4°C for 18 hr before inoculation on to L cell monolayers. The end point was determined graphically as the serum dilution giving a 50 per cent. reduction in plaque count.

RESULTS

Whole serum and the various serum fractions were examined for virus neutralising activity in the usual way. They were also examined for inhibitory activity by incorporating them in the medium of infected cultures and estimating the production of extracellular and intracellular virus.

Virus-neutralising activity

Fractionation of serum on DEAE-cellulose separates the γG globulin from the remainder of the serum proteins. Fig. 1 shows the relation between dilution and plaque count obtained in the standard neutralisation test in the case of whole sheep serum and the 2 fractions obtained by DEAE chromatography. The 50 per cent. end-points obtained from these curves are included in the table together with those similarly obtained with fractions prepared on
Sephadex G-200. In the latter case 5 fractions were examined corresponding to the γM (19S) globulin, γG (7S) globulin and albumin peaks and the 2 intermediate zones. Pooled batches of serum were used for all fractionation procedures. In both cases virus-neutralising activity was confined to the γG globulin-containing fraction. The table also shows that virus-neutralising activity was diminished, but not destroyed, by mercaptoethanol treatment, and unaffected by dialysis. These results suggest that the neutralisation of reovirus infectivity is due to the presence of antibody in sheep serum. Similar results were obtained with calf serum. On the other hand, both foetal calf and foetal sheep serum were devoid of neutralising activity.

**Table**

*Virus-neutralising activity of sera after various forms of treatment*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fraction</th>
<th>50 per cent. end-point serum dilution in neutralisation test with</th>
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<tr>
<td></td>
<td></td>
<td>foetal sheep serum was 1 in</td>
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<tr>
<td>Nil</td>
<td>Whole serum</td>
<td>&lt;4</td>
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<tr>
<td>Dialysis</td>
<td>Whole serum</td>
<td>&lt;4</td>
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<tr>
<td>DEAE chromatography</td>
<td>I γG globulin</td>
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<td></td>
<td>Ⅱ Remaining proteins</td>
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<tr>
<td>Sephadex G-200 chromatography</td>
<td>I γM globulin (19S)</td>
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<td></td>
<td>Ⅱ Intermediate zone</td>
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<td>Ⅲ γG globulin (7S)</td>
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<td></td>
<td>V Albumin</td>
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<tr>
<td>Mercaptoethanol</td>
<td>Whole serum</td>
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</tr>
</tbody>
</table>

*Inhibitory activity*

Fig. 2 shows that the inhibitory activity of the pooled calf serum resided in the γG globulin fraction, as would be expected if the inhibition is mediated by neutralising antibody. The γM globulin fraction had no effect on virus yield, and the albumin and two intermediate fractions were similarly inactive.

The opposite situation was observed in the case of sheep serum. Of the 5 fractions obtained by Sephadex G-200 chromatography only the γM globulin fraction exhibited inhibitory activity. The γG globulin fraction, though possessing neutralising activity, did not reduce the virus yield during the course of a single multiplication cycle. Similar results were obtained with the γG globulin and residual serum protein fractions obtained by DEAE-cellulose.
The inhibitory activity occurred in the fraction containing the residual proteins and not in the γG globulin-containing fraction.

Fig. 3 shows the titres of free and cell-associated virus during the course of a single multiplication cycle in the presence of the DEAE-cellulose fractions of sheep serum. The curve for whole sheep serum was similar to that for the residual protein fraction and is not shown in fig. 3. In the presence of the γM globulin-containing fraction the titre of cell-associated virus rose initially and then gradually declined. There was no corresponding rise in the titre of

![Graph showing the effect of calf serum fractions on virus yield.](image)

**Fig. 2.**—The effect of calf serum fractions obtained by Sephadex G-200 chromatography on virus yield.

(a) Whole calf serum; (b) γG globulin; (c) serum-free control; (d) γM globulin. ●—● Titre of virus in supernatant; ○—○ titre of cell-associated virus.

![Graph showing the effect of sheep serum fractions on virus yield.](image)

**Fig. 3.**—The effect of sheep serum fractions obtained by DEAE-cellulose chromatography on virus yield.

○—○ γG globulin, titre of virus in supernatant; ×⋯× γG globulin, titre of cell-associated virus; ●—● γM globulin containing fraction, titre of virus in supernatant; ○⋯○ γM globulin containing fraction, titre of cell-associated virus.
virus in the medium. Cytopathic changes were delayed and became apparent only at 96 hr after infection. In the absence of serum (not shown) or in the presence of the yG globulin fraction the titres of cell-associated virus and free virus rose in parallel and cytopathic changes were apparent at 24 hr after infection. The inhibitory activity of sheep serum was unaffected by exposure to 56°C for 30 min.

DISCUSSION

Both the neutralising and inhibitory activities of calf serum reside in the yG globulin fraction and both activities are therefore probably mediated by specific antibody. On the other hand, these activities are dissociated in the case of sheep serum where the inhibitory activity resides in the yM fraction.

Substances that neutralise virus infectivity, but are not specific antibodies, are known for several viruses (Allen, Finkelstein and Sulkin, 1958; Ginsberg, 1960; Bögel, 1966; McFerran et al., 1968). A poliovirus-neutralising substance that resembles yM globulin in molecular size and physical properties has been found in normal bovine serum (Svehag, 1964; McFerran et al.). The inhibitor of reovirus multiplication present in sheep serum also resembles yM globulin and may be another manifestation of the serum component. The inhibitor is absent in foetal serum and McFerran (1962) has shown that these poliovirus-neutralising substances do not appear in cattle until 6 mth after birth. There are a number of differences, however, between the two situations.

Firstly, although the reovirus inhibitor did not neutralise extracellular virus, it did not affect intracellular replication either, which suggests that the inhibitor does not penetrate the cell. In this latter respect the inhibitor resembles an antibody. It appears to prevent the release of virus from infected cells. Infected cells do not undergo cytopathic changes in the presence of the inhibitor at a time when the viral content of the cells reaches a level at which cytopathic changes are observed in the absence of inhibitor (fig. 3).

In the poliovirus case the reaction is believed to be non-specific, since cattle are not susceptible to experimental infection with this virus (McFerran et al.). Reovirus infection on the other hand is prevalent in domesticated animals (Rosen, Abinanti and Hovis, 1962; Lamont, 1966). The pooled calf and sheep sera examined in these experiments contained high titres of neutralising antibody in the yG globulin fraction indicating extensive exposure of the donor animals to reovirus infection. The inhibitor in the yM globulin fraction could therefore also be a specific antibody with a unique mode of action, i.e., able to act at the cell surface, blocking virus release, although unable to neutralise free virions.

All batches of sheep serum obtained locally possessed inhibitory activity. Only one batch from another source was exceptional in that it had no inhibitory properties. The yM globulin of sheep serum therefore cannot be inherently inhibitory for reovirus, and this finding supports the hypothesis of the specific antibody nature of the inhibitor.
The difference between the results obtained with calf serum and sheep serum might be either a species difference, or a difference in the nature of the exposure to virus infection and the level of antibody attained. Unfortunately nothing is known of the age of the donor animals. The pooled calf serum was of high titre with no neutralising or inhibitory activity in the γM globulin fraction. The pooled sheep serum was of lower titre with inhibitory activity in the γM globulin fraction. It is possible that the sheep serum was obtained from younger animals at an early stage of infection before waning of the 19S "early" antibody response.

Mutants of poliovirus have been isolated that were resistant to bovine serum inhibitor (Takemori et al., 1957, 1958), although in immunological properties they were identical with the parent strain. The intention of the reovirus experiments was to establish whether similar inhibitor-resistant mutants could be obtained in the case of this virus. In the event it proved impossible to isolate inhibitor-resistant mutants of reovirus either by direct selection or by alternating selection and treatment with mutagenic agents.

**SUMMARY**

Yields of reovirus type 3 were depressed and cytopathic changes delayed when calf or sheep serum was present in the culture medium. Both sera also neutralised free reovirus. Fractionation of pooled serum samples by DEAE-cellulose and Sephadex G-200 chromatography showed that in the case of calf serum the inhibitory and neutralising activities were associated with the γG globulin fractions. Therefore it is likely that the inhibitory activity of calf serum is due entirely to the presence of neutralising antibody. In the case of sheep serum, however, neutralising and inhibitory activities were dissociated. Virus-neutralising activity in this case was maximal in the γG globulin fractions, whilst the inhibitory activity resided in the γM fraction. The inhibitory component of sheep serum appears to act by blocking virus release.

The technical assistance of Miss P. Butler is gratefully acknowledged.

**REFERENCES**


