# Demonstration by Plaque Reduction Technique of Immunologic Relationship between Canine Herpes Virus and Herpes Simplex Virus* (32721)

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Recently, a virus morphologically similar to herpesviruses, and designated canine herpes virus (CHV) has been isolated from fetal and neonatal pups suffering from a fatal septicemic disease (1–3).

CHV develops, at least in part, in the nucleus of infected dog kidney cells (2, 3). On the basis of neutralization tests, no immunologic relationship between this virus and certain other members of the herpesvirus group could be established (2). The objective of this communication is to report that CHV is immunologically related to herpes simplex virus.

*Materials and Methods. Solutions and media.* PBS-A: phosphate buffered saline made according to Dulbecco and Vogt (4) but containing 0.2% bovine albumin (Fraction V, Armour Labs., Chicago, Illinois) used as diluent in virus assays. 199-Mc: semisolid overlay containing methyl cellulose and used for virus assay consists of mixture 199 (Grand Island Biological Co., Grand Island, New York) containing 1% calf serum and 10 gm of methyl cellulose (4000 cps) per liter of medium.

*Cells.* The continuous line of dog kidney cells (DK) was originally obtained from Dr. M. D. Hoggan, Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, NIH. Human Epidermoid Carcinoma No. 2 (HEp-2) cells were originally obtained from Microbiological Associates, Bethesda, Maryland.

*Viruses.* Canine herpes virus (CHV) strain F-205V originally obtained from Dr. L. E. Carmichael, Veterinary Virus Research Institute, New York State Veterinary College, Cornell University, Ithaca, New York, serially passaged in our laboratory in DK cell cultures; the macroplaque strain of herpes simplex virus (HSV-MP) the properties of which have been described in detail by Roizman and Roane (5, 6).

*Antibody.* The rabbit anti-CHV serum was prepared as follows: DK cells harvested 48 hours after infection with CHV were frozen and thawed three times to release virus and were mixed with complete Freund's adjuvant (Difco Labs., Inc., Detroit, Michigan) to yield a 50% (v/v) suspension. Approximately 1.5 ml of this suspension was injected into rabbits subcutaneously, once a week for 3 weeks. The subcutaneous injections were followed 1 week later by one intravenous injection of the cell extract without adjuvant. The serum used in this study was obtained 8 days after the last injection. It was adsorbed repeatedly with uninfected DK cells, decomplemented by heating at 56°C for 30 min and stored at —20°C. The rabbit anti-HSV-MP serum was prepared as described elsewhere (7).

*Infectivity assays.* The plaque assay for HSV-MP has been described elsewhere (7). CHV was assayed by a plaque method as follows: DK monolayer cultures prepared in 30-ml Falcon flasks were each inoculated with 1 ml of virus diluted in PBS-A, then placed on a rotary shaker at 37°C for 2 hours. At that time the virus was removed and each culture flask received 5 ml of 199-Mc overlay and was further incubated at 37°C. After 2 days the overlay was removed by suction, the cells were washed with PBS-A, fixed with methanol, stained with Giemsa stain and air dried. The plaques produced by CHV in monolayer cultures of DK cells consist of small clumps of rounded cells staining more intensely than the uninfected cells (Fig. 1). These plaques are similar to those produced by a variety of strains of herpes simplex virus (7,8). Virus titers are expressed in terms of plaque forming units (pfu) per ml of fluid.

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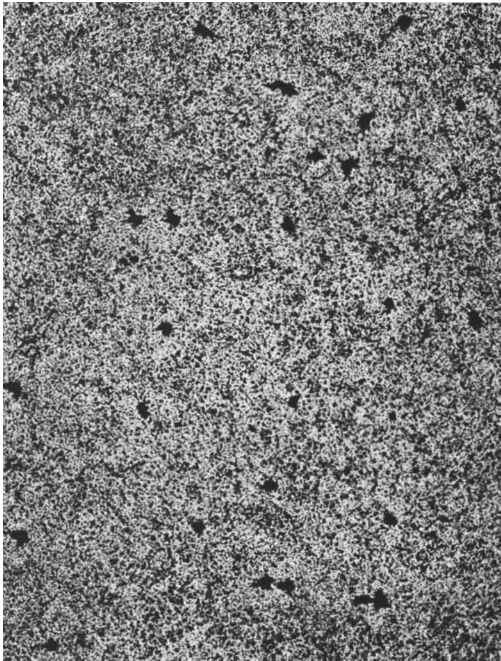

FIG. 1. Morphology of plaques produced by CHV in DK cells. Giemsa stain; × 15.

*Results.* The experimental design consisted of adding 0.5-ml amounts of an artificial mixture of CHV and HSV-MP of approximately equal titers to equal volumes of PBS-A containing increasing amounts of rabbit anti-CHV serum. After 1 hour of incubation at 37°C, the neutralization mixtures were diluted and plated on DK and HE-2 monolayer cultures. The residual titers of CHV and HSV-MP were determined on the basis of the following considerations: HSV-MP does not grow and does not produce plaques on DK cells (9) whereas CHV does not produce plaques on HEp-2 cells (Aurelian, unpublished data). Therefore the titer of CHV surviving neutralization was obtained from the plaque count in DK cells, whereas the titer of HSV-MP surviving neutralization was obtained from the plaque count on HEp-2 cell cultures. The results of one such set of experiments are summarized in Fig. 2. As expected, CHV is neutralized more readily by anti-CHV serum than is the heterologous virus.

In a reciprocal set of experiments, 0.5-ml volumes of PBS-A containing increasing amounts of rabbit anti-HSV-MP serum were added to 0.5-ml amounts of an artificial mixture of CHV and HSV-MP of approximately equal infectivities. The results summarized in Fig. 3 show that CHV is neutralized by anti-HSV-MP serum even though the serum neutralizes the homologous virus to a much greater extent.

As a control, normal rabbit serum was tested in place of immune serum and was found to neutralize neither CHV nor HSV-MP.

*Discussion.* The results described in this

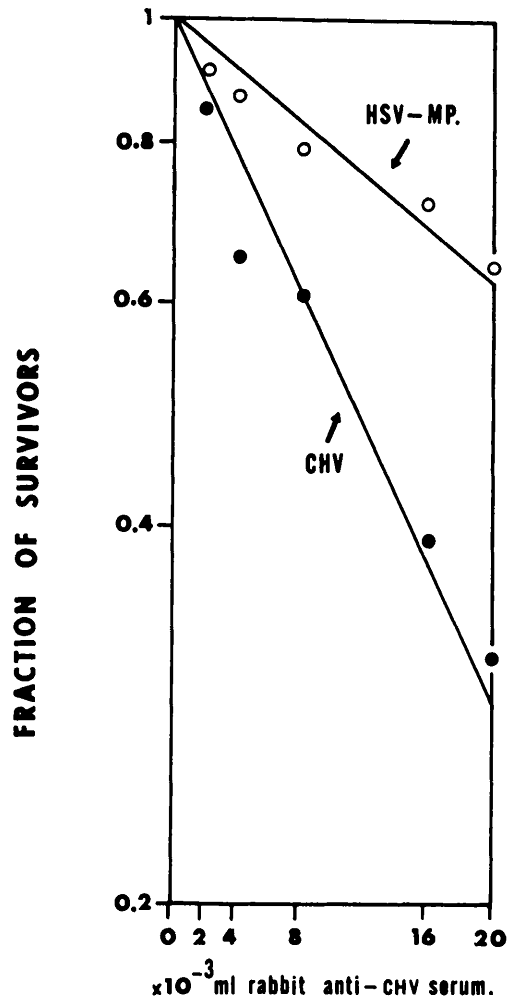


FIG. 2. Multiplicity analysis comparing neutralization of CHV and HSV-MP viruses by rabbit anti-CHV serum. The residual fractions of pfu are plotted as a function of antiserum concentration.

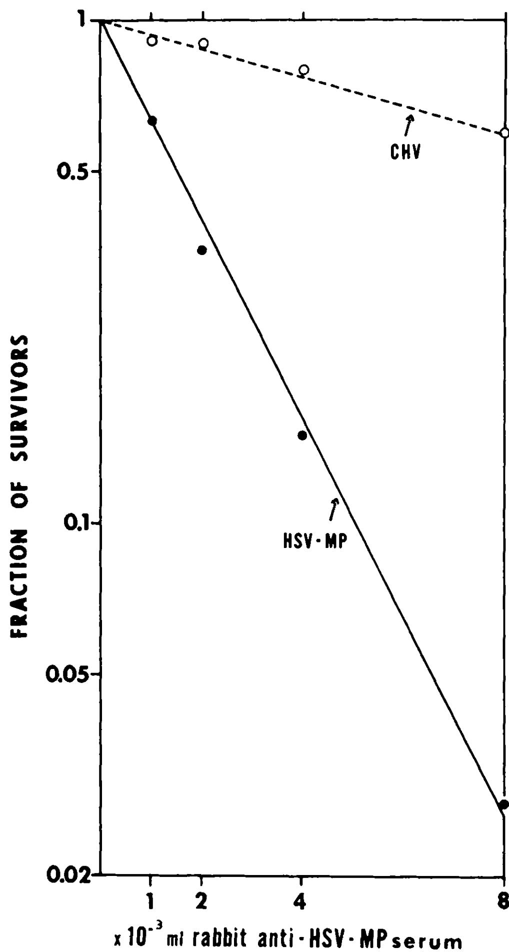


FIG. 3. Multiplicity analysis comparing neutraliza- anti-HSV-MP serum. The residual fractions of pfu are plotted as a function of antiserum con- tion of CHV and HSV-MP viruses by rabbit centration.

communication show that CHV and HSV-MP are immunologically related. Two points should be stressed with regard to these findings.

First, there is considerable discrepancy in the reports from different laboratories as to the immunologic relationship of various members of the herpesvirus group. Thus, Sabin has described the occurrence of *in vivo* cross-neutralization between B virus and herpes simplex as well as pseudorabies virus (10). More recently, on the basis of complement fixation tests it has been reported that herpes simplex is antigenically related to pseudorabies (11). Contrary results, however, were obtained by Kaplan and Vatter (12) using an *in vitro* plaque reduction techhnique, and by Watson *et al.* (13) who also reported the absence of *in vitro* cross-neutralization between herpes simplex and pseudorabies viruses. On the other hand, Watson *et al.* (13) did report the presence of a common precipitin band between herpes simplex and pseudorabies viruses in immunodiffusion tests. On the basis of these data they suggested the possibility that the two viruses share a group antigen that cannot be detected by the *in vitro* neutralization technique. The conclusion to be drawn from these reports is that the discrepancies in the data presented by the various investigators might be due to the variety of methods employed in the study of the antigenic relationship between various members of the herpesvirus group.

Second, it should be noted that the plaque reduction technique used in this study is an extremely sensitive one which has been previously used to differentiate between two closely related strains of herpes simplex virus (6). The cross-reactivity observed between herpes simplex and canine herpes virus appears to be due to similarities in the structure of specific antigenic determinant groups in the two virions.

It is of interest that Stewart *et al.* (2) using an *in vitro* neutralization technique have shown that CHV is antigenically distinct from two other members of the herpesvirus group, namely pseudorabies virus and infectious bovine rhinotracheitis virus both of which have been reported to be immunologically unrelated to herpes simplex virus in cross-neutralization tests (12, 13, 14).

*Summary.* Canine herpes virus and herpes simplex virus have been shown by neutralization tests (multiplicity analyses) to be immunologically related, but not identical. Previous investigators have failed to demonstrate an antigenic relationship between canine herpes virus and two other herpes viruses: pseudorabies virus and infectious bovine rhinotracheitis virus. The results are discussed in terms of the immunologic relationship between various members of the herpesvirus group.

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# *In Vitro* Studies on Mechanism of Action of Thyrotropin Releasing Factor* (32722)

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Recent investigations have been undertaken to discover the mechanisms whereby hypothalamic thyrotropin releasing factor (TRF) stimulates thyrotropin (TSH) secretion from the anterior pituitary. It has been shown that TRF stimulation of TSH release can be blocked competitively by thyroid hormones both *in vivo* and *in vitro* (1–3). Moreover, thyroid hormone inhibition requires intact protein synthesis, whereas TRF stimulated TSH secretion itself is not dependent upon protein synthesis (2,4,5). The recent development of a radioimmunoassay for rat TSH in this laboratory (6) has provided a sensitive and precise technique for extending studies concerning the mode of action of TRF. *In vitro* studies using this method are reported herein, and data are presented which indicate that metabolic energy is required for TRF mediated TSH release.

*Materials and Methods.* Bisected adenohypophyses were rapidly removed from 100-gm male Sprague-Dawley rats under a dissecting microscope and incubated singly in 5-ml Erlenmeyer flasks at 37°C in a metabolic shaker. Each flask contained 1 ml of TC-199 medium with 10% calf serum and was gassed continuously with 95% $O_2$–5% $CO_2$. For incubations with oligomycin and 2,4-dinitrophenol, Krebs–Ringer bicarbonate buffer without glucose was used to preclude substrate phosphorylation from glycolysis.

Hemipituitaries were "preincubated" for a period of 4 hours prior to experimental manipulations to remove the TSH liberated from nonviable cells. The medium was then replaced, and the glands were incubated for two successive 1-hour periods from which media was collected separately. During the first hour, no additions were made in order to determine basal TSH release. During the second hour, one hemipituitary of each pair was incubated in the presence of porcine TRF alone (kindly provided by Drs. A. Cohen and W. White of Abbott Laboratories) at a dose of 2 μg/ml medium. This TRF preparation contains 1 μU TSH/μg. The other hemipituitary was incubated with TRF plus one of the following test materials: L-thyroxine, 1 or 10 μg/ml; oligomycin, 2 μg/ml; 2,4-dinitrophenol, $10^{-4}$ *M*; cyclohexamide, 100 μg/ml, or ouabain, $10^{-4}$ *M*. Four or five hemipituitary pairs were em-

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