

# Biophysical Comparison of Two Canine Adenoviruses

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The two canine adenoviruses, infectious canine hepatitis (ICH) virus and infectious canine laryngotracheitis (ICL) virus (also designated as Toronto A26/61 virus), were studied with respect to their morphology and the biological properties of their soluble components. The two viruses were found to be composed of soluble components similar to, and carrying biological activities corresponding to, those of the human adenoviruses after fractionation by rate zonal centrifugation and anion-exchange chromatography. The following soluble components were identified: hexons (carrying a common group-specific complement-fixing antigen), penton oligomers (complete soluble hemagglutinin), and penton and fiber monomers (incomplete soluble hemagglutinins). The latter were indicated by hemagglutination enhancement with selected antisera directed against human adenovirus soluble components. Elution characteristics of corresponding components of the two viruses in anion-exchange chromatography experiments were distinctly different. Electron microscopic examination of purified virions and soluble components revealed the fiber component of ICL virus to be 35 to 37 nm in length, and that of ICH virus to be 25 to 27 nm long.

Several papers have been published (2-5, 15), in which the authors have given evidence on the degree of immunological relatedness of the two canine adenoviruses, infectious canine hepatitis (ICH) virus, and the isolate referred to as infectious canine laryngotracheitis (ICL) virus (19, 20), originally designated as Toronto A26/61 (2). Results of serological testing with crude virus materials and sera directed against these materials have proved to be inconclusive. Most workers have hesitated to refer to ICL virus as a separate canine adenovirus serotype and, instead, have postulated that it might be a variant of ICH virus (2, 4). Obvious differences in the biological activities of the two viruses—ICH virus being associated with a hepatic disease (12) and ICL virus with upper respiratory tract infections (1, 2)—and the fact that the two virus types do cross-react in neutralization, hemagglutination-inhibition, and complement-fixation tests (2, 15) led this group to believe that the two viruses were probably distinct, though closely related, serotypes.

To investigate the relationship of ICH and ICL viruses, a systematic biophysical and serological study of the two virus types was carried out. The part of the study reported here has shown that with the use of such techniques, ICH and ICL viruses can be readily distinguished from one another. The elution of the corresponding kinds of soluble components of each type from anion-

exchange chromatography has been shown to differ remarkably, and electron microscopy has revealed that ICH and ICL viruses differ morphologically, with the fiber component of ICL virus being considerably longer than that of ICH virus.

## MATERIALS AND METHODS

**Cell cultures and virus.** Strains of ICH virus and ICL virus were originally obtained from W. Ditchfield, Guelph, Ontario, Canada, and from T. Yamamoto, Edmonton, Alberta, Canada. The virus stocks were prepared in monolayer cultures of Madin-Darby canine kidney cells, obtained from Flow Laboratories Ltd, Scotland, and an established dog kidney cell line, originally obtained from R. C. Parker, Toronto, Ontario, Canada. Both cell lines were maintained on Eagle's minimal essential medium, Earle's salts, containing 1 to 3% inactivated calf serum. Three different methods were employed for preparing crude virus material: (i) concentration of degenerated cell monolayers 10 to 20 times against polyethylene glycol (PEG 6000) followed by freeze-thawing three times, (ii) treatment of degenerated cell monolayers with 0.2% sodium deoxycholate, pH 9.0, and (iii) freeze-thawing three to five times at  $-70^{\circ}\text{C}$  and room temperature. Cellular debris was removed from all preparations by low-speed centrifugation.

**Serological techniques.** Hemagglutination tests for detection of soluble hemagglutinin and virions, hemagglutination-inhibition antibody-consumption (HIC) tests for detection of fiber material, and hemagglutination-enhancement (HE) tests for detection of penton monomers and fiber monomers were per-

formed as described by Norrby et al. (7), except that, in all cases, freshly washed human O erythrocytes were used, and incubation was at room temperature for 2 hr. All tests were performed by using microdilution loops and disposable plastic plates.

Complement-fixation tests were performed using a micromodification of the technique described by Svedmyr et al. (14). Group complement-fixation activity, for the detection of hexon material, was determined by using an antiadenovirus type 3 hexon serum.

**Separation techniques.** For CsCl density gradients, virus-soluble components were prepared in preformed nonlinear gradients of CsCl consisting of 5 ml of density 1.40 g/ml, 5 ml of 1.32 g/ml, and 5 ml of 1.20 g/ml, overlaid with 15 ml of crude virus material and centrifuged at  $41,000 \times g$  for 90 min in a Spinco SW25.1 rotor. The region above the resultant virus band was pooled and stored at  $-20^\circ\text{C}$  as viral soluble components. To further purify virions from the nonlinear gradients, 1 ml of collected virions was layered onto 4 ml of a preformed linear gradient of density 1.32 to 1.40 g/ml, and the gradient was centrifuged at  $100,000 \times g$  for 3 hr in a Spinco SW39L rotor.

For further separation of viral soluble components by zonal centrifugation in sucrose linear gradients, 2 ml of material from the top region of nonlinear CsCl gradients was layered on 28 ml of a preformed linear gradient of 5 to 20% sucrose in 0.15 M NaCl. The gradients were centrifuged at  $52,000 \times g$  for 40 hr in a Spinco SW25.1 rotor, and fractions were collected dropwise from the bottom of the tube.

Anion-exchange chromatography on diethylaminoethyl-Sephadex A25 (Pharmacia, Uppsala, Sweden) columns (1.4 by 32 cm), equilibrated with 0.04 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 8.4) and eluted with the required gradient of NaCl in the same buffer, was also used to separate the soluble components.

In trypsin treatment, viral components were incubated with 0.4% (w/v)  $2\times$  crystallized trypsin (Fluka AG, Buchs, Switzerland) for 1 hr in a water bath at  $37^\circ\text{C}$ . The buffer system consisted of 0.05 M Tris-hydrochloride, 0.15 M NaCl, and 0.001 M  $\text{CaCl}_2$  (pH 8.1,  $37^\circ\text{C}$ ). Trypsin activity was stopped by the addition of equal amounts of soy-bean-trypsin inhibitor (National Biochemical Corp., Cleveland, Ohio).

**Electron microscopy.** Material for electron microscopy was dialyzed versus 1% ammonium acetate buffer, followed by negative staining with 2% sodium silicotungstate, pH 6.8, and examination in a Philips EM200 electron microscope. Magnification calibration was performed with a carbon grating with 54,864 lines per inch (Ladd Research Industries Inc., Burlington, Vt.).

## RESULTS

**Separation of soluble components.** Zonal centrifugation on sucrose gradients was used for separation of various soluble components from each other and from intact virions. A distribution of activity as shown in Fig. 1 was obtained. Components carrying soluble complete hemagglutinin,

incomplete hemagglutinin (two populations), and group-specific complement-fixing activities were separated from each other.

Of major interest, is the manner in which the incomplete hemagglutinins were detected. Antisera directed against human adenovirus pentons and fibers were tested for their enhancing effect on selected fractions from 40-hr sucrose gradients, which, by comparison with certain human adenoviruses (17, 18), would contain incomplete hemagglutinin. The presence of a similar antigenic determinant on pentons and fibers, respectively, from each species of adenovirus would be indicated by a positive HE test (6). Among the various antisera tested, it was found that certain sera directed against subgroup III human adenovirus pentons gave satisfactory enhancement and were subsequently used in most tests (detailed results will be reported in a separate communication). The rapidly sedimenting incomplete hemagglutinin was detected with antisera directed against penton material from all three human adenovirus subgroups, whereas the slowly sedimenting incomplete HA was detected only with antisera against fiber material from human adenovirus subgroups II and III. The most rapidly sedimenting peak of HE in Figure 1 is the result of an enhancement of the soluble complete HA activity, the middle peak is due to the presence of rapidly sedimenting incomplete HA, and the highest peak in the gradient is due to slowly sedimenting incomplete hemagglutinin.

To characterize the two incomplete hemagglutinins, which by comparison with results obtained with certain human adenoviruses (17, 18) would be penton monomers and fiber monomers, suspected penton monomer material from sucrose gradients (identified by HE tests) was treated with 0.4% trypsin, which, in the case of human adenoviruses, degrades the penton base and leaves only fiber components (9, 10, 16, 18). Table 1 gives the results obtained. Testing of trypsin-digested rapidly sedimenting incomplete hemagglutinin with sera directed against fibers of human adenovirus subgroup III members, i.e., those human adenoviruses with long fibers, gave positive results, whereas antisera against subgroup I members gave no enhancement. Antisera against human adenovirus pentons, which gave positive HE with the rapidly sedimenting incomplete hemagglutinin before trypsin digestion, failed to do so after the trypsin treatment. Such results would infer that the common antigenic determinant on the penton base has been destroyed by the trypsin digestion.

Anion-exchange chromatography was used as an additional procedure for separating and studying the characteristics of the soluble components.

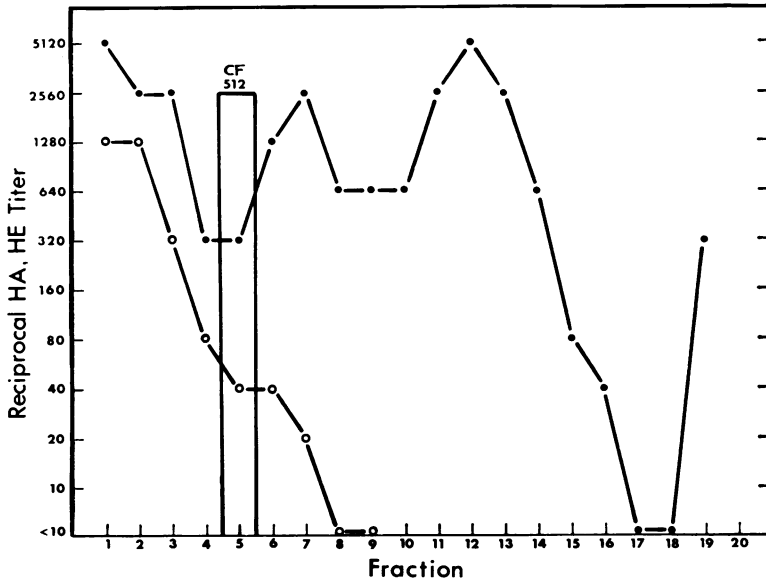


FIG. 1. Distribution of biological activities after centrifugation of ICL virus-soluble component material on a linear 5 to 20% sucrose gradient, at  $52,000 \times g$  for 40 hr. (○) Complete hemagglutination activity, (●) HE activity as measured with human adenovirus penton antiserum. The column represents the position of maximum hexon CF activity within the gradient.

TABLE 1. Trypsin digestion of canine adenovirus rapidly sedimenting incomplete soluble hemagglutinin<sup>a</sup>

Antisera against	Hemagglutination-enhancement titer			
	Before digestion		After digestion	
	ICL	ICH	ICL	ICH
Human adenovirus type 11 penton.....	80	160	8	8
Human adenovirus type 6 fiber.....	160 <sup>b</sup>	40 <sup>b</sup>	512	512

<sup>a</sup> Incomplete soluble hemagglutinin was prepared by zonal centrifugation on sucrose gradients, as described. Control experiments with slowly sedimenting incomplete hemagglutinin gave negative enhancement titers before trypsin digestion with adenovirus type 11 penton antisera and no reduction in enhancement titers after trypsin digestion with adenovirus type 6 fiber antisera.

<sup>b</sup> High HE titers with fiber antisera before trypsin digestion are possibly due to hemagglutinating fiber aggregates (serum mediated), which result from the presence of free fibers in the rapidly sedimenting incomplete hemagglutination preparation.

It was found that a linear NaCl gradient of 0 to 0.25 M for ICH virus soluble components and one of 0.05 to 0.4 M for ICL virus-soluble components gave optimal conditions for separation of the various components. Figure 2 is a composite representation of the elution profiles of the biological activities of the soluble components of each type. It is obvious that there is a distinct difference in the positions of the eluting components, with those of ICH virus eluting before a reference albumin peak and those of ICL virus after the albumin. The relative positions of the eluting material were reproducible and consistent, but recoveries of the fiber material (as indicated by the HIC test) varied from experiment to experiment.

**Ultrastructure of purified virions and soluble components.** Virions purified by  $2 \times$  centrifugation in CsCl were examined by electron microscopy. Figure 3 shows an ICL virus particle and an ICH virus particle with projecting fiber components. Measurement of virion-attached, fully extended fibers gave measurements as follows: ICL virus fiber, 35 to 37 nm, and ICH virus fiber, 25 to 27 nm. The measured lengths and differences in fiber lengths between the two types are consistent, and the length of the ICL virus fiber verifies an earlier report by Yamamoto and Marusyk (21).

Electron microscopy of anion-exchange and sucrose gradient prepared group-specific comple-

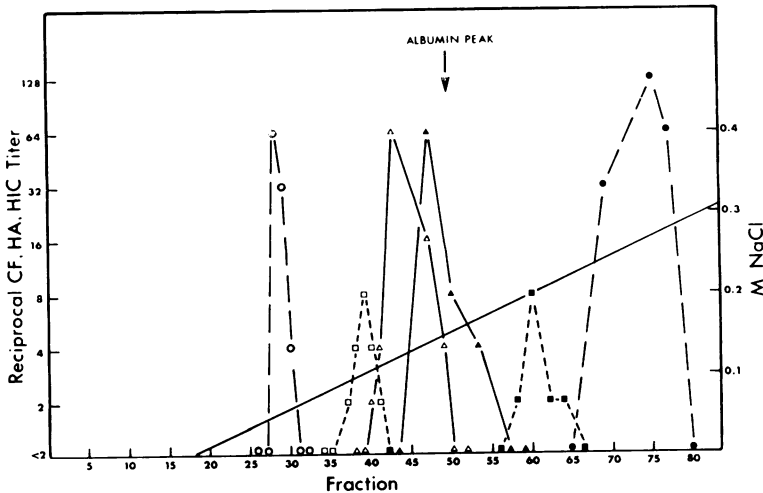


FIG. 2. Composite representation of anion-exchange chromatography elution profiles on diethylaminoethyl-Sephadex A25, 0.04 M Tris-hydrochloride (pH 8.4), of canine adenovirus soluble components. The open symbols represent ICH virus components, and the closed symbols represent ICL virus components. (○, ●) Penton material, indicated by complete hemagglutination; (◻, ◼) fiber material, indicated by the HIC test; (△, ▲) hexon material, indicated by group-specific complement-fixation test.

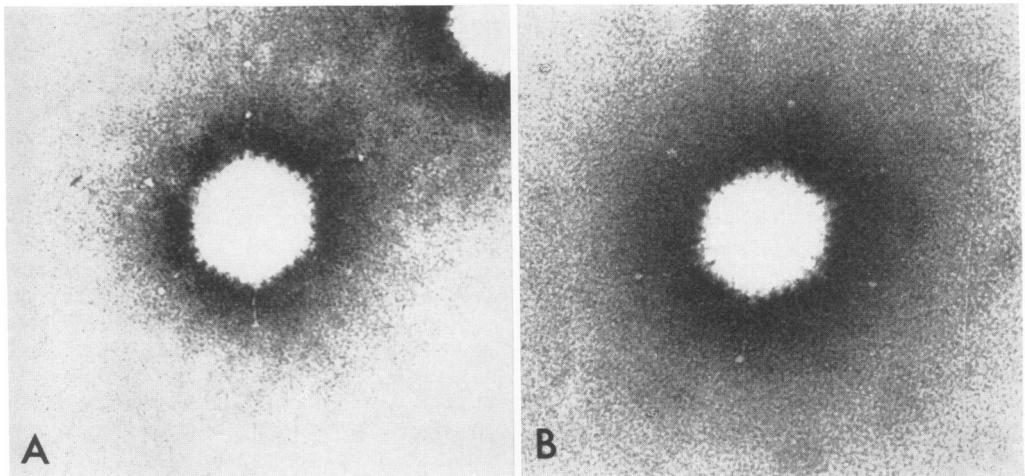


FIG. 3. Electron micrograph of (A) ICH virus and (B) ICL virus, negatively stained with 2% sodium silicotungstate (pH 6.8). Magnification,  $\times 233,000$ .

ment-fixing antigen showed hexons similar to those found in preparations of human adenovirus material (11).

Non-virion-associated fiber components (pentons, penton aggregates, and free fibers) were seen only with great difficulty. Figure 4 illustrates the purity of the preparations obtained. Measurement of the fiber length in such preparations, verified the measurements made on virion-attached fibers.

## DISCUSSION

Even though there was an early establishment of the differences in the pathogenicity of the two canine adenoviruses, ICH virus, which causes a hepatic disease (12), and ICL virus, which has been associated with an upper respiratory infection (1), similarities on the level of serological relatedness have led many authors to state that

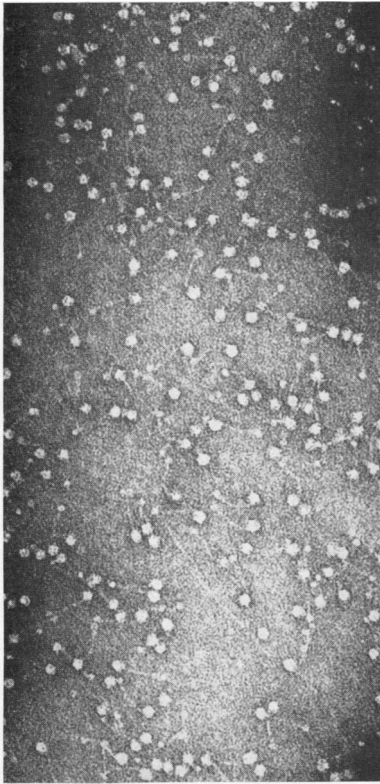


FIG. 4. Electron micrograph of sucrose gradient-prepared ICL virus penton monomers. Negatively stained with 2% sodium silicotungstate (pH 6.8). Magnification,  $\times 139,800$ .

ICH and ICL viruses may be variants of the same serotype (2-4), with ICH virus possibly being the wild type. One proposal is that ICL virus is a vaccine variant of ICH virus (4).

Distinct differences between the two canine adenoviruses are demonstrated in the present study, with regard to their morphology and to the biophysical properties of their soluble components.

Anion-exchange chromatography was used as a method to separate and purify soluble components of ICH and ICL viruses, and it was shown that the relative elution profile of each set of components was markedly different. The difference in position of the complete soluble hemagglutinin and incomplete soluble hemagglutinin (detected by HE or HIC tests) would indicate that the components are differently charged. The results of the anion-exchange separation of ICH virion components differed from those reported by Sugimura and Yanagawa (13). These authors were able to demonstrate only the presence of hexons and a

"soluble" hemagglutinin, the anion-exchange elution profile of which was opposite to that found in this study.

Long-time centrifugation of the soluble components of each virus type on sucrose gradients allowed separation and purification of each of the components. A complete hemagglutinin and two incomplete hemagglutinins were found, as well as the major structural component, the hexon. The incomplete hemagglutinins were detected in HE tests, in which sera against pentons and fibers of human adenoviruses and simian adenoviruses were used. The nature of the HE test (6) requires that the enhancing serum be directed against shared antigenic determinants for a reaction to take place and allow formation of a hemagglutinating entity. The positive results obtained in this study, using human and simian adenovirus penton antisera to detect canine adenovirus pentons and fibers, would indicate that there is a common antigen not only on the hexon component, as was previously shown (16), but also on the vertex capsomere and proximal part of the fiber component. Furthermore, other results from this laboratory (*unpublished data*), by utilizing highly purified components and component-specific antisera from all three mentioned adenovirus groups, have indicated that this is the case. It should be emphasized that demonstration of this antigenic relationship among the adenovirus group can only be carried out when ample amounts of highly purified components and component-specific antisera are available.

The results of the trypsin digestion experiments (Table 1), in which the rapidly sedimenting incomplete soluble hemagglutinin and the slowly sedimenting incomplete soluble hemagglutinin were identified as penton monomer and fiber monomer, respectively, would make the canine adenoviruses similar to certain members of the human adenovirus subgroup III (17, 18), with regards to the occurrence and behavior of the soluble components.

The morphology of the human adenoviruses has been well elucidated (16) and the biological properties of each of the soluble components determined (6). It was possible in this study to demonstrate that the two canine adenoviruses are composed of similar soluble components, hexons, penton, and fibers, and that these components carry biological properties corresponding to those of the human serotypes: that is, hexons which carry a group-specific complement-fixing antigen demonstrable by using hexon antisera from any other adenovirus regardless of species; pentons, which may occur in oligomeric or monomeric form; and fibers, detected only in monomeric

form, both of which may agglutinate human O erythrocytes under certain conditions.

Electron microscopic examination of the canine adenoviruses has shown that the viruses are similar except for the length of the fiber component. The fiber component of ICL virus was found to be 35 to 37 nm long, in agreement with earlier reports (21), whereas that of ICH virus was found to be 25 to 27 nm in length. Such a difference in length could easily constitute a difference in typing the two viruses, if heed is paid to the data presented by Norrby (6, 7), in which specific fiber lengths of the human adenoviruses have been shown to conform to their subgrouping by biological activity.

Further serological and biochemical work is now underway to verify the distinctiveness of each of the virus types and to discover the extent of their immunological relatedness. Antisera prepared against each of the viral components will be used to determine the degree of cross-reaction on a component level and to determine the number of shared antigenic sites. Nucleic acid hybridization studies will also be used to gain further information on the relatedness between ICH and ICL viruses.

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