Summary. (1) Urinary calcium excretion and creatinine clearance were measured in 8 patients before and after hypophysectomy for advanced breast cancer. (2) Mean creatinine clearance for the group was lower than in patients in an earlier stage of metastatic breast cancer. (3) No consistent changes in urinary calcium excretion could be correlated either with polyuria, spontaneous interphase, or vasopressin-induced reduction in urinary volume.

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Secondary Serological Response to Infectious Canine Hepatitis Virus Produced by Adenovirus Type 4.* (27108)

LELAND E. CARMICHAEL AND JAMES A. BAKER
Veterinary Virus Research Institute, New York State Veterinary College at Cornell University, Ithaca, N. Y.

Comparison of infectious canine hepatitis (ICH) virus with human adenoviruses indicates that ICH virus is a member of the adenovirus group(1,2,3). Dogs have been shown to be susceptible to human adenovirus infection, and they develop complement-fixing and neutralizing antibodies following a single inoculation(3,4). These findings actuated further studies on the relationship between ICH virus and human adenoviruses, with the specific aim of determining the clinical and immunological response of dogs to adenovirus type 4 and to ICH virus following adenovirus inoculation. The results of these studies are presented below.

Virus used. Adenovirus type 4 stock was obtained from Lt. R. R. Gutekunst (Naval Medical Research Unit 4, U. S. Naval Training Center, Great Lakes, Ill.) Stock virus was transferred once in HeLa cell cultures maintained in a media composed of medium 199 (Difco), 15% tryptose-phosphate broth, 5% agammaglobulin calf serum and antibiotics. When complete cell degeneration occurred, the cultures were frozen and thawed 6 times and centrifuged at 1500 rpm for 10 minutes to remove cellular debris. The clarified virus suspension was then distributed in sealed vials and stored at −50°C. The virus titer of the pooled suspension was $10^{4.0}$ $TCID_{50}$ per 0.1 ml.

For animal inoculation Cornell strain 1 ICH virus was stored at −50°C as small portions of liver from an infected dog. In the preparation of inoculum, a portion of stored liver was made into a 10% suspension with 0.15 M phosphate-buffered saline (pH 7.4). The virus content of the suspensions ranged from $10^{5.5}$ to $10^{5.8}$ per ml.

For neutralization tests, Cornell 1 strain

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ICH virus that had been transferred 58 serial passages in dog kidney cells was used. Virus was harvested from infected cultures 36 hours after inoculation, clarified by centrifugation, and stored at −50°C in sealed ampules. Stock virus possessed a titer of $10^{6.8}$ per 0.1 ml.

Animal inoculations. Except in one instance, litters of puppies 4 to 8 months of age were obtained from the purebred beagle colony maintained by the Institute. These dogs were reared in strict isolation and were known to be susceptible to ICH virus when the study began. The exceptional litter came from a farm near Ithaca when they were approximately 2 months of age. Tests of sera for complement-fixing and neutralizing antibodies showed no antibodies to ICH virus or adenovirus type 4 at the time the puppies were used.

Each litter was divided into 2 groups. Then each puppy of a litter group was inoculated either intranasally or subcutaneously with 1 ml of adenovirus type 4, prepared as mentioned above. The other litter groups were left uninoculated and either placed in separate isolation units or else placed in contact with those that had been inoculated. In all, 7 dogs were inoculated subcutaneously, 3 intranasally, and of the 9 kept as uninoculated controls, 4 were exposed by contact. After inoculation, temperatures were taken daily, total and differential leukocyte counts were made, and the dogs were observed for other signs of illness.

Following adenovirus inoculation, the groups were inoculated with ICH virus in the following manner: (1) Two weeks after receiving adenovirus subcutaneously, a group of 3 dogs and 3 uninoculated littermates each was given a 1 ml inoculum of ICH virus suspension intravenously; (2) five weeks after subcutaneous adenovirus inoculation, 4 dogs and 4 uninoculated littermates each was given 1 ml of ICH virus suspension intravenously; (3) five weeks after intranasal inoculation, 3 inoculated dogs and 2 uninoculated littermates each received 1 ml of ICH virus inoculum intravenously.

In addition, each of 3 littermate dogs, that had received ICH virus 4 weeks previously, was given 1 ml of adenovirus type 4 suspension intravenously.

Virus isolations. Specimens of blood, feces, and nasal and throat swabs were taken daily for one week from 3 puppies that had been inoculated intranasally and from 4 puppies inoculated subcutaneously with adenovirus. Swabs were placed in sterile vials containing medium 199, 1000 units of penicillin and 2 mg of streptomycin. These were stored at −50°C until tested for virus. After inoculation of ICH virus, blood was collected daily from all dogs for one week. Blood was allowed to clot and serum was collected in sterile vials and stored at −20°C until tested for the presence of virus and antibody.

For adenovirus isolations, 3 tubes of HeLa cells were inoculated, each with 0.2 ml of a test specimen. Dog kidney cell cultures were used for ICH virus isolation and were similarly inoculated. After inoculation, the cell cultures were observed for 7 to 10 days for cytopathic changes. If none were observed, a second transfer of clarified frozen-thawed culture fluid was made into fresh cell cultures and observed for an additional 7 to 10 days.

Serological tests. At the time of initial inoculation with adenovirus, all dogs were bled for serum and then at weekly intervals until they were inoculated with ICH virus. At time of inoculation of ICH virus dogs were bled again and thereafter at daily intervals for one week, then at weekly intervals for varying periods of time.

For virus neutralization tests, serial 4-fold or 5-fold dilutions of heat-inactivated serum were mixed with an equal volume of virus suspension diluted in medium 199 to contain 100 to 300 TCD_{50} per 0.1 ml. After incubation for one hour at 36°C (for ICH virus) or 12 hours at 4°C (for adenovirus), 0.2 ml of a virus-serum mixture was inoculated into each of 4 tubes of cells. Dog kidney cells were used for ICH virus tests and HeLa cell cultures were used for adenovirus neutralization tests. Results were finally read when at least 50% degeneration was evident in the virus control tubes showing 100 TCD_{50}. If there was any evidence of specific degeneration it was considered that the serum did not neu-

tralize. Endpoints were calculated by the Reed-Muench method.

Complement-fixation (CF) tests for ICH virus and adenovirus antibodies have been described in detail(3). Antigens were prepared from infected cell cultures when complete cytopathic changes had occurred, usually 5-6 days after inoculation. ICH virus was grown in dog kidney cells and adenovirus type 4 was grown in HeLa cells. In all tests, serial 2-fold dilutions of heat-inactivated serum in 0.25 ml amounts were incubated for 2 hours at 36°C with 4 units of either ICH virus or adenovirus antigen in 0.25 ml and 2 full units of complement contained in 0.5 ml. Antigen units had been previously determined by titration. Following incubation of the test system, 0.5 ml of the hemolytic system was added. The hemolytic system consisted of a mixture in equal volumes of 2 units of hemolysin and of 2% sheep erythrocytes. The test was read after further incubation for 30 minutes and endpoints were recorded as the highest serum dilution showing at least 50% fixation by visual observation. Appropriate controls of each test component were included.

Results. Clinical response. After inoculation with adenovirus, no signs of illness were observed in any of the inoculated or uninoculated dogs.

Following inoculation with ICH virus all dogs showed increased temperatures, leukopenia, anorexia, and enlarged tonsils. The temperature response of the dogs that had been initially inoculated with adenovirus persisted 5 to 7 days, while the temperatures of the controls generally remained elevated 7 to 8 days. Corneal opacities developed in 5 of the 9 dogs given ICH virus only, but no corneal opacities were observed in any of the dogs that initially received adenovirus.

Virus isolation. Adenovirus was isolated from the throat and nasal swabs of only 2 inoculated animals. This occurred one day after inoculation intranasally. Repeated attempts to isolate virus from blood or feces failed.

After inoculation with ICH virus, all dogs showed virus in the blood on the day after inoculation. Dogs inoculated with only ICH virus showed viremia lasting from 4 to 6 days. In contrast, dogs that had been given adenovirus and then ICH virus 5 weeks later showed viremia only on the first day following inoculation.

Serological findings. Adenovirus complement-fixing antibody was detected in the serum of dogs inoculated subcutaneously 14 days after inoculation, while dogs that were inoculated intranasally showed antibody on the 21st day but not on the 14th day. Maximum antibody titers were attained by the 28th day and thereafter declined (Fig. 1,2). Sera from dogs inoculated with adenovirus also fixed complement with ICH viral antigen, but antibody titers were lower when this test antigen was employed. Neutralizing antibody for adenovirus was first detected 14 days after inoculation.

Following inoculation of ICH virus, all dogs that had been given adenovirus developed complement-fixing antibodies for ICH virus antigen and also for adenovirus antigen. A marked secondary-type rise in adenovirus antibody occurred in the adenovirus inoculated groups when ICH virus was given (Fig. 1, 2). Moreover, adenovirus CF titers persisted at levels of 1:16 to 1:32 in these dogs for at least 11 months. In a like manner, complement-fixing antibodies for ICH virus antigen developed in an accelerated manner when compared to control dogs given ICH virus only. Dogs that had been inoculated only with ICH virus did not develop antibody for the adenovirus antigen, with the exception of one contact animal that had been placed with the group given virus intranasally at time of inoculation.

Following ICH virus inoculation, there was an apparent rise in adenovirus-neutralizing antibodies in those dogs that had received a prior inoculation of adenovirus. The response was not marked, however, and antibody titers tended to decline to the level reached before ICH virus inoculation. In one litter of dogs retained for prolonged observation adenovirus-neutralizing antibody was detectable at a titer of $\geq$ 1:4 340 days after inoculation.

All dogs inoculated with adenovirus and then inoculated 4 or 5 weeks later with ICH

virus showed ICH virus-neutralizing antibody in the blood 2 days after inoculation.

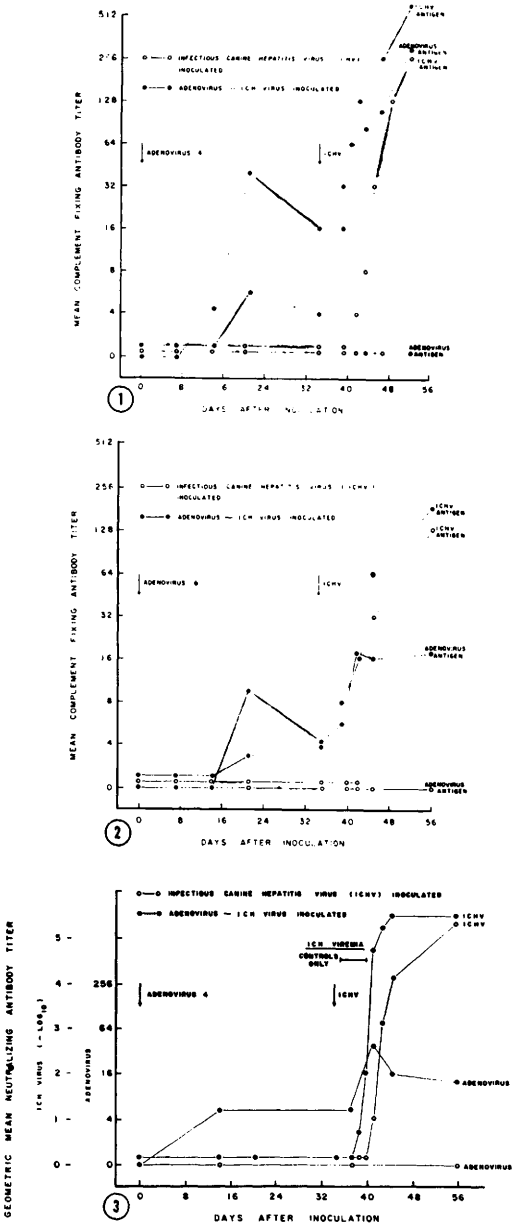

FIG. 1. Complement fixing antibody response of dogs inoculated with adenovirus type 4 subcutaneously followed by infectious canine hepatitis virus intravenously.

FIG. 2. Complement fixing antibody response of dogs inoculated with adenovirus type 4 intranasally followed by infectious canine hepatitis virus intravenously.

FIG. 3. Neutralizing antibody response of dogs inoculated with adenovirus type 4 followed by infectious canine hepatitis virus intravenously.

TABLE I. Antibody Response to Adenovirus Type 4 Shown by ICH-Immune Dogs.

Dog	Test	Test antigen	Days after adeno. inoculation 0	8	32
C1	Complement fixation	ICHV	16	16	16
C2			32	32	32
C3			16	16	16
C1		Adeno.	<4	16	8
C2			<4	4	4
C3			<4	16	4
C1	Virus neutralization	ICHV	32,000	50,000	50,000
C2			2,500	3,200	3,200
C3			10,000	10,000	10,000
C1		Adeno.	<4	<4	8
C2			<4	<4	16
C3			<4	<4	8

This is in contrast to the response of those dogs given ICH virus only, for neutralizing antibody then appeared 4-6 days later (Fig. 3). Time of antibody appearance was generally correlated with duration of viremia; however, in some instances, low antibody levels (1:2 to 1:8) were present in sera yielding ICH virus.

The serological response was variable in the group given ICH virus 14 days after adenovirus, for only one of the 3 dogs that received adenovirus showed accelerated ICH virus complement-fixing and neutralizing antibody responses and failed to develop ICH viremia. Other dogs in the group responded to the ICH virus inoculation in the same manner as the controls. With the exception noted, adenovirus antibody responses did not appear to be of the secondary type. Such antibody persisted however for at least 3 months following the ICH virus inoculation.

There was no change in the ICH virus complement-fixing or neutralizing antibody levels of ICH-immune dogs subsequently inoculated with adenovirus (Table I). Adenovirus complement-fixing antibodies developed in these dogs, but they did not appear to persist. Adenovirus neutralizing antibodies also developed and were present at the last test one month later.

Discussion. Dogs given adenovirus type 4 developed complement-fixing and neutralizing antibodies against the adenovirus and complement-fixing antibodies, but not neu-

tralizing antibodies, against ICH virus. Dogs given ICH virus developed complement-fixing and neutralizing antibodies only against the homologous virus. When dogs were given adenovirus and later ICH virus, a secondary response of complement-fixing antibodies developed to adenovirus and to ICH virus antigens. Also, production of neutralizing antibody to ICH virus was accelerated. When dogs were given adenovirus after ICH virus, however, usual serological responses were observed.

It appears that inoculation of dogs with adenovirus type 4 acts like a single inoculation of an homologous inactivated virus vaccine by creating a secondary response state. Heterotypic responses have also been noted within the human adenovirus group following vaccination(5,6) and complement-fixing antibody responses to poliovirus have been observed in individuals following ECHO 9 infection(7). Similar findings have recently been observed between virus diarrhea virus of cattle and hog cholera virus and, in this instance, the resistant state created by virus diarrhea virus protected pigs against lethal doses of hog cholera virus(8). Also, cross protection among certain of the group A arthropod-borne viruses has been found(9,10,11, 12) and other groups of viruses may have this relationship.

The relationship between ICH virus and adenovirus type 4 probably has epidemiological implications. For example, since adenovirus complement-fixing antibodies have been found in street dogs(3,4) this might partially account for the variable clinical and serological responses seen when dogs become infected with ICH.

Summary. Dogs inoculated with adenovirus type 4 developed complement-fixing and neutralizing antibodies following an asymptomatic course of infection. When such dogs were later inoculated with ICH virus, a secondary-type complement-fixing antibody response occurred to both viral antigens, and antibodies persisted for at least 340 days. Accelerated production of neutralizing antibody for ICH virus also occurred in these dogs. The duration of ICH viremia correlated with the development of neutralizing antibody. This secondary response to ICH virus created by heterotypic adenovirus may partially account for varied clinical manifestations observed in dogs with infectious canine hepatitis.

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