lymphomas(10).

Summary. Electrophoretic analyses of sera from 92 ferrets revealed 38 to have *gamma* globulin values above 20%, 27 above 30%, and 12 above 40%. Sixteen of the ferrets with hypergammaglobulinemia were necropsied and found to have systemic plasmacellular infiltrates with lymph node and frequent thymic hypertrophy. Seven of the ferrets had a monoclonal type of hypergammaglobulinemia.

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The Virus Watch Program. IV. Recovery and Comparison of Two Serological Varieties of Adenovirus Type 5.* (31530)

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From January 1961 through March 1965, during the Virus Watch program(1,2,3), a continuing surveillance of viral infections in metropolitan New York families, adenovirus type 5 was found in 66 respiratory and fecal specimens. Isolates from 32 specimens resembled previously described type 5 viruses (4,5,6) in that they exhibited at most a minor hemagglutination-inhibition (HI) cross-reactivity with rabbit antiserum against adenovirus type 1. The 34 remaining specimens, however, yielded type 5 isolates which showed considerably increased HI cross-reactivity with the same type 1 antiserum. The observation of two such readily distinguishable varieties of adenovirus type 5 prompted this comparative study.

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Materials and methods. Specimen collection and processing. Routine surveillance of each Virus Watch family consisted of a regularly scheduled bi-weekly collection of a family illness history and simultaneous collection of respiratory and fecal specimens from one or more family members designated as index persons. In addition, "special" respiratory and fecal specimens were obtained from as many family members as possible when suspected viral illness occurred in any family members. Respiratory samples generally were inoculated into tube cultures of primary rhesus monkey kidney (MK) and HEP-2 cells within a few hours of collection, while fecal specimens were extracted overnight as previously described(2) and usually were inoculated into both cell cultures the day after collection. For serologic studies, serum samples usually were obtained from all family members on admission to the program and roughly twice a year thereafter.

Typing antiserum. Stock antiserum to adenovirus 1 was produced in four 8½ to 10 lb female rabbits of non-pure basically New Zealand white breed which were bled and

then immunized with the second HEp-2 tissue culture passage of strain Adenoid 71 from the American Type Culture Collection (ATCC). Virus was grown on HEp-2 cell monolayers in stationary 32 oz prescription bottles at 37°C. Cell growth medium was Eagle's basal medium in Earle's salt solution (EBME) with 5% heat-inactivated chicken serum plus 0.11% $NaHCO_3$; maintenance medium for virus growth was the same except for 0.22% $NaHCO_3$. Infected bottles were harvested on day 7 when cellular destruction was complete. After one cycle of freezing and thawing, the fluids were clarified by low speed centrifugation and stored frozen at —20°C for 15 months before rabbit immunization was begun. Each rabbit was given 1.0 ml of antigen intravenously on day 1, followed by 0.5 ml on days 7, 12 and 28. Exsanguination was performed on day 35.

Stock antiserum to type 5 was produced in rabbits similar to those above by a single intravenous injection of the second HEp-2 passage of virus strain Adenoid 75 from the ATCC. Exsanguination was performed on day 21. All rabbit antisera were provided by Mr. David A. Stevens of this Department and were stored at —20°C.

Hemagglutination-inhibition. HI tests were performed by standard Microtiter methods with 4 HA units of virus(6,7). Both human and rabbit antisera routinely were absorbed with kaolin and rat erythrocytes. All human sera and most rabbit sera also were absorbed with monkey erythrocytes. Our stock type 1 antiserum had a mean HI titer of 1:1280 against type 1 adenoviruses and 1:10 to 1:20 against various stocks of type 5 virus including strains derived from ATCC Adenoid 75. No other HI cross-reactivity of our type 1 antiserum was detected on extensive testing with 25 of 31 human adenoviruses (types 12, 18, 20, 25, 28 and 31 lacked sufficient hemagglutinin for testing). By tube neutralization test (Stevens, unpublished), this type 1 antiserum had a homologous titer of 1:640 and was free of cross-reacting activity with 30 human adenoviruses except for a titer of 1:10 to 1:20 with ATCC adenoviruses types 2 and 5, and 1:10 with ATCC adenovirus type 14.

Neutralization kinetics. Plaque-purifier stocks of virus were obtained from plates of HeLa cell monolayers by methods which have been described(8). A capillary tube was used to pick well-isolated plaques, and the agar plug with adherent plaque was discharged into 2 ml of pH 7.4 phosphate buffered saline. Following 3 cycles of freezing and thawing, this material was plaqued again. Virus from the fourth plaque-purification was added to HeLa cells suspended in Eagle's Minimal Essential Medium supplemented with 5% calf serum (Agamma, Hyland Laboratories) and 10 ml $NaHCO_3$ (7.5%) per liter. After 48 hours at 37°C on a roller drum the fluids were frozen and thawed 3 times and centrifuged to remove cellular debris. The supernatant then was treated briefly with difluorodichloroethane in a homogenizer.

Each antiserum was prepared by injecting 2 rabbits intravenously with 1.0 ml of a plaque-derived stock of virus, followed by 5.0 ml intraperitoneally on days 7 and 14. The animals were bled from the marginal ear vein on day 20 and the sera pooled. Neutralization kinetics were determined by the procedure previously described(9).

Results. Virus differentiation. The two varieties of adenovirus type 5 isolates were very reproducibly differentiated by absorbed stock adenovirus type 1 antiserum into normal reactors (HI reactivities of $<$ 1:20, 1:20, or, rarely, a weak $\pm$ reaction at 1:40) and cross-reactors (strong HI reactivities of 1:40 through 1:320). Furthermore, when both varieties of virus were tested in parallel, HI titers with stock type 1 antiserum were approximately 4- to 8-fold higher with the cross-reacting than the normal reacting isolates. Adenovirus type 5 antiserum gave indistinguishable HI titers with both type 5 varieties, while pre-immunization serum from rabbits which were used to prepare stock type 1 antiserum did not react with either type 5 variety.

In repeated tests, conducted over a period of 3 years and with several different absorption batches of the same type 1 stock antiserum, all type 5 isolates from the same person and all such isolates from members of the same family fell into a single type 1 reacting category. This category was maintained regardless of the number of MK and/or HEp-2 tissue culture passages the virus iso-

lates had undergone, or whether the viruses were isolated from throat or fecal samples, and regardless of how early or late in an infection the viruses had been obtained (Tables I and II). Intermittent excretion continuing for more than 100 days was observed in 4 individuals, including one subject infected with a cross-reacting strain. The longest detected excretion was 313 days.

Although special parallel observations have not been made to detect minor differences in cytopathic effects produced by these 2 virus varieties in tissue culture, viruses of both reacting types were readily recognized as

TABLE I. Normal Reacting Type 5 Adenoviruses Isolated from Paired Respiratory and Fecal Specimens.

Family	Person	Age (yr)	Sex	Date of initial isolation	Subsequent days virus excretion detected	Positive isolations from subject: By cell type MK	By cell type HEp-2	By excretion route Resp.	By excretion route Fecal	Illness −7 through +14 days of initial isolation
ST 5	3	8	♂	9/ 5/61	—	—	1	—	1	None
ST 43	4	4	♀	11/21/61	1,115,127,204, 272,313	3	5	—	7	"
	5*	1	♀	1/ 2/62	—	—	1	—	1	"
ST 1	2*	1	♀	12/ 8/61	—	—	1	1	—	Resp.
SH 14	5	2	♂	2/ 2/62	—	—	1	—	1	Resp., enteric
ST 84	4†	5	♀	6/25/62	196	1	1	1	1	Enteric, other
	5	4	♂	12/23/62	—	—	1	—	1	Resp.
ST 20	3	6	♀	1/23/63	—	—	1	—	1	Resp.
ST 114	3	3	♀	12/13/63	—	—	1	—	1	Resp.
PS 40	CT*	7	♂	12/18/63	—	—	1	1	not taken	Resp.
ST 136	3	2	♀	7/ 1/64	15,127	—	3	—	3	Resp.
	4	1	♂	7/ 1/64	—	—	1	—	1	Resp.
ST 117	3	1	♀	7/ 7/64	9	1	2	—	2	Other
ST 137	3	1	♂	7/29/64	—	1	1	—	1	"
ST 66	3	4	♂	2/ 2/65	1,29	—	3	3	—	Resp.
	4‡	1	♀	2/ 2/65	13,16,16	—	4	2§	2	Resp.
	5‡	1	♀	2/15/65	—	—	1	—	1	Resp.
Totals	17					6	29	8	24	

* Not an index person. † Became an index person 56 days after initial excretion. ‡ Twins.
§ Two throat swabs taken and found positive on day 16.

TABLE II. Cross-Reacting Type 5 Adenoviruses Isolated from Paired Respiratory and Fecal Specimens.

Family	Person	Age (yr)	Sex	Date of initial isolation	Subsequent days virus excretion detected	Positive isolations from subject: By cell type MK	By cell type HEp-2	By excretion route Resp.	By excretion route Fecal	Illness −7 through +14 days of initial isolation
ST 111	4	½	♂	3/10/64	9,15,15,56,84	—	6	2	4	Resp.
	3	2	♀	3/10/64	9,14,29	—	4	2	2	Other
	2	27	♀	3/19/64	6,6,20,175	—	5	3	2	Resp.
ST 128	2	24	♀	3/18/64	—	—	1	—	1	None
	3	1	♂	3/18/64	—	—	1	—	1	"
ST 127	3	3	♀	4/19/64	3,24,51	1	4	2	2	Resp.
	4	1	♂	4/22/64	6,6,9,9,19,56,57	2	8	3	5	Resp., other
ST 139	2	31	♀	5/27/64	—	—	1	1	—	Resp., other
	4	3	♂	5/27/64	—	—	2	1	1	Other
	5	1	♀	7/ 6/64	—	—	1	—	1	None
ST 148	4	¼	♀	8/ 2/64*	—	—	1	—	1	"
Totals	11					3	34	14	20	

* Subject not sampled prior to this date.

adenoviruses on initial isolation and passage. In accordance with usual experience, heteroploid cells, in this case HEp-2 cells, were a much more sensitive indicator of both varieties of type 5 adenovirus than were MK cells; if anything, the differences in the respective cell-sensitivities were accentuated when cross-reactors were isolated. All type 5 viruses isolated in the Virus Watch produced hemagglutinin, and HI typing of some viruses of both varieties has been confirmed by neutralization tests. Both varieties readily produced adenovirus group complement-fixing antigen.

In agreement with our observations on the adenoviruses as a group(2), fecal samples yielded type 5 adenoviruses more commonly than did respiratory samples. Even though respiratory symptoms were present in approximately 70% of both groups of excretors who were ill, and only 7% of all the detected excretors presented with definite enteric symptoms, 75% of normal and 58% of the cross-reacting type 5 isolates were detected in fecal samples.

Epidemiology. The data in Tables I and II reveal additional points of possible epidemiologic interest. Although the differences lack statistical significance, illness association was slightly greater (14/17 or 82%) with infections with normal reacting strains than with those due to variant strains (7/11 or 64%) whereas the order was reversed with respect to intra-familial spread (11/15 index persons or 73% excreted variant virus as compared with 13/22 or 59% who shed normal virus). That the variant virus may possess an enhanced potential for spread is suggested more strongly by evidence from the community. Whereas cross-reacting virus produced a single distinct outbreak of excretion by 11 index persons in 5 different families (more than 10% of all families then under study) and mostly initiated in a 3 month period March-May 1964, excretion of normal virus was initiated sporadically in only 3 or 4 persons per year.

The different capacities for spread may be related to differences in virus excretion patterns. Index persons infected with the variant strain averaged 3.0 positive specimens per person as compared to 2.1 for normal virus, and, probably of greater importance, a higher proportion of variant isolates were from respiratory specimens (14/34 or 41% *vs* 8/32 or 25%). The proportion of persons with at least one detected excretion *via* the respiratory tract was 7/11 (64%) for cross-reactors compared to 5/17 (29%) for normal reactors.

Serologic response. The available relevant sera from all members of families yielding type 5 isolates were tested by HI for antibody to adenovirus 1 through 7. Although no type 1 adenovirus was isolated from persons within the 5 families excreting cross-reacting viruses, some rise in antibody to type 1 could have been expected as the result of heterotypic responses(10). Interestingly, no rise in type 1 titer was detected in any of the 8 excretors of variant virus who could be examined serologically. Four of these excretors had type 5 HI seroconversions from $< 1{:}10$ to $\geqq 1{:}20$, and one young child had no pre-excretion serum, but had a titer of 1:160 three months following initial excretion. Three excretors did not show any type 5 HI response post-infection, though one of these (the only one tested) converted from $< 1{:}4$ to 1:16 by neutralization test.

The serologic data also permitted a more complete definition of virus spread within households by revealing instances of infection not detected by virus isolation. Individuals were considered to have been infected if they yielded a type 5 isolate and/or had a significant ($< 1{:}10$ to $\geqq 1{:}10$, or $\geqq$ 4-fold) type 5 HI response. Persons with no positive evidence of infection were considered to have been uninfected, except that non-index persons having neither serologic tests nor a type 5 isolation were excluded from tabulation. By these criteria, within families in which at least one excretion was detected by virus isolation, normal-reacting virus infected 20 of 36 (56%) of the family members, and cross-reacting virus infected 12 of 18 (67%) of the family members.

Isolate HI with other rabbit antisera. When cross-reacting type 5 isolates first were encountered in our HI typings, their reactivity was suggestive of a variant type 5 or, less likely, of a variant type 1 adenovirus. Identification as a type 5 virus was easily confirmed by tests with absorbed types 1 and 5 reference antiserum(4) produced for the National In-

TABLE III. Neutralization of Viruses by Stock Anti-1 and Anti-5 Sera.

Virus	Serum*	% Surviving PFU after 8 min
ATCC Adeno 1	Anti-1	5.6
	Anti-5	100.0
ATCC Adeno 5	Anti-1	100.0
	Anti-5	39.2
34592†	Anti-1	48.1
	Anti-5	42.8
33018†	Anti-1	29.2
	Anti-5	23.9

* Serum dilution 1:100.
† Cross-reacting isolate.

stitutes of Health. These tests also showed that not all type 1 antisera have the property of differentiating our type 5 variants. NIH reagent type 1 antiserum had a homologous titer of 1:640, but with both cross- and normal-reacting type 5 isolates, gave HI titers of about 1:40 to 1:80. A type 1 rabbit antiserum produced at Tulane University has also been tested. After absorption, it had a homologous titer of 1:320-640, but gave no cross-reactivity at 1:20 or above with either type 5 variety.

Neutralization kinetics. To determine if the varieties of adenovirus 5 differed in their surface antigens, neutralization kinetics were measured using stock anti-1 and anti-5 sera and plaque-purified viruses. The reaction mixtures contained serum in a final dilution of 1:100 and approximately 5×10^6 PFU/ml of virus. Slow virus inactivation and a lack of linear components characterized the curves obtained. Though the inactivations could have been accelerated by the use of more concentrated sera, this would have entailed some danger of non-specific inactivation. Curvilinear responses precluded the calculation of meaningful rate constants. However, comparison of the survival of PFU after 8 minutes in the presence of antiserum (Table III), when neutralization had levelled off, shows clearly that each cross-reacting isolate was equally susceptible to anti-1 and anti-5 serum, though they differed in their respective affinities for the sera. At the dilution tested, there was no cross-reaction between ATCC viruses and heterologous antisera. Neutralization kinetics were not measured with NIH reference

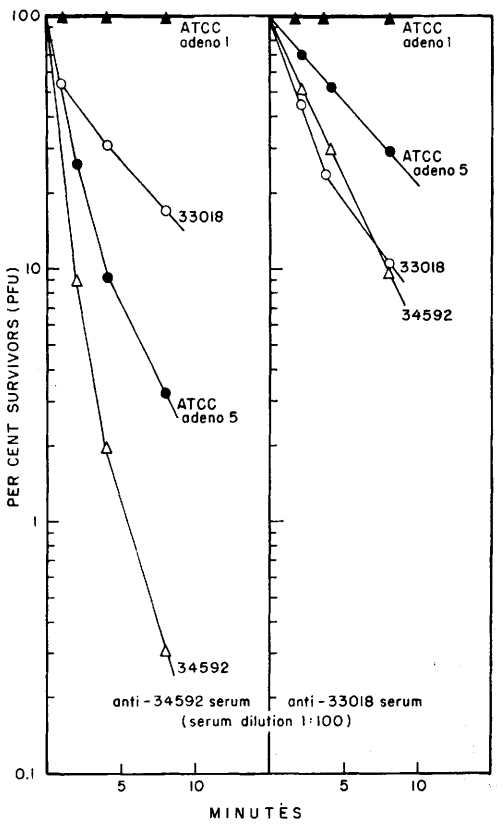

FIG. 1. Neutralization kinetics of virus strains by rabbit antisera prepared against cross-reacting type 5 isolates. In a 37°C waterbath, approximately 5×10^7 PFU/ml were diluted 1:10 into buffered saline containing antiserum at dilution 1:100. At the indicated times, samples were removed, rapidly diluted into chilled buffered saline, and subsequently plated on HeLa cell monolayers. The values shown represent the average of two independent neutralizations, each point plated in duplicate.

antisera, as these reagents were in too short supply.

When cross-reacting isolates and stock viruses were tested with sera prepared in rabbits immunized with plaque-purified cross-reactors, the resulting inactivation curves (Fig. 1) reveal that both antisera neutralized the homologous, the heterologous, and the stock strain of adenovirus 5; in addition, ATCC adenovirus 1 was not inactivated by either serum. The inactivation curves indicate that anti-34592 serum apparently can distinguish between the heterologous isolate and stock adenovirus type 5, while, within the limits of experimental error, anti-33018 serum

cannot. Since similar comparative neutralizations have not been made with sera which had been massively absorbed with heterologous virus, and since anti-33018 serum generally was of lower activity than anti-34592, it does not seem useful to speculate at this time regarding a likely explanation for the observation.

Discussion. Regardless of passage level in HEp-2 or MK cells, all type 5 viruses from the same specimen, the same person, and the same family were of one reacting variety with a particular stock type 1 antiserum. From January 1961 until March 1964, positive specimens yielded type 5 isolates only of the normal variety. During March through June 1964, 31 specimens yielded the cross-reacting variety, and no normal isolates were recovered. In July of 1964, the normal virus reappeared as cross-reacting virus was dying out, and in 1965 only the normal type was detected.

Antisera produced in rabbits injected with cross-reacting virus did not show neutralizing antibody to adenovirus type 1, though plaque-purified cross-reacting strains were equally sensitive to neutralization by stock type 1 and type 5 antiserum. In addition, no type 1 virus was isolated from any persons within families excreting cross-reacting virus. Furthermore, cross-reacting type 5 viruses did not induce demonstrable type 1 antibody in those excretors who could be examined serologically. These observations and the fact that variant type 5 virus was isolated 37 different times clearly shows that the cross-reacting variants do not represent dual infections with type 1 and type 5 viruses.

Only one of 3 type 1 antisera was capable of delineating type 5 viruses on the basis of differential cross-reactivity in an HI test. That this did not result from an immunizing event prior to rabbit inoculation is indicated by the absence of preimmunization antibody against either type 5 variety.

Four persons excreted the same variety of adenovirus 5 intermittently for more than 100 days, and one person excreted for at least 313 days. Prolonged excretion of the same reacting variety of virus argues against appreciable genetic instability, at least relative to this factor, and also argues against the selection of antigenically altered "survivors" following the development of type-specific circulating antibody. Excretion of an adenovirus for more than 300 days is not an isolated event. During the Virus Watch program, 18 additional individuals excreted for at least this period of time while experiencing infections with other adenovirus types(2).

Summary. During continuing virologic surveillance of family groups observed excretion of type 5 adenovirus comprised two antigenically distinct varieties, one which reacted normally in HI and neutralization tests and a variant which, with a particular stock type 1 antiserum, cross-reacted extensively in tests of both types. The variant strains were isolated during a distinct outbreak and appeared to spread more readily within families and the community than did normal reacting strains, possibly because of more abundant (especially respiratory) shedding. All type 5 isolates from members of the same family, regardless of interval after first isolation, were of the same variety. In 4 individual instances, including one variant infection, excretion persisted intermittently for more than 100 days (313 maximum). The prolonged excretion of but one variety argues for persisting infection rather than reinfection as the source of the intermittent excretion and also suggests relative genetic stability of the variant.

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