A Hemagglutination Test for Detection of Adenovirus Antibodies. (23062)

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Adenoviruses have received considerable clinical and laboratory attention since their initial isolations by Hilleman(1) and Rowe *et al.*(2). The number of viruses in this group has grown to 14 as reported by Huebner *et al.*(3) and certain types have been associated with acute respiratory disease (ARD) in the civilian(4,5,6) and military (1,6,7,8) populations. The importance of adenovirus types 3, 4, and 7 in the military populations has been emphasized by development of adenovirus vaccines to reduce ARD in recruits(9,10). To justify the use of vaccines or to investigate the epidemiology of these agents in the military or civilian population the prevalence of adenovirus infection must be determined. This may be done by isolation of the agent, or by serological evidence relating to presence of this virus group since ARD may be influenced by a variety of organisms and conditions. Virus isolation and serological methods, employing neutralization and complement-fixation tests, have been used to determine incidence of adenovirus infection in military and civilian populations. These methods have been described in detail by Hilleman(7) and Rowe *et al.* (11). Many laboratories that lack isolation and tissue culture facilities must rely entirely on the complement-fixation test as a means of determining presence of adenovirus infection in a population. This test is limited because it is not type specific and previous infections with any one type many result in acute antibody titer level which may obscure a more recent infection with a new type.

To provide a simple laboratory *in vitro* test for diagnosis of adenovirus infection a modified hemagglutination test has been developed to detect antibody to adenoviruses. Although type specificity is not achieved, this test appears to be superior to the CF test as the titer rises are extremely high and elevated acute antibody levels are not found generally to interfere with the diagnostic interpretation.

Materials and methods. Hemagglutination (HA) test. Fresh sheep red blood cells (SRBC) suspended in Alsever's solution were washed 3 times with phosphate buffered saline at pH 7.2, and resuspended as a 4% packed cell concentration. Sensitization of the SRBC with dilute tannic acid was similar to the method of Boyden(12), as modified by Witebsky and Rose(13). An equal volume of tannic acid diluted 1:20,000 in buffered saline was added to the 4% suspension of SRBC. The mixture was held at room temperature (22-25°C) for 30 minutes. The cells were washed 3 times in buffered saline by centrifugation at 1500 rpm for 10 to 15 minutes and resuspended as 2% cell concentration by volume. Tannic acid sensitized SRBC were subject to slight hemolysis upon standing and were discarded 2 or 3 days after sensitization. An equal volume of adenovirus antigen diluted in buffered saline was added to the tannic acid sensitized SRBC and kept at room temperature for 30 minutes. The antigen treated cells then were washed in 1:200 normal rabbit serum by centrifugation at 1500 rpm for 15 minutes and resuspended in 1:200 normal rabbit serum at a 1% cell concentration. Acute and convalescent human sera were obtained from U.S. Naval recruits stationed at Great Lakes Naval Training Center. Acute and 21 day convalescent blood specimens were drawn from both afebrile, and febrile recruits who were hospitalized with upper respiratory illness. Zero and 21 day sera were drawn from recruits who had no recent history of acute upper respiratory infection and served as controls. All sera were inactivated at 56°C for 30 minutes before use. The dilution range was from 1:10 through 1:81,960. Occasionally, hemolysis of sensitized cells was observed in the 1:10 and 1:20 dilutions of sera. All dilutions were made in 0.2 ml volume of buffered saline with 0.2 ml

serological pipettes in 13 x 75 mm Kahn tubes rinsed in distilled water. In the final step 0.2 ml of adenovirus antigen treated SRBC was added to the diluted sera giving a final volume of 0.4 ml per tube. The tubes were kept at room temperature unless otherwise noted and read after 2 hours. *Antigen.* HeLa cells were grown in 1 l bottles containing 35 ml of Eagle's basal medium(14) with 10% human serum. After 3 to 5 days growth the cells were washed and the nutrient fluid replaced with Eagle's medium containing 3% horse serum. Each bottle was inoculated with 1 to 3 ml of stock adenovirus. The virus was harvested after all HeLa cells had fallen from the surface of the bottle, *i.e.* in 3 to 4 days incubation. The fluid was frozen and thawed 5 times and centrifuged at 2,000 rpm for 10 minutes. The supernatant which served as antigen was decanted and stored at –70°C. Serum titration endpoints were recorded as the reciprocal of highest serum dilution which produced a sedimentation pattern at 1+ hemagglutination according to the method of grading described by Stavitsky(15). Controls were carried out using the lowest serum dilution with normal SRBC and tannic acid treated SRBC without antigen. In addition, tannic acid treated cells with antigen were also employed as controls in each test. Unless otherwise noted, all hemagglutination tests were performed with adenovirus type 4 antigen. *Complement-fixation (CF) test.* The CF test was performed by making serial 2-fold dilutions of 0.2 ml aliquots of acute and convalescent sera. To these dilutions, 0.2 ml containing 2 units of adenovirus type 4 antigen was added followed by 0.2 ml containing two 75% hemolytic units of complement. The mixture was incubated at 37°C for 1 hour. Then 0.4 ml of sensitized SRBC was added, and the tubes were further incubated at 37°C for 30 minutes. The sensitized SRBC mixture consisted of equal volumes of 2% SRBC and 2 full units of amboceptor. Following this incubation all tubes were centrifuged at 1000 rpm for 1 minute and read for complement-fixation. Titration endpoints were determined as the reciprocal of highest serum dilution producing a 3+ fixation. All dilutions were carried out in physiological saline containing 0.01% $MgSO_4$ and 0.003% $CaCl_2$. The neutralization test was carried out in accordance with the method described by Rowe *et al.*(11) using procedure 2.

TABLE I. HA Antibody Titers with 2 Lots of Type 4 Antigens at Various Dilutions.

Antigen	Reference sera		Antigen dilution 1/4	1/8	1/16	1/32
Lot I	A	a*	640	160	40	<10
		c	40860	10280	2560	"
II		a	320	320	160	"
		c	81920	5120	1280	"
I	B	a	<10	<10	<10	"
		c	10240	2560	1280	"
II		a	<10	<10	<10	"
		c	10240	2560	1280	"

* a = acute serum; c = convalescent serum.

Results. To employ the proper dilution of antigen for the test, 2 adenovirus type 4 antigen lots were diluted and incubated with tannic acid treated cells for sensitization. After sensitization, the cells were incubated at room temperature with dilutions of acute and convalescent sera from 2 Naval recruits who had ARD and from whom type 4 adenoviruses were isolated. Table I shows the relation between antigen dilution and serum titer. The data indicate that higher antigen concentrations will result in higher titers in both the acute and convalescent sera of Sera A. It has also been observed that undiluted antigen produces high acute antibody levels which obscure convalescent titer rises in persons having adenovirus infections. By reducing the antigen concentration, it is possible to determine the antigen dilution which will detect acute antibodies in a reference serum (A in Table I) and which will show at least a 4 tube titer rise with the convalescent serum specimen. The antigen was similarly standardized in another dilution test with another reference serum (B, Table I) without acute antibodies. Using these 2 reference sera it is possible to determine the highest dilution of antigen which will react with 2 such reference sera in the above manner. This dilution is defined as one unit. In Table I the 1/16 antigen dilution is by definition one unit of antigen. In all subsequent tests

TABLE II. HA Antibody Titers to Heterologous and Homologous Adenovirus. HA antigens of recruits infected with adenovirus Type 4.

Patients		Antigen 1	2	3	4	5	7
XX	a*	80	40	<40	<40	40	80
	c	1280	80	2560	5120	>320	2560
PP	a	<40	320	<40	<40	<40	<40
	c	320	640	160	5120	"	80
QQ	a	<40	80	80	<40	160	<40
	c	"	"	640	1280	320	160
GG	a	"	320	<40	<40	<40	<40
	c	5120	2560	"	2560	"	"
JJ	a	160	<40	80	<40	80	320
	c	1280	"	640	640	40	1280
KK	a	160	320	80	<40	320	<40
	c	320	1280	40	2560	"	40
LL	a	80	320	<40	<40	80	<40
	c	320	1280	"	160	320	40
MM	a	<40	<40	160	<40	<40	<40
	c	40	"	320	1280	"	80
RR	a	<40	"	640	80	"	320
	c	"	"	1280	2560	"	"

* a = acute serum; c = convalescent serum.

one unit of antigen was used routinely. Pending further investigations on the stability of the HA antigen, it may be desirable to use 2 units of antigen in carrying out the test.

Specificity of the HA reaction was determined by titrating the sera of Naval recruits from whom adenovirus type 4 had been isolated with antigens of the other types. One unit of heterologous antigen was determined by antigen dilution and sera titration with sera A and B as reference standards and using this antigen dilution to determine heterologous antibody response. The data in Table II show homologous (Type 4) and heterologous reactions with acute and convalescent sera. It demonstrates that the HA test is non-specific since titer rises occurred to other antigens. Most of the cross reactions (4-fold rises) occurred with the type 7 antigen (67%), while types 1 (56%), 3 (45%) and 2 (33%) also showed crossing in a decreasing order. Type 5 demonstrated the least amount of crossing (22%) with sera of recruits who had been infected with type 4. These heterotypic crossings are somewhat in variance with the information obtained by Grayston *et al.* (16) which showed less crossing in the neutralization test with types 1, 5, and 7 than with types 2 and 3 in recruits infected with type 4 virus.

Stability of the antigen was determined by heating the type 4 (Lot II) preparation for 30 minutes at 56°C. The heated and unheated antigen was diluted to one unit, adsorbed to tannic acid treated cells and incubated at room temperature with serum dilutions (acute and convalescent) of recruits. Adenovirus type 4 was isolated from some of these hospitalized recruits. Heating the antigen to 56°C for 30 minutes does not appear to reduce antigenic activity of the preparation. The data in Table III show that heating the antigen increases some antibody titer endpoints, and, in addition, provides a means of inactivating live virus in the preparation (11).

Studies on effect of incubation temperature on hemagglutination reaction were carried out by incubating the sensitized cells and serum dilutions at 8°C, 25°C, and 35°C. The test was read after a 2 hour incubation. The results presented in Table IV show that the higher temperatures increase the serum titers. However, titer increases from 25°C to 35°C are not significant and 25°C was used routinely as a matter of convenience. Readings

TABLE III. HA Titers with One Unit of Type 4 Unheated Antigen and Heated Antigen.

Patient		Hospitalized	Virus isolation	Serum titer Unheated antigen	Heated antigen
C	a*	Yes	None	40	160
	c			81920	81920
D	a	"	Type 4	20	40
	c			5120	5120
E	a	"	"	80	20
	c			1280	2560
F	a	"	"	<10	<10
	c			160	1280
G	a	"	"	80	40
	c			2560	5120
H	a	"	"	20	<10
	c			40960	20480
I	a	Not†	None	40	80
	c			"	40
J	a	"	"	"	"
	c			20	80
K	a	"	"	40	320
	c			"	"

* a = acute serum; c = convalescent serum.
† Men symptomatic and not hospitalized.

TABLE IV. HA Antibody Titers at Various Incubation Temperatures with Sera from Recruits with and without Adenovirus Isolations.

Patient		Type 4 virus isolate	Incubation temp. 8°C	25°C	35°C
1	a*	Yes	40	40	40
	c		640	1280	1280
2	a	"	40	40	40
	c		320	640	1280
3	a	"	80	80	40
	c		640	5120	10240
4	a	No	20	40	80
	c		40	"	40
5	a	"	10	20	"
	c		20	"	80

* a = acute serum; c = convalescent serum.

were also made at 5 hour and 18 hour intervals at room temperature and antibody titers were not significantly altered from the initial 2 hour observation.

Titrations were carried out for neutralization, CF and HA test with 13 pairs of acute and convalescent sera to compare titer levels and rises in both febrile (ARD) and afebrile Naval recruits. Neutralization and CF tests were performed with type 4 virus antigens since this agent was the only type isolated at the time these sera were collected. Results of the test comparisons are presented in Table V. The data suggest that the HA antibody is distinct from the CF or neutralizing antibody.

In asymptomatic cases (R, S and T) there was no CF or neutralizing antibody rise, nor was there an HA titer rise. Where a virus has been isolated, there was a neutralizing, HA and CF antibody rise in every instance. However, in cases where there is a neutralizing antibody rise without a CF rise, an HA antibody titer rise is demonstrated. This is shown with the sera of patients Y and Z. There was no corresponding rise in CF antibodies presumably due to presence of antibodies in the acute sera resulting from previous adenovirus infections. The HA test did demonstrate an antibody rise thus confirming the neutralizing antibody rise. If the CF test had been used, exclusively, these men would have been regarded as serologically negative. Conversely, patients U, V, W and X who had elevated acute CF antibodies showed no change in CF titer, and it would not have been possible to determine whether a present adenovirus infection had occurred. Since the HA test showed no titer rise, it may be presumed that these patients were not infected. This is confirmed by the neutralization tests.

It was of interest to compare time of appearance of CF antibodies and HA antibodies in recruits from whom adenoviruses type 4 and 7 had been isolated. Ten nasal washing specimens were obtained from these individuals at various times during their recruit training period. These samples were taken for adenovirus isolation in HeLa cells when the men became febrile (100°F or greater). The data in Table VI show the relation between virus isolation, CF and HA serology. There is a similarity in time of appearance of CF antibody and HA antibody which indicates that HA antibody may be produced simultaneously with CF antibody although the 2 antibodies appear to be quite different.

TABLE V. Neutralization, CF and HA Antibody Titers of Febrile and Afebrile Recruits.

Patient		Fever†	Virus isolation	Neutralization	CF	HA
N	a*	F	Type 4	<4	<4	<10
	c			64	128	320
O	a	F	"	4	<4	<10
	c			64	16	640
P	a	F	"	<4	<4	<10
	c			32	64	2560
Q	a	F	"	<4	16	<10
	c			64	256	640
R	a	AF	Negative	16	<4	2560
	c			"	"	1280
S	a	AF	"	<4	"	<10
	c			"	"	10
T	a	AF	"	"	"	<10
	c			"	"	"
U	a	AF	"	"	8	"
	c			"	16	"
V	a	AF	"	"	"	"
	c			"	"	"
W	a	AF	"	"	"	"
	c			4	"	"
X	a	AF	"	4	"	640
	c			4	"	"
Y	a	AF	"	<4	"	"
	c			8	"	2560
Z	a	AF	"	4	64	40
	c			16	128	640

* a = acute serum; c = convalescent serum.
† F = Temp. 100°F or greater; AF = Temp. less than 100°F.

TABLE VI. Virus Isolation, CF and HA Antibody Titers by Date in Naval Recruits.

Patient	Date (serum)	Date virus isolation	Titer CF	Titer HA
AA	1- 6		<4	<10
	1-17	1-16 negative	"	"
	2- 2	1-30 Type 4	32	1280
	2-18		128	5120
	3-10		256	2560
BB	1- 6		8	10
	1-27	1-18 Type 7	4	5
	3-10		16	80
CC	1- 6	1- 7 negative	<4	"
	1-27		"	"
	2- 5	2- 7 Type 7	4	160
	2-18		<4	"
	3-10		128	5120
DD	1- 6	1-24 Type 7	16	320
	1-27		"	2560
	2-18		64	10240
	3-10		"	"
EE	1- 6		"	640
	1-27	1-27 Type 4	32	"
	2-18		64	20240
	3-10		128	"
FF	1- 6	1- 5 negative	8	160
	1-27	1-18 "	4	"
	2-18	2- 3 Type 7	256	40960
	3-10		"	20480

Discussion and summary. The information presented shows that the HA test can be utilized for determining adenovirus antibody response. The test may be carried out with heated adenovirus antigen and yields results which relate to a current adenovirus infection. Hemagglutination antibody titrations can be performed at room temperature and readings may be made up to 18 hours after the initial 2 hour incubation without change in titration endpoints. Although the HA test does not achieve type specificity, this test appears to be more specific than the CF test. Huebner *et al.*(17) have reported 74%-90% heterotypic crossing in the CF test. The HA test reported here deals only with types 4 and 7 adenovirus infections and by extending these studies to include other adenovirus type infections it may be possible to utilize this test more specifically to determine infections to other types.

Based on preliminary data the HA test appears to be superior to the CF test in regard to determining infections in persons having CF antibodies in their acute serum due to previous adenovirus infections. The HA test and CF test show close correlation with respect to antibody titer rises and time of antibody appearance so that the HA test appears to be comparable to the CF test in its diagnostic value. Studies are in progress comparing HA, neutralization, and CF antibody titer responses in Naval recruits who have received the adenovirus vaccine.

Application of this HA technic to the immunology of other viruses should be considered. The ease of performing this test may provide a simple laboratory tool for determining serological responses to virus infections.

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Progesterone-Like Activity of a Series of 19-Nortestosterones. (23063)

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Hertz *et al.*(1) reported that 17α-ethynyl-19-nortestosterone is an orally effective progestational agent in rabbits, having an activity about 5 times that of 17α-ethynyltestosterone. Greenblatt(2) found the norcompound was also effective in women, when administrated orally. Ferin(3) has recently reported that 17-methyl-19-nortestosterone has progestational properties in women, and Overbeek(4) found this compound to be active in rabbits. Pincus *et al.*(5) extended the series, observing that 17-ethyl-19-nortestosterone also has progestational effects in rabbits.

In this laboratory, extensive studies on the biological properties of 19-nortestosterones carried out over the past several years, indicated a marked progestational activity of these compounds (Drill and Saunders).† The present paper deals with the progestational effects of a series of 19-nortestosterones in which the 17α side chain ranges from H to C_8H_{17}, with both saturated and unsaturated linkages.

Methods. White rabbits weighing about 2 kg were ovariectomized. After a 2 week recovery period, they were injected subcutaneously, daily for 6 days, with 5 μg estrone. On the day following the last injection the abdomen was opened and a 1-inch segment of each uterine horn was isolated after the method of McGinty *et al.*(6). The test substance, in 0.1 ml corn oil, was injected into one uterine horn while a like amount of corn oil was injected into the opposite horn as a control. Three days later, the animals were sacrificed, cross-sections of the uteri were fixed in Zenker's and prepared for histology. The degree of glandular proliferation was graded from +1 to +4. Additional studies were made, using intact, immature rabbits which had been primed with 6 daily injections of 5 μg estradiol each. They were then treated orally or subcutaneously with the test compound in oil, daily for 5 days. On the day after the last injection the animals were sacrificed and the uteri were studied histologically.

Results. Intrauterine assay. The average responses to the various doses are shown in Figs. 1-3. The ordinates are arbitrary scales on which 1 denotes that the endometrial glands are only small straight indentations, similar to those usually found in the estrogen-primed controls. Further development with slight branching of a few glands is designated 2, while 3 indicates that there are many branched glands. Grade 4 is reserved for those uteri which show so much branching that, in cross-section, many portions appear detached. The number of animals at each dose is also indicated. The average rating for 545 control, estrogen-primed uteri was 1.04. Only 2 of these control uteri had ratings of 3 and 4 respectively. In both of these cases the contralateral horn had been injected with a high dose of an active compound. A systemic effect, or leakage, may account for these false positives.

The activity of progesterone is shown in Fig. 1. It will be seen that a dose of 0.5 μg is adequate to produce a definite response, with an average value of 2.3. The data for

† Drill, V. A., and Saunders, F. J., *Proc. of a Conference on Anabolic Agents.* G. D. Searle & Co., Chicago, Apr. 9, 1956.