Immunization of Volunteers with Soluble Antigens of Adenovirus Type 1. (29531)

JULIUS A. KASEL, MARGRET HUBER, FRANK LODA,
PETER A. BANKS AND VERNON KNIGHT
Department of HEW, USPHS, National Institutes of Health, National Institute of Allergy and Infectious Diseases, Laboratory of Clinical Investigations, Bethesda, Md.

The importance of adenoviruses as causal agents of disease in military and civilian populations has been well established(1,2). Although administration of inactivated adenovirus vaccines to military recruits has been followed by a reduced incidence of illness, there has been variation in antigenic potency of different lots and failure to obtain consistently the desired high protection ratios(3). Moreover, acceptable vaccines are not yet available for infants and children.

The production of neutralizing antibody in rabbits by immunization with purified and concentrated preparations of adenovirus soluble antigens was reported recently. This was demonstrated with both the type-specific and group-specific soluble antigens from adenovirus type 5(4). The observation was confirmed in this laboratory by the use of type-specific antigen derived from adenovirus type 1 and type 15 suspensions(5).

In view of the inadequacy of inactivated whole virus vaccines, studies were undertaken to determine the effectiveness of these soluble antigens as immunizing agents in humans. The study consisted of: 1) demonstration that the soluble antigens from adenovirus type 1 stimulated production of neutralizing antibody in man and 2) observations on a protective effect of these immunizations against live virus challenge with adenovirus type 1.

Materials and methods. Volunteer selection and clinical procedures. The study was performed at the Clinical Center, National Institutes of Health, with volunteers from federal correctional institutions. They were selected on the basis of the absence of measurable neutralizing antibody ($<1:4$) to adenovirus type 1 and evidence from a careful medical examination that no contra-indications to inoculation existed.

Urine analysis, complete blood count, chemical tests of liver and kidney function, and bacterial cultures of nose, throat and rectum were done prior to inoculation and repeated at weekly intervals or more frequently when indicated.

Volunteers were housed 3 per room and except for exercise periods were kept there during the course of the experiments. Oral temperature, pulse and respiratory rates were determined 4 times daily by the nursing staff. Daily examinations were performed by a team of 2 physicians and a report of findings was recorded before leaving each room. The examining physicians were unaware of the inoculation schedule of the volunteers during the period of immunization and infectious virus challenge.

Antigen preparations and immunization schedule. Type-specific and group-specific soluble antigen preparations were prepared from fourth passage adenovirus type 1 throat specimen* in primary human embryonic kidney cultures (HEK). These cultures† were maintained with Eagle's basal medium(6) containing 250 units of penicillin and 250 μg of streptomycin per ml. Antigens were obtained by fractionation of virus suspensions by chromatography with DEAE-cellulose as previously described(7,8). Several fractionation runs were required in order to obtain sufficient volume of each antigen. Only the fraction which contained the bulk of each antigen from each run was used. Eluates which contained the type-specific antigen (0.0 M NaCl) *i.e.,* buffer solution, were pooled after a single cycle of chromatography. Eluates which contained the group-specific antigen (0.25 M NaCl) were rechromato-

* Received through the courtesy of Dr. Andrew Vargosko, Children's Hospital, Washington, D. C.

† Pathologic material was obtained through the Courtesy of Dr. W. Jaffurs, Columbia Hospital for Women, Washington, D.C.

graphed under identical conditions before pooling. Prior to use, each antigen preparation was subjected to the following procedures: the fluid was centrifuged twice in a Spinco model L ultracentrifuge at 105,400 *g* for 90 minutes, the supernatant fluid was then heated in sealed and submerged glass ampules at 57°C for 30 minutes, and filtered through a millipore filter with a 450-millimicron pore diameter. Safety tests(9) with samples taken before and after heating indicated the absence of adventitious agents and infectious type 1 adenovirus. In addition, the HEK cultures were kept 75 days beyond the required observation period and were negative for infectious virus. Protein determinations were done by a previously described method(10); the type-specific and group-specific antigen preparations contained .185 and .174 mg of protein per ml, respectively. These materials had complement-fixing titers of 1:128 and 1:64 when tested by a previously described procedure(11).

Six volunteers, on days 1 and 8, were injected with 1 ml of type-specific antigen intramuscularly. In addition, on day 1, 0.1 ml was given intradermally. Three volunteers were given the group-specific antigen as described above except that the second dose was given approximately 3 weeks after the initial dose.

Inoculum. Viral inoculum consisted of nonfractionated adenovirus type 1 described above.

Virus isolation. Virus isolation studies were done with HEK cultures using specimens as described in the text.

Neutralization test. Neutralization tests were done with rhesus kidney cultures according to "procedure 2" as described by Rowe *et al*(11).

Results and discussion. No local reactions occurred following intramuscular inoculation of either antigen. Intradermal inoculations in the 3 men given group-specific antigen and in 4 of the 6 men who received type-specific antigen were followed in 48 hours by mild local reactions with small areas of erythema and induration. Eight days after the first injection of type-specific antigen, 2 men exhibited rises in serum transaminase values, serum glutamic oxalacetic transaminase to 152 and 275 (normal <50), serum glutamic pyruvic transaminase to 312 and 375 (normal <50). Alkaline phosphatase and thymol turbidity test values showed slight transient elevations at this time as well. There were no further increases in these values after the second injection, and they returned to essentially normal values in 3 weeks. The significance of these elevations could not be evaluated in these experiments. A survey of the occurrence of abnormal liver chemistries in approximately 200 volunteers during residence at the Clinical Center revealed that an appreciable frequency of abnormal liver function tests occurred both in men who had been experimentally infected with a number of different viruses and in non-infected volunteers(12).

TABLE I. Distribution of Neutralizing Antibody Titers in Antibody-Negative Volunteers Following Immunization with Adenovirus Type 1 Soluble Antigen Preparations.

Immunizing antigen	No. of volunteers	No. of men with indicated level of neutralizing antibody* (25 day sera)					
		<2	4	8	16	32	64
Type-specific	6			2	2		2
Group-specific	3	1†	1	1			

* Expressed as reciprocal of dilution.
† Partial neutralization of dilution of 1:2.

The distribution of neutralizing antibody titers with sera obtained from volunteers approximately 25 days after the initial administration of antigen is shown in Table I. Significant levels of neutralizing antibody developed in all men vaccinated with type-specific antigen and in 2 men with group-specific antigen. These post-vaccine antibody titers were comparable to those elicited in adults following natural and experimental infection with type 1 and other adenovirus serotypes (13-15). It is of interest that, following a single injection of type-specific antigen, 4 men developed antibody as early as the seventh day; moreover, after 2 doses of antigen, all were antibody-positive by the fourteenth day. Furthermore, 2 of the volunteers given group-specific antigen developed neutralizing

antibody by the seventh day. In a separate experiment, 12 of 15 volunteers similarly vaccinated with highly purified adenovirus 1 type-specific antigen developed significant levels of neutralizing antibody(16).

Virus isolation studies done with HEK cultures using throat and rectal specimens obtained prior to immunization and twice weekly for 3 weeks thereafter, were consistently negative. These cultures were observed for 28 days.

Twenty-six days after the initial intramuscular dose, 4 volunteers who had been vaccinated with type-specific antigen, 2 volunteers who had received group-specific antigen, and 3 volunteers without demonstrable neutralizing antibody were challenged with adenovirus type 1. Volunteers were inoculated by stroking the lower conjunctival sac with a cotton applicator moistened in a 1:10 dilution of a virus suspension having an infectivity titer of $10^{8.5}$ $TCID_{50}$/ml. The other eye was similarly swabbed with Eagle's basal medium.

In order to compare severity of illness a grading system was devised. The severity of conjunctivitis was measured through a range of 1 to 3 points, with periorbital edema, pain and/or preauricular adenopathy representing the maximum response. Extraocular involvement was given a maximum of 4 points, one point for each of the following: pharyngitis and cervical adenopathy; fever; cough; and systemic complaints of myalgias, malaise and anorexia. Thus, illness response could vary from 1 to 7 points on any one day. The sum of responses during a period of illness was designated the illness index.

The responses of volunteers following ocular challenge with adenovirus type 1 are shown in Table II. Conjunctivitis, when it occurred, was always limited to the inoculated eye. Significant illness occurred in 2 of 3 control volunteers and in 1 of 2 volunteers vaccinated with group-specific antigen. Control volunteer W.M. developed pharyngoconjunctival fever with temperatures of 37.5°C or more for 4 days, 6 days of moderately severe conjunctivitis, 5 days of pharyngitis and anterior cervical adenopathy, and several days of cough. Control volunteer L.S. had palpebral erythema for 9 days, with bulbar conjunctival injection for 5 days and asymptomatic pharyngeal injection for 3 days. Control volunteer A.E., despite marked virus shedding, failed to develop illness.

Volunteer G.H., who was vaccinated with group-specific antigen, experienced a biphasic illness: the first, a mild conjunctivitis which began on day 4 and lasted through day 10; the second, a recurrence of conjunctivitis in the inoculated eye on day 12 which was accompanied by pharyngitis, cervical adenitis, systemic complaints and low-grade fever. This second phase lasted for 11 days. This man showed only transient virus infection in the eye and no shedding from the throat or rectum despite his long illness. There was no increase in type 1 antibody from the low initial level. These findings give reason to doubt that the illness represented a direct manifestation of infection, although no other infectious agents were isolated and no other explanations are available to explain the occurrence. Volunteer J.P., who also was given this antigen, developed a mild conjunctivitis of 3 days duration.

In contrast, the volunteers who had been vaccinated with type-specific antigen developed only mild and transient conjunctivitis.

Infection of antibody-negative volunteers resulted in marked virus shedding from inoculated eye, oropharynx and rectum. In contrast, virus excretion from all vaccinated volunteers was limited to the inoculated eye, except for 4 positive rectal cultures from one man given type-specific antigen and 5 positive throat cultures from one man given group-specific antigen. Shedding from throat and rectum of the 6 men given one of the soluble antigens was significantly less than that from the 3 non-immune controls ($p = .024$, $u = 0$, Wilcoxon test). Shedding from inoculated eyes was slightly less in the immunized men but the difference was not significant ($p > .10$, permutation test). It should be noted that in previous experiments with other adenovirus serotypes it was not unusual to recover virus from inoculated eyes of antibody-positive volunteers challenged with low dilutions of virus(14).

Following ocular challenge with type 1 adenovirus, all of the antibody-negative volunteers developed significant titers of neutralizing antibody. One of the 2 volunteers

TABLE II. Comparison of Indices of Infection in Non-vaccinated and Vaccinated Volunteers Following Ocular Challenge with Adenovirus Type 1.

Volunteer	Immunizing antigen	Neutralizing antibody titer	Illness severity*	Virus isolation† Eye 1	Eye 2	Eye 3	Eye 4	Eye Total	Throat 1	Throat 2	Throat 3	Throat 4	Throat Total	Rectum 1	Rectum 2	Rectum 3	Rectum 4	Rectum Total
W.M.	None	<2‡	33	2/4	1/2	0/1	ND§	3/7	1/7	7/7	2/2	0/5	10/21	0/7	3/7	1/2	0/5	4/21
		128																
L.S.	"	<2	17	1/3	1/4	0/3	ND	2/10	1/7	3/7	5/7	ND	9/21	0/7	5/7	2/7	ND	7/21
		16																
A.E.	"	<2	0	2/3	1/4	0/2	ND	3/9	0/7	6/7	3/4	0/6	9/24	0/7	4/7	3/4	0/6	7/24
		16																
J.H.	Type-specific	64	4	1/3	1/4	0/2	ND	2/9	0/7	0/7	0/4	0/6	0/24	0/7	1/7	3/4	0/6	4/24
		64																
H.S.	" "	64	3	1/3	1/4	0/2	ND	2/9	0/7	0/7	0/4	0/6	0/24	0/7	0/7	0/4	0/6	0/24
		64																
D.F.	" "	16	3	1/3	1/4	0/2	ND	2/9	0/7	0/7	0/4	0/6	0/24	0/7	0/7	0/4	0/6	0/24
		16																
G.D.	" "	16	5	0/3	1/4	0/2	ND	1/9	0/7	0/7	0/4	0/6	0/24	0/7	0/7	0/4	0/6	0/24
		16																
G.H.	Group-specific	4	42	1/3	1/4	0/4	0/3	2/14	0/7	0/7	0/7	0/7	0/28	0/7	0/7	0/7	0/7	0/28
		4																
J.P.	" "	8	3	1/3	1/4	0/2	ND	2/9	0/7	1/7	4/4	0/6	5/24	0/7	0/7	0/7	0/7	0/28
		64																

* See text.
† No. of positive specimens/no. of specimens tested during 4-week post-challenge period.
‡ Expressed as reciprocal of dilution; first line indicates pre-challenge titer; second line indicates post-challenge titer (day 21).
§ ND = not done.

previously vaccinated with group-specific antigen also demonstrated a rise in antibody titer. In contrast, no further increases in antibody titer occurred in any of the volunteers who had been vaccinated with type-specific antigen. It is noteworthy that the vaccine-induced neutralizing antibody titers were comparable to those which developed in the antibody-negative volunteers following challenge with infectious adenovirus type 1.

Summary. Vaccination with non-infectious, type-specific and group-specific soluble antigens of adenovirus type 1 induced neutralizing antibody in adult volunteers. As compared with non-immunized controls, ocular challenge of volunteers immunized with both preparations was followed by a significant reduction in throat and rectal virus shedding. There was almost complete suppression of illness in volunteers receiving type-specific antigen. It is difficult to interpret the illness pattern in the man who was given group-specific antigen. These findings suggest that soluble antigens of adenovirus type 1 may provide effective immunization in this disease.

1. Huebner, R. J., Rowe, W. P., Chanock, R. M., *Ann. Rev. Microbiol.*, 1958, v12, 49.

2. Jordan, W. S., *Arch. Intern. Med.*, 1958, v101, 54.

3. Meiklejohn, G., *Am. Rev. Resp. Dis.* (Suppl)., 1963, v88, 372.

4. Wilcox, W. C., Ginsberg, H. S., PROC. SOC. EXP. BIOL. AND MED., 1963, v114, 37.

5. Kasel, J. A., Huber, M., unpublished data.

6. Eagle, H., *Science*, 1955, v122, 501.

7. Wilcox, W. C., Ginsberg, H. S., *Proc. Nat. Acad. Sci.*, 1961, v47, 512.

8. Kasel, J. A., Huber, M., PROC. SOC. EXP. BIOL. AND MED., 1964, v116, 16.

9. Couch, R. B., Chanock, R. M., Cate, T. R., Lang, D. J., Knight, V., Huebner, R. J., *Am. Rev. Resp. Dis.* (Suppl.), 1963, v88, 394.

10. Lowry, O. H., Rosenbrough, N. J., Farr, A. L., Randall, R. J., *J. Biol. Chem.*, 1951, v193, 256.

11. Rowe, W. P., Huebner, R. J., Hartley, J. W., Ward, T. G., Parrott, R. H., *Am. J. Hyg.*, 1955, v61, 197.

12. Loda, F., Douglas, G., unpublished data.

13. Huebner, R. J., Rowe, W. P., Ward, T. G., Parrott, R. H., Bell, J. A., *New Engl. J. Med.*, 1954, v251, 1077.

14. Kasel, J. A., Evans, H. E., Spickard, A., Knight, V., *Am. J. Hyg.*, 1963, v77, 265.

15. Kasel, J. A., Loda, F., Knight, V., PROC. SOC. EXP. BIOL. AND MED., 1963, v114, 621.

16. Kasel, J. A., Knight, V., unpublished data.

Received June 11, 1964. P.S.E.B.M., 1964, v117.

A New Inhibitor of *in vitro* Cholesterol Biosynthesis. (29532)

J. F. DOUGLAS

Wallace Laboratories, Division of Carter Products, Inc., Cranbury, N. J.

Cholesterol biosynthesis, a normal body function, can be inhibited by a number of chemical entities. Included in the compounds which have been studied extensively in this regard are triparanol(1); benzmalecene(2); β-diethylaminoethyl diphenylpropylacetate hydrochloride, SKF-525A(3); fluoromevalonic acid(4); an azasterol, 20α-(2-dimethylaminoethyl)amino-5α-pregnan-3β-01 dihydrochloride(5); and vanadyl sulfate(6). Cholesterol itself also regulates its own formation through a feedback mechanism which acts prior to the mevalonate stage and probably on the conversion of hydroxymethyl glutaryl-coenzyme A (HMG-CoA) to mevalonic acid (7).

Benzyl N-benzyl carbethoxyhydroxamate (W-398) which is effective in reducing hypercholesteremia and plaque formation in animals has recently been described by Berger *et al*(8). This compound has now been investigated for its effect on the *in vitro* biosynthesis of cholesterol and has been found to inhibit this conversion prior to mevalonic acid. These findings are presented here.

Experimental procedure. The rat liver homogenate of Bucher(9) was employed as an enzyme source for biosynthesis of cholesterol.