

Response of Volunteers to Inoculation with Hemagglutinin-positive and Hemagglutinin-negative Variants of Coxsackie A₂₁ Virus *

DAVID J. LANG, THOMAS R. CATE, ROBERT B. COUCH, VERNON KNIGHT,†
AND KARL M. JOHNSON

(From the National Institute of Allergy and Infectious Diseases, Laboratory of Clinical Investigations, Bethesda, Md., and the Middle America Research Unit, Balboa Heights, Canal Zone)

Coxsackie A₂₁ virus has been associated with naturally occurring human respiratory illness (1-4), and in this laboratory many of the responses to inoculation with this agent have been studied in volunteers (5, 6).

In a previous report (7) the recognition and separation of hemagglutinating and nonhemagglutinating Coxsackie A₂₁ particles were described. Evidence was presented that these were genetic variants distinguishable by differences in their capacity to grow in primary and continuous cell line tissue cultures. Passage in continuous cell lines led to a predominance of hemagglutinin-negative (HA -) particles, whereas passage in primary tissue culture slightly favored growth of hemagglutinin-positive (HA+) particles.

To determine whether the HA + and HA - variants of Coxsackie A₂₁ virus caused differing human responses, volunteer inoculations with separated pools of these variants were performed. The results of these inoculations will be described.

Methods

Preparation of inocula. The virus used for volunteer inoculations was derived from a throat swab specimen obtained from a Marine recruit with an acute upper respiratory illness (no. 48560, Bouma) (7). This strain of Coxsackie A₂₁ was serologically indistinguishable from the original Coe strain described by Lennette, Fox, Schmidt, and Culver (1). Virus was initially isolated in primary human embryonic kidney (HEK) tissue cultures. Separation of the HA + and HA - particles was accomplished by differential adsorption on human type O erythrocytes at 4° C (7). The separated

erythrocyte and supernatant fractions were further purified by four terminal dilution passages in HEK tissue cultures. These separated pools which contained virus that had been passaged nine times in HEK tissue cultures were centrifuged at 2,000 rpm for 20 minutes, filtered through gradocol membranes (830 mμ), and stored at -70° C until used.

An additional, further purified pool of each hemagglutinin variant was prepared from individual plaque harvests of the pools described above. Semicontinuous diploid fibroblast tissue cultures derived from human embryonic lung (WI-26) (8) were used for this procedure in a plaque assay technique similar to that described elsewhere (9). Medium containing neutral red in a final dish concentration of 1:50,000 was added after 60 hours of incubation, and plaques were counted at 68 to 72 hours. The individual plaque harvests of each hemagglutinin variant were passaged an additional time in WI-26 tissue culture tubes to obtain sufficient volume for volunteer inoculations. These pools were centrifuged, submitted to treatment with trifluorotrichlorethane (Genetron) in a ratio of 3:1 (pool:Genetron) with a high speed Virtis homogenizer, separated from the trifluorotrichlorethane by centrifugation, filtered, and stored at -70°.

Each of these four virus pools was safety tested (10) before volunteer inoculations and was reidentified as Coxsackie A₂₁ in neutralization tests with hyperimmune guinea pig serum prepared against the original Coe strain that had been passaged in human epidermoid carcinoma (KB) tissue cultures. Virus titrations of these pools on tissue culture revealed that each contained at least 10^{6.5} TCID₅₀¹ of virus per 0.2 ml, and hemagglutination tests performed as described previously (11) indicated that the virus in each pool was of the expected hemagglutinin type.

Serum inactivated inocula were prepared containing the same concentrations of virus used for other inoculations, but in addition containing a 1:100 dilution of specific hyperimmune guinea pig serum. After 3 hours incubation at room temperature, these mixtures were administered to volunteers and were subsequently shown to be free of infectivity for tissue culture.

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† Address requests for reprints to Dr. Vernon Knight, Clinical Director, National Institute of Allergy and Infectious Diseases, Bethesda, Md. 20014.

¹ TCID₅₀ = that dose of virus which will infect 50% of tissue culture tubes.

Procedure for inoculations. The HEK pools were administered nasopharyngeally by spraying 0.5 ml of virus suspension into each nostril with a hand nebulizer (DeVilbiss no. 5) and instilling an additional 0.5 ml into each nostril by pipette. Each pool was diluted with veal infusion broth before inoculation. Simultaneous titrations of the inocula indicated that the 2.0-ml dose per man contained $10^{2.3}$ to $10^{2.7}$ TCID₅₀ of HA + virus or $10^{2.8}$ to $10^{3.2}$ TCID₅₀ of HA - virus.

Inoculation by aerosol was performed with the plaque derived pools as described elsewhere (6). The aerosol consisted of particles ranging in size from 0.2 to 3.0 μ in diameter. Estimates based on studies with other strains of Coxsackie A₂₁ suggest that about two-thirds of the virus was carried by particles ranging from 1 to 2 μ in diameter. Each volunteer inhaled 10 ± 0.2 L of aerosol in 9 to 12 consecutive deep inspirations—a total of $10^{2.2}$ TCID₅₀ of virus. The serum inactivated virus inocula were also administered in aerosol.

Volunteers. Participants in these studies were volunteers from federal correctional institutions. After an indication of willingness to participate, subjects were selected on the basis of absence of measurable serum neutralizing antibody.

Volunteers were isolated three per room 3 days before and 7 to 14 days following inoculation. Physicians who were unaware of the specific virus administered or the route of inoculation examined the volunteers daily. Temperatures were recorded four times a day after inoculation. Oral values above 37.5° C were confirmed by rectal temperatures and were considered to be fever. Peripheral blood counts, liver function tests, chest X rays,

electrocardiograms, urinalyses, and nose and throat bacterial cultures were obtained pre- and postinoculation.

Specimens for virus. Throat, nose, and anal swab specimens were collected daily from volunteers inoculated via the nasopharyngeal route. In the aerosol experiments only throat swab specimens were collected. Swabs were twirled in 2.5 ml of veal infusion broth containing 0.5% bovine albumin. The liquid was stored at -20° C until tested; then 0.4 ml of the fluid was added to a culture of HEK tissue. Cultures were placed on a revolving drum, incubated at 33 to 34° C, and observed for 9 to 14 days for cytopathic effect. Cultures inoculated with anal swab specimens were washed with fresh medium after 12 hours to minimize contamination.

The identity of the first, the last, and an intermediate isolate from each volunteer was checked by neutralization with specific hyperimmune antiserum. All other isolates were assumed to be Coxsackie A₂₁ if they produced characteristic cytopathic effect.

Hemagglutination tests (11) were performed on all virus isolates. To ensure that adequate virus was present for hemagglutination (10^8 TCID₅₀ per 0.2 ml) all tests were performed on isolates from specimens in HEK tubes in which the cytopathic effect had progressed to 100%, and all isolates with questionable hemagglutination results were titered, and reisolation was performed if necessary.

Measurement of antibody response. Sera obtained before and 3 to 4 weeks after inoculation were tested in both neutralization and hemagglutination-inhibition tests as previously described (1). The Coxsackie A₂₁ virus pool used for these tests contained both HA + and HA -

TABLE I
Illness following inoculation of volunteers with HA + and HA - Coxsackie A₂₁*

Inoculum and dose (TCID ₅₀)	Route	Volunteers inoculated	Volunteers ill	Volunteers with clinical findings				Average duration of illness days
				Upper respiratory symptoms	Systemic symptoms	Fever		
HEK ₉ , HA +, 10 ^{2.3} -10 ^{2.7}	Nasopharyngeal	11	9	8	5	5	4.4	
HEK ₉ , HA -, 10 ^{2.3} -10 ^{3.2} †	Nasopharyngeal	9	8	8	8	6	5.3	
HEK ₉ , WI ₂ , HA + 10 ^{2.2}	Aerosol	6	5	5	3	5	5.3	
HEK ₉ , WI ₂ , HA - 10 ^{2.2}	Aerosol	5	5	5	5	4	4.6	
HEK ₉ , WI ₂ , HA + 10 ^{2.2} neutralized	Aerosol	3	0	0	0	0	0	
HEK ₉ , WI ₂ , HA - 10 ^{2.2} neutralized	Aerosol	3	0	0	0	0	0	

† Three volunteers received $10^{3.2}$ TCID₅₀ of virus, the remainder receiving $10^{2.3}$ to $10^{2.5}$ TCID₅₀. No differences in response according to dose were detected.

* HA + and HA - = hemagglutinin-positive and negative particles; HEK = human embryonic kidney tissue cultures; WI = human embryonic lung tissue cultures.

TABLE II
Virus shedding by volunteers inoculated with HA+ and HA- Cocksackie A₂₁

Inoculum and route	Volunteers inoculated	Volunteers with virus recovery (ies)				Anal swab specimens, total	Positive anal swab specimens/number tested*
		Pharyngeal and/or nasal swab specimens					
		Total	Days 1-7	Days 8-14	Days 15-21		
HEK ₉ , HA+, nasopharyngeal	11	11	11	10	7†	7	13/86 (15%)
HEK ₉ , HA-, nasopharyngeal	9	9	9	9	6†	9	28/106 (26%)
HEK ₉ , WI ₂ , HA+, aerosol	6	4	4	4	4	ND‡	ND
HEK ₉ , WI ₂ , HA-, aerosol	5	4	4	4	4	ND	ND

* Number tested refers only to specimens from volunteers with at least one anal isolate.

† Three volunteers from group discharged on day 12.

‡ Not done.

virus particles but was hemagglutinin positive. In addition, each of the purified hemagglutinin variants was used in neutralization tests with several sera.

Results

Illness. The number of volunteers who became ill and the characteristics of their illnesses are shown in Table I for each of the six inoculum groups. As can be seen in the first four rows of Table I, upper respiratory illness often with fever and systemic symptoms occurred in similar high proportions of volunteers receiving one or the other hemagglutinin types of Cocksackie A₂₁, whether administered nasopharyngeally or by aerosol. There were no statistically significant differences in frequency of illness, upper respiratory symptoms, systemic symptoms, or fever among these groups. Represented in the bottom two rows of Table I are the volunteers who received serum inactivated virus pools. None of these latter men became ill.

Volunteers who became ill displayed a slight granulocytosis and lymphopenia during the acute phase of illness. There were no significant changes in liver function tests, urinalyses, sedimentation rates, or bacterial cultures of the throat.

Two men given HA+ variant by aerosol did not shed virus or show antibody rise. One of these became ill. It was found, however, that sinusitis had been present before inoculation, and it is presumed that this illness was unrelated to Cocksackie A₂₁ administration. One man in the HA- aerosol group also developed an unex-

plained illness without evidence of infection by virus shedding or rise in antibody titer. It was discovered that, although the latter volunteer possessed no serum neutralizing antibody to Cocksackie A₂₁, his serum hemagglutination-inhibiting titer was 1:20 before inoculation. None of the other volunteers selected for absence of detectable neutralizing antibody possessed preinoculation hemagglutination-inhibiting antibody.

Virus shedding. In Table II are shown virus shedding patterns for the four inoculum groups of volunteers who received infectious virus. There was a similar high frequency of virus recovery from the respiratory tract in each group regardless of the hemagglutinin type of virus received, and positive cultures were obtained with considerable frequency throughout the 3 weeks of observations.

The amount of virus in several throat swab specimens collected during the first 5 days after inoculation was determined by plaque assay on WI-26 cells that are equally sensitive for detection of the two hemagglutinin variants of Cocksackie A₂₁ (12). Observations were available for seven men given the HA+ variant and six men given the HA- variant. The average titer of Cocksackie A₂₁ per 0.2 ml of specimen fluid was 10^{0.911} (SE, 0.163) plaque forming units for the HA+ group, and 10^{1.12} (SE, 0.312) units for the HA- group. These values were not significantly different (Wilcoxon two-sample test, U = 16.5, p = 0.20; t test, t = < 1, p > 0.30).

However, virus recovery from anal swab specimens did differ according to the hemagglutinin

TABLE III
Serologic response by volunteers inoculated with HA + and HA - Coxsackie A₂₁

Inoculum and route	Volun- teers inocu- lated	Volun- teers shedding virus	Neutralizing antibody		HI antibody	
			Volun- teers with rise in titer*	Geometric mean titer postinoc.†	Volun- teers with rise in titer*	Geometric mean titer postinoc.†
HEK ₀ , HA +, nasopharyngeal	11	11	3	1:6	5	1:13
HEK ₀ , HA -, nasopharyngeal	9	9	9	1:64	9	1:218
HEK ₀ , WI ₂ , HA +, aerosol	6	4	2	1:8	2	1:14
HEK ₀ , WI ₂ , HA -, aerosol	5	4	4	1:54	4	1:320

* Fourfold or greater, preinoculation versus 3- to 4-week sera.

† Postinoculation titers only from volunteers who shed virus, all of whom were free of both neutralization and HI antibody preinoculation. Comparison of geometric mean antibody titers (*t* test): HA + versus HA -, nasopharyngeal inoculation—neutralizing $p < 0.01$, HI $p < 0.01$; HA + versus HA -, aerosol inoculation—neutralizing $p = 0.17$, HI $p < 0.01$.

reaction of the inoculum (Table II). Among volunteers given the HA + variant nasopharyngeally virus was recovered from anal swab specimens of only seven of the eleven infected volunteers, and from these seven volunteers only 15% of the specimens were positive. In contrast, virus was recovered from anal swab specimens from all nine volunteers who similarly received the HA - variant, and 26% of their specimens were positive. These differences were statistically significant when analyzed by the Wilcoxon two-sample test ($U = 13$, $p = 0.01$).

Antibody response. The frequency of fourfold or greater rises in neutralizing and hemagglutination-inhibiting antibody titers and the geometric mean values of convalescent titers are described in Table III. The three men from whom no virus was recovered exhibited no rise in antibody titer with either type of antibody assay and were not included in these calculations. Only three of eleven and two of four men who were infected following inoculation with HA + virus by the nasopharyngeal and aerosol routes, respectively, displayed both neutralizing and hemagglutination-inhibiting antibody responses, and an additional two volunteers developed a rise in hemagglutination-inhibiting antibody titer alone. In contrast, all men who were infected following inoculation with the HA - virus by these routes exhibited fourfold rises with both types of antibody assay. These differences are further emphasized by the

substantially greater geometric mean titers of postchallenge antibody from infected men who received HA - virus. In this calculation less than measurable titers were considered as positive at one-half the value of the lowest measurable titer. The increases in postchallenge titers for the HA - groups over the HA + groups were 7- to 10-fold for neutralizing antibody tests and 15- to 23-fold in hemagglutination-inhibition tests, differences that were highly statistically significant, except for neutralizing antibody titers in men inoculated by aerosol where the numbers are small.

Although volunteers who received HA - virus nasopharyngeally also yielded more virus recoveries in anal swab specimens than those who received HA + variant, no correlation existed within either group between the frequency of anal swab virus recoveries of individual volunteers and final serum antibody titer. Similarly, there was no correlation of final antibody titers with the number of virus recoveries from nasal or pharyngeal swab specimens, nor with titers of virus in pharyngeal swab specimens.

Duplicate neutralization titers were obtained on sera from three HA + volunteers and two HA - volunteers employing the HA + and HA - inocula as test viruses. The titers were identical in all cases.

Comparison of the relationship between hemagglutination-inhibiting and neutralizing antibody titers in all men given the two variants is shown

in Figure 1. If all titers are considered without regard to the kind of variant given, there is a close correlation between hemagglutination-inhibiting and neutralizing antibody titers ($r = 0.697$, $p < 0.01$). There is also a significant correlation between the two titers in men given HA + virus ($r = 0.646$, $p < 0.01$). No significant relationship exists between the two titers in men given HA - virus, but it should be noted that maximal titers were not determined in some tests and that the positive tests were all of relatively high titer so that only a narrow range of variation in titers was available for the comparison.

Pattern of shedding of the HA + and HA - virus particles. The hemagglutination reactions of 294 virus isolates from volunteers inoculated by the nasopharyngeal route were determined. All but 16 of these were of the same hemagglutinin type as the inoculum the volunteers received. From Table IV it can be seen that one isolate from one volunteer changed from HA + to HA -. The remaining 15 isolates that differed in hemagglutinin type from the inoculum were composed of one or more isolates from seven of nine volunteers who received HA - virus.

For the first week following inoculation all isolates from each volunteer were of the same hemagglutinin type as the inoculum. When shifts in hemagglutinin type occurred, they were often so late that the final pattern of virus shedding in regard to hemagglutinin type could not be determined. For example, the single isolate conversion from HA + and HA - was from the last specimen obtained from that particular volunteer. Four volunteers with a single isolate conversion from HA - to HA + had at least one subsequent isolate that was of the original type, but conversion

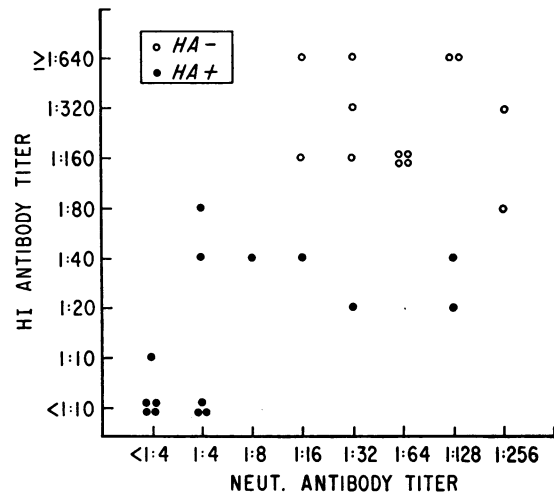


FIG. 1. COMPARISON OF HEMAGGLUTINATION-INHIBITION (HI) AND NEUTRALIZING SERUM ANTIBODY TITERS FOLLOWING INOCULATION WITH HA + AND HA - COXSACKIE A₂₁.

in hemagglutinin type of virus isolates from three volunteers appeared to persist. Shifts in hemagglutinin type were usually confined to isolates from one specimen site (nose, throat, or anal) per man, and a majority were seen in isolates from nasal specimens.

The hemagglutination reactions of 70 isolates from volunteers inoculated by aerosol were determined, and there were no conversions in hemagglutinin type. However, this was not significantly fewer than the conversions in isolates from volunteers who received nasopharyngeal inoculation ($p > 0.10$).

There was no difference in antibody response between men whose specimens remained unchanged in hemagglutinin type and those whose

TABLE IV
Alteration in hemagglutination type of virus isolates from volunteers inoculated with HA + and HA - Cocksackie A₂₁

Inoculum and route	Volunteers inoculated	Volunteers shedding virus	Volunteers with shift in HA type of isolates	Isolates with shift in HA type/ isolates from all volunteers	
				Days 1-7	After Day 7
HEK ₉ , HA +, nasopharyngeal	11	11	1	0/68	1/77*
HEK ₉ , HA -, nasopharyngeal	9	9	7	0/82	15/67*

* $p < 0.01$, Chi square test.

isolates exhibited this late change in hemagglutinin type.

Discussion

This study has described the response of volunteers to inoculation with purified pools of HA + and HA - variants of Coxsackie A₂₁. Upper respiratory illness, often with fever and systemic symptoms, occurred in a large majority of volunteers whether inoculated by the nasopharyngeal route or by a small-particle aerosol. There were no significant differences in the clinical responses to the two variants, nor did they differ appreciably from responses to a wild strain of Coxsackie A₂₁ tested earlier (5, 6).

In contrast, antibody responses after infection with the two hemagglutinin variants differed to a very considerable extent. Men who received HA - inocula had significantly higher postchallenge titers of both neutralizing and hemagglutination-inhibiting antibody than those who received HA + inocula.

The fact that anal swab specimens from volunteers who received HA - virus yielded more virus recoveries than anal swab specimens from volunteers who received HA + virus suggested that increased multiplication of HA - virus in the intestines might result in increased antigenic mass. However, among individuals receiving one or the other variant, there was no correlation between frequency of virus recovery from anal swab specimens and final serum antibody titer. Virus recoveries from nasal and pharyngeal swab specimens and the amount of virus in several of the latter specimens were similar for the two hemagglutinin variants, and individual antibody titers did not correlate with any of these measurements of virus shedding. Thus the contrasting serological responses do not appear to be explained by differences in multiplication of the two variants and the resultant differences in antigenic mass that this would imply.

Simon and Dömök (13) have reported separation of two hemagglutinin variants of ECHO virus 6 and have demonstrated an antigenic difference between the two. Antiserum prepared in animals against an HA - strain of ECHO virus 6 was of higher titer against the HA - variant than against the HA + variant; antise-

rum prepared against the HA + variant did not distinguish between the two. In the present study the neutralizing antibody in human serum following an induced infection with one or the other hemagglutinin variant of Coxsackie A₂₁ virus was of similar titer when either variant was used as the test virus. The sum of these studies suggests that the HA + and HA - particles of Coxsackie A₂₁ virus possess differing capacities to stimulate the production of antibody directed against antigens that are common to both.

The finding of nearly uniform shedding of the hemagglutinin type administered during several days after inoculation suggests that these inocula were true genetic variants rather than HA + virus, the hemagglutinin of which had been masked by inhibitors in the suspending medium as was suggested in studies by Schmidt, Fox, and Lennette (14). These investigators found that treatment of HA - populations with Genetron developed or increased hemagglutinin reactions in a number of strains of Coxsackie A₂₁. Treatment of the present HA + and HA - pools used for aerosol inoculation with Genetron did not alter these properties of the inocula, nor did it appear to alter the volunteer responses to the inocula.

There was a definite shift toward HA + variants in men given HA - virus beginning 8 days after inoculation. The reciprocal event was detected in only a single specimen. Although there is a slight growth advantage of the HA + particle in HEK tissue (7), which was used to test specimens for virus, this advantage alone could not account for the shift towards HA + variants in the absence of a true shift occurring in the volunteers. The late development of this change in hemagglutinin type may merely represent the fact that HA + variants were present in very small numbers in the HA - inoculum and required several days to increase to detectable concentrations. In a study in 1960 among military recruits (3), 227 of 238 (95%) isolates in HEK tissue culture were HA +. It is possible that this is indicative of a predominance of HA + variants in natural infection, and the present results may reflect a return to the normally predominant variant.

One question of possible practical interest is

whether the HA - variant of Coxsackie A₂₁ would prove to be a potent vaccine. The natural disease is apparently not often serious, but use for vaccine might be found in military populations, and increased knowledge of the epidemiology of the disease may disclose new areas for its application.

Summary

Volunteers without measurable antibody were inoculated by the nasopharyngeal route or by inhalation of a small-particle aerosol with hemagglutinin-positive (HA +) and hemagglutinin-negative (HA -) variants of Coxsackie A₂₁ virus. Mild upper respiratory illness often with slight fever occurred in similar high proportions of both groups. Virus recoveries from nasal and pharyngeal swab specimens were frequent and continued about equally in both groups throughout 3 weeks of observation, and pharyngeal swabs taken during the acute phase of illness from both groups yielded about the same quantities of virus. Anal swab specimens from volunteers who received the HA - variant more frequently yielded virus than those from volunteers who received the HA + variant. There was a definite shift to HA + variants in men given HA - virus beginning on the eighth day that rarely occurred reciprocally. Neutralizing and hemagglutination-inhibiting antibody responses were many times higher in men given the HA + variant. Within each group there was no correlation between antibody response and frequency of virus isolates from the various specimens. The two strains gave identical titers when used as antigen in neutralizing antibody tests of volunteer sera.

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References

1. Lennette, E. H., V. L. Fox, N. J. Schmidt, and J. O. Culver. The Coe virus: an apparently new virus recovered from patients with mild respiratory disease. *Amer. J. Hyg.* 1958, **68**, 272.
2. Pereira, M. S., and H. G. Pereira. Coe virus: properties and prevalence in Great Britain. *Lancet* 1959, **2**, 539.
3. Johnson, K. M., H. H. Bloom, M. A. Mufson, and R. M. Chanock. Acute respiratory disease associated with Coxsackie A-21 virus infection. I. Incidence in military personnel: observations in a recruit population. *J. Amer. med. Ass.* 1962, **179**, 112.
4. Bloom, H. H., K. M. Johnson, M. A. Mufson, and R. M. Chanock. Acute respiratory disease associated with Coxsackie A-21 virus infection. II. Incidence in military personnel: observations in a nonrecruit population. *J. Amer. med. Ass.* 1962, **179**, 120.
5. Spickard, A., H. Evans, V. Knight, and K. Johnson. Acute respiratory disease in normal volunteers associated with Coxsackie A-21 viral infection. III. Response to nasopharyngeal and enteric inoculation. *J. clin. Invest.* 1963, **42**, 840.
6. Couch, R. B., T. R. Cate, P. J. Gerone, W. F. Fleet, D. J. Lang, W. R. Griffith, and V. Knight. Production of illness with a small-particle aerosol of Coxsackie A₂₁. *J. clin. Invest.* 1965, **44**, 535.
7. Johnson, K. M., and D. J. Lang. Separation of hemagglutinating and nonhemagglutinating variants of Coxsackie A-21 virus. *Proc. Soc. exp. Biol. (N. Y.)* 1962, **110**, 653.
8. Hayflick, L., and P. S. Moorhead. The serial cultivation of human diploid cell strains. *Exp. Cell Res.* 1961, **25**, 585.
9. Kisch, A. L., P. A. Webb, and K. M. Johnson. Further properties of five newly recognized picornaviruses (rhinoviruses). *Amer. J. Hyg.* 1964, **79**, 125.
10. Couch, R. B., R. M. Chanock, T. R. Cate, D. J. Lang, V. Knight, and R. Huebner. Immunization with types 4 and 7 adenovirus by selective infection of the intestinal tract. *Amer. Rev. resp. Dis.* 1963, **88** (suppl. 3), 394.
11. Johnson, K. M., H. H. Bloom, L. Rosen, M. A. Mufson, and R. M. Chanock. Hemagglutination by Coe virus. *Virology* 1961, **13**, 373.
12. Lang, D. J., and K. M. Johnson. In preparation.
13. Simon, M., and I. Dömök. Studies on intratypic variants of Echoviruses. I. Separation of haemagglutinating and non-haemagglutinating virus particles from Echovirus type 6. *Acta microbiol. Acad. Sci. hung.* 1963, **10**, 293.
14. Schmidt, N. J., V. L. Fox, and E. H. Lennette. Studies on the hemagglutination of Coe (Coxsackie A₂₁) virus. *J. Immunol.* 1962, **89**, 672.