Serologic Relationships of the B632 and ECHO-28 Rhinovirus Strains. (30844)

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The original descriptions of the JH(1) and 2060(2) viruses were followed by confirmation of their association with the common cold syndrome(3,4). Cross-protection between the two agents was demonstrated(5), and they were therefore recognized to be 2 strains of the same picornavirus, designated ECHO-28(6). More recently, because of their acid lability(7) and their lack of multiplication in the lower intestinal tract, they have been grouped with the rhinoviruses(8).

As more and more types of rhinoviruses are described, most are said to be totally distinct from all others. One of the few examples of a serologic relationship is found between the ECHO-28 strains and B632 virus(9). Since the existence of such a large number of serotypes has created numerous problems in dealing with this group of agents it was decided to determine further the extent of this relationship in neutralization tests using human and animal serums.

Materials and methods. Seeds and serums used. Seeds of B632 virus and of 2060 and JH strains of ECHO-28 were obtained from the Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, and were passaged in KB cell cultures for production of infectious pools. Another strain of ECHO-28, 52992, had been isolated at Camp Lejeune, N. C. in January, 1961. This agent was recovered and passaged 3 times in human embryonic fibroblast cultures (strain WI-26) after which an infectious pool was prepared in HeLa cell cultures. These 4 strains were all further passaged once in HeLa cell cultures which were maintained on Eagle's Minimum Essential Medium (MEM) containing 2% chicken serum with 100 μg of streptomycin and 100 units of penicillin per ml. The cells were disrupted by freezing and thawing, the fluids clarified by low speed centrifugation and the resulting pools used for animal inoculation. These pools titered between $10^{-5.7}$ and $10^{-6.4}$ $TCID_{50}$ per ml.

Adult male guinea pigs were prebled and were inoculated intramuscularly with these strains according to the following schedule: Day 0, 0.5 ml of immunizing viral pool emulsified with an equal volume of complete Freund's adjuvant; Day 7, 0.5 ml viral pool; Day 14, 0.5 ml of viral pool emulsified with an equal volume of incomplete Freund's adjuvant. On Day 28 the animals were bled and then rested for a 3-week interval. On Day 49, immunization was resumed with 3 weekly injections using either the same or different viral strains. The first inoculation consisted of 0.5 ml of the immunizing pool emulsified with 0.5 ml of incomplete Freund's adjuvant, while the last 2 injections were 0.5 ml of the viral pool alone. The animals were again bled on Day 70, one week after the last injection.

Paired serums were also obtained from a group of individuals of all ages, resident for life on the Isthmus of Panama. The first specimen of each pair was collected in May 1963 and the second in January 1964.

Neutralization tests. All tests were performed in WI-26 cell cultures. These had been grown on Eagle's minimum essential amino acids and vitamins in Hanks' salts with 10% calf serum and were maintained on Eagle's MEM with 2% calf serum. Virus and serum dilutions were made in Hanks' Balanced Salt Solution (BSS) with 0.5% gelatin. All media contained 100 units of penicillin and 100 μg of streptomycin per ml.

The infectious pools described above were used in tests of guinea pig serums at a planned dose of 32-100 $TCID_{50}$ per 0.1 ml. For tests with human sera, a strain each of ECHO-28 and B632 isolated on the Isthmus was employed. These had been isolated and passed in WI-26 cell cultures and were used

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TABLE I. Comparison of B632 and 3 ECHO-28 Strains by Neutralization Test Using Guinea Pig Antisera.

	Reciprocal titer with indicated guinea pig serum*							
	B632		JH		2060		52992	
Virus	A†	B‡	A	B	A	B	A	B
B632	*80*	*≥12040*	<10	113	<10	<10	<10	<10
JH	<10	224	*20*	*5120*	14	2560	40	1280
2060	<10	220	56	2560	*<10*	*2560*	68	2560
52992	<10	240	28	1810	<10	1530	*56*	*1280*

* All preimmune sera had reciprocal titer <10 with all 4 virus strains.
† Serum collected after 3 inoculations.
‡ Serum collected after 6 inoculations of same viral strain.

at the second and third passage level respectively at a planned dose of 10-32 $TCID_{50}$ per 0.1 ml.

All serums were inactivated at 56°C for one-half hour. The guinea pig serums were run in 2-fold master dilution steps. The human serum pairs were first tested at a 1 in 2 dilution. Many serum pairs were retested at 1 in 2 dilution or were run in serial dilution. To 0.3 ml of the diluted serum, 0.3 ml of the appropriate virus dilution was added and the mixture was incubated at room temperature for 1½ hours. Thereafter, 0.2 ml of each serum virus mixture was inoculated into each of 2 cell culture tubes. The tubes were placed in a roller drum and incubated at 33°C.

End-points for those serums run in serial dilutions were calculated according to the method of Reed and Muench(10). In tests involving human serum pairs, antibody was considered present in the initial specimen, when at a 1 in 2 dilution, there was neutralization in both cell culture tubes. Those initial specimens which produced neutralization in one of the two cell culture tubes were called antibody positive only if the serum was run in a replicate test and neutralization was again demonstrated. A significant titer rise in a serum pair was considered present if, in those specimens run only at 1 in 2 dilution, there was no neutralization at all with the initial serum and neutralization in both tubes with the second specimen. For pairs tested in serial dilution, a demonstrated 4-fold rise in titer was required for significance.

Results. Homologous immunization of guinea pigs. Guinea pigs were bled and were given 3 weekly injections of the 4 viral strains and were then rebled (Serum A). After a rest of 3 weeks, immunization was resumed for a 3-week period using the same strains and the animals were again bled (Serum B). These sera were prepared in master dilution and were run in cross-neutralization tests against 32-100 $TCID_{50}$ of virus. The results in Table I demonstrate a cross-reaction between B632 antiserum and all three ECHO-28 strains. However, this was non-reciprocal, except with JH antiserum. Other than this cross-reaction, it was impossible to distinguish the three ECHO-28 strains from one another.

Cross immunization of guinea pigs. Another group of guinea pigs was immunized for 3 weeks with B632 or 2060 virus and Serum A was obtained. After the rest period, the animals given B632 were inoculated with 2060 virus while those previously given 2060 were switched to B632. When the second three-injection course was completed, Serum B was collected. The sera were tested in cross neutralization tests against 32-100 $TCID_{50}$ of B632 and 2060 virus and the results are given in Table II. The initial series of injections with 2060 produced only low level antibody against that virus. Subsequent inoculation of the same animals with B632 resulted in production of B632 antibody in moderate titer, but there was no change in 2060 antibody level. Animals given B632 virus initially developed low level homologous antibody but no detectable antibody against 2060. Changing to 2060 virus produced a rise in 2060 antibody much greater than would be anticipated had these animals not been immunized previously with a related virus. There was also a significant increase in the titer of B632 neutralizing antibody.

TABLE II. Antibody Titers of Guinea Pigs Sequentially Immunized with Both B632 and 2060 Viruses.

	Reciprocal titer of serum from guinea pigs immunized in indicated sequence*			
	B632 followed by 2060		2060 followed by B632	
Virus	A†	B‡	A	B
B632	48	250	<10	450
2060	<10	≥5120	10	10

* Preimmune serums had reciprocal titer <10 with both virus strains.

† Serum collected after 3 inoculations with first virus.

‡ Serum collected after 3 inoculations with first virus and 3 inoculations with second virus.

Antibody prevalence in human sera. A human population is exposed repeatedly to rhinoviruses of differing serotypes. Because of the relationship demonstrated between ECHO-28 and B632 viruses, it was of interest to determine if antibody against one of these types is acquired in a similar fashion as antibody against the other. Blood specimens collected in May 1963 from 363 residents of a single community in the Canal Zone were run in neutralization tests against 3-32 $TCID_{50}$ of a locally isolated strain of each virus. The results in Fig. 1 demonstrate the antibody prevalence in a series of age groups. The 2 upper curves indicate the total number of individuals with B632 or with ECHO-28 antibody. The lower curve represents the number of persons with antibody against both viruses. The area between the lower curve and either of the upper curves therefore consists of those individuals with antibody against only one type. B632 antibody was more prevalent in this population than ECHO-28 antibody and there were individuals with antibody against only one of the strains up to the 50-69 age group.

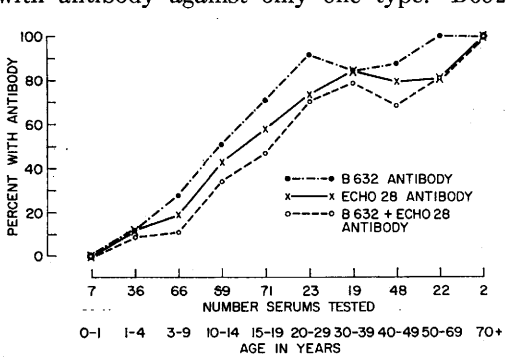

FIG. 1. Age-specific prevalence of B632 and ECHO-28 virus antibodies. Canal Zone, 1963.

It was possible to collect a second follow-up blood specimen in January 1964 from 354 of the 363 persons bled the previous May. During the interval, strains of both B632 and ECHO-28 had been isolated locally and were used in the test. Significant titer rises to one or both virus types were found in 41 individuals (Table III). The quantity of one serum was not adequate for a complete study. Of the remaining 40 pairs, only 13, or 32.5%, showed a rise in antibody titer against both strains. Only one of the 23 pairs with no antibody in the first specimen showed a rise to both viruses, but in contrast, 12 or 70.6% of the 17 pairs with prior antibody to B632 and/or ECHO-28 had a dual rise. All of these 12 had prior antibody to B632 and 3, in addition, had prior ECHO-28 antibody.

In 16 of the 40 pairs, a rise in titer against a strain occurred in spite of prior homologous antibody. Only those pairs with initial antibody which were most likely to show a rise in titer were re-run in serial dilution. These were specimens which had shown a suggestion of titer rise in the first test or which were obtained from individuals in whose families serologic conversions had occurred. Therefore, there may well have been additional undetected rises in pairs with initial antibody.

Discussion. Although cross protection occurs between the JH and 2060 strains of ECHO-28(5), a slight antigenic variation has been demonstrated in particular sera(3,11, 12). In the present study, when guinea pigs were inoculated 6 times with these strains and with a more recent isolate of ECHO-28, and the sera produced were run in cross neutralization tests, no significant antigenic difference was observed. Only when B632 virus was tested against these antiserums could the variation be demonstrated. JH antiserum neutralized B632 virus while 2060 and 52992 antiserums did not. B632 antiserum neutralized all 3 strains in similar titer. These results are in agreement with the findings of Taylor-Robinson and Tyrrell, who observed, using JH as the representative ECHO-28

TABLE III. Number of Individuals with Rises in B632 and/or ECHO-28 Antibody Titer.

Antibody status of initial specimen	Rise in titer of indicated antibody						
	B632 alone	%	ECHO-28 alone	%	B632 and ECHO-28	%	Total
No prior antibody to either type	4	17.4	18	78.3	1	4.3	23
B632 antibody only	0		1		9		10
ECHO-28 antibody only	0		1		0		1
B632 and ECHO-28 antibody	2		1*		3		6
B632 and/or ECHO-28 antibody	2	11.8	3	17.6	12	70.6	17
Grand total	6	15.0	21	52.5	13	32.5	40

* Quantity of one additional specimen not sufficient to titer against B632.

strain, a reciprocal cross reaction between that virus and B632(9).

Because of the relatively low antigenicity of the rhinoviruses, a protracted period of immunization has usually been necessary for production of antisera of adequate titer. Little homologous antibody was produced by the guinea pigs after the first series of injections, but the second series of inoculations with the homologous virus resulted in a high level of antibody. When 3 inoculations of 2060 virus followed 3 injections of B632, as much 2060 antibody resulted as in animals given a full course of 6 injections of 2060. At the same time, there was also a significant rise in B632 antibody. After the reverse sequence of inoculations, B632 antibody was produced in moderate titer but this was higher than that which would have followed only 3 injections. This demonstrates the sharing of antigens even between B632 and the ECHO-28 strain from which it is most distinct.

The observed similarity in antigenic structure might mean that human infection with one virus would produce antibody to both. It also raises the question of cross protection by pre-existing antibody. The study of human sera showed an age-specific pattern of antibody prevalence for both B632 and ECHO-28 similar to that found for all rhinoviruses thus far evaluated in this manner(2,13,14). As the adult age group was reached, there were few who did not have antibody to both viruses. Since the antibody prevalence curves for both types are similar, this can mean that many individuals developed heterologous antibody after infection with only one type. It may equally imply that antibody was acquired independently following infection with the two viral types which happened to behave in a similar fashion.

Both B632 and ECHO-28 viruses were isolated in the interval between the collection of the serum pairs. A rise in titer to one or both types was observed in 41 of the 354 pairs tested. Since there had been no large scale isolation attempt, and the period was long enough to allow serial infection of one individual, the recognition of contact with a specific rhinovirus can only be made on presumptive serologic evidence. An observed titer rise to both viruses might represent the reported cross reaction from a single agent(8, 15), or actual infection with both. Nevertheless, the titer rise was directed against only one virus in 67.5% of the 40 individuals adequately tested. There was also a definite suggestion that prior antibody influenced the type of immune response. While only one of the 23 individuals with no initial antibody had a titer rise to the 2 strains, 12 or 70.6% of 17 with prior antibody to one or both strains had a dual response. All had initial B632 antibody and 3 had, in addition, prior ECHO-28 antibody. It would be unlikely that those with pre-existing antibody would be more subject to actual infection with both virus types than those with no pre-existing antibody. These dual responses are therefore most likely due to a recent infection with one type and are similar to the guinea pig data, in which animals previously immunized with B632 showed a significant anamnestic response to that virus when given ECHO-28.

In at least 16 individuals, the immune response occurred in the presence of initial homologous antibody, in titer sometimes as high

as 1 in 32. Although prior antibody did not prevent infection, no information is available about the presence or type of clinical illness accompanying the rise in titer. Since even homologous antibody was so ineffective in preventing reinfection, nothing can be said about the role of heterologous antibody. The evidence is strong, however, that repeated infections are important in broadening of the serologic response.

The recent description of additional rhinovirus serotypes which have distinct similarity to previously characterized types(16) has given added importance to the study of any antigenic interrelationships that might exist. Grouping these agents, if possible, would be a great aid in handling and identifying them. Evaluation of these relationships in terms of protection from clinical disease will require the use of volunteers or the epidemiologic study of a significant number of natural infections.

Summary. The antigenic relationships of the ECHO-28 strains and B632 were studied in guinea pigs. The cross reaction demonstrated between B632 and JH was reciprocal, while the one between B632 and 2060 was not. Inoculation of guinea pigs with both B632 and 2060 virus in different order brought out the antigenic relationship of these types. In human sera, the age-specific prevalence of ECHO-28 and B632 antibody was compared. A titer rise to one or both types was found in 41 of 355 paired sera. This increase in antibody occurred in some cases in spite of homologous prior antibody. In most cases, the rises in antibody level occurred against only one virus, but there was a strong suggestion that with prior antibody, an increase in antibody to both viruses was more likely.

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Glycogen Synthesis in Rat Liver Slices: Role of Glucose Concentration, Mesothelium and Insulin.* (30845)

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Glucose uptake has been studied extensively under *in vitro* conditions(1,2), but little attention has been given to the actual extent to which glucose penetrates the tissue under

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