**AN APPLICATION OF FACTOR ANALYTICS ON ANTIGENIC
DRIFT, AND CHROMATOGRAPHIC ANALYSIS
ON INTRATYPIC VARIATION OF
ENTEROVIRUS TYPE 70**

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SUMMARY: An attempt was made to reveal the antigenic determinant site of enterovirus (EV) 70 by application of Harman's factor analytics. The normalized neutralization rate constant (NK) values obtained by the kinetic-neutralization tests (NT) with 12 strains of EV70 isolated during the period from 1971 to 1976 were used in the principal factor analysis. From the analytic results, it was suggested that at least two antigenic determinant sites, composing to variable antigenic factor (VAF) and common one (CAF), might be closely related to the antigenic makeup. There were no significant differences in CAF score among the strains, and they were numerically classified into prototype-like and prime-like virus groups from the VAF scores. The drift appeared here could be explained as successive alteration of VAF scores.

Chromatographic analyses were carried out on intratypic variable and common particles with two virus strains, i.e. the prototype J 670/71 and prime G-2/74. The intratypic variable and common particles were eluted in low and high ionic concentrations at neutral pH, respectively. It was concluded that the virus populations of two strains were heterogenic in the proportion of variable to common virus particles.

INTRODUCTION

Intratypic variation of EV70 has been considered to be an antigenic drift successively occurring from prototype-like viruses to prime-like ones since its introduction into Japan in 1971. As a probable aspect, it was proposed that EV70 might be such a type of virus of antigenic heterogeneity in a previous report (Kawamoto, 1979). In facts, acute hemorrhagic conjunctivitis (AHC) caused by a prototype-like strain has rarely occurred in Japan since 1974 (Ishii, Nakazono and Sawada, 1977; Kawamoto, 1976, 1979). Such a heterogeneity is well known to exist widely among enteroviruses and to be an established character of a given population of virus particles (Abraham, 1967; Choppin and Eggers, 1962; Dömök and Simon, 1966; Gaudin et al., 1971; Margalith et al., 1968; Simon and Dömök, 1963). In the present study, at first, an attempt was made to reveal the antigenic determinant sites of EV70 by application of Harman's

factor analytics (1967). This analytics was developed originally in psychology. In this report, the drift phenomenon of EV70 was analyzed by comparing the strains' factor scores assigned significances in respective antigenic characteristics. Secondly, the elution behavior in adsorption chromatography was analyzed to find the heterogeneity of EV70 among strains.

MATERIALS AND METHODS

Experiment 1: Numerical analytics of neutralization activities among EV70 strains

Introduction into factor analytics: The 72 normarized neutralization rate constant (NK) values calculated for serum-virus combinations of 12 isolates obtained during the period from 1971 to 1976 and six rabbit hyperimmune sera by a modification of McBride's neutralization kinetics (1959) were used as principal data introduced into factor analytics. The data have been reported in a previous report (Kawamoto, 1979). The NK value was useful as a summary measure showing either antigenic similarity or diversity. Although factor analytics applied here was developed originally as a numerical procedure for multivariate analysis in psychology, it has often been applied to epidemiological studies from the public health aspect e.g. that by Nakamura et al. (1978). However, it has scarcely been applied to virological study. As a factor model in the present study, an experimental variable, i.e. NK values on antigen-antibody reactions, could be explained in one dimensional structure of elemental subvariables.

For example, the equation is as follows:

$$z_{ij} = a_{ji}f_{i1} + a_{j2}f_{i2} + \cdots + a_{jm}f_{im} + d_j u_{ij}$$

where a_{jm} is coefficient, so-called "factor loading", which related a variable factor with a common one, whereas f_{im} is a hypothetic common variable, so-called "factor score". It was defined that antigenic characteristics of each strain could be mathematically expressed as a factor score in varying proportional possession of individual antibody makeup which was designated in the place of different antigenic determinant sites on virus particles. Hence, their mathematical details will be after illustrated in basics of factor analytics.

Basics of factor analytics: As shown in Table I, the kinetic results are summarized as principal data matrix X . Correlation coefficients, calculated from possible combinations of columns in the matrix X , are summarized as correlation matrix R shown in Table II. The matrix R was used to calculate factor loading matrix A explained by the following equation:

$$R = AA' + D^2$$

$$R^* = AA' \quad (R^* = R - D^2)$$

where matrix A' is transposed matrix, and matrix D of unknown factor is diagonal matrix composed of uniqueness (d_j^2) as elements. That is to say, matrix R can be approximated to a product AA' , whereas data matrix X can be stand-

ardized by the following equation:

$$Z = (X - 1m') \sum^{-1}$$

where m is vector composed of mean of each column in matrix X as element, and $\sum^{-1}$ is diagonal matrix composed of standard deviation of each column in matrix X as element. Accordingly, factor score matrix F can be expressed by the following equation:

$$Z = FA' + UD$$

where matrix UD is product of factor loading and factor score of unknown factor. It can be neglected in this part, if it is small enough. Additionally, if original matrix X is used, factor score matrix F should be further arranged as the following equation:

$$F = (X - 1m') \sum^{-1} (A')^{-1}$$

where matrix $(A')^{-1}$ is inverted matrix, and it is defined as weight coefficient matrix to be used to estimate factor score. Accordingly, distribution of factor score given here will naturally follow standardized distribution.

Practice of analytics: Factor analysis applied here was carried out according to the modification of original Harman's procedure reported by Shiba (1976).

Experiment 2: Adsorption chromatography with hydroxyl apatite gel

Viruses: Two seed viruses, selected from two antigenic subgroups, i.e. the prototype J 670/71 and prime G-2/74, were passaged in HEK₃, MK₃ and HeLa₇, and in MK₃ and HeLa₇, respectively. They were plaque purified three times, and then propagated once in HeLa cells in maintenance medium without bovine plasma albumin (Fraction V, Armour). The cultures, which showed cytopathic effect after virus inoculation, were freeze- and -thawed three times. The supernatants of culture fluids were divided into 2-ml portions and stored at -20°C as virus stock.

Sera: Two kinds of rabbit hyperimmune serum prepared with two EV70 strains, J 670/71 and G-2/74, by the procedure already described (Kawamoto, 1976, 1979), were used for the neutralization test (NT) and kinetic-NT.

Cell cultures and media: HeLa cells, kindly offered by Dr. H. Matsumiya (Hokkaido University School of Medicine, Sapporo), were used in this part. The cells were propagated in Roux bottles to multiply virus stock, and the growth medium used here was original Dulbecco's YLE supplemented with 10% newborn calf serum and 0.075% bicarbonate.

Infectivity assay: The fractionated viruses as well as the original ones were titrated with the micro tissue culture (MTC) system and by the plaque technique described previously (Kawamoto, 1976, 1979).

Analysis of neutralization activity: Cross-NT and kinetic-NT accorded those described previously (Kawamoto, 1976, 1979).

Adsorption chromatography with hydroxyl apatite gel: Hydroxyl apatite

gel (Nippon Chem. Co., Ltd.) was used instead of calcium phosphate gel. Chromatographic operation was the modified procedures previously described by Ozaki et al. (1965) and by Gaudin et al. (1971). The hydroxyl apatite gel was equilibrated with 0.005 M phosphate buffer at neutral pH by decantation three times with the same buffer, and stored at 4 C until chromatographic operation. Prior to chromatography, the gel was further washed with the same ionic strength buffer by centrifugation at 2,000 rpm for 5 min. The virus stock was diluted fourfold with 0.0005 M phosphate buffer at neutral pH, and the dilution was mixed with gel precipitate prepared from an equal volume of gel suspension. Viruses were allowed to be adsorbed at 25 C for 90 min by shaking at 25 oscillations/min. The supernatant was removed by centrifugation, and then the remaining gel was washed with 0.005 M phosphate buffer at neutral pH of the same volume to eliminate non-adsorbed particles. The adsorbed particles were stepwisely eluted from the gel with the buffer at neutral pH of the same volume by increasing the ionic strength from 0.05 M to 0.5 M. The eluted fractions were designated as free virus, including unadsorbed and washed viruses and the sequential fractions from F1 (0.05 M) to F6 (0.5 M) in the ascending order of high ionic strength. In preliminary tests, it was found that more than 90% of the input of each virus were adsorbed to the gel at a concentration of 20 mg/ml at 25 C for 90 min by 25 oscillations/min.

Consequently, operation of chromatography was carried out with a 2-ml aliquot of the diluted virus stock under these conditions. Each fraction except F1 (0.05 M) and F2 (0.1 M) was appropriately diluted to be equilibrated to the ionic strength of 0.1 M buffer with sterile distilled water after passage through 0.22 μ millipore filter, and then diluted twofold with a usual maintenance medium. Each of the treated fractions was stored at -20 C until analysis. A portion of untreated F1 (0.05 M) from two viruses was purified by re-chromatography in the same manner as mentioned above, and then eluted with the same ionic strength (0.05 M) at neutral pH.

RESULTS

Experiment 1. Numerical Analysis of Neutralization Activities of 12 Strains of EV70

The results of kinetic-NT are shown as principal data matrix *X* in Table I. The correlation coefficients, calculated from possible combinations with six hyperimmune sera, are shown as correlation coefficient matrix *R* in Table II. Antigenic determinant factors and their loadings, stepwisely calculated from matrix *R*, are shown as matrix *A* in Table III. Matrix *A* is composed of four different factors and their loadings were each under each variable, i.e. immune serum. The influences of different factors among six variables can be compared with individual factor contributions each of which is sum of factor loadings. The contribution by the primary factor showed a significantly higher value than any

TABLE I

Normalized neutralization rate constants (NK values) for twelve EV 70 strains isolated the period from 1971 to 1976

EV 70 isolates	Rabbit immune serum against					
	J 670/71		HK 3454/71	G-10/72	# 137/73	G-2/74
	A	B				
J 670/71	100	100	70	84	69	89
HK 3454/71	98	71	100	100	63	92
Rabat 6/71	100	73	73	94	76	91
# 99/72	94	93	98	98	95	94
G-10/72	66	56	14	100	70	98
# 137/73	80	65	72	91	100	94
# 141/74	38	25	12	38	46	89
HP 23/74	44	12	15	98	68	84
G-2/74	50	44	13	74	41	100
J 150/75	50	18	57	84	65	91
HP 83/75	50	40	19	67	67	100
G-6/76	43	40	16	59	50	100

* Each number expresses the average NK value of each serum-virus combination. The underline indicates the NK value for isologous serum-virus combination. (Referred to the data published by Kawamoto, 1979)

TABLE II

Correlation coefficient matrix R calculated for possible combinations of six antisera from NK values obtained by kinetic NT

Antiserum	Antiserum					
	J 670/71-A	J 670/71-B	HK 3454/71	G-10/72	# 137/73	G-2/74
J 670/71-A	—	0.907	0.912	0.631	0.599	-0.175
J 670/71-B	0.907	—	0.812	0.472	0.512	0.079
HK 3454/71	0.912	0.812	—	0.510	0.660	-0.179
G-10/72	0.631	0.472	0.510	—	0.610	0.098
# 137/73	0.599	0.512	0.660	0.610	—	-0.172
G-2/74	-0.175	0.079	-0.179	0.098	-0.172	—

Numbers express correlation coefficients among serum combinations.

of the sequent ones. All of them, except anti-G-2/74 serum, were approximately equal among the primary factor loadings, whereas the contributions by sequent three different factors were gradually decreased after the secondary factor. In this connection, there were no significant differences among the antisera in individual factor loadings. Consequently, it was considered that the primary factor might be closely related to intratypic variability among 12 strains, whereas sequent others, i.e. the secondary factor as the representative, might be associated with common antigen. In other word, the former one was designated as variable.

TABLE III

Factor loading matrix A of anti-EV 70 serum by factor analytics

Antiserum	Factor loading				Communarity
	A ₁	A ₂	A ₃	A ₄	
J 670/71-A	0.888	0.229	0.020	-0.021	0.841
J 670/71-B	0.860	0.195	0.159	0.070	0.808
HK 3454/71	0.839	0.359	0.130	0.035	0.851
G-10/72	0.717	0.055	-0.017	0.007	0.517
# 137/73	0.683	0.027	0.180	0.113	0.512
G-2/74	-0.108	0.272	0.080	-0.007	0.092
Contribution	3.879	1.137	0.552	0.197	—

TABLE IV

Factor score against each antigenic determinant factor on EV 70 particle

EV 70 isolates	Factor score			
	VAF	CAF ₁	CAF ₂	CAF ₃
J 670/71	1.65	0.48	0.28	0.14
HK 3454/71	4.46	0.79	0.19	0.05
Rabat 6/71	4.52	0.54	0.24	0.11
# 99/72	1.64	0.97	0.67	0.33
G-10/72	-1.06	0.01	-0.01	0.00
# 137/73	0.07	0.51	0.50	0.27
# 141/74	-1.74	-1.03	-0.54	-0.28
HP 23/74	0.06	-1.02	-0.53	-0.09
G-2/74	-2.07	-0.21	-0.32	-0.11
J 150/75	-0.95	-0.32	-0.23	-0.08
HP 83/75	-2.45	-0.16	-0.04	-0.07
G-6/76	-2.33	-0.30	-0.23	-0.18

VAF, variable antigenic factor; CAF, common antigenic factor.

antigenic factor (VAF), while the latter three were designated as common antigenic factor (CAF). As shown in Table III, an individual communality, expressed as sum of squares of factor loadings under each variable, was used as embodying parameter to elucidate the antibody makeups of VAF and CAF in varying proportions. Consequently, it was considered that anti-G-2/74 serum, which was different from other five antisera, might be composed of proportional makeup with CAF rather than VAF. As shown in Table IV, 12 strains were compared from one to another with respect to CAF and VAF scores as numerical measure of antibody makeup. HK 3454/71 and Rabat 6/71 were similar each other with respect to VAF and CAF scores, and, especially, their VAF makeups were significantly higher than those of other Japanese isolates.

Therefore, it became clear that they could be differentiated from Japanese

strains with respect to the VAF makeup. In contrast, it was found that VAF scores had successively drifted from prototype-like virus to prime-like one along VAF axis. CAF scores, on the contrary, were scarcely changeable after 1971, because there were no significant differences in CAF scores among 12 strains.

Experiment 2. Chromatographic Analysis of Intratypic Variabilities of EV70

Elution behaviors of input viruses: The J670/71 and the G-2/74 viruses, adsorbed onto hydroxyl apatite gel, were stepwisely fractionated with phosphate buffer at neutral pH by increasing ionic strength. As shown in Table V, the virus inputs of J 670/71 and G-2/74 were adsorbed in 90% and 91%, respectively. There was no significant difference in the adsorption rate between two strains. In comparison of the infectivities of the eluted fractions, 46.8% and 21.7% of virus inputs of J 670/71 and G-2/74 were recovered in F1 (0.05 M), while 21.5% and 46.5% of them were recovered in F2 (0.1 M), respectively. A large amount of adsorbed viruses were thus recovered in low ionic strength buffer. However, less than 3 to 4% of virus inputs were still eluted in sequent fractions of high ionic strength, F3 (0.2 M) through F6 (0.5 M). In the elution pattern, the recovery peak of J 670/71 appeared at lower ionic strength than did G-2/74, but there was no significant difference in the elution behavior at high ionic concentrations between two intratypic viruses.

Cross-NT of fractionated viruses with rabbit sera: Neutralization of the fractionated viruses as well as the original ones was analyzed by cross-NT with two rabbit immune sera. As shown in Table VI, F1 (0.05 M) and F2 (0.1 M) of J 670/71 against homologous antiserum showed fourfold higher virus-neutralizing (VN) titers than those against heterologous antiserum. Accordingly, it was

TABLE V

Recovery of prototype and primetype EV 70 strains from hydroxyl apatite chromatography

Fraction	Prototype J 670/71		Primetype G-2/74	
	TCID ₅₀ /25 μ l	Recovery (%)	TCID ₅₀ /25 μ l	Recovery (%)
Original virus fluid	1.0 $\times$ 10 ⁶		2.2 $\times$ 10 ⁵	
Free virus after adsorption	1.0 $\times$ 10 ⁵	—	2.0 $\times$ 10 ⁴	—
F 1 (0.05 M) ^a	4.7 $\times$ 10 ⁵	(46.8)	4.7 $\times$ 10 ⁴	(21.7)
F 2 (0.1 M)	2.2 $\times$ 10 ⁵	(21.5)	1.0 $\times$ 10 ⁵	(46.5)
F 3 (0.2 M)	3.2 $\times$ 10 ⁴	(3.2)	1.0 $\times$ 10 ⁴	(4.7)
F 4 (0.3 M)	4.7 $\times$ 10 ³	(0.5)	4.7 $\times$ 10 ³	(0.2)
F 5 (0.4 M)	3.2 $\times$ 10 ³	(0.3)	4.7 $\times$ 10 ³	(0.2)
F 6 (0.5 M)	3.2 $\times$ 10 ³	(0.3)	4.7 $\times$ 10 ³	(0.2)

Note: a, F1 (0.05 M) is the first fraction eluted with 0.05 M phosphate buffer at neutral pH.

TABLE VI

Cross neutralization with rabbit immune sera against original viruses and their chromatographic fractions

Fraction	Challenge of J 670 origin TCID ₅₀ /25 μ l	Antiserum against		Challenge of G-2 origin TCID ₅₀ /25 μ l	Antiserum against	
		J 670/71	G-2/74		J 670/71	G-2/74
Original virus fluid	100	2048 ^a	256	200	1024	1024 ^b
F 1 (0.05 M)	47	2048	512	316	512	512
F 2 (0.1 M)	47	2048	512	47	1024	2048
F 3 (0.2 M)	47	2048	1024	47	1024	2048
F 4 (0.3 M)	47	512	512	100	512	512
F 5 (0.4 M)	47	512	512	47	512	512

Note: a and b, homologous 50% virus neutralizing (VN) titers as reciprocals of serum dilution step.

possible that F1 (0.05 M) of two strains proportionally composed of more variant particles than intratypic common particles of EV70, while the sequent fractions after F3 (0.2 M) predominantly involved common particles. It was considered that antigenic makeups of virus inputs were segregated into two intratypic antigenic groups with respect to the elution behavior in low and high ionic strength at neutral pH, respectively.

Kinetic-NT of re-chromatographic fractions with rabbit immune sera: Neutralization of re-chromatographic F1 (0.05 M) fractions of two viruses was analyzed by kinetic-NT with rabbit hyperimmune sera. As shown in Table VII, J 670/71 showed no significant differences in NK value between re-fractionated F1 (0.05 M) and its original virus against two antisera, while G-2/74 showed highly significant differences ($p < 0.01$) in NK value by the chi square test between re-fractionated F1 (0.05 M) and its original virus against two antisera. As the results, with anti-J 670/71 serum, re-fractionated F1 of G-2/74 showed a lower NK value than did its original virus. Consequently, it was concluded that the virus populations of two strains were heterogenic in the proportion of variable virus particle to common ones.

TABLE VII

NK values of kinetic neutralization of original viruses and their re-chromatographic fraction, F1 (0.05 M), with rabbit immune sera

Virus	Fraction	Antiserum against	
		J 670/71	G-2/74
J 670/71	Original virus fluid	<u>100</u>	89
	Re-fractionated F 1 (0.05 M)	<u>100</u>	102
G-2/74	Original virus fluid	50	<u>100</u>
	Re-fractionated F 1 (0.05 M)	28	<u>100</u>

The underline indicates NK value for isologous serum-virus combination.

DISCUSSION

From factor analysis in *experiment 1*, it was considered that two antigenic determinant sites in varying proportions, i.e. VAF and CAF, might exist as the antigen makeup of EV70, and VAF might be closely associated with intratypic antigenic drift. Furthermore, the drift could be explained by the alteration hypothesis in variation of factor scores given to 12 strains.

It was suggested that factor analytics applied here was useful as an embodying technique for quantitative estimation in virological studies. A similar concept was recently reported by others.

Iwasaki et al. (1976, 1977) attempted numerical classification of A/Hong Kong influenza virus current strains using average distant coefficient obtained by cross-hemagglutination inhibition (HI) tests.

They considered that viral HA antigens might be composed of various antigenic determinant sites, and intratypic variation among type A influenza viruses might depend upon the proportion of HA antigenic sites. We proposed in the present study that EV70 might be a virus type with heterogeneity, and the drift might have occurred by its heterogeneity. Such heterogeneity has been investigated by chromatographic analysis in many viruses, i.e. in polioviruses with diethylaminoethyl-cellulose columns (Hodes, Zepp and Ainbender, 1960), with Al (OH)₃ gel (Woods and Robbins, 1961) and with calcium phosphate gel (Ozaki et al., 1965), and in echovirus type 11 with calcium phosphate gel (Gaudin et al., 1971). In the present chromatographic analysis, intratypic variabilities of EV70 specifically showed in low ionic strength at neutral pH, and the segregated virus populations were shown to be stable in various ionic strength solutions. It was demonstrated that EV70 was composed of four different structural proteins like polioviruses (Esposito and Obijeski, 1976). Recently, it was noted that several group A coxsackieviruses (e.g. A-13, 15, 18, and 21) were rapidly inactivated in low ionic strength concentration at neutral pH, and the phenomenon was due to inhibiting adsorption onto susceptible cells by specific structural alteration in the coxsackievirus capsid induced under conditions of low ionic strength (Cords, James and McLaren, 1975).

However, we considered that antigenic alteration of EV70 presented here was quite different from that of several group A coxsackieviruses mentioned above.

In conclusion, it was confirmed that original virus populations of two intratypic strains used here were heterogeneous as proportional mixtures composed of neutralization-sensitive and -insensitive particles, and the former ones were equilibrated in high ionic strength concentration and the latter ones in low ionic strength concentration at neutral pH, respectively. Therefore, it was considered that neutralization-insensitive particles of EV70 might become the predominant population as a result of the sequent antigenic drift.

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