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GENETIC RELATEDNESS OF THE KEMEROVO SEROGROUP VIRUSES: I. RNA—RNA BLOT HYBRIDIZATION AND GENE REASSORTMENT *IN VITRO* OF THE KEMEROVO SEROCOMPLEX

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Summary. — The dsRNA profiles of the Czechoslovakian and Siberian serotypes of the Kemerovo serocomplex viruses examined were similar in agarose, while their dsRNA profiles were distinct in polyacrylamide gel. Blot hybridization studies of the Kemerovo serocomplex viruses demonstrated that the genes were highly conserved among the members within each type, but not between types. Gene reassortment *in vitro* was demonstrated among selected pairs of the Kemerovo serocomplex viruses by intra- and inter-typic crosses. The majority of the reassortant progeny from inter-typic crosses were single gene replacements, whereas the majority of the reassortant progeny from intra-typic crosses were multiple gene replacements suggesting that certain gene combinations were restrictive under conditions of the experiment.

Key words: Kemerovo serocomplex, Orbivirus, genetic relatedness

Introduction

Following the classification scheme proposed by Casals (1968), the Kemerovo serogroup was subdivided into three serocomplexes based on antigenic relationships in complement-fixation (CF) tests; these serocomplexes included Chenuda, Kemerovo, and Seletar (Wad Medani) (Casals, 1971). A fourth serocomplex, Cape Wrath-Great Island, was recognized later (Main *et al.*, 1976); and in recent years, the number of virus isolates in this serocomplex has increased such that it represents the largest serocomplex. Members of the different Kemerovo serocomplexes have been isolated from different geographic regions, and they are vectored by either argasid or ixodid ticks.

Viruses unrelated to tick-borne encephalitis were isolated from *Ixodes persulcatus* ticks and from a human in Western Siberia in 1962. These virus

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Table 1. Kemerovo Serocomplex Virus Isolates

Isolate	Isolation Source	Geographical Origin	Year[a]
Siberian Serotype			
EgAn 1169-61	*Pheonicurus pheonicurus*	Burg El Arab, U.A.R.	1961
KM 3	*Ixodes persulcatus*	Kutchum, U.S.S.R.	1962
L 75	human	Kutchum, U.S.S.R.	1962
R 9	*Ixodes persulcatus*	Romanovka, U.S.S.R.	1962
Kemerovo (R 10)	*Ixodes persulcatus*	Romanovka, U.S.S.R.	1962
R 54	*Ixodes persulcatus*	Romanovka, U.S.S.R.	1962
Czechoslovakian Serotype			
Kol 42	*Ixodes ricinus*	Koliba, Czechoslovakia	1963
Kol 152	*Ixodes ricinus*	Koliba, Czechoslovakia	1963
Kol 156	*Ixodes ricinus*	Koliba, Czechoslovakia	1963
Lip 10	*Ixodes ricinus*	Lipovník, Czechoslovakia	1963
Lip 11	*Ixodes ricinus*	Lipovník, Czechoslovakia	1963
Lip 53	*Ixodes ricinus*	Lipovník, Czechoslovakia	1963
Lipovnik (Lip 91)	*Ixodes ricinus*	Lipovník, Czechoslovakia	1963
Tribeč	*Ixodes ricinus*	Tribeč Mountains, Czechoslovakia	1963

[a] Represents the year in which the isolate was collected in the field.

isolates were designated Kemerovo virus (Chumakov *et al.*, 1963; Libíková *et al.*, 1964). Additional virus isolates with biologic and antigenic properties similar to Kemerovo virus were isolated from *I. ricinus* ticks and small mammals in Czechoslovakia and from a migratory redstart, *Phoenicurus phoenicurus*, captured in Egypt (Libíková *et al.*, 1965; Grešíková *et al.*, 1965; Schmidt and Shope, 1971). These virus isolates constitute the currently recognized Kemerovo serocomplex within the Kemerovo serogroup.

In the Kemerovo serocomplex, the isolates are cross-reactive in serologic tests and they are difficult to distinguish by serologic criteria. Nevertheless, systematic and reproducible differences between selected virus strains from the geographic regions of Czechoslovakia and Siberia were demonstrated by complement-fixation and plaque reduction neutralization tests (Libíková and Casals, 1971; Libíková and Buckley, 1971). Thus, the Czechoslovakian and Siberian isolates have been regarded as two distinguishable serotypes (Libíková and Casals, 1971). The Egyptian EgAn 1169—61 bird isolate was a close serologic relative to the Siberian isolates and considered a Siberian serotype virus (Schmidt and Shope, 1971)

In this study, Kemerovo serocomplex viruses were examined by RNA--RNA blot hybridization and gene reassortment to determine their sequence and functional relatedness. Blot hybridization has been used to assess sequence relatedness among members of other *Orbivirus* serogroups and to identify serotype-specific genes for members (Bodkin and Knudson, 1985*b*, 1986, 1987; Gonzalez and Knudson, 1987*a*). Recently, gene reassortment *in vitro* has been correlated with hybridization data in an effort to

predict functional relatedness of virus isolates (Gonzalez and Knudson, 1987*b*). The taxonomic significance of these hybridization and reassortment data are discussed relative to the antigenic relationships of the Kemerovo serocomplex viruses.

Materials and Methods

Virus stocks and tissue culture. Viruses used in this study are listed in Table 1. The viruses were grown in BHK-21 cells (Gonzalez and Knudson, 1987*a*).

Plaque assay and in vitro reassortment. Virus stocks were titrated and reassortant progeny viruses were plated following a plaque assay procedure utilizing an agarosenutrient overlay (Buckley, 1974; Gonzalez and Knudson, 1987*b*). Briefly, viral suspensions containing the two parental virus stocks in varying multiplicity of infection (m.o.i.) ratios were prepared. Day-old BHK-21 cell monolayers grown in microtest plates (96 well) were infected with the viral suspensions, and the infected plate was incubated at 32 °C. After 24 hr incubation, the infected BHK-21 cells were harvested and frozen at − 70 °C. The m.o.i. for each well or mixed-virus ratio was determined by plaque assay titration of the parental viruses on the day of the reassortant experiment. The infected cell harvests were thawed, serially diluted, and plated in plaque assays. The plaques were picked, and progeny stocks were prepared for inoculation in a 24-well cluster plate. Viral RNA was extracted from infected BHK-21 cells, electrophoresed in a polyacrylamide gel and stained with ethidium bromide, and the dsRNA profiles were scored for the parental origin of the segments. The dsRNA of selected reassortants was end-labelled and electrophoresed in a polyacrylamide gel.

Agarose and polyacrylamide gel electrophoresis. Viral dsRNA isolated from virus infected BHK-21 cells grown in a well of a 24-well cluster plate (Travassos da Rosa *et al.*, 1984) was labelled, and the RNA was electrophoresed through either a 1% agarose gel or a Tris-glycine buffered 10% polyacrylamide gel (Bodkin and Knudson, 1985*b*). Polyacrylamide gels used for electrophoretic transfer of RNA were prepared and electrophoresed for 30 hr at 20 mAmps. The dsRNA in the gel was stained with ethidium bromide (5 μg/ml), visualized with UV light, and photographed. The selected labelled reassortants were electrophoresed for 20 hr at 10 mAmps in 10% PAGE gels which were prepared using 0.5 mm spacers and a shark's tooth comb.

Preparation of RNA probes. Virus was was extracted from infected cells (Ramig *et al.*, 1977). The dsRNA was extracted from the virus and precipitated (Gaillard and Joklik, 1982). The dsRNA was electrophoresed through low melting temperature agarose (Marine Colloids, Rockland, ME) to further purify the dsRNA (Bodkin and Knudson, 1985*b*). The dsRNAs which were used for probes were 3′ end-labelled with 1.48 MBq of [5′-^{32}P] pCp (Donis-Keller, 1979; Knudson, 1981; Gonzalez and Knudson, 1987*a*) and prepared for hybridization (Bodkin and Knudson, 1986).

RNA-RNA blot hybridization. Procedures used in the electrophoretic transfer of RNA and in the hybridization at $Tm_{(R\cdot R)}$-36 were described previously (Bodkin and Knudson, 1985*a*). Blots were washed at an equivalent effective temperature of hybridization (Gonzalez and Knudson, 1987*a*).

Results

Gel electrophoresis

The agarose profiles of selected dsRNA genomes of the Kemerovo serocomplex viruses were similar (Fig. 1). In contrast, these isolates exhibited unique polyacrylamide gel (PAGE) dsRNA profiles (Fig. 2). While the PAGE dsRNA profiles were distinct, the profiles of the Czechoslovakian isolates were more heterogeneous than the Siberian isolates. The original Tribeč isolate contained several genetic types (or genotypes) as evidenced by the appearance of twelve segments in its dsRNA PAGE profile (data not shown). This virus stock was plaque-purified, and four different geno-

Table 2. In Vitro Reassortment of Czechoslovakian and Siberian isolates

Minimum Number of Genes Distinguishable[a]	MOI Ratio (PFU/cell) (P1 : P2)	Observed Progeny (P1 : P2) (Reassortant)	Reassortant Genotype (Segment)[b]									
			1	2	3	4	5	6	7	8	9	10
			Cross: 75 X EgAn 1169 61									
6	18 : 18	5 : 2 : 11	X	L 75	X	EgAn	L 75	X	L 75	L 75	L 75	X
			X	L 75	X	EgAn	L 75	X	EgAn	L 75	L 75	X
			EgAn	EgAn	X	EgAn	EgAn	X	EgAn	L 75	EgAn	L 75
			L 75	L 75	X	L 75	L 75	X	EgAn	L 75	L 75	EgAn
			X	EgAn	X	EgAn	EgAn	X	L 75	L 75	L 75	X
			X	EgAn	X	L 75	L 75	X	L 75	L 75	L 75	X
			X	EgAn	X	L 75	L 75	X	L 75	L 75	L 75	X
			EgAn	L 75	X	EgAn	EgAn	X	EgAn	L 75	EgAn	L 75
			L 75	EgAn	X	EgAn	L 75	X	EgAn	L 75	EgAn	L 75
			X	EgAn	X	EgAn	L 75	X	L 75	L 75	L 75	X
			X	EgAn	X	L 75	L 75	X	L 75	L 75	L 75	X
			Cross: Tribeč (TRB) X Kol 152 (Kol)									
8	23 : 29	3 : 3 : 12	TRB	Kol	Kol	TRB	TRB	Kol	TRB	TRB	TRB	TRB*
			Kol	Kol	Kol	Kol	Kol	Kol	Kol	Kol	TRB	Kol*
			TRB	TRB	TRB	Kol	TRB	TRB	Kol	Kol	TRB	TRB*
			TRB	TRB	TRB	TRB	TRB	TRB	TRB	Kol	TRB	TRB*
			TRB	TRB	TRB	TRB	TRB	TRB	Kol	Kol	TRB	TRB*
			TRB	Kol	Kol	Kol	Kol	X	X	TRB	TRB	TRB
			TRB	Kol	Kol	Kol	Kol	X	X	Kol	TRB	TRB
			TRB	TRB	TRB	TRB	TRB	X	X	TRB	Kol	TRB
			TRB	TRB	Kol	TRB	TRB	X	X	TRB	TRB	TRB
			Kol	TRB	Kol	Kol	Kol	X	X	TRB	Kol	Kol
			TRB	TRB	TRB	TRB	TRB	X	X	TRB	Kol	TRB
			Kol	TRB	TRB	Kol	Kol	X	X	TRB	Kol	TRB

Table 2 continued

			Segment[b]									
			1	2	3	4	5	6	7	8	9	10
			Cross: L 75 X Tribeč (TRB)									
7	65 : 20	21 : 9 : 2	L 75	L 75	L 75	TRB	X	L 75	L 75	X	L 75	X
			L 75	L 75	L 75	TRB	X	L 75	L 75	X	L 75	L 75
	65 : 40	13 : 41 : 6	L 75	L 75	L 75	L 75	TRB	L 75	L 75	L 75	L 75	L 75
			TRB	TRB	TRB	TRB	TRB	TRB	TRB	TRB	L 75	TRB
			L 75	L 75	L 75	TRB	L 75	L 75	L 75	L 75	L 75	L 75
			L 75	L 75	L 75	L 75	L 75	L 75	L 75	L 75	L 75	TRB
			TRB	TRB	TRB	TRB	TRB	TRB	TRB	TRB	L 75	TRB
			L 75	L 75	TRB	TRB	L 75	L 75	L 75	L 75	L 75	TRB

[a] The number of segments which have a significantly different mobility when the two parental viruses are electrophoresed in polyacrylamide. Thus, it reflects the number of genes for which the parental origin could be determined in progeny viruses. This represents a minimum number because resolution was often improved using end-labelled dsRNA.

[b] The parental origin of each segment is indicated by a single letter or number abbreviation, and segments of undetermined origin are indicated by the letter X.

* Polyacrylamide gel profiles of reassortants indicated are shown in Fig. 5.

types of 10 dsRNA segments were found (Lavender, R., 1980, Yale University M.PH. Thesis; and unpublished data).

RNA-RNA blot hybridization

The EgAn 1169-61 probe hybridized strongly to the Siberian isolates, and it hybridized to a lesser extent with the Czechoslovakian isolates (Fig. 3 and data not shown for additional Czechoslovakian isolates). In contrast, the Tribeč probe hybridized weakly to members of the Siberian serotype isolates and strongly to the Czechoslovakian isolates (Fig. 4 and data not shown for additional Siberian isolates). One isolate, Lip 53, was variant gene 4 with respect to the Tribeč probe. In reciprocal hybridizations, gene 3 among the Siberian and Czechoslovakian serotype isolates hybridized with a weaker signal than did the other genes. The labelled Tribeč probe represented the predominant genotype (clone 1) in the Tribeč virus population. The four clones of Tribeč virus exhibited high conservation in their sequence by hybridization (data not shown).

Gene reassortment

Fifty percent of the progeny from the mixed infection (or cross) of the two Siberian serotype isolates, L 75 and EgAn 1169-61, were reassortants (Table 2), and 67% of the progeny from the cross between the two Czechoslovakian isolates, Tribeč (clone 1) and Kol 152, were reassortants (Table 2). Selected reassortants from the cross between the two Czechoslovakian isolates are presented in Figure 5. Gene reassortment was also demonstrated between the two isolates representing the Czechoslovakian and Siberian types. A difference was observed in the progeny from the cross between the Siberian isolate, L 75, and the plaque-purified Czechoslovakian isolate, Tribeč. For example, only eight reassortants (9%) were observed in progeny from this cross, and seven were single gene replacements. In contrast, more than half of the reassortant progeny from intra-typic crosses had multiple gene replacements (Table 2). While gene reassortment was demonstrated in both intra- and inter-typic crosses, the inter-typic reassortment yielded fewer reassortant progeny with only a few gene substitutions.

Discussion

The dsRNA agarose profiles of virus isolates within serogroups which have been examined are generally similar (Bodkin and Knudson, 1985*b*; Gonzalez and Knudson, 1987*a*). Although some isolates from different serogroups may also exhibit similar agarose profiles, agarose profiles are useful in the initial screening of new isolates. For example, the agarose profile of a new isolate may be compared to the profiles of the recognized *Orbivirus* serogroups, allowing an assignment of the new isolate to its likely serogroup. In contrast, dsRNA PAGE profiles for isolates within an *Orbivirus* serogroup are usually distinct (Bodkin and Knudson, 1985*b*, 1986; Gonzalez and Knudson, 1987*a*). The heterogeneity in dsRNA PAGE pro-

files within the serogroup reflects the extent of genetic diversity within the serogroup.

Isolates within each type of the Kemerovo serocomplex viruses exhibited similar dsRNA agarose profiles, and they were generally indistinguishable by serologic tests (Libíková and Casals, 1971; Libíková and Buckley, 1971) and by blot hybridization with one exception. Lip 53 was the only isolate which was distinguishable within the Czechoslovakian isolates by blot hybridization because its gene 4 was variant when compared to the other isolates. Nevertheless, the distinct dsRNA PAGE profiles for the Siberian and Czechoslovakian serotype isolates were indicative of genetic drift in both types with members of the Siberian type being relatively more homogeneous when compared to the Czechoslovakian isolates.

While the genes of Tribeč virus were conserved among the four plaque-purified clones by blot hybridization (data not shown), the presence of the different genotypes in the original virus stock may explain the "broad antigenic structure" observed for Tribeč in complement-fixation tests (Libíková and Buckley, 1971). Whether the original isolate contained the four different genotypes, or the presence of the four resulted from reassortment during passage of the virus stock is not known. These data, however, suggest that at least two genetic types were co-circulating in the Tribeč region.

In these hybridization experiments, light signals identify variant genes. At T_m(RNA)-36 hybridization conditions, sequences among variant genes approach the lower limit of 74% homology required for the formation of stable hybrids. While the two types cross-hybridized in all ten genes, the hybridization signals indicated that the sequence relatedness between types approached the lower limit of 74% sequence homology.

Although EgAn 1169-61 was isolated from a migratory redstart captured in Egypt in 1961, this isolate was indistinguishable from the Siberian isolates by serology, blot hybridization, and gene reassortment. Thus, the redstart may have acquired the virus in Siberia and this suggestion would be consistent with ornithological data on the summer and winter range of redstarts (Schmidt and Shope, 1971). Since the isolation of EgAn 1169-61 predates the Siberian isolates, Kemerovo virus may have been active in Siberia before its recorded date of isolation. Further, these data implicate migratory birds in the dispersal of the virus over vast distances.

I. persulcatus ticks, the vector of the Siberian isolates, and *I. ricinus*, the vector of the Czechoslovakian isolates, are many-host ticks which utilize a variety of vertebrate hosts including birds, small rodents, and mammals (James and Harwood, 1969). The potential for mixed infections in the same tick or vertebrate host, therefore, exists. Although the geographic distribution of the *I. persulcatus* and *I. ricinus* vector species may overlap, microclimatic conditions separate their ecological niches (James and Harwood, 1969). Contact between the two virus populations would be, at best, an infrequent event resulting from the chance movement of the vertebrate host of the tick. Thus, the Czechoslovakian and Siberian types may represent

two distinct and separate virus populations evolved from a recent common ancestor. While the contribution of inter-typic reassortment in introducing new genes to the population may be small, intra-typic reassortment could promote genetic diversity in the population by a continual shuffling of the drifted genes. The Kemerovo serocomplex within the Kemerovo serogroup, therefore, is characterized as two viral types which correlate with their geographic sites of isolation. When the barrier to inter-typic reassortment is removed as in gene reassortment *in vitro*, the types will interact genetically.

The Kemerovo serogroup is the only *Orbivirus* serogroup for which serocomplex designations have been established based on low level cross-reactivities in complementfixation tests. The bluetongue, epizootic haemorrhagic disease of deer (EHD), and Eubenangee viruses also cross-react at low levels in serologic tests (Borden *et al.*, 1971), and yet, they have been assigned to different serogroups. Intra- and inter-scrocomplex hybridization and gene reassortment data on the Kemerovo serogroup viruses are required to evaluate the taxonomic significance of the serocomplex designation relative to other *Orbivirus* serogroups.

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Explanation of Figures (Plates XLVII–LI):

Fig. 1. Autoradiogram depicting the profiles of representative isolates of the Czechoslovakian and Siberian serotypes in 1% agarose. Genomic RNA was end-labelled with [5′-^{32}P]pCp and electrophoresed through a 1% agarose gel. Lanes are from left to right reovirus 3 Dearing strain (Lane 1), R 10 (Lane 2), KM3 (Lane 3), L 75 (Lane 4) EgAn 1169–61 (Lane 5), Lip 91 (Lane 6), Tribeč (Lane 7), Kol 156 (Lane 8). Kol 152 (Lane 9), Lip 53 (Lane 10), and Lip 10 (Lane 11).

Fig. 2. Autoradiogram depicting the profiles of representative isolates of the Czechoslovakian and Siberian serotypes in a 10% polyacrylamide gel. Genomic RNA was end-labelled with 148 kBq [5′-^{32}P]pCp and electrophoresed through a Tris-glycine buffered 10% polyacrylamide gel. Lanes are from left to right: reovirus 3 Dearing strain (Lane 1), R 10 (Lane 2), KM 3 (Lane 3), L 75 (Lane 4), EgAn 1169–61 (Lane 5), Lip 91 (Lane 6), Tribec (Lane 7), Kol 156 (Lane 8), Kol 152 (Lane 9), Lip 53 (Lane 10), and Lip 10 (Lane 11).

Fig. 3. Autoradiogram depicting the hybridization of the 3′ end-labelled genomic dsRNA probe EgAn 1169–61 to a Zeta-Probe membrane (Bio-Rad, Richmond, CA) containing profiles of members of the Siberian serotype, Lip 91, and Tribeč. The lanes are from left to right: uninfected BHK-21 control (Lane 1), reovirus 3 Dearing strain (Lane 2), R 10 (Lane 3), R 9 (Lane 4), R 54 (Lane 5), KM 3 (Lane 6), L 75 (Lane 7), EgAn 1169–61 (Lane 8), Lip 91 (Lane 9), and Tribeč (Lane 10).

Fig. 4. Autoradiogram depicting the hybridization of the 3′ end-labelled genomic dsRNA probe Tribeč to a Zeta-Probe membrane containing the profiles of members of the Czechoslovakian serotype and EgAn 1169–61. Lanes are from left to right uninfected BHK-21 control (Lane 1), reovirus 3 Dearing strain (Lane 2), Kol 156 (Lane 3), Kol 152 (Lane 4), Kol 42 (Lane 5), Lip 53 (Lane 6), Lip 11 (Lane 7), Lip 10 (Lane 8), Lip 91 (Lane 9), Tribeč (Lane 10), and EgAn 1169–61 (Lane 11).

Fig. 5. Autoradiogram depicting the resolution of the dsRNA genomes of Kemerovo serocomplex viruses and selected reassortant progeny viruses. Labelled dsRNA was electrophoresed through Tris-glycine buffered 10% polyacrylamide gels. Each reassortant virus profile is flanked by

the two parental profiles for ease of comparison. The genotypes of the reassortant are listed in Table 2. Lanes are from left to right reovirus 3 Dearing strain (Lane 1), Tribeč (Lane 2), reassortant Tribeč X Kol 152 (Lane 3), Kol 152 (Lane 4), reassortant Tribeč X Kol 152 (Lane 5), Tribeč (Lane 6), reassortant Tribeč R Kol 152 (Lane 9), Tribeč (Lane 10), reassortant Tribeč X Kol 152 (Lane 11), and Kol 152 (Lane 12).

Book Review

Vaccines 87

Robert M. Chanock, Richard A. Lerner, Fred Brown, and Harold Gingsberg (Eds): Vaccines 87: Modern Approaches to New Vaccines. Prevention of AIDS and other Viral, Bacterial and and Parasitic Diseases. Cold Spring Harbor Laboratory, 1987, 461 pages, price $95.—

The specific profile of the edition (Vaccine 87) is related with significant and valuable extension of the presented problems on all aspects of AIDS; on this deadly disease occupying attention of scientific groups and of all human beings in the world. It is the very positive general aspect of the edition, that paying the attention to AIDS problems it is also concentrating on several most dynamic problems in immunology and pathology, both of basic molecular and direct utilitarian value.

Out of the large number of over eighty presentations it is worth of mentioning the problems which are especially attached by several authors, and with great accumulation of experimental data and/or ideas opening next future possibilities:

- immunological role of viral, microbiological and parasitic structures;
- genetic monitoring of immunologically active components and prospective applicability;
- relations and development of pathological processes as a consequence of viral and microbial infections, including pre- and postexposure specific behaviour in HIV-infected persons;
- Antitumour immunity including gene manipulations controlled and in natural conditions, also in virus participation;
- aspects of epidemiology, therapy and prophylactic treatments especially in molecular categories.

Summing up the book may be very recommended both for research workers involved in basic and in utilitarian problems, for scientists and practioners interested in new methods applied as well in theoretical considerations, for virologists, immunologists, immunochemists, clinicians and epidemiologists.

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M. Kańtoch, Warsaw