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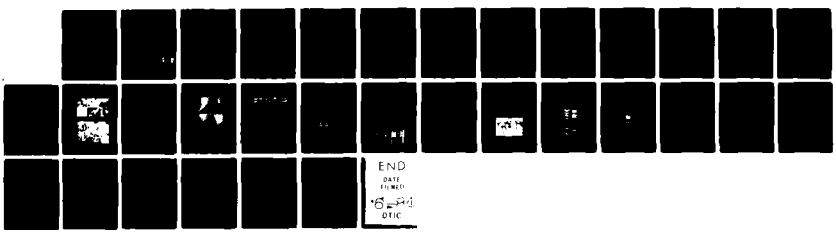

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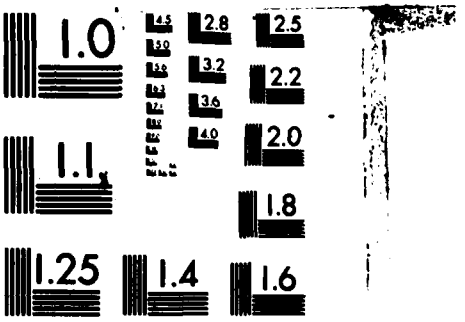

GENETIC AND MOLECULAR STUDIES OF THE PHLEBOTOMUS FEVER GROUP OF VIRUSES

ANNUAL REPORT

DR. DAVID H.L. BISHOP
AUGUST 1982

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In the current reporting period we have made the following progress relevant to the aims of the contract.

We have:

(1). Undertaken heterologous recombination analyses involving dual infections with wild-type PT and SFS viruses, PT and CHG viruses, PT and ICO viruses, or PT and KAR viruses. We have not detected recombinant viruses. Similar studies with different PT virus varieties yielded recombinant viruses.

(2). Performed analyses of the homologous and heterologous virus interference capabilities of PT (or ICO) virus preparations that contain DI particles. These studies did not detect heterologous virus interference with KAR, SFS, SFN, CHG, CDU or ITP phleboviruses under conditions in which homologous PT (or ICO) interference was demonstrated. We conclude that these heterologous viruses are distantly related to PT (or ICO) viruses, and (by this test) not genetically compatible.

(3). ~~Current data~~ indicate that at the structural level the tick-borne uukuviruses (Uukuvirus genus, Bunyaviridae family) closely resemble phleboviruses. The 3' end sequences of the RNA species, and virion polypeptides and RNA sizes of several non-assigned tick-borne members of the family (including the Congo strain of Crimean-Congo hemorrhagic fever virus) have been characterized and found to conform to neither the phleboviruses/uukuviruses, nor to the mosquito-borne bunyaviruses. Because of their unique characteristics these other viruses have been constituted into a new genus, the Nairovirus genus. Member viruses have 3' AGAGUUUC.. end sequences, in contrast to those of bunyaviruses (3' UCAUCACAUGA...), or Uukuniemi virus (3' UGUGUUUUCUGG...), or PT, or BUE, phleboviruses (3' UGUGUUUCG..). The nairovirus viral RNA species and virion polypeptide sizes also differ significantly from those of uukuviruses, phleboviruses, or bunyaviruses.

(4). ~~Characterization of~~ characterized the unassigned tick-borne Dhori (DHO) virus, a virus that heretofore has been considered as a possible member of the family, has been undertaken in order to determine if this virus has the structural attributes of phleboviruses (or members of other defined genera of the family). The data obtained indicate that DHO virus does not conform to any other member of the family, rather it resembles an orthomyxovirus.

and (5). In order to clone, sequence and thereby determine the complete coding content the RNA species of PHL group viruses, the Principal Investigator spent a 10 month sabbatical in the laboratory of Dr. G.G. Brownlee in Oxford, England. During this time he cloned and sequenced complete copies of influenza A/RR/8/34 RNA segments 2 and 3, as well as bunyavirus SSH S RNA species.

I. SUMMARY

The reporting period represents the last 12 of the 54 months since the inception of the project. During the previous reporting periods we have: (1) characterized the major structural components of several Phlebotomus fever (PHL) group viruses (Robeson, et al, 1979; Bishop et al., 1980: Cash et al., 1981), (2) isolated temperature sensitive (ts), conditional lethal, mutants of Punta Toro (PT) virus and categorized them by complementation-recombination analyses, (3) demonstrated the feasibility of using oligonucleotide fingerprinting to distinguish both Dengue (DEN) virus serotypes using cloned Aedes albopictus cells to produce labeled virus (Vezza et al., 1980) and Rift Valley fever (RVF) virus isolates obtained from various parts of Africa (Uganda, Rhodesia and South Africa, including the recent isolates from Egypt; Cash et al., 1981), (4) undertaken electron microscopic analyses on various phleboviruses; (5) analyzed, by radioimmune assays (RIA), the antigenic relationships of Karimabad (KAR), Chagres (CHG) and sandfly fever virus, Sicilian, (SFS) glycoproteins (G1, G2) and nucleocapsid (N) proteins (Klimas et al., 1981), (6) initiated genetic analyses on Icoraci (ICO) phlebovirus, (7) characterized at the biochemical level the major viral induced intracellular antigens of KAR, Aguagate (AGU), SFN, CDU, BUE and SFS viruses; we have also characterized alternate isolates of PT virus that have different virulence markers (the viruses were originally obtained from various parts of Panama and were made available through the courtesy of USAMRIID personnel); (8) obtained reassortant intertypic PT viruses from prototype PT virus and an alternate PT strain; (9) used such reassortants to demonstrate that the phlebovirus S RNA codes for the viral N polypeptide and, from biochemical studies, shown that the S RNA also codes for a viral induced nonstructural polypeptide (NS_c); (10) analyzed the reassortant virus capabilities of PT, ICO and BUE phleboviruses; (11) developed a convenient assay for demonstrating phleboviral interactions that may be useful in screening for genetically compatible phleboviruses (the procedure involves assays of the interference capabilities using ICO and PT DI virus stocks); (12) characterized DI preparations of ICO and PT virus stocks, demonstrating that they have new RNA species representing deleted L RNA segments; and (13) characterized the 3' end RNA sequences of several phleboviruses. A synopsis of the results of the prior reporting periods is presented below:

(1). Analyses of the major structural components of PHL group viruses have established that KAR, PT, CHG, Candiru (CDU), ICO, PHL 3, Itaporanga (ITP), Buenaventura (BUE), and the Sicilian and Naples sandfly fever (SFS, SFN) viruses, each has a tripartite RNA genome and three major structural polypeptides (two external glycoproteins, G1 and G2, 57-69x10^3 daltons, and an internal, nucleocapsid associated, protein N, 20-24x10^3 daltons). Both the mol. wt. of the major structural polypeptides and the virion RNA segments of the different PHL group viruses can be easily distinguished from those of bunyaviruses (e.g. the California serogroup, CAL, viruses) and, to various extents (depending on the virus), from each other. Tryptic peptide analyses of ^{35}S and ^{3}H methionine labeled G1 and G2 polypeptides of KAR have established that these two polypeptides have distinguishable sequences. The behaviour of reduced KAR G1 and G2 polypeptides on polyacrylamide gel electrophoresis was found to be aberrant by comparison with unreduced preparations.

(2). Twenty four ts mutants of PT virus were isolated following mutagenesis of the wild-type virus by growth in the presence of 5-fluorouracil. Recombination assays with these mutants have allowed them to be categorized into 3 non-overlapping

recombination groups (Group I has 8 ts mutants; Group II has 5 ts mutants; Group III has 1 ts mutant - so strictly is not a group - and 1 ts mutant is probably a double mutant).

(3). Oligonucleotide fingerprint analyses of prototype DEN 1, 2, 3 and 4 viruses involving both single and mixed coelectropherograms of ribonuclease T1 digests of ^{32}P labeled 40S viral RNA samples showed that each has a unique fingerprint that is easily distinguished from that of another prototype DEN virus (these procedures have now been adopted by WRAIR personnel for their Dengue virus studies). Evidence was obtained indicating that the 5' sequence of DEN 2 RNA is m7GpppAmpXp...

(4). Electron microscopic analyses of the surface structure arrangments of PHL group viruses have been undertaken and indicate that unlike bunyaviruses, but like uukuviruses (2 of the other Bunyaviridae genera) PHL group viruses have a particular "chimney-pot" arrangements of their surface glycoproteins as evidenced by glutaraldehyde fixation prior to staining.

(5). Competition RIA assays using iodinated nucleocapsid and glycoprotein preparations of KAR virus, KAR antisera and the competing antigens of KAR, CHG and SFS indicate that the KAR and SFS N polypeptides have more antigenic determinants in common than have the N polypeptides of KAR and CHG. Also the KAR and SFS G polypeptides share more antigenic determinants than the G polypeptides of KAR and CHG. No shared antigenic determinants were detected between KAR and vesicular stomatitis virus (VSV), or the bunyaviruses La Crosse (LAC), Oriboca (ORI), or Bunyamwera (BUN) viruses.

(6). ICO virus has been adapted to produce plaques in Vero cell monolayers at 39.8°C. The original virus stock, which gave 10^5 plaques at 35°C, gave none at 39.8°C. By high temperature passaging of the virus stock and cloning at 39.8°C, a stock of ICO virus has been derived which gives 8×10^4 PFU at 35°C and 2.3×10^3 PFU at 39.8°C.

(7). The viral induced polypeptides, (immune precipitated from infected cell extracts by their homologous antisera), have been characterized for KAR, AGU, BUE, CDU, SFS and SFN viruses. Several alternate isolates of PT virus obtained from the eastern, central and western regions of Panama have been cloned and their L, M and S RNA species fingerprinted and shown to be distinct/related to each other (depending on the virus isolate).

(8). By dual wild-type virus crosses, reassortant viruses have been obtained between prototype PT and an alternate PT isolate (PT-ada). The genotypes of 2 such reassortants were shown by fingerprint analyses to be the L/M/S combinations of ada/PT/ada and PT/PT/ada.

(9). The N polypeptides of PT-ada and prototype PT viruses can be distinguished by tryptic peptide analyses allowing the N polypeptides of the reassortants to be

(9). The N polypeptides of PT-ada and prototype PT viruses can be distinguished by tryptic peptide analyses allowing the N polypeptides of the reassortants to be analyzed. By such analyses it has been shown that their S RNA codes for the virion N polypeptide. From S mRNA in vitro translation analyses, this conclusion has been confirmed, with results that also indicate that the S mRNA directs the synthesis of a non-structural polypeptide (NS_S) that appears to be unrelated by primary sequence to the viral N polypeptide.

(10). Dual wild-type virus infections have failed to detect reassortant virus formation between PT and BUE viruses, or BUE and ICO viruses.

(11). An assay of the homologous and heterologous virus interference capabilities using by stocks of PT, or ICO, viruses that contain their respective defective interfering (DI) virus has been developed. Using such assays, homologous virus interference has been demonstrated. No heterologous virus interference has been detected from ICO DI and PT coinfections, or ICO DI and BUE, or ICO DI and snowshoe hare (SSH) bunyavirus coinfections.

(12). The stocks of PT and ICO DI virus have been shown to contain new RNA species not found in virus stocks lacking DI virus. Fingerprint analyses have shown that for both viruses the new RNA species represent deletion derivatives of their respective L RNA species.

(13). The 3' end sequence of the 3 RNA species of both PT and BUE viruses have been shown to be like those of the uukuvirus, Uukuniemi, i.e., $_{HO}UUC$.

In the current reporting period we have continued certain of the projects described above (as outlined in last year's proposal). Also we have established conditions for cloning and sequencing phlebovirus genes (see this year's proposal). In part, this was achieved during the sabbatical year of the Principal Investigator in Oxford, England. The current reporting period therefore includes some of these sabbatical studies.

(14). Heterologous recombination attempts involving dual infections with wild-type PT and SFS viruses, PT and CHG viruses, PT and ICO viruses, or PT and KAR viruses have failed to yield recombinant viruses. Similar studies with different PT virus varieties (see last year's Report) yielded recombinant viruses.

(15). Further analyses of the homologous and heterologous virus interference capabilities of PT (or ICO) virus preparations that contain DI particles have failed to detect heterologous virus interference with KAR, SFS, SFN, CHG, CDU or ITP phleboviruses under conditions in which homologous PT (or ICO) interference was demonstrated. We conclude that these heterologous viruses are distantly related to PT (or ICO) viruses, and (by this test) not genetically compatible.

(16). Current data indicate that at the structural level the tick-borne uukuviruses (Uukuvirus genus, Bunyaviridae family) closely resemble phleboviruses. The 3' end sequences of their RNA species, and the virion polypeptides and RNA sizes are similar, as are their viral-induced non-structural polypeptides. The structural components of several non-assigned tick-borne members of the family have been

characterized and found to conform to neither the phleboviruses/uukuviruses, nor to the mosquito-borne bunyaviruses. Because of their unique characteristics these other viruses have been constituted into a new genus, the Nairovirus genus. Member viruses have 3' AGAGUUUC.. end sequences, in contrast to those of bunyaviruses (3' UCAUCACAUGA...), or Uukuniemi virus (3' UGUGUUUUCUGG...), or PT, or BUE, phleboviruses (3' UGUGUUUCG..). The nairovirus viral RNA species and virion polypeptide sizes also differ significantly from those of uukuviruses, phleboviruses, or bunyaviruses.

(17). Characterization of the unassigned tick-borne Dhori (DHO) virus, a virus that heretofore has been considered as a possible member of the family, has been undertaken in order to determine if this virus has the structural attributes of phleboviruses (or members of other defined genera of the family). The data obtained indicate that DHO virus does not conform to any other member of the family, rather it resembles an orthomyxovirus.

(18). In order to clone, sequence and thereby determine the complete coding content the RNA species of PHL group viruses (see Proposal), the Principal Investigator spent a 10 month sabbatical in the laboratory of Dr. G.G. Brownlee in Oxford, England. During this time he cloned and sequenced complete copies of influenza A/RR/8/34 RNA segments 2 and 3, as well as bunyavirus SSH S RNA species.

The results of the current reported period are therefore as follows:

II. REPORT

A. Introduction.

The objectives of this contract are to determine the infection strategy of members of the PHL group viruses (Phlebovirus genus, Bunyaviridae) and develop protocols for vaccine development. Since this group of exotic viruses includes agents that cause illnesses in epidemic proportions (e.g., RVF) in different parts of the world, and therefore are of military importance, our objectives relate to the question of deriving vaccines that will be useful in protecting military personnel against virus infections.

To realize these objectives, part of our research has been directed towards determining if reassortant PHL viruses can be used for preparing vaccines. We have sought to determine which PHL group viruses are genetically compatible (capable of producing reassortant viruses with other members of the genus, or members of other genera of the family). Studies conducted in our laboratories have demonstrated that certain virus serotypes of the CAL serogroup of the Bunyavirus genus (another genus of the Bunyaviridae) are capable of producing reassortant viruses with other CAL group members (Gentsch & Bishop, 1976; Gentsch, et al., 1977; Gentsch et al., 1979; Rozhon et al., 1981). However, not all CAL group bunyavirus crosses yield reassortants, indicating that their gene pool is limited. Also no crosses between viruses representing different bunyavirus serogroups (e.g., CAL and the Group C, or Bunyamwera, serogroups) have yet yielded reassortant viruses.

Results reported previously from this contract have documented that PHL group viruses have a tripartite RNA genome with RNA segments that are designated L, M and S.

From dual PT ts mutant, or wild-type, virus coinfections recombinant PT viruses have been obtained. However, as reported below, we have so far failed to obtain reassortants between different PHL group viruses. Although not all PHL virus combinations have been tested, this negative result suggests (but does not prove) that there are restrictions at the genetic level between different PHL group viruses. Our DI interference studies (see below) also suggest that there are restrictions on genetic interactions between many of the PHL group viruses. The impact of this conclusion on our future studies will be discussed at the end of this Report.

An alternate stategy for vaccine development is to identify and prepare adequate quantities of the viral antigen(s) that elicit protective antibodies. Central to this issue is knowledge of the RNA segment coding assignments (see Gentsch & Bishop, 1978, 1979). Some of our studies have been directed towards defining the RNA segment coding assignments of phleboviruses. We have already demonstrated that the S RNA segment codes for the N and NS_S polypeptides. Proof that the M RNA codes for the glycoproteins (as shown for bunyaviruses) has not yet been obtained, and remains a goal of our continuing efforts. Studies by USAMRIID personnel with monoclonal antibodies indicate that the glycoproteins interact with neutralizing sera. Therefore it is important to determine which PHL viral RNA species codes for the glycoproteins.

This present report describes both analyses of the genetic potential of selected PHL group viruses, and molecular studies of unassigned members (or possible members) of the family that are of military importance. Also included are the results obtained by the Principal Investigator while on sabbatical in Oxford, U.K., since they concern cloning techniques that are proposed for future analyses of the coding strategies of PHL group viruses.

The report covers items 14-18 listed in the Summary. It will not detail the results of items 1-13 that were given in previous reports.

B. Results from this Reporting Period.

(14). Recombination between heterologous phleboviruses

Rather than produce ts mutants of each of the available phleboviruses and perform heterologous virus genetic recombination analyses beween their ts mutants we have elected to perform dual wild-type virus coinfections and seek reassortant viruses among selected progeny virus clones. Another reason not to use ts mutants stems from our recent observations (in studies with bunyaviruses), that ts mutants often have silent, attenuating, mutations in RNA segments that do not have the change specifying the ts phenotype (Rozhon et al., 1981). Such attenuating mutations may be transferred to "wild-type" progeny of dual ts mutant virus crosses causing these progeny to be attenuated. Since one of the objects of our studies has been to determine which viral gene products are the major determinants of virulence, attenuating mutations would complicate our analyses.

We documented in last year's Report the feasability of using dual wild-type infections to recover reassortant intertypic PT viruses. Provided care is taken to use multiplicities of infection (MOI) that in single virus infections yield equivalent numbers of progeny viruses, reassortant viruses can be obtained in high enough frequencies from dual virus infections to be detected by oligonucleotide fingerprinting the cloned progeny viruses.

We have employed two approaches to investigate the question of reassortment between different phlebovirus serotypes. In one approach we used dual wild-type virus infections (PT x SFS, PT x CHG, PT x ICO, PT x KAR) in non-selective infections to produce reassortants; in the other we used PT ts mutants in coinfections with wild-type KAR virus (assaying the progeny at the non-permissive temperature) to screen for reassortants.

For the dual wild-type virus infections Vero cells were infected with prototype PT virus (MOI=10) and the alternate phlebovirus (MOI=1-10) and incubated at 35° - each coinfection representing experimental conditions which preliminary single virus infections indicated should have yielded essentially equivalent numbers of both viruses by 36 hr post-infection. In all cases the yields from the dual virus infections were, within experimental error, similar to the expected values and represented about a 1000-fold increase over the desorbed inoculum viruses (i.e., those detected after washing the infected cells 2 hr. post-adsorption). Some 20 randomly selected clones of progeny virus were recovered from each dual virus infection. The polypeptides induced by the individual cloned progeny in infected Vero cells were then analyzed and compared to those induced by the parental viruses. This was undertaken to take advantage of differences in the N polypeptide migration characteristics of the different phleboviruses (see previous Reports of this contract). Not only did the results provide evidence that both parental viruses grew to equivalent levels, but also they provided data on their protein phenotypes. This was of value in discriminating against the occassional contaminated progeny clone (that on subsequent recloning yielded viruses of either one or the other N protein phenotype), and for recovering progeny representative of the 2 parental phenotypes. From each of the coinfections 8 progeny clones were then selected. Four of the progeny were recovered that had a PT N protein phenotype and 4 that had the N protein phenotype of the alternate phlebovirus. The composite L+M+S RNA fingerprints were then obtained for all 8 viruses. In every case fingerprint analyses demonstrated that the cloned progeny that induced a PT-type N polypeptide had an L/M/S genotype of PT/PT/PT. Likewise the fingerprints of the viruses that had the N protein phenotype of the other virus were identical to the L/M/S genotype of the alternate phlebovirus.

The second strategy we used to investigate the question of whether different phleboviruses could produce reassortants took advantage of the fact that wild-type PT virus produces plaques at both 35° and 39.8°, whereas wild-type KAR virus and ts mutants of PT virus give plaques at 35°, but not at 39.8° (see previous year's Report). Assuming that the inability of the KAR virus to produce plaques at 39.8° is not a property of the gene products of each of its RNA segments, we assayed for reassortant viruses that would grow at 39.8° because they lacked the segment coding for the conditional lethal mutation of the PT ts mutant (i.e., ones that possessed a compensating KAR viral RNA and its gene products).

The results of crosses of wild-type KAR and ts mutants of PT representing Group I (ts 1, ts 8), or Group II (ts 2, ts 6), or Group III (ts 8) indicated that no more wild-type progeny viruses were obtained from the mixed virus infections than from the single virus infections (i.e., viruses representing revertants, or virus that leaked through the plaque assays; %R less than 0.01 %).

In conclusion, no evidence for recombination between PT and SFS, PT and CHG, PT and ICO, or PT and KAR, has been obtained.

(15). The use of interference as a screening procedure for genetically compatible phleboviruses.

Although reassortant, intertypic, PT viruses have been obtained from dual infections involving prototype PT and an alternate PT isolate, (see previous Report), our inability to detect recombination between PT and SFS, CHG, ICO or KAR viruses suggests that the gene pool of these viruses may be limited. To define the PHL viruses that contribute to a common gene pool using the procedures outlined in the previous section is a time consuming process. We have therefore used an alternate method to screen for genetically interactive phleboviruses.

As outlined in last year's Progress Report, preparations of PT and ICO viruses have been obtained that contain defective interfering particles as judged by (1) interference of alternate homologous virus stocks and (2) the presence of a deletion (L) RNA species in viral RNA preparations.

Heterologous virus interference analyses were undertaken in order to determine if by this test the viruses could be shown to be genetically interactive. We recognise that such tests even if positive do not prove that the viruses would be capable of reassorting their RNA segments, however we have proceeded on the assumption that the lack of heterologous interference under conditions of demonstrable homologous interference probably means that the viruses are not genetically interactive.

We have experienced difficulty in obtaining high titered PT, BUE and ICO virus stocks upon successive undiluted passage. Such a phenomenon is typical of the amplification of dl virus (Huang & Baltimore, 1977). For example, a second passage ICO virus derived from an initial virus clone gave titers in Vero cells of 2×10^8 pfu/ml, while a fifth passage ICO virus stock yielded 9×10^5 plaques/ml. Likewise, a second passage PT virus derived from an initial virus clone yielded 1×10^8 pfu/ml while a sixth passage virus stock gave only 5×10^5 pfu/ml.

In continuation of the initial ICO x ICO dl, PT x ICO dl and SSH x ICO dl coinfection analyses reported last year, we have undertaken additional heterologous virus infections using PT, or ICO, dl virus preparations. The results are recorded in Tables I and II. In every case the input multiplicity of infection (MOI) was adjusted to 0.1 pfu/cell, however no estimate of the actual number of infecting dl viral particles present in the dl preparations was made.

As documented in last year's report the greatest level of homologous ICO virus interference was obtained with the highest concentration of ICO dl virus used for the coinfections (MOI = 0.1). Higher MOI values could not be employed due to the low titers of the dl virus stocks. Likewise, the highest levels of PT virus interference were obtained with the highest levels of dl PT virus that were used.

The yields of virus from the various coinfections, as a function of the infection timecourses, r ached plateau levels by 48-60 hr post-infection. They did not decrease by 72 hr post-inf tion.

TABLE I

Heterologous and homologous virus interference by ICO dl preparations.

	Infecting virus	Inoculum MOI	Yield at 60 hr. post-infection
A.	ICO dl	0.1	4.0×10^{4}
B.	ICO	0.1	1.1×10^{8}
C.	PT	0.1	2.1×10^{8}
D.	KAR	0.1	2.0×10^{8}
E.	SFS	0.1	4.2×10^{7}
F.	SFN	0.1	5.4×10^{7}
G.	CHG	0.1	8.0×10^{7}
H.	CDU	0.1	6.2×10^{7}
I.	ITP	0.1	4.0×10^{7}
b.	ICO + ICO dl	(as above)	4.5×10^{5}
c.	PT + ICO dl	(as above)	1.6×10^{8}
d.	KAR + ICO dl	(as above)	1.7×10^{8}
e.	SFS + ICO dl	(as above)	3.0×10^{7}
f.	SFN + ICO dl	(as above)	5.0×10^{7}
g.	CHG + ICO dl	(as above)	8.0×10^{7}
h.	CDU + ICO dl	(as above)	5.6×10^{7}
i.	ITP + ICO dl	(as above)	5.0×10^{7}

TABLE II

Heterologous and homologous virus interference by PT dl preparations.

	Infecting virus	Inoculum MOI	Yield at 60 hr. post-infection
A.	PT dl	0.1	5.0×10^{5}
B.	PT	0.1	1.0×10^{8}
C.	ICO	0.1	1.4×10^{8}
D.	KAR	0.1	4.6×10^{8}
E.	SFS	0.1	3.0×10^{7}
F.	SFN	0.1	4.3×10^{7}
G.	CHG	0.1	6.9×10^{7}
H.	CDU	0.1	1.0×10^{8}
I.	ITP	0.1	6.0×10^{7}
b.	PT + PT dl	(as above)	4.0×10^{6}
c.	ICO + PT dl	(as above)	1.2×10^{8}
d.	KAR + PT dl	(as above)	5.0×10^{8}
e.	SFS + PT dl	(as above)	3.1×10^{7}
f.	SFN + PT dl	(as above)	5.2×10^{7}
g.	CHG + PT dl	(as above)	6.0×10^{7}
h.	CDU + PT dl	(as above)	8.9×10^{7}
i.	ITP + PT dl	(as above)	5.3×10^{7}

We conclude from these experiments that ICO and PT viruses are not capable of interacting at the genetic level with each other, or with KAR, SFS, SFN, CHG, CDU, or ITP viruses.

In addition to the homologous virus interference documented above, we have been able to obtain intertypic virus interference using different PT virus varieties. As reported in last year's Progress Report we have characterized alternate PT virus isolates made available by USAMRIID personnel and originally obtained at various times from either eastern Panama (PT-Bayano, 1975; PT-Bayano, 1976; PT-Adamas, 1974), or western Panama (PT-Aguacate, 1970). Each virus stock, representing a second, or third passage from a virus clone, was used in single, or dual virus coinfections with the dI PT virus stock and the yields of infectious virus determined. The results are shown in Table III.

TABLE III

Intertypic homologous virus interference by PT dI preparations.

	Infecting virus	Inoculum MOI	Yield at 60 hr. post-infection
A.	PT dI	0.1	4.0×10^5
B.	PT-proto., 1966	0.1	1.0×10^8
C.	PT-Bayano, 1975	0.1	2.5×10^8
D.	PT-Bayano, 1976	0.1	7.0×10^7
E.	PT-Adamas, 1974	0.1	3.2×10^8
F.	PT-Aguacate, 1970	0.1	9.4×10^7
b.	PT-proto + PT dI	(as above)	5.7×10^5
c.	PT-Bay.'75 + PT dI	(as above)	4.7×10^5
d.	PT-Bay.'76 + PT dI	(as above)	2.0×10^6
e.	PT-Ada.'74 + PT dI	(as above)	5.6×10^5
f.	PT-Agu.'70 + PT dI	(as above)	8.0×10^5

We conclude from these studies that prototype PT virus dI preparations are capable of interfering with the productive infection capabilities of alternate PT virus isolates. This is in agreement with the demonstration of intertypic PT recombinant virus formation we reported last year for some of the viruses.

(16). Analyses of alternate members of the Bunyaviridae family that are of interest for vaccine development.

We have continued our studies of unassigned members of the Bunyaviridae family in order to determine whether they can be categorized at the biochemical level to the Phlebovirus, Uukuvirus, or Bunyavirus genera. In particular, we have investigated viruses that are of interest for vaccine development. Included among such agents is the tick-borne Crimean-Congo hemorrhagic fever virus that each year is responsible for human disease and death in eastern Europe and the Middle East (Bishop & Shope, 1979; Hoogstraal, 1979).

Previous studies have shown that, other than vector preferences, the tick-borne Uukuniemi virus and members of the Phlebovirus genus share structural and morphogenic attributes. In view of the large number of unassigned tick-borne viruses that have heretofore been considered as probable/possible members of the family, we have obtained representative isolates and characterized them at the biochemical level.

Viruses representing six antigenic groups of arthropod borne viruses (Crimean-Congo hemorrhagic fever (CCHF), Nairobi sheep disease (NSD), Qalyub (QYB), Sakhalin (SAK), Dera Ghazi Khan (DGK), and Hughes (HUG) serogroups), previously categorized as bunyavirus-like viruses, or ungrouped, were obtained and grown into virus stocks. Molecular studies of the virion RNA and viral polypeptides were undertaken (Clerx et al., 1981) with representative members of the different virus serogroups (Hazara, HAZ, and Congo, CON, viruses, CCHF group; Dugbe, DUG, virus, NSD group; QYB, Omo and Bandia, BDA, viruses, QYB group; Avalon, AVA, virus, SAK group; DGK and Abu Minah, AM, viruses, DGK group; and HUG virus, HUG group).

Shown in Fig. 1 are growth curves of CON, DUG, BDA and QYB viruses.

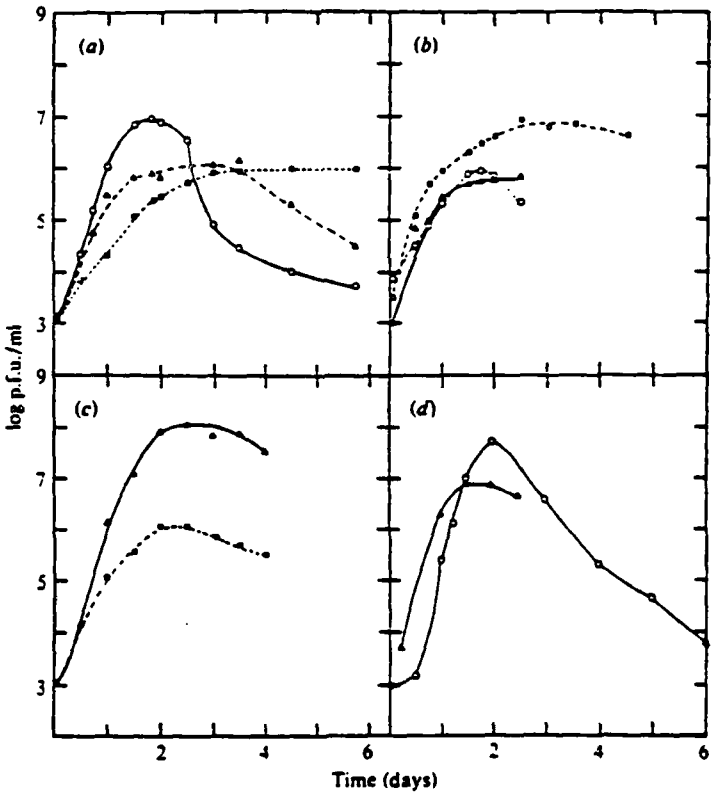

Fig. 1. Growth curves of the nairoviruses (*a*) Congo Ib Ar 10300 (CON) in Vero (□---□), BHK (▲——▲) and CER (○——○) cells at 33 °C; (*b*) Dugbe Ib Ar 1792 (DUG) in Vero (□——□), BHK (○---○) and CER (▲——▲) cells at 33 °C; (*c*) Bandia RV 611 (BDA) in BHK (▲——▲) and Vero (□---□) cells at 35 °C; (*d*) Qalyub Ar 370 (QYB) in BHK cells (▲——▲), and Hazara JC 280 (HAZ) in CER cells (○——○), both at 35 °C. Ninety-five to ninety-eight percent confluent monolayers of BHK-21, Vero or CER cells were infected with 10% baby mouse brain suspensions. Approx. m.o.i.s in BHK and CER cells were 0·5 (CON), 3 (DUG), 0·5 (BDA), 0·2 (QYB) and 0·1 (HAZ). The m.o.i. in Vero cells was approx. 3 times lower. CON, DUG, BDA and HAZ viruses were assayed on CER cells; QYB virus was assayed on Vero cells.

In Fig. 2 are shown electron micrographs of HAZ, DUG, QYB, BDA and HUG viruses. The bars represent 100 nm. In Fig. 3 are presented electron micrographs of thin sections of nairovirus infected BHK21 cells (HAZ, BDA, QYB), or Vero cells (DUG), again the bars represent 100 nm.

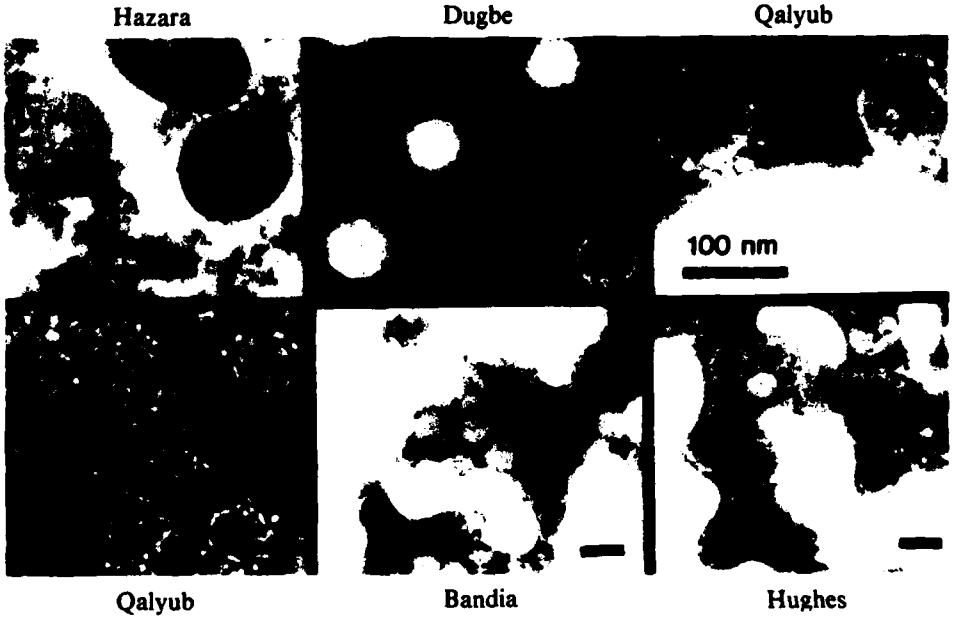

Fig. 2. Electron micrographs of HAZ, DUG, QYB, BDA and HUG viruses. Bars represent 100 nm.

Fig. 3. Electron micrographs of thin sections of HAZ, QYB, and BDA virus infected BHK21 cells, or DUG infected Vero cells 48 hr post-infection. Bars represent 100 nm.

In Fig. 4 are presented profiles of the viral RNA species of HAZ, QYB, BDA, DUG and SSH (the latter is a bunyavirus that was included as a reference). The data obtained for seven of these viruses are summarized in Table IV.

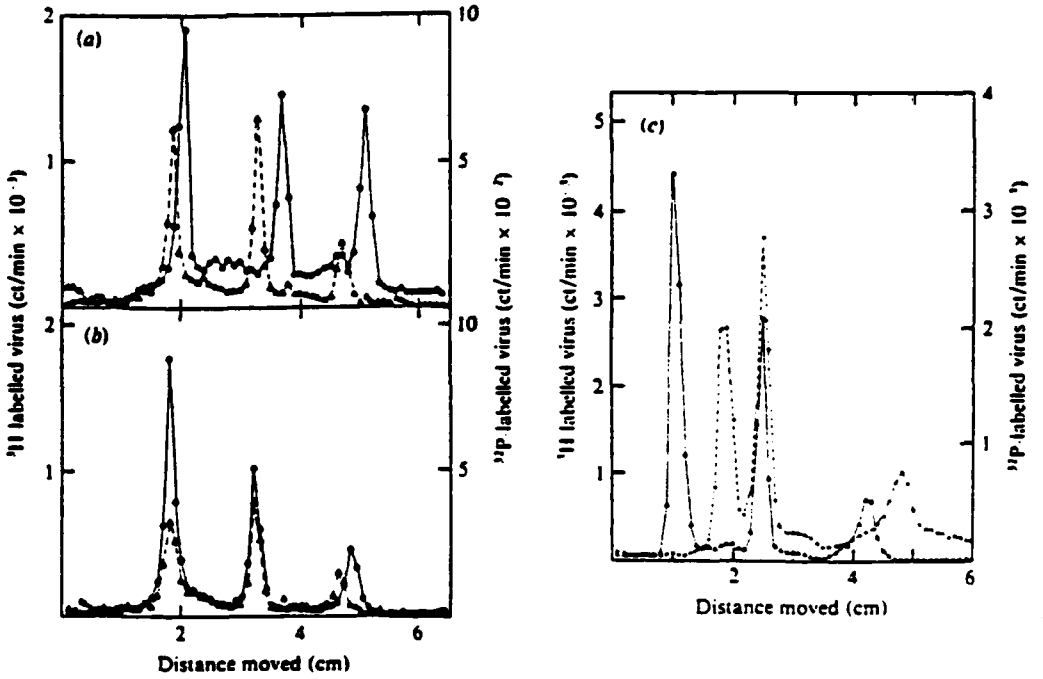

Fig. 4. Electrophoresis of RNA species recovered from purified virions of HAZ, QYB, BDA, DUG and SSH. RNA species were resolved by electrophoresis in 1% slab agarose gels (*a*, *b*) according to Wiesiander (1979), or in 2·4% polyacrylamide gels (*c*) as described by Roy & Bishop (1972). (*a*) ^{3}H-labelled Hazara (●——●). ^{32}P-labelled Qalyub (▲——▲); (*b*) ^{3}H-labelled Bandia (●——●). ^{32}P-labelled Qalyub (▲——▲); (*c*) ^{3}H-labelled Dugbe (▲——▲), ^{32}P-labelled Snowshoe hare (●---●).

Table N *Mol. wt.* ($\times 10^{-6}$) *estimations of nairovirus RNA species*[*]

Virus serogroup	Virus	RNA species L	M	S	Gel system
Crimean–Congo haemorrhagic fever	HAZ	4·3	1·5	0·6	Agarose
Dera Ghazi Khan	AM	4·6	1·5	0·6	Agarose
Hughes	HUG	4·9	1·6	0·6	Agarose
Nairobi sheep disease	DUG	4·5	1·9	0·6	Polyacrylamide
Qalyub	QYB	4·7	1·7	0·7	Polyacrylamide
	QYB	4·7	1·9	0·7	Agarose
	BDA	4·7	1·9	0·6	Agarose
Sakhalin	AVA	4·1	1·5	0·6	Agarose

[*] Mol. wt. estimates were obtained in either 2·4% polyacrylamide cylindrical gels (DUG, QYB), or 1% agarose slab-gels (AM, AVA, BDA, HAZ, HUG, QYB) using the published mol. wt. of SSH viral RNA (Gentsch *et al.*, 1977) and eukaryotic ribosomal RNA species as standards in both gel systems. QYB M RNA migrates more slowly in agarose gels than in polyacrylamide gels when compared to SSH M RNA.

In Fig. 5 are presented the oligonucleotide fingerprints of the individual and composite L, M and S RNA species of BDA virus, providing proof that the 3 RNA species have distinct sequences.

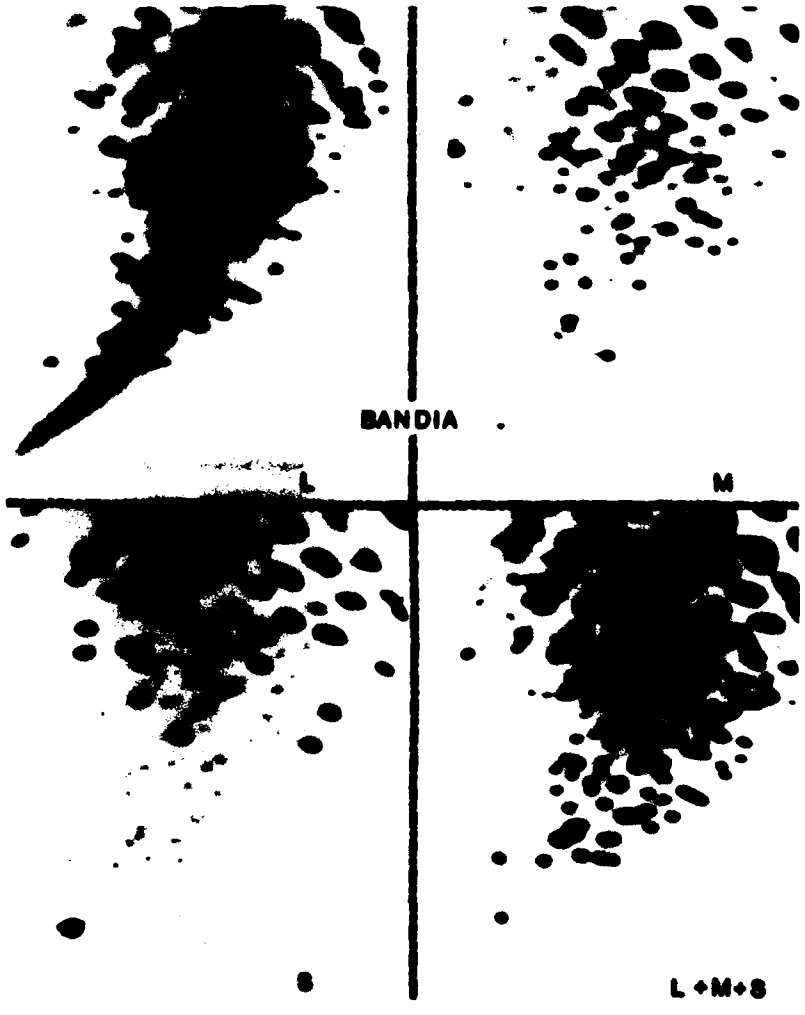

Fig. 5 The oligonucleotide fingerprints of BDA L, M, S and L+M+S RNA species.

The virus particle polypeptides of five nairoviruses (AM, BDA, DUG, HAZ and QYB) were analyzed by slab polyacrylamide gel electrophoresis with representative results shown in Fig. 6 and the data summarized in Table V. For each virus preparation three major polypeptides were identified. Although similar data has not been developed for AM and HAZ viruses, it was concluded from the preferential incorporation of ^{3}H-glucosamine and their susceptibility to removal by protease as shown previously for QYB virus (Clerx & Bishop, 1981), that two of the viral polypeptides (G1,G2) are glycoproteins that form the surface spikes on virus particles.

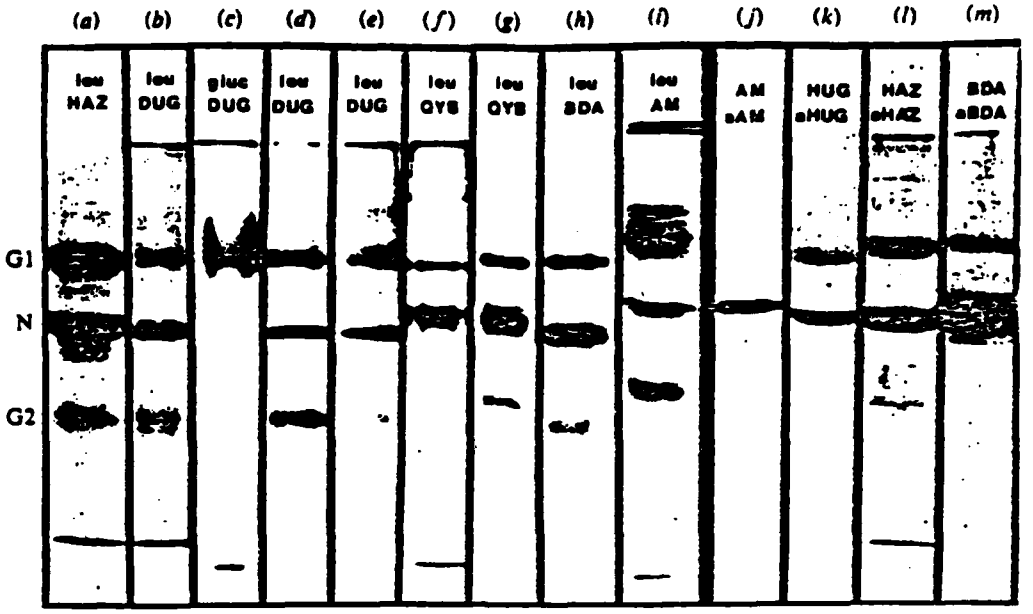

Fig. 6. Electrophoresis of nairovirus polypeptides in 10% SDS–polyacrylamide slab gels. [^{3}H]leucine (leu)- or [^{3}H]glucosamine (gluc)-labelled polypeptides from purified virus (lanes *a* to *i*) were electrophoresed as described in Methods and visualized by fluorography (Bonner & Laskey, 1974). Lanes (*a*) and (*b*) were adjacent lanes from one gel, lanes (*c*) to (*f*) came from a second gel, lanes (*g*) and (*h*) from a third, and lane (*i*) from a fourth gel. To obtain the immune precipitates of pelleted extracellular material (lanes *j* to *m*), homologous virus antisera were used for the AM virus (aAM), HUG virus (aHUG), HAZ virus (aHAZ), and BDA virus (aBDA) extracts. The AM sample was run in the same gel as lane (*i*). The other three samples were run on a separate gel.

Table V. *Mol. wt.* ($\times 10^{-3}$) *estimations of the major viral polypeptides of nairoviruses*[*]

Virus serogroup	Virus	Virus particle polypeptide G1	N	G2
Crimean–Congo haemorrhagic fever	HAZ	75	50	30
Dera Ghazi Khan	AM	84	53	35
Nairobi sheep disease	DUG	76	49	30
Qalyub	QYB	75	54	40
	BDA	75	51	33

[*] Mol. wt. were estimated by comparing the electrophoretic mobility of virus proteins with marker proteins (BDH) using 10% SDS–polyacrylamide slab gels (see also Fig. 6).

For several nairoviruses (CON, DGK, AVA, HUG) we were unsuccessful in obtaining sufficient quantities of purified virus for polypeptide analyses using sucrose, or glycerol tartrate, gradient purification procedures. In order to identify the principal viral polypeptides of CON, DGK and HUG viruses, the materials present in extracellular fluids were pelleted and immune precipitated with homologous viral antiserum. The results obtained for AM, BDA, HUG and HAZ viruses are shown in Fig. 6), and the data are summarized in Table VI for AM, BDA, CON, DGK, HAZ, HUG and QYB viruses. Attempts to characterize AVA viral polypeptides by this method were not successful. As exemplified by the results shown for AM and BDA viruses, not all the viral polypeptides that were identified in preparations of purified virus, were precipitated by the immune precipitation procedure. Presumably this reflects the absence of the respective antibodies in the antisera used.

Since for AVA virus we were not able to identify its viral polypeptides by purifying virus, or by immune precipitation of extracellular material, an alternate approach, involving immune precipitation of viral induced material from infected cells, was used. Shown in Fig. 7 are the homologous antisera-immune precipitated extracts of AM, or AVA, or BDA, or CON, or DGK, or DUG, or HAZ, or HUG, or QYB infected cells. The data are summarized in Table VII. For each of the viruses analyzed, including AVA virus, a major polypeptide, with an estimated mol. wt. of 48-54x10^3 daltons, was specifically immune precipitated by the respective homologous antiserum.

Table VI *Mol. wt. (x 10^{-3}) estimations of the major nairovirus-induced polypeptides identified in immune precipitates of pelleted extracellular material**

Virus serogroup	Virus	Immune-precipitated extract of extracellular material: G1	N	G2
Crimean–Congo haemorrhagic fever	CON	75	50	
	HAZ	75	50	30
Dera Ghazi Khan	DGK	75	50	
	AM		53	
Hughes	HUG	72	49	
Qalyub	BDA	75	51	
	QYB	75	54	40

* Mol. wt. were estimated (as in Table 2) for labelled polypeptides immune-precipitated from extracellular pelleted material by homologous virus antisera (see also Fig. 6).

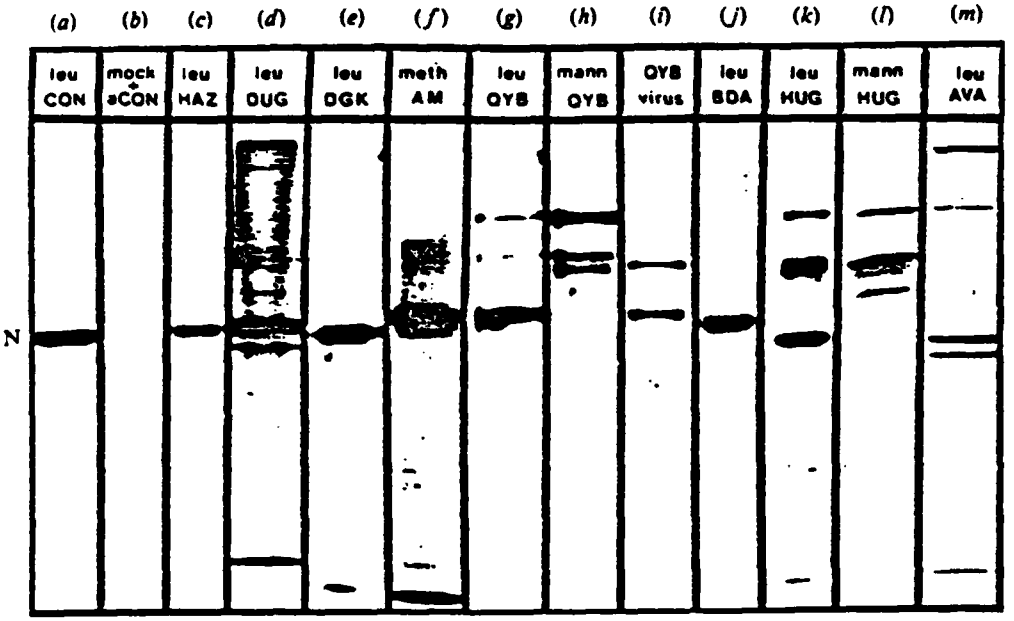

Fig. 7. Electrophoresis of immune precipitates of nairovirus-infected cells. [^{3}H]leucine (leu)-, [^{35}S]methionine (meth)- or [^{3}H]mannose (mann)-labelled virus-infected cell extracts, or mock-infected cell extracts, were incubated with homologous viral mouse antisera or ascitic fluids, and the immune complexes recovered as described previously for QYB virus (Clerx & Bishop, 1980). Lanes (*a*) to (*c*) came from one gel, lanes (*d*) to (*f*) and lane (*j*) each came from separate gels, and lanes (*g* to *i*) came from a single gel, as did lanes (*k*) to (*m*). As indicated in the text, a [^{3}H]leucine-labelled QYB virus preparation was run alongside the QYB virus-infected cell extracts.

Table V. *Mol. wt. ($\times 10^{-3}$) estimations of the major nairovirus-induced polypeptides identified in immune precipitates of infected cell extracts**

Virus serogroup	Virus	Immune-precipitated cell extract: G1	N	G2	Other
Crimean–Congo haemorrhagic fever	CON		50		
	HAZ		50		
Dera Ghazi Khan	DGK		50		
	AM		53		
Hughes	HUG	72	49		115, 78
Nairobi sheep disease	DUG		49		
Qalyub	QYB	75	54		115, 84
	BDA	75	51		
	OMO	75	54		
Sakhalin	AVA		48		

* Mol. wt. were estimated (as in Table 2) for the major labelled polypepatides immune-precipitated (Clerx & Bishop, 1981) by homologous antisera from infected cell extracts (see also Fig. 7). The 115 x 10^3 and 78 x 10^3 to 84 x 10^3 mol. wt. polypeptides are intracellular virus-specified glycopolypeptides as shown by [^{3}H]mannose incorporation studies.

Other than for QYB and HUG virus infected cells, the glycosylated viral polypeptides were not easily identified in the immune precipitates of infected cells, especially when using extracts from cells which were labelled for prolonged periods of time. However, for each of the viruses, coelectrophoresis (in cylindrical gels) of immune precipitated ^{35}S-methionine labelled cell extracts with immune precipitated ^{3}H-glucosamine, or ^{3}H-mannose, labelled infected cell extracts, demonstrated the presence of a virus specified glycosylated polypeptide with the same migration as the viral G1 polypeptide (see Tables V-VII), as well as another, 5-10x10^3 daltons larger, glycopolypeptide. With the exception of AVA virus, for which we do not have similar data, this pattern of intracellular glycosylated polypeptides was consistently observed for all the infected cell extracts analyzed.

We have used immunoprecipitation to demonstrate serologic relationships between representative members of the six Nairovirus serogroups. The results obtained are exemplified in Fig. 8, and summarized in Table VIII. Not all the possible combinations of antisera and virus induced antigens have been analyzed due to difficulties in obtaining sufficient viral antigens. However, the results clearly confirm the serologic relationships between nairoviruses representing the six serogroups, and lack of serologic relationships with selected members of the Phlebovirus genus (PT), Bunyavirus genus (LAC) and Uukuvirus genus (GA).

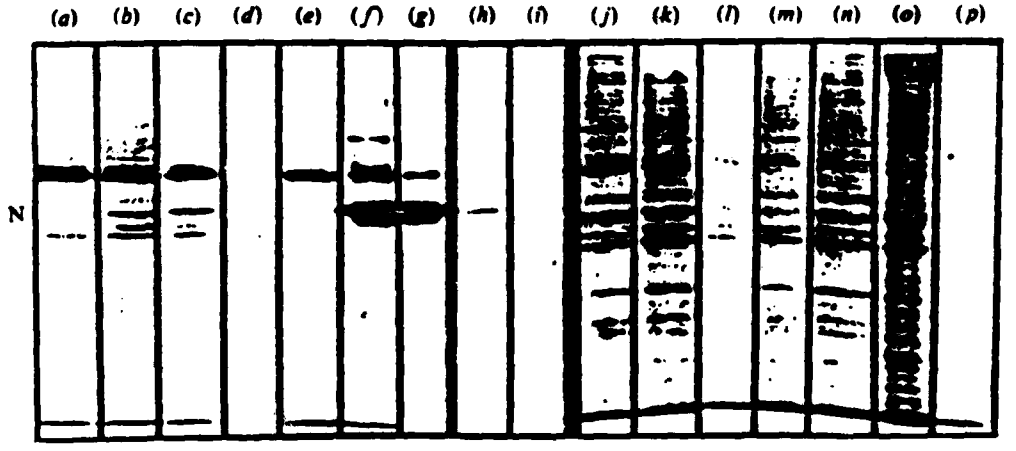

Fig. 8. Electrophoresis of immune precipitates of [^{3}H]leucine-labelled QYB virus-infected BHK cell extracts precipitated with antisera against (*a*) CON, (*b*) HAZ, (*c*) DUG, (*d*) DGK, (*e*) AM, (*f*) QYB, (*g*) BDA, (*h*) HUG, (*i*) SOL and (*j*) AVA nairoviruses, and (*k*) DHO 1-611313, (*l*) LAC, (*m*) PT and (*n*) GA viruses. The other two lanes are the total, untreated, QYB-infected cell extract (*o*) and an uninfected cell extract (*p*) treated with anti-QYB serum. Samples were run on two different gels [lanes (*a*) to (*i*) and lanes (*j*) to (*p*) respectively].

Table VIII *Cross-reactivities between nairoviruses as determined by gel electrophoresis of heterologous antiserum–antigen immune precipitates*[a]

Antiserum raised against		Cross-reactive antigen in extracts of cells infected with									
		HAZ		DGK		AM		QYB		BDA	
Serogroup	Virus	N	G1	N	G1	N	G1	N	G1	N	G1
Crimean–Congo haemorrhagic fever	CON	+	−	−	−	−	−	−	+	−	−
	HAZ	+	+	−	+	+	−	+	−	+	−
Dera Ghazi Khan	DGK	−	−	+	+	+	−	−	−	−	−
	AM	−	−	+	−	+	+	−	−	−	−
Hughes	HUG	−	−	+	+	−	−	+	−	+	−
	SOL	−	−	−	+	−	−	−	−	−	−
Nairobi sheep disease	DUG	+	−	−	+	−	+	+	+	+	−
Qalyub	QYB	−	−	−	+	+	+	+	−	+	+
	BDA	−	−	−	−	−	−	+	+	+	+
Sakhalin	AVA	−	−	−	−	+	−	+	+	+	−
Others (DHO. LAC. PT. GA)		−	−	−	−	−	−	−	−	−	−

[a] The cross-reactive N or G1 viral polypeptides identified by heterologous antiserum immune precipitation of [^{3}H]leucine- or [^{35}S]methionine-labelled infected cell extracts (see also Fig. 8) are indicated by a (+). No G2 polypeptide was immune-precipitated from any infected cell extract.

In agreement with a recent study of QYB virus (Clerx & Bishop, 1981), and, in part, an earlier report on DUG virus (David-West 1974), the results of these molecular analyses indicate that nairoviruses have: (1) three virion RNA species (large, L, medium, M, and small, S) with apparent mol. wt. of 4.1-4.9x10^6(L), 1.5-1.9x10^6(M) and 0.6-0.7x10^6(S); (2) a 48-54x10^3 dalton nucleocapsid (N) polypeptide; and (3) two external glycopolypeptides, 72-84x10^3 daltons (G1) and 30-40x10^3 daltons (G2). Cross-immune precipitation analyses have confirmed that these viruses share antigenic determinants and are antigenically distinct from representative members of the Bunyavirus, Phlebovirus and Uukuvirus genera (Bunyaviridae). The data obtained has been used to justify the formation of a new genus in the Bunyaviridae family, named the Nairovirus genus (Bishop, et al., 1980).

Studies undertaken by Parker and Hewlett (1981) have established that the 3' end sequences of Uukuniemi L, M, and S RNA species are: 3'UGUGUUUUCUGGAG. Studies we have undertaken with PT and BUE phlebovirus L, M and S RNA species indicate that they each have the sequence 3' UGUGUUUCG. Analyses of the 3' end sequences of 7 nairoviruses indicate that they have quite different 3' end sequences. The 3' ends of QYB virus are AGAGAUUCUUUA... (L and M), AGAGAUUCUGCC... (S); those of BDA are AGAGAUUCUC... (L), AGAGAUCCCCU... (M), and AGAGACNGCGUC... (S); for HUG and AVA viruses they are AGAGUUUCUUU... (L, M, and S); for HAZ they are AGAGUUUCUNU... (L, M and S); for DUG they are AGAGUUUCUNU... (L), AGAGUUUCUGU... (M), and AGAGUUUCUUU... (S); and for AM virus they are AGAGUUUCAUU... (L and S), and AGAGUUUCANU... (S) (Clerx-van Haaster et al., 1982). The observation that the 3' end sequences of nairoviruses are distinct from those of uukuviruses, or phleboviruses, as well as those of bunyaviruses (generally, UCAUCACAUGA..., Clerx-van Haaster et al., 1982), agree with their categorization into a different genus in the Bunyaviridae family.

(17). Analyses of Dhori virus.

Based on data obtained by Dr. Chumakov's group in Moscow, DHO virus was initially considered to be morphologically and biochemically similar to Crimean-Congo hemorrhagic fever virus, although antigenically distinct (Bishop et al., 1981). Because no antigenic relationship could be demonstrated between DHO virus and members of the newly formed Nairovirus genus, we undertook to characterize the virus further. The virion RNA species and polypeptides of various DHO virus isolates have been studied and the conclusion drawn that this virus is totally unlike any of the accepted members of the Bunyaviridae family, rather it resembles an orthomyxovirus.

Dhori virus isolates have been recovered from ticks collected in India (Andersen & Casals, 1973), ticks, birds and mosquitoes in the USSR (Butenko & Chumakov, 1971), and ticks collected in Egypt (Williams et al., 1973) and Portugal (Filipe & Casals, 1979). Electron micrographs of thin sections of DHO virus infected Vero cells (Fig. 9), reveal virus particles budding from cell surface plasma membranes and pleomorphic viral particles in interstitial spaces. Characteristically members of the Bunyaviridae bud at intracellular membranes and not at the surface of infected cells (Bishop & Shope, 1979). The latter is the preferred site for budding of ortho/paramyxoviruses, rhabdoviruses and oncornaviruses. Productive infections of BHK21, Vero, LLC-MK2, BS-C1 and $CV_5$1 cells by DHO viruses have been obtained with progeny virus titers of the order of 10^5-$6x10^8$ pfu/ml. However, no allantoic, or amniotic infection of embryonated eggs has been detected, nor has chick erythrocyte hemagglutination been observed.

Fig. 9 Interstitial spaces between DHO virus infected Vero cells showing virus budding from cell surface plasma membranes and accumulating in interstitial spaces.

When the viral RNA species of DHO virus were analyzed by gel electrophoresis 6 size classes of RNA were initially identified, as exemplified in Fig. 10. The size ranges of the DHO viral RNA species broadly correspond to those of influenza A/WSN virus, although there are evident differences. Under alternate electrophoretic conditions (e.g., in acid-urea gels) the slowest migrating DHO viral RNA species can be resolved into 2 distinct species.

Fingerprint analyses show that the 7 DHO RNA species have different fingerprints and lack a poly A sequence. Analyses of the 3' terminal sequences of several of the DHO viral RNA segments indicate that they have common end sequences, generally: 3' UC$^{G}_{A}$UUNUU$^{G}_{A}$UUC$^{G}_{A}$UCA (where N is A, or G, or U). This sequence differs from those of bunyaviruses, nairoviruses, or phlebo/uukuviruses, but in some respects is similar to those of orthomyxoviruses (influenza A: 3' UCGYUUUCGUCC, influenza B: 3' UCGUCUUCGC$^{U}_{G}$UC, and influenza C: UCGUYUUCGUCCC, where Y is U or C).

Fig. 10 The viral RNA species of DHO and influenza A/WSN resolved by electrophoresis in 3% polyacrylamide - 7M urea.

DHO virus infections of Vero, or BHK21, cells are sensitive to the presence of actinomycin D. At 0.1 ug actinomycin D per ml of infected BHK21 cell culture fluids, DHO virus yields are decreased by 99%; at 5 ug/ml they are decreased by 99.9%. Also virus yields are sensitive to the presence of α-amanitin. In both of these respects DHO virus resembles an orthomyxovirus.

The DHO viral polypeptides resolved by polyacrylamide gel electrophoresis also are totally unlike those of accepted members of the Bunyaviridae. In Fig. 11 (left 2 lanes) are shown reference sera-immune precipitates of cell extracts obtained after infection by prototype DHO virus, or an alternate Portugese DHO isolate. In the right 2 lanes of Fig. 11 are shown the polypeptides of extracellular virus before, or after immune precipitation. The slowest migrating immune precipitated virion polypeptide is the only one that incorporates ^{3}H-glucosamine. In many respects (e.g., the size, and type) the viral and viral-induced polypeptides resemble those of orthomyxoviruses. However, no antigenic relationship has been detected between DHO virus and influenza viruses as judged by unsuccessful attempts to immune precipitate DHO infected cell extracts by reference influenza A, B, or C antisera.

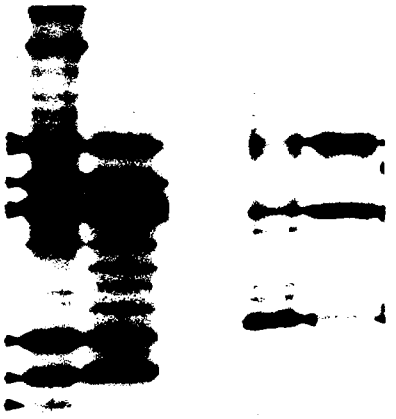

Fig. 11. DHO polypeptides. Shown in lane 1 (right to left) are reference sera-immune precipitates of prototype DHO virus infected BHK21 cells, immune precipitates of DHO POTi461 virus infected BHK21 cells and, lanes 3 and 4, viral polypeptides before, or after, immune precipitation.

In summary, we conclude that DHO virus does not resemble any of the accepted members of the Bunyaviridae. As a tick-borne virus it would be unique for an orthomyxovirus. However all the structural attributes point to it being a member of that family of segmented genome RNA viruses.

(18). Cloning viral RNA species.

The Principal Investigator spent 10 months on sabbatical leave in the laboratories of Dr. G.G. Brownlee, Oxford, during the current reporting period. One purpose of selecting Dr. Brownlee's laboratory was to learn some of the procedures of cloning viral RNA so that they could later be applied to viruses of interest to our laboratory (see Proposal). Since many of the viruses we work on are exotic to the U.K., and would require special permission, it was more convenient (and appropriate to the host laboratory) to work on questions of interest to Dr. Brownlee. Therefore the time was spent cloning and sequencing complete copies of influenza A/NT60/68 RNA segments 2 and 3 (Bishop et al., 1982a,b) as well as the S RNA species of SSH bunyavirus (Bishop et al., 1982c) for which permission was eventually obtained. Note, the bunyavirus S RNA sequencing was supported by this contract since the NIH bunyavirus grant held by the Principal Investigator ended Dec. 31, 1981 and was not renewed until July 1 1982.

The stategy employed to sequence the SSH S viral RNA species was to synthesis an oligonucleotide complementary in sequence to the first 18 residues of SSH S RNA species and use it to make a cDNA copy. The complete cDNA copy was then recovered after resolution of the products on a 3% polyacrylamide-7 M urea gel, tailed with poly dA, back primed with oligo dT, and cloned into the unique PVU II site of pBr322. Clones containing the viral DNA sequence were identified by hybridization to short copy cDNA made from the viral RNA using the original primer and limiting amounts of dATP, and sequenced by Maxam and Gilbert chemical digestion procedures using end-labelled, strand-separated, restriction fragments. Details of the procedures employed have recently been published (Bishop et al., 1982a,b,c) and consequently will not be reiterated. The sequencing strategy used to analyze both strands of the SSH DNA insert is shown in Fig. 12.

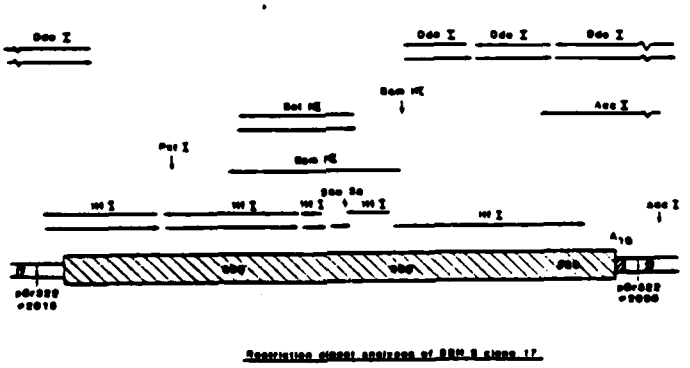

Fig. 12. Sequence analyses of SSH S clone 17. The restriction enzyme digestion products (enzyme names over the arrows e.g. Hfl = Hinf I) used to sequence the clone were either strand separated (arrow pairs of opposite polarity), or recut with an alternate enzyme (vertical arrow in line with a horizontal arrow e.g. Acc I product recut with Bam HI). The orientation of the viral complementary DNA in the pBr 322 vector is indicated as is the stretch of the 16 adenylic acid residues at the 3' end of the clone presumably originating from the cDNA polyA tailing.

The sequence of the cloned DNA, depicted as the viral complementary (plus sense strand), and its encoded large primary gene products are given in Fig.13.

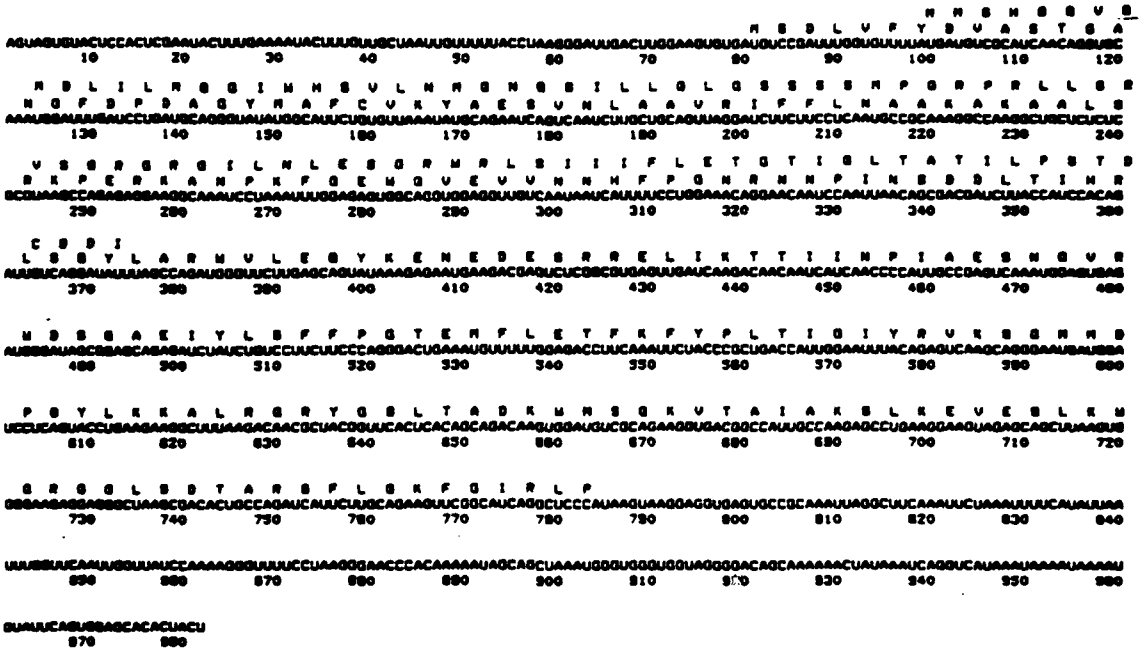

Fig. 13. The nucleotide and amino acid sequences deduced for SSH S viral RNA species. The RNA sequence, written as the complementary (plus sense) strand, was deduced from analyses of a DNA copy cloned in pBr322 (S clone 17), in addition to direct RNA and dideoxy sequence data (9,10). The nucleotides are numbered below their respective residues. Amino acids of polypeptides (coded by contiguous open reading frames and initiated by methionine codons) are centered over the corresponding nucleotide triplets.

From the analyses of the cloned DNA, and earlier SSH S direct RNA sequencing analyses (Clerx-van Haaster et al., 1982) it was deduced that the SSH S RNA is 982 nucleotides long (3.3×10^5 daltons) with complementary 5' and 3' ends. The viral RNA (negative sense strand) has a base composition of 30.5%U, 25.8%A, 24.9%C and 18.7%G. In the viral complementary (plus sense) RNA there are 2 overlapping open reading frames initiated by methionine residues. The first reading frame codes for a 235 amino acid polypeptide (mol. wt., 26,768) with the composition shown in Table IX. The composition of the gene product of the second reading frame (92 amino acids, mol. wt., 10,478) is given in Table X. .

Table IX. Amino acid composition of the 235 amino acid SSH S RNA gene product.

Ala	(A)	21	Leu	(L)	20
Arg	(R)	15	Lys	(K)	18
Asn	(N)	13	Met	(M)	6
Asp	(D)	12	Phe	(F)	14
Cys	(C)	1	Pro	(P)	10
Gln	(Q)	8	Ser	(S)	14
Glu	(E)	15	Thr	(T)	10
Gly	(G)	16	Trp	(W)	5
His	(H)	2	Tyr	(Y)	10
Ile	(I)	12	Val	(V)	13

Table X. Amino acid composition of the 92 amino acid SSH S RNA gene product.

Ala	(A)	1	Leu	(L)	14
Arg	(R)	7	Lys	(K)	0
Asn	(N)	3	Met	(M)	6
Asp	(D)	3	Phe	(F)	1
Cys	(C)	1	Pro	(P)	3
Gln	(Q)	12	Ser	(S)	12
Glu	(E)	2	Thr	(T)	5
Gly	(G)	5	Trp	(W)	2
His	(H)	2	Tyr	(Y)	0
Ile	(I)	10	Val	(V)	3

Genetic and molecular studies of SSH virus have established that the S RNA codes for the viral nucleoprotein, N, as well as a nonstructural protein NS_S (Bishop & Shope, 1979; Gentsch & Bishop, 1978; Cash et al., 1979; Fuller & Bishop, 1982). A review of the S RNA sequence reveals that in the viral complementary RNA there is only one continuous open reading frame of sufficient size (234 amino acids, 26.8×10^3 daltons) to code for N. The N polypeptide recovered from virions, or from infected cell extracts, has an estimated size in SDS polyacrylamide gels of 21×10^3 daltons (Gentsch et al., 1977). There is no available information on the sequence of SSH S mRNA species so that it is not known whether there are spliced S mRNA species derived, as in the case of certain mRNA species of DNA viruses, or influenza orthomyxovirus, from non-contiguous regions of the genome. However, it has been shown that, unlike influenza virus, bunyaviruses do not require a cell nucleus for developing a productive infection (Pennington, et al., 1977), nor is the synthesis of their mRNA species particularly sensitive to actinomycin D, or a-amanitin (Vezza et al., 1979). Preliminary amino acid and tryptic peptide analyses of SSH N protein agree with the postulate that N represents the gene product of the 235 amino acid open reading frame (Fuller & Bishop, unpublished data). However, whether the mature N protein has conserved the amino and carboxy terminal amino acid sequences of the primary gene product is not known.

The recent analyses which established that the SSH S RNA codes for NS_S, also demonstrated that N and NS_S do not share either arginine, or leucine, labelled tryptic

peptides (Fuller & Bishop, 1982). Lysine, although an efficient precursor of N, does not appear to be incorporated into NS_S. By contrast, methionine is efficiently incorporated into both N and NS_S.

Preliminary peptide sequence analyses of NS_S indicate that it has a tryptic peptide that has the sequence LLSR (Fuller & Bishop, unpublished data). The only place such a peptide is predicted from the primary RNA sequence is in the viral complementary RNA sequence in the second reading frame (i.e. read from nucleotide residues 231-242).

If it is correct that N and NS_S are coded by overlapping reading frames then the question of the identity of the mRNA species responsible for their synthesis is raised. No direct analyses of the S mRNA sequences have been reported so that it is not known whether one mRNA species functions as a template for the synthesis of both gene products, or whether there are distinct mRNA species for each product. In this connection, it will also be important to determine exactly where translation of N and NS_S initiate. In addition to later positions, there are methionine codons at residues 80-82 and 149-151 (first reading frame) and 99-101, 102-104, 123-125, 138-140 and 168-170 (second reading frame).

C. Summary of Progress Report.

The research supported by this contract has shown that representative PHL group viruses (Phlebovirus genus) have a tripartite RNA genome consisting of 3 unique RNA species (L, M and S). The L and M RNA species have mol. wts. like those of Bunyavirus and Uukuvirus genus members. Like uukuviruses, the S RNA species of phleboviruses is significantly larger than that of bunyaviruses. Members of all three of the above genera have a major $20\text{-}24 \times 10^3$ dalton nucleocapsid protein N. Our analyses have shown that the size ranges of phlebovirus glycoproteins, their surface arrangement and the 3' end sequences of their RNA species are similar to those of uukuviruses, and unlike those of Bunyavirus genus viruses. Studies of a variety of unassigned tick-borne viruses have established that biochemically they are unlike bunyaviruses, phleboviruses, or uukuviruses. From biochemical and serologic analyses these tick-borne viruses have been categorized as a new genus in the Bunyaviridae family (the Nairovirus genus). Dhori virus, another tick-borne virus we have analyzed appears to be an orthomyxovirus and unlike members of the Bunyaviridae.

Genetic studies initiated with Punta Toro (PT) virus have shown that high frequency intertypic genetic recombination can result from certain PT mixed virus infections (ts, or dual wild-type coinfections using alternate isolates of PT virus). From analyses of the reassortants generated from dual infections involving prototype PT and an alternate PT strain, PT-ada, it has been demonstrated that the viral S RNA codes for the N polypeptide. In vitro translation by subgenomic polysome mRNA indicates that N is coded by a small mRNA. Some evidence has been obtained which suggests that a non-structural polypeptide, p30, is also coded by a small subgenomic mRNA representing an S cRNA.

So far no recombination has been detected between PT and BUE, or PT and SFS, or PT and CHG, or PT and ICO, or PT and KAR, or BUE and ICO viruses, either by analyses of the progeny of dual wild-type virus infections, or from dual infections involving PT ts mutants and the wild-type alternate virus (neither of which give plaques at 39.3°) and screening for reassortant progeny at the non-permissive temperature. These results suggest that these phleboviruses do not belong to the same gene pool.

In addition to the heterologous phlebovirus recombination assays, we have screened for genetic interaction between phleboviruses based on heterologous virus interference assays. We have failed to detect heterologous virus interference using PT (or ICO) dI virus preparations and BUE, KAR, SFS, SFN, CHG, CDU or ITP phleboviruses under conditions in which interference of homologous PT, or alternate PT varieties, (or ICO) viruses could be demonstrated.

We conclude from these results that the phleboviruses are genetically diverse. From the vaccine development point of view it now appears unlikely that heterologous phleboviruses could be used to produce reassortants for vaccine development. Evidently, however, intertypic reassortants could be obtained and may be of use for vaccine development.

The BARD contract we have recently been awarded addresses the question of RVF vaccine development by (1) attenuating a virulent RVF strain through consecutive high level mutagenesis, (2) identifying the location of the attenuating mutations by reassortment with an alternate virulent RVF strain that has distinguishable RNA fingerprints, and (3) constructing intertypic reassortant RVF viruses that have attenuating defects in each RNA segment. Because of these objectives, and in light of the evidence against heterologous phlebovirus recombination, we propose to discontinue our effects on investigating the recombination potential of phleboviruses sponsored by this contract. Rather, we propose to concentrate on defining the strategy of phleboviruses by analyses of the viral RNA sequences and their encoded gene products (see Proposal).

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