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Generation and transmission of Rift Valley fever viral reassortants by the mosquito *Culex pipiens*

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Reassortant viruses containing heterologous S and M genomic RNA segments were obtained from both mosquito and vertebrate hosts that had been co-infected with Egyptian and Senegalese strains of Rift Valley fever (RVF) virus. The origin of the S and M RNA segments in each plaque-cloned virus was determined with monoclonal antibodies capable of differentiating the nucleocapsid protein (S segment marker) or the G1 glycoprotein (M segment marker) of the parental strains. In the mosquito *Culex pipiens*, reassortants were detected after sequential ingestion of parental viruses by interrupted feeding on two infected hamster hosts, after feeding on a single host that had been infected with both parental strains, and from individual mosquitoes inoculated intrathoracically with both parental strains. Reassortant viruses replicated efficiently in mosquitoes and were readily transmissible by bite to hamsters. Replication of a second infecting strain of RVF virus was, however, completely inhibited if that virus was inoculated into a mosquito $\geqslant$48 h after the first viral strain. Genetic reassortment may provide a mechanism for increased heterogeneity, and thus affect the epidemiology and evolution of RVF virus.

Introduction

For many arboviruses, strains have been isolated that exhibit significant phenotypic heterogeneity relative to prototype strains and, in some cases, these characteristics can be related to specific changes in RNA sequence. Although heterogeneity presumably arises from selection and accumulation of point mutations, genetic reassortment among viruses with multisegmented genomes has also been suggested to contribute to these divergent phenotypes (Bishop & Shope, 1979; Beaty *et al.*, 1985; Jones *et al.*, 1987; Davies *et al.*, 1987; Saluzzo & Smith, 1990). As is well documented with other viruses containing segmented genomes (Buckler-White *et al.*, 1985), genetic reassortment among arboviruses could occur in a vertebrate host that was exogenously infected with two strains of a virus within a relatively short time interval by being bitten by two vectors carrying different strains of virus. Alternatively, reassortants could be formed within a vertebrate host among spontaneous variants arising during infection of that host. Likewise, genetic reassortment could occur in a single vector that fed on two infected vertebrate hosts, or could occur between spontaneous mutants arising in an infected vector.

The development of monoclonal antibodies against gene products of the S and M segments of Rift Valley fever (RVF) virus that are able to distinguish between viral strains collected in Egypt and sub-Saharan Africa (Saluzzo *et al.*, 1989) provided an opportunity to examine the potential of segment reassortment to serve as a mechanism for generating genetic diversity among RVF viruses. We investigated the potential for reassortants to occur in mosquitoes inoculated with two different strains of RVF virus, in mosquitoes that fed on a vertebrate host infected with two different strains of RVF virus, or in mosquitoes that fed partially on each of two RVF virus-infected vertebrate hosts. Additionally, we determined whether reassortants could be transmitted by bite to a susceptible vertebrate host and also the extent to which prior infection with one viral strain could interfere with replication of a second strain.

Methods

Mosquitoes. Culex pipiens (El Gabal strain), derived from specimens collected in Egypt in 1981 (Gargan *et al.*, 1983), were used in the 115th to 120th generation of colonization. This species is a competent vector of RVF virus (Gargan *et al.*, 1983; Turell *et al.*, 1984) and was identified as an epizootic vector of RVF virus during the 1977 outbreak of RVF in Egypt (Meegan *et al.*, 1980).

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Viruses. The ZH501 strain of RVF virus was isolated in Egypt in 1977 from the serum of a patient with a fatal haemorrhagic infection (Meegan, 1979) and was passaged twice in foetal rhesus lung cells prior to its use in this study.

The RVF MP-12 strain was derived from the ZH548 strain (also isolated in Egypt in 1977) of RVF virus by 12 successive cycles of 5-fluorouracil mutagenesis and replication in MRC5 cells (Caplen *et al.*, 1985). This strain is attenuated for adult mice (Caplen *et al.*, 1985) and hamsters (Rossi & Turell, 1988) and is temperature-sensitive (ts) at 41 °C (Rossi & Turell, 1988).

The ArD38661 strain of RVF virus was isolated from *Aedes dalzieli* collected in Senegal in 1983 (Saluzzo *et al.*, 1989). This strain was passaged three times in suckling mice by intracerebral inoculation, once in Vero cells, and then plaque-purified twice in Vero cells prior to its use in these studies.

Determination of genome segment origin in plaque-cloned viruses. Two monoclonal antibodies raised against the ZH501 strain of RVF virus, an anti-nucleocapsid (R1-P2E7) and an anti-G1 (R1-3B4), did not react in immunofluorescence assays with gene products of the ArD38661 strain, but did react strongly with those of both the homologous (ZH501) and RVF MP-12 strains (Saluzzo *et al.*, 1989). Thus, these antibodies could be used to determine the origin of the S and M RNA segments, respectively. Each plaque-purified clone was grown in Vero cells at 35 °C; the cells were removed by trypsinization, washed in phosphate-buffered saline and dried onto glass slides. The antigenic phenotype of each of these clones was determined by immunofluorescence assays with the appropriate monoclonal antibodies. An anti-G2 (R4-10G10), which reacted with both the ZH501 and the ArD38661 strains, was used as a positive control to confirm the presence of viral antigen. We did not determine the origin of the L RNA segment in these clones owing to the lack of an unambiguous differentiating marker.

Mosquito inoculation experiments. To determine the potential for RVF virus to reassort in a mosquito, 3- to 5-day-old female *C. pipiens* were inoculated intrathoracically (Rosen & Gubler, 1974) with a suspension containing $10^{6.5}$ p.f.u./ml (10^3 p.f.u./mosquito) of a mixture of the ZH501 and ArD38661 strains. Inoculated mosquitoes were placed in an incubator maintained at 26 °C with a 16 : 8 h (light : dark) photoperiod and were provided apple slices as a carbohydrate source. Mosquitoes were triturated individually in 1 ml of diluent (10% foetal bovine serum in Medium 199 with Hanks' salts plus antibiotics and $NaHCO_3$) at 1, 2, 3, 4 and 25 days after inoculation with virus. The mosquito suspensions were centrifuged at 1000 *g* for 10 min and assayed for virus by plaque assay on Vero cell monolayers (Gargan *et al.*, 1983) and individual plaques were selected at the terminal dilution and analysed to determine the antigenic phenotype of each virus clone.

Interference assay. Adult female *C. pipiens* mosquitoes were inoculated with 10^2 p.f.u. ($10^{3.5}$ ID_{50}) of the ts RVF MP-12 strain and subsequently inoculated with 10^2 p.f.u. of the ArD38661 strain at 1 day intervals after the original inoculation. In addition, some mosquitoes were inoculated with a suspension containing both the RVF MP-12 and ArD38661 strains. Mosquitoes were harvested 7 days after their second inoculation, triturated individually and assayed at 35 °C and 41 °C. Individual plaques were selected from cells grown at 35 °C and analysed for the presence of reassortant viruses.

Interrupted feeding studies. To study a situation in which mosquitoes ingest blood from two different viraemic sources, hamsters were inoculated intraperitoneally (i.p.) with 10^4 p.f.u. of either the ZH501 or the ArD38661 strain of RVF virus. These hamsters were anaesthetized 24 h later and female *C. pipiens* were allowed to feed on a hamster inoculated with the ArD38661 strain. When these mosquitoes had consumed approximately half of a complete blood meal, they were disturbed, captured and allowed to complete feeding on a hamster infected with the ZH501 strain. Immediately after feeding on the second hamster, two engorged mosquitoes were triturated individually in 1 ml of diluent. These suspensions were assayed on Vero cell monolayers by a plaque assay for infectious virus and individual plaques were analysed to determine the antigenic phenotype of each virus clone.

The remaining engorged mosquitoes were provided with apple slices, incubated for 1 week at 26 °C and then cold-anaesthetized. For each mosquito, the legs were removed and triturated in 1 ml of diluent and the midguts were dissected, washed twice in diluent, and then triturated in 1 ml of diluent. This procedure enabled us to distinguish mosquitoes with a non-disseminated infection (i.e. mosquitoes with an infection limited to the midgut) from those with a disseminated infection (i.e. those in which virus had escaped from the midgut into the haemocoel) (Turell *et al.*, 1984). Each suspension was assayed on Vero cells and plaques were picked to determine the antigenic phenotype of each virus clone.

Dually infected vertebrate studies. To simulate the situation in which mosquitoes might take a blood meal from a vertebrate host circulating two viral strains (i.e. a vertebrate host that had been fed on by two infectious vectors within a short time period), mosquitoes were allowed to feed on a hamster that had been inoculated i.p. 26 h previously with 10^4 p.f.u. of the ArD38661 strain and 10^4 p.f.u. of the ZH501 strain. The two strains were inoculated approximately 2 h apart. After feeding, the engorged mosquitoes were handled as described above for the interrupted feedings.

Transmission experiments. To determine the ability of reassortant viruses to replicate in and be transmitted by mosquitoes, two reassortants, R1 (which contained the M and S segments of ArD38661 and ZH501, respectively) and R42 (which contained the M and S segments of ZH501 and ArD38661, respectively) produced in the experiments described above, were inoculated intrathoracically into separate groups of *C. pipiens*. These mosquitoes were assayed individually at selected time intervals after inoculation. At 7 days after inoculation, mosquitoes were allowed to feed on uninfected female hamsters. Samples of liver suspension from hamsters dying after mosquito feeding were assayed for virus, individual plaques were expanded and examined to determine the antigenic phenotype of each virus clone by immunofluorescence procedures as described above.

Results

Demonstration of segment reassortment in inoculated mosquitoes

To determine the potential for genetic reassortment of RVF virus in mosquitoes, female *C. pipiens* were inoculated with a suspension containing $10^{6.5}$ p.f.u./ml (10^3 p.f.u./mosquito) of a mixture of the ZH501 and ArD38661 strains. Of 10 plaque clones derived from mosquitoes immediately after inoculation with this suspension, four were typed as ZH501 and six as ArD38661. Reassortant viruses were readily recovered from these mosquitoes and M and S segment reassortants accounted for 27% of the 119 individual clones examined (Table 1). Reassortants were detected at similar rates from 1 to 25 days after inoculation.

Because of the relatively high rate of spontaneous mutation that occurs in RNA viruses, we monitored the

Table 1. *Genetic reassortment of RVF virus in* C. pipiens *after intrathoracic inoculation with the ZH501 and ArD38661 strains of RVF virus*

Time after inoculation (days)	Antigenic phenotype of virus clones*				Number (%) of reassortants
	++	+−	−+	−−	
1	0	1	3	14	4 (22)
2	1	0	2	6	2 (22)
3	16	6	13	28	19 (30)
4	2	0	2	5	2 (22)
25	5	3	2	10	5 (25)
Totals	24	10	22	63	32 (27)

* Number of each antigenic phenotype (++, ZH501, ZH501; +−, ZH501, ArD38661; −+, ArD38661, ZH501; −−, ArD38661, ArD38661 for the M and S RNA segments, respectively) of viruses cloned from mosquitoes harvested at selected days after inoculation.

Table 2. *Homologous interference in* C. pipiens *after sequential inoculation with 2 strains of RVF virus**

Day†	Number tested	Number (%)‡ non-ts	Number (%)§ reassortant
0	10	10 (100)	10 (100)
1	10	9 (90)	9 (90)
2	10	1 (10)	1 (10)
3	10	0 (0)	0 (0)
6	10	0 (0)	0 (0)
8	10	0 (0)	0 (0)
10	10	0 (0)	0 (0)

* Mosquitoes were inoculated with the ts RVF MP-12 strain of RVF virus and at various times afterwards with the non-ts ArD38661 strain.

† Number of days between inoculation with the RVF MP-12 strain and subsequent inoculation with the ArD38661 strain. All mosquitoes were harvested 7 days after inoculation with the ArD38661 strain.

‡ Number (percentage) of mosquitoes from which non-ts viruses were recovered. All mosquitoes that contained non-ts virus also contained reassortant viruses.

§ Number (percentage) of mosquitoes from which reassortant viruses were recovered. Ten plaque clones were tested from each mosquito.

stability of the G-1 and nucleocapsid markers used in this study by determining the antigenic phenotype of 43 plaque-purified viral clones recovered from mosquitoes that had been inoculated with only one of the four different antigenic phenotypes of RVF virus. All 43 clones contained only the same phenotype as had been inoculated initially into the mosquito from which it was recovered (data not shown). The stability of these markers has also been shown in a previous study (Saluzzo & Smith, 1990).

Demonstration of homologous interference

To determine the extent to which prior infection with one strain of RVF virus might interfere with the replication of a second strain and thus prevent genetic reassortment, mosquitoes were inoculated with the ArD38661 strain either simultaneously with, or at various times after, inoculation of the ts RVF MP-12 strain. The RVF MP-12 strain is known to contain multiple ts lesions and the ts phenotype was found to be stable in previous studies (Saluzzo & Smith, 1990). Non-ts and reassortant viruses were recovered from all (10/10) mosquitoes inoculated simultaneously with ArD38661 and the ts RVF MP-12 strains and from nine of 10 mosquitoes inoculated with the ArD38661 strain 24 h after inoculation with the RVF MP-12 strain (Table 2). By contrast, non-ts virus was recovered from only one of 10 mosquitoes inoculated with ArD38661 2 days after inoculation with RVF MP-12 and from none of 40 mosquitoes inoculated 3 or more days after inoculation of the ts virus. The one mosquito with non-ts virus from day 2 also contained reassortant viruses.

Reassortment in mosquitoes after interrupted feeding

Mosquitoes that obtained part of a blood meal from a hamster infected with the ArD38661 strain (viraemia equivalent to $10^{8.3}$ p.f.u./ml) and then continued feeding on a hamster infected with the ZH501 strain (viraemia equivalent to $10^{8.6}$ p.f.u./ml), ingested approximately 10^6 p.f.u. of virus. Examination of 37 plaque clones obtained from mosquitoes triturated immediately after feeding indicated that both parental strains were ingested in approximately equal amounts (Table 3). Of five engorged mosquitoes that survived 7 days, four were infected. One of these, mosquito 1, contained all possible phenotypes, and only the ZH501 strain was recovered from the other three mosquitoes (Table 3).

Reassortment after feeding on a dually infected vertebrate

Mosquitoes were also allowed to feed on a single hamster (viraemia equivalent to $10^{6.2}$ p.f.u./ml) that had been infected with both the ArD38661 and ZH501 strains of RVF virus the previous day. These mosquitoes ingested a mean of $10^{3.7}$ p.f.u. of RVF virus. Based on an analysis of 41 individual clones, obtained from three mosquitoes harvested immediately after feeding, approximately equal numbers of each of the parental phenotypes were ingested as well as a small proportion of reassortant viruses (7%, 3/41) (Table 4). Of eight engorged mosquitoes that survived 7 days, only three were infected (Table 4). However, both phenotypes of reassortant viruses were recovered from each of these three mosquitoes. Interestingly, both parental phenotypes were recovered from only one of these mosquitoes. Recovery of virus from the legs of two mosquitoes indicated that the virus had escaped the midgut and established a disseminated

Table 3. *Genetic reassortment of RVF virus in* C. pipiens *after interrupted feeding on hamsters infected with the ZH501 and ArD38661 strains of RVF virus*

	Antigenic phenotype of virus clones*									
	Midgut					Legs				
Mosquito	Titre	++	+−	−+	−−	Titre	++	+−	−+	−−
Time 0†										
1	6·3‡	12	0	0	7			Not tested		
2	5·5	9	0	0	9			Not tested		
Total		21	0	0	16			Not tested		
Day 7†										
1	3·8	5	3	13	4	<0·7	0	0	0	0
2	4·1	10	0	0	0	<0·7	0	0	0	0
3	4·6	10	0	0	0	<0·7	0	0	0	0
4	3·6	19	0	0	0	3·5	10	0	0	0

* Number of each antigenic phenotype (++, ZH501, ZH501; +−, ZH501, ArD38661; −+, ArD38661, ZH501; −−, ArD38661, ArD38661 for the M and S RNA segments, respectively) of viruses cloned from the midgut and legs of the mosquitoes.

† Time 0, mosquitoes assayed immediately after the infectious blood meal (i.e. virus ingested by mosquitoes) and Day 7, mosquitoes harvested 7 days after the infectious blood meal.

‡ $\log_{10}$ p.f.u. per specimen.

Table 4. *Genetic reassortment of RVF virus in* C. pipiens *after feeding on a hamster infected with both the ZH501 and ArD38661 strains of RVF virus*

	Antigenic phenotype of virus clones*									
	Midgut					Legs				
Mosquito	Titre	++	+−	−+	−−	Titre	++	+−	−+	−−
Time 0†										
1	3·5‡	4	0	1	8			Not tested		
2	3·7	5	0	1	5			Not tested		
3	3·9	6	0	1	10			Not tested		
Total		15	0	3	23			Not tested		
Day 7†										
1	4·1	0	7	1	13	<0·7	0	0	0	0
2	4·3	1	7	5	13	2·4	0	0	0	31
3	4·5	22	5	3	0	3·5	30	0	0	0

*†‡ Footnotes: see Table 3.

infection. Although the midgut from each of these mosquitoes contained a variety of phenotypes, only a single phenotype was recovered from the legs of each mosquito (the ArD38661 phenotype from mosquito 2 and the ZH501 phenotype from mosquito 3; Table 4).

Transmission experiments

To determine the transmission potential of reassortant RVF viruses, two reassortants obtained from the study described in Table 1 (R1 and R42, see Methods) were inoculated into adult female *C. pipiens*. Both of these reassortants replicated efficiently in *C. pipiens* after intrathoracic inoculation. Titres in excess of 10^5 p.f.u. per mosquito were recovered from all inoculated mosquitoes by 4 days after inoculation and all 10 mosquitoes, five inoculated with each of the two strains, transmitted virus by bite to hamsters. All clones recovered from inoculated mosquitoes were antigenically identical to the strain inoculated into those mosquitoes. Likewise, all clones recovered from hamster livers exhibited the same antigenic phenotype as the strain inoculated into the mosquito that infected the hamster (data not shown).

Discussion

Several studies have demonstrated the potential for the production of reassortants among multisegmented arboviruses in either vertebrate or vector hosts as well as in cell culture (Bishop & Shope, 1979; Beaty *et al.*, 1985; Jones *et al.*, 1987; Davies *et al.*, 1987; Saluzzo & Smith, 1990). The present study extends these findings by demonstrating reassortment between two naturally occurring strains of RVF virus in a natural vector species after interrupted feeding on two different infected vertebrate hosts. In addition, the recovery of three reassortants from mosquitoes immediately after a blood meal (time 0) from the dually infected hamster indicates that RVF virus can also form reassortants in vertebrate hosts. Finally, the number and distribution of phenotypes in these time 0 mosquitoes, compared with the number and distribution in the infected mosquitoes 7 days later, suggests that reassortment must also have continued to occur in the mosquitoes that fed on the dually infected hamster.

Reassortant viruses were able to replicate well in mosquitoes after intrathoracic inoculation and all inoculated mosquitoes tested transmitted virus by bite to hamsters. These results are similar to those observed with the parental strain, ZH501 (Turell *et al.*, 1984). Thus, reassortant RVF viruses presumably have the potential to be transmitted in nature. The ability of a virus to produce reassortants either between spontaneous mutations in an infected host, or within a host infected with two different strains of virus, may help to explain the natural diversity of viral strains. Homologous interference, however, may reduce the potential for genetic reassortment. We observed nearly complete inhibition of replication of a homologous virus if that virus had been inoculated $\geqslant 48$ h after inoculation of the first strain. This is similar to the situation described by Beaty *et al.* (1983) for La Crosse virus in *Aedes triseriatus*.

When a mosquito ingests an arbovirus, a number of steps must occur before that mosquito can transmit the virus biologically. The virus must infect the cells of the midgut, escape into the haemocoel, infect the salivary glands and then be secreted into the saliva. In the interrupted feeding portion of the present study, only one mosquito became infected with more than one phenotype of virus, although all ingested both parental phenotypes. Similarly, in the dual feeding portion of the study, only a single phenotype was recovered from the legs of each mosquito, although several phenotypes were recovered from the midguts of the same mosquitoes. Although numerous infectious particles are ingested in a given blood meal, only a relatively small number of them may adsorb to midgut cells. Likewise, escape of virus from the midgut to the haemocoel probably does not occur simultaneously for many infected cells. Thus, both infection of the midgut and dissemination of virus to the haemocoel may act as a highly selective filter through which a virus must pass before it is transmitted. Because of the large numbers of mosquitoes involved with virus transmission, even a relatively rare viral phenotype may occasionally be selected by this process and introduced into a susceptible vertebrate host. Thus, selection in and by a vector (mosquito) may allow for the appearance of new strains of virus, which may differ significantly from the parental strains.

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