Establishment and Characterization of Three Cell Lines from *Aedes triseriatus* (Diptera: Culicidae)

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ABSTRACT Three cell lines (*A.t.* GRIP-1, 2, and 3) were established from *Aedes triseriatus* (Say) embryonated eggs or neonate larvae and their morphology, growth, karyotype, and isozyme pattern were studied. The isozyme alleles observed in the 3 cell lines also were found in adults of the original mosquito colony. Each cell line differed in enzymatic, morphological, and karyotypical patterns. La Crosse encephalitis (LAC) and snowshoe hare (SSH) viruses, members of the California encephalitis virus group, were able to replicate in these 3 cell lines. Furthermore, these cell lines, especially *A.t.* GRIP-1, were more sensitive than the *Aedes aegypti* (L.) (ATC 10) cell line for detection of small amounts of delta-endotoxin of *Bacillus thuringiensis* serovar. *israelensis* (de Barjac).

KEY WORDS *Aedes triseriatus*, *Bacillus thuringiensis* serovar. *israelensis*, cell lines, arboviruses, California encephalitis

Aedes triseriatus (SAY) is distributed widely in North America, where it is a major vector of La Crosse virus (LAC) of the California encephalitis virus group (Watts et al. 1972). Because of the association of this virus with human disease and the abundance of *Ae. triseriatus*, the relationship between the virus and the vector has been studied extensively. Virus transmission can occur by biting (Watts et al. 1972) and transovarially (Watts et al. 1973, Miller et al. 1977). Despite intensive investigation, LAC virus has not been detected in Canada, but another member of the California encephalitis virus group, snowshoe hare virus (SSH), has been associated with human cases of encephalitis (Fauvel et al. 1980, Belloncik et al. 1982) and is the most prevalent arbovirus in Québec Province (Belloncik et al. 1982, 1983). SSH virus was isolated from *Ae. triseriatus* adults (Artsob 1983, Grayson and Calisher 1983) and larvae (Bourassa et al. 1992), demonstrating the importance of this mosquito species in both vertical and horizontal transmission.

Since the first mosquito cell line was established by Grace (1966), numerous cell lines have been established from various species of mosquitoes (Kurtti and Munderloh 1984). However, to date there has been only 1 derived from *Ae. triseriatus* (Rowley et al. 1984). Since its establishment in 1984, no other work using this cell line has been published. Moreover, the eggs used for starting this cell line originated from a mosquito colony from the midwestern United States (Rowley et al. 1984). We required a cell line from a cold-temperate *Ae. triseriatus* population from Canada. As stated by Rowley et al. (1984), most research on mosquito cell lines has been with arboviruses isolated in mice or mammalian cell lines and may have missed the isolation of some strains of viruses. The *Ae. albopictus* cell system was shown to be superior to newborn mice or mammalian cell culture for the isolation of dengue-2 virus (Rowley et al. 1984). Nevertheless, mammalian cell cultures like Vero cells remain necessary for virus titration, because usually there are no cytopathic effects on mosquito cell lines. Furthermore, the C6/36 clone of *Ae. albopictus* cells routinely used for the isolation of tropical arboviruses recently was found to be infected with a parvolike virus (Jousset et al. 1993).

This paper reports the establishment of 3 cell lines derived from embryonic or neonate larval tissues of Canadian *Ae. triseriatus* that are suitable for isolation of arboviruses from cold-temperate mosquito species, for in vitro studies of SSH and LAC viruses, and for cytotoxicity assays of *Bacillus thuringiensis* serovar. *israelensis* toxins.

Materials and Methods

Primary Culture. The primary cultures were initiated from embryonated eggs or neonate larvae following standard protocols (Lynn et al. 1988). The eggs of *Ae. triseriatus* were from the GRIP colony started from adult mosquitoes originally collected in the Trois-Rivières area (Québec, Canada) and reared in the laboratory for several generations at the frequency of 1 generation every 27–28 d. The eggs were surface-sterilized by immer-

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sion into White's solution (White 1931) for 8 min and then washed 3 times with sterile distilled water containing 100 μg/ml of penicillin and 100 μg/ml of streptomycin. Eggs then were placed for hatching into a vacuum bell jar for 1 h or directly triturated in medium. Neonate larvae were triturated into Mitsuhashi-Maramorosch (M.M.) culture medium supplemented with 20% fetal calf serum (FCS) inactivated by heating for 1 h at 56°C. The larval fragments were transferred into a sterile flask (25 cm^2) and incubated at 28°C. The primary cultures were maintained by adding fresh medium once a week. Subcultures were initiated by detaching cells from the wall surface of the culture flask by repeated gentle pipetting and transferring 1 ml of this cell suspension to a new flask containing 4 ml of fresh medium.

Measurement of Cell Growth. For evaluation of the doubling time of each cell line, 2 aliquots were taken daily from each of 3 cultures flasks and counted after staining with trypan blue.

Electron Microscopy. After 7 d of subculture, cells were fixed in 1% glutaraldehyde in phosphate buffer and postfixed in 1.33% osmium tetroxide in S-collidine buffer plus 5% sucrose, embedded in vestopal, ultrasectioned, stained with uranyl acetate and lead citrate (Reynolds 1963), and observed under an electron microscope (Philips) (Alain et al. 1989).

Karyotype Analysis. Chromosome number was determined for each cell line using a protocol similar to that of Schneider (1973). Cells in the logarithmic growth phase were incubated for 6 h at 28°C in a medium containing 50 μg/ml of colchicine (Sigma), fixed with glacial acetic acid and methanol (1:3), spread on a microscope slide, and then stained with Giemsa. Chromosomes were counted for each cell in metaphase. The percentages of cells with various ploidies were calculated from 300 metaphase spreads.

Isozyme Analysis. For isozyme analysis, we compared the 3 *Ae. triseriatus* cell lines with *Aedes aegypti* (L.) ATC 10 (Singh 1967), *Euxoa scandens* (Riley) IAF *Es* 1 (Belloncik et al. 1985), *Spodoptera littoralis* (Boisduval) SPC *Sl* 52 (Mialhe et al. 1984), and *Spodoptera frugiperda* (J. E. Smith) *Sf* 9 (Vaughn et al. 1977) cell lines. Centrifugation was carried out with 5×10^6 cells. After 2 washes with phosphate-buffered saline, 25 μl of homogenization buffer (8 m*M* Tris-citrate at pH 7.1, 292 m*M* sucrose, 1% Triton X100, and 0.02 m*M* bromophenol blue) was added to the pellet, and the cell extracts were frozen at −80°C. We also compared our cell lines with the GRIP colony of *Ae. triseriatus*. For that, individual adult female mosquitoes older than 48 h were homogenized at 4°C in 25 μl of buffer and then analyzed. The major bands of isozymes remain during the development of mosquitoes (Pasteur et al. 1988) and adults are easier to anesthetize.

Electrophoresis on 5% polyacrylamide gel in Tris-citrate at pH 7.1 or Tris-borate EDTA at pH 8.9 was performed according to the standard protocol of Munstermann (1979). The enzyme systems are listed in Table 1. The specimens were centrifuged using a micro centrifuge (Eppendorf model 5415) at 16,000 × *g* for 5 min. Depending on the enzymes tested, 1–3 μl of mosquito supernatant, 4–6 μl of the supernatant of *A.t.* GRIP-2 and 3, 3–5 μl of cell lines *A.t.* GRIP-1 and ATC 10, and 1–3 μl of lepidopteran cell lines were put in each well (except for EST 6 where 1, 10, 8, and 6 μl were used, respectively).

Lysates of 10 cell lines together with lysates of 8 adult female *Ae. triseriatus* were compared on the same gel. Migration was performed at 4°C, at 250 volts during 2–3 h depending on the enzyme tested. After staining (Munstermann 1979, Steiner and Joslyn 1979, Richardson et al. 1986), the gels were fixed with an acetic acid–methanol solution, photographed, and analyzed.

The migration value of the most abundant allele of female *Ae. triseriatus* was selected for each locus as reference. The migration values of the other alleles were compared with the reference by the ratio between their migration distance and that of the selected allele multiplied by 100.

Arbovirus Assays. LAC (strain M 3123) and SSH (strain WML 55-59-75) viruses were used. Mosquito cells were seeded at a concentration of 10^5/ml in 25-cm^2 flasks; 4 d later, the medium was removed and 1 ml of SSH or LAC virus at $10^{6.3}$ and $10^{5.9}$ TCID (tissue culture infectious dose) $_{50/ml}$ was added, respectively. After 1.5 h of viral adsorption, the virus was removed and the cell monolayer was washed with M.M. medium. Then 4 ml of medium supplemented with 10% FCS were added and the cells were incubated at 28°C. At various times from 1.5 h to 10 d after infection, each cell line flask was frozen at −80°C. The experiment was done in duplicate, and the virus titer was determined using the $TCID_{50}$ technique (Kärber 1931, Payment and Trudel 1989) on the Vero cells.

Cytotoxicity Tests of Toxins from *Bacillus thuringiensis* serovar. *israelensis*. A commercial formulation of *Bacillus thuringiensis* serovar. *israelensis* (Teknar-HPD, provided by Zoecon Canada) was compared with a blank formulation of the inert ingredients of the Teknar-HPD but not the active part (also provided by Zoecon Canada [composition not given by company]). The parasporal crystals were dissolved following a procedure modified from Thomas and Ellar (1989). The *B. thuringiensis* serovar. *israelensis* crystal delta-endotoxin was solubilized by addition of an equal volume of 0.5 *M* Na_2CO_3-HCl (pH 10.5) to 100 mg/ml of Teknar-HPD and incubation at 37°C for 24 h. The solution then was neutralized by HCl (5.8 *M*) and centrifuged in a Sorvall RC5 (head SS-34) at 17,369 × *g* for 30 min to remove insoluble material. The supernatant was filtered with a 0.45-μm filter (Millipore). The same protocol was applied to the blank solution. Protein concentration was determined for each solubilized solution by Bio-Rad

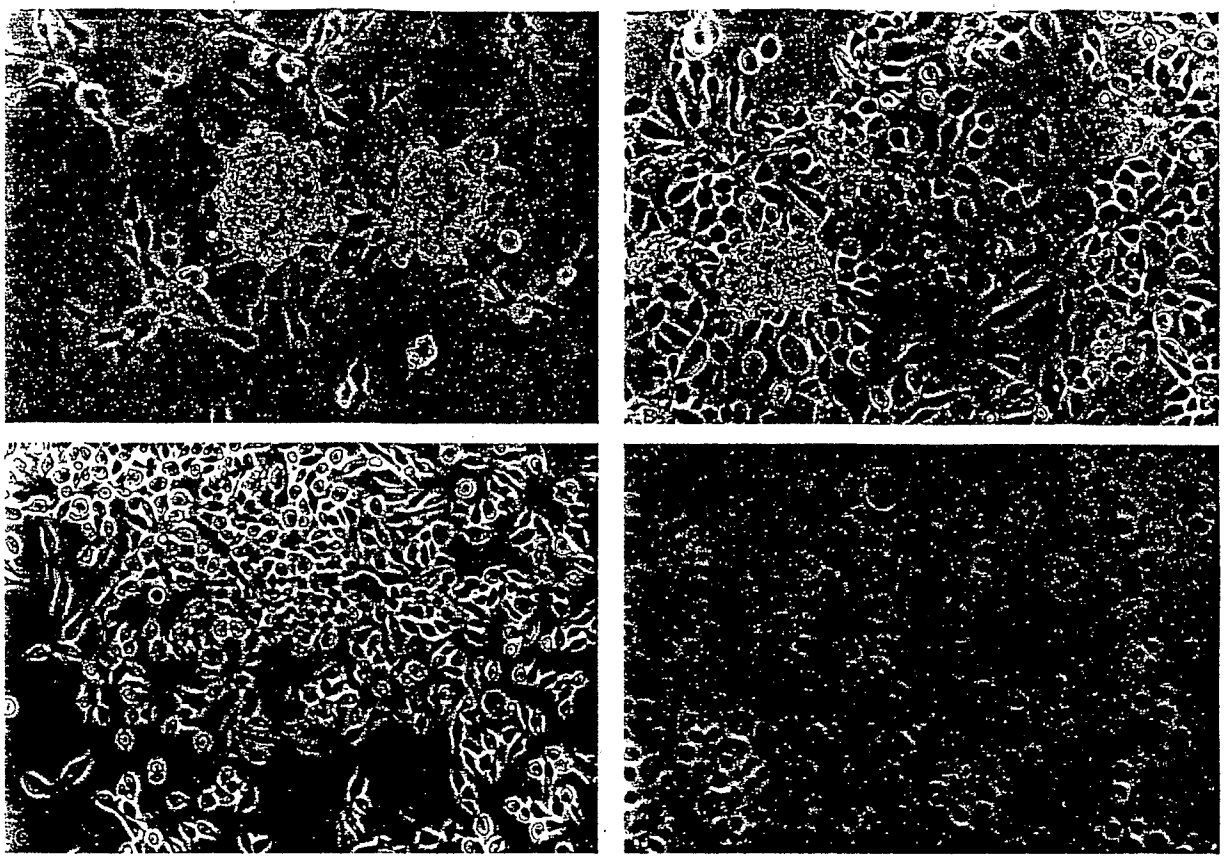

Fig. 1. Phase-contrast light micrographs of the 3 cell lines from *Aedes triseriatus*. A, *A.t.* GRIP-1 cells at passage 155 showing clumps (left arrow) and fibroblastic (right arrow) and epithelial (center arrow) cell types (×820). B, *A.t.* GRIP-2 cells at passage 122 showing the same cell types, a giant cell (right arrow), and a clump (left arrow) (×820). C, *A.t.* GRIP-3 cells at passage 122 showing the same 2 cell types (×820). D, *A.t.* GRIP-2 cells at passage 153 after a 4-h treatment with solubilized toxins of *B. thuringiensis* serovar. *israelensis* at dilution 1:500. Note the many degenerated cells (left and right arrows) and some refringent ones still alive (center arrows). All the cells are rounded, losing their fibroblastic or epithelial morphology (×700).

protein assay (Bio-Rad Laboratories) using bovine serum albumin as a standard.

The cells from a log-phase flask of different cell lines (*Ae. aegypti* ATC 10, *A.t.* GRIP-1, 2, and 3) were detached from the plastic by gentle pipetting, and 150 μl of a cell suspension of 35,000 cells/ml was inoculated into each well of a 96-well tissue culture microplate (Falcon). The microplates were incubated 24 h at 28°C before the addition of 50 μl of different dilutions of Teknar-HPD or blank solubilized sterile solutions. The protein concentration causing cell lysis in 50% of the wells was calculated after 24 h using the $TCTD_{50}$ (tissue culture toxic dose) technique (Kärber 1931, Payment and Trudel 1989). Experiments were done in triplicate.

Results

Primary Culture. Two types of growth were observed in primary cultures. During the 1st week after fragmentation of the larvae, some vesicles emerged from the extremities of the larval fragments. Light microscopy revealed that vesicles were formed by a monolayer of epithelial cells surrounding an empty space. During the following 2 mo, some foci of cells appeared with variable morphology. These foci extended continuously, forming a monolayer attached to the bottom of the flask.

Cell Lines. During the 1st passages, cell morphology and growth were highly variable. Also, the cells were very fragile at the time of the early passages. Only after the 25th passage did the cell lines reach a uniform stage. After 10–15 passages, the serum concentration of the medium was reduced from 20 to 10% (FCS) for the 3 cell lines and then to 5% for the *A.t.* (*Ae. triseriatus*) GRIP-1 cell line.

The characteristics of the *A.t.* GRIP-1 cell line derived from 7-d-old embryonated eggs (the total length of embryogenesis in our laboratory conditions is 12 d before hatching) were different from those of *A.t.* GRIP-2 and 3 originating from neonate larvae. Even after 155 passages, the *A.t.* GRIP-1 cell line continued to demonstrate the morphological characteristics of a primary culture. These cells (Fig. 1A) were predominantly fibroblastic with few epithelial forms. Clumping was

frequent. The clumps detached frequently from the plastic substrate and started the typical cell monolayer when passaged. The doubling time was 30 h.

The 2nd and 3rd cell lines were started from triturated fragments of neonate larvae. Both *A.t.* GRIP-2 (Fig. 1B) and *A.t.* GRIP-3 (Fig. 1C), at passage 122, had a cell population composed in near equal proportion of fibroblastic and epithelial cell types. Giant cells were seen, as well as some clumps (Fig. 1B). The doubling time of each of these 2 cell lines was 60 h.

The cell lines now are maintained routinely by subculturing once a week. The 3 cell lines have been congelated and kept at −80°C in medium containing 10% glycerol (Payment and Trudel 1989). They were recultivated successfully after 3 mo of storage.

At the electron microscopy level, the 3 cell lines showed many vacuoles in the cell cytoplasm and a well-developed endoplasmic reticulum (Figs. 2A–C). Electron micrographs confirmed the absence of microbial (bacteria, fungi, and mycoplasm) as well as California encephalitis or other viral contaminants.

The 3 cell lines were characterized further by the usual karyotype and isozyme analysis mandatory for full description of newly established cell lines and for the rejection of cross-contamination with other insect cell lines maintained in the same laboratory (Lee and Hou 1992).

Karyotype Analysis. For the *A.t.* GRIP-1 cell line, 42% of the cells were diploid and 47% tetraploid, but for *A.t.* GRIP-2 and 3, 63, and 59% were diploid and 31 and 37% were tetraploid, respectively (Fig. 3).

Isozyme Analysis. The 3 cell lines of *Ae. triseriatus* showed different patterns for 3 enzyme systems, IDH2, ME, and PGM (Table 1). For IDH2, *A.t.* GRIP-1 was homozygous for allele 100, *A.t.* GRIP-2 was homozygous for allele 118, and *A.t.* GRIP-3 was homozygous for allele 105. For ME, *A.t.* GRIP-1 was homozygous for allele 93 and *A.t.* GRIP-3 for allele 100. The cell line *A.t.* GRIP-2 was heterozygous for alleles 93 and 100. For PGM, *A.t.* GRIP-1 was heterozygous for alleles 100 and 109, and *A.t.* GRIP-3 for 100 and 118; *A.t.* GRIP-2 was homozygous for allele 100.

Furthermore, EST 6 and GPI could differentiate *A.t.* GRIP-1 from the 2 other cell lines (Table 1). For EST 6, *A.t.* GRIP-1 was homozygous for allele 94, whereas *A.t.* GRIP-2 and 3 were homozygous for allele 92. For GPI, *A.t.* GRIP-1 was homozygous for allele 100, whereas the other 2 cell lines were heterozygous for 91 and 100, respectively.

For the other enzyme systems (IDH 1, MDH 2, HBDH, ACON, and HK 3), the 3 cell lines were homozygous for allele 100.

The enzyme profiles for each cell line remained constant between passages 58 and 107 for *A.t.* GRIP-1, between passages 66 and 75 for *A.t.* GRIP-2, and between passages 32 and 76 for *A.t.* GRIP-3, demonstrating the absence of contamination among these cell lines.

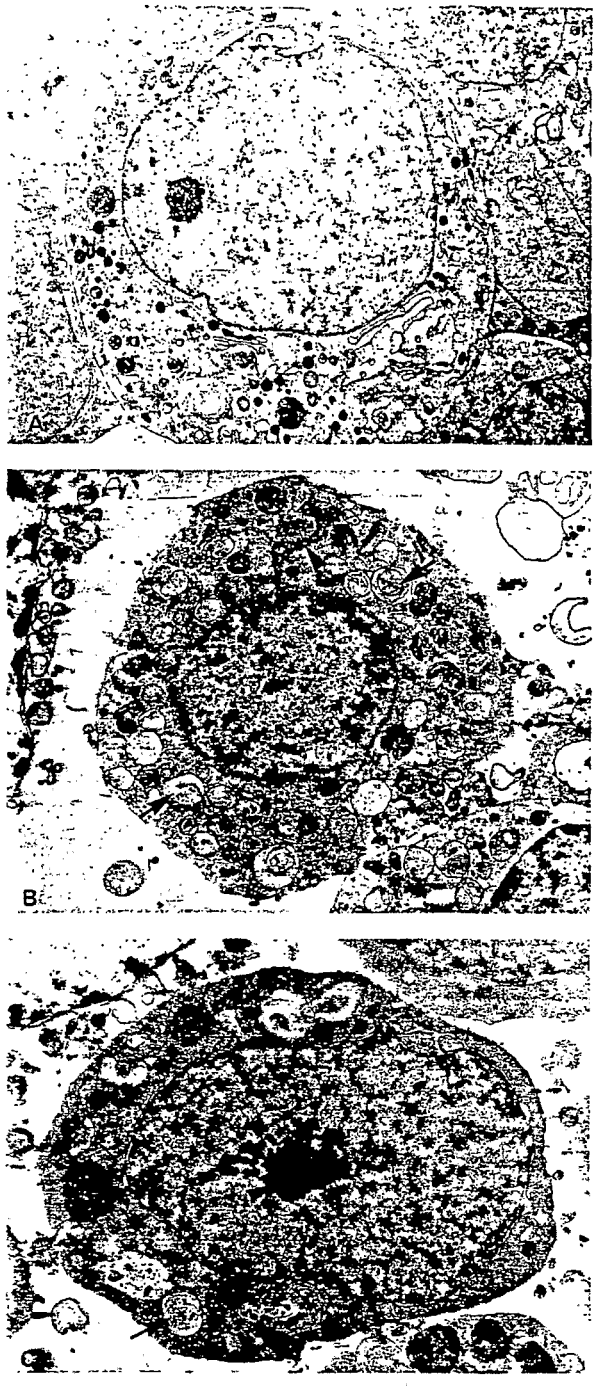

Fig. 2. Electron micrographs of the 3 cell lines from *Aedes triseriatus*. A, *A.t.* GRIP-1 at passage 155 (×6,250). B, *A.t.* GRIP-2 at passage 122 showing many vacuoles (near top and bottom arrows) and a well-developed endoplasmic reticulum (top arrow) (×10,500). C, *A.t.* GRIP-3 at passage 122 showing some vacuoles (bottom arrow) and an enlarged nucleolus (top arrow) (×10,800).

The 3 GRIP cell lines were compared with the ATC 10 cell line of *Ae. aegypti* and 3 cell lines from Lepidoptera and clearly showed different isoenzyme profiles. Furthermore, all the alleles ob-

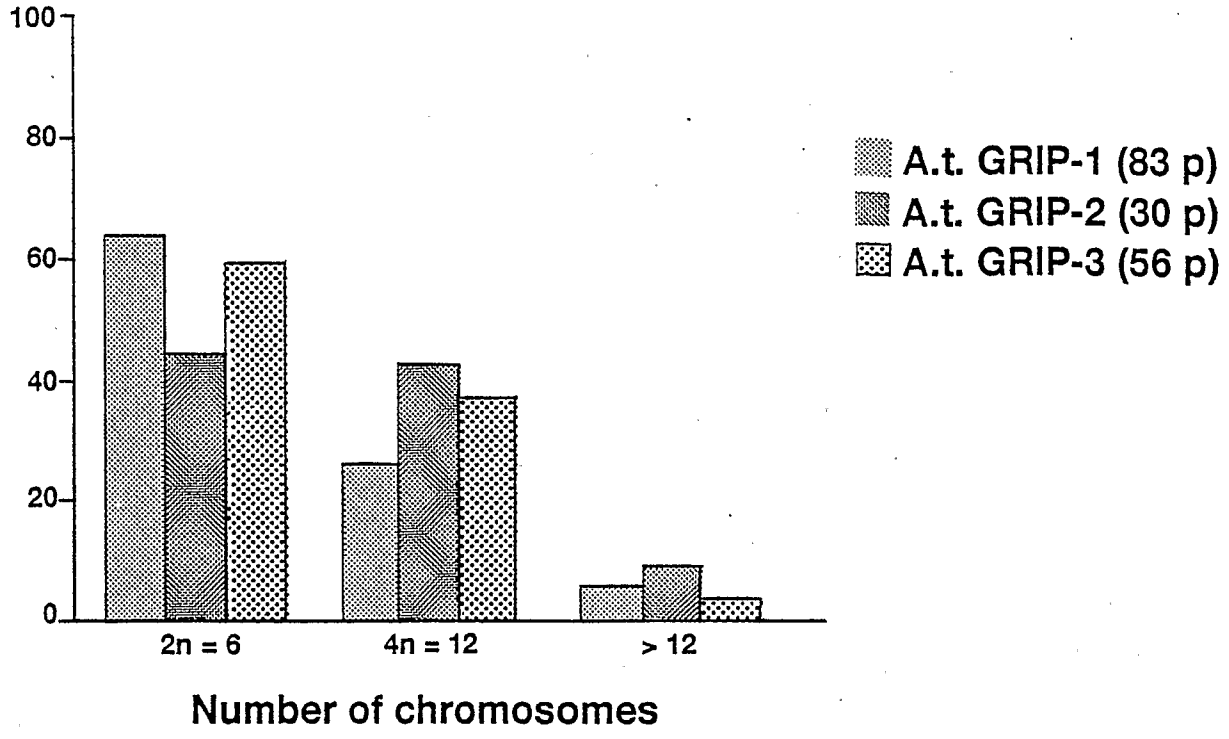

Fig. 3. *Aedes triseriatus* cell karyotype.

served in the 3 cell lines also were found in adults from the parent colony (Table 1).

Arbovirus Assays. Growth curves of SSH and LAC viruses are shown in Figs. 4 a and b. The titers ($TCID_{50}$ [tissue culture infectious dose]/ml) of SSH virus increased from $10^{3.6}$, $10^{3.6}$, and $10^{3.2}$ for *A.t.* GRIP-1, 2, and 3, respectively, at the end of the adsorption period to $10^{6.35}$ on day 3 after infection for *A.t.* GRIP-1, to $10^{5.8}$ on day 7 for *A.t.* GRIP-2, and to $10^{6.7}$ on day 10 for *A.t.* GRIP-3. For the same length of adsorption time, the titers of LAC virus increased from $10^{3.55}$, $10^{3.15}$, and $10^{3.15}$ for *A.t.* GRIP-1, 2, and 3, respectively, to $10^{6.4}$ on day 10 after infection for *A.t.* GRIP-1 and to $10^{7.1}$ and $10^{6.8}$ for *A.t.* GRIP-2 and 3, respectively, on day 7 after infection.

Table 1. Relative mobility of enzymes expressed in cell lines

Enzymes[a]	Alleles of female mosquito	Cell lines									
		A.t. GRIP-1		*A.t.* GRIP-2		*A.t.* GRIP-3		ATC 10	IAF *Es* 1	SPC *Sl* 52	*Sf* 9
		58°p	107°p	66°p	75°p	32°p	76°p				
ACON	100, 106, 109	100	100	100	100	100	100	98	98	94	91, 102
EST 6	84, 88, 89, 90, 92, 93, 94, 95, 96, 98, 100, 101, 102, 103, 105	94	94	92	92	92	92	71, 78	83	102, 108	78
GPI	91, 100, 102, 106	100	100	91, 100	91, 100	91, 100	91, 100	89	N[b]	N	N
HBDH	78, 100, 141, 147, 180	100	100	100	100	100	100	125, 202	N	N	N
HK 3	100, 107	100	100	100	100	100	100	105, 115	115	93, 102	105, 112
IDH 1	100	100	100	100	100	100	100	70	100	N	N
IDH 2	100, 105, 118, 121	100	100	118	118	105	105	100	89	68, 89	68, 79
MDH 2	82, 86, 118, 121	100	100	100	100	100	100	78	N	N	N
ME	93, 100	93	93	93, 100	93, 100	100	100	98	81, 108	N	73
PGM	91, 100, 109, 118	100, 109	100, 109	100	100	100, 118	100, 118	109, 125	100	114	134

Enzymatic mobilities are expressed by the ratios between their migration distance and that of the most abundant allele of female *Aedes triseriatus* designated as migration distance 100.

[a] ACON, aconitate hydratase (ES no. 4.2.1.3); EST 6, esterase (EC no. 3.1.1.1); GPI, glucose phosphate isomerase (EC no. 5.3.1.9); HBDH, beta-hydroxybutyrate dehydrogenase (EC no. 3.1.1.31); HK 2, 3, 4, hexokinase (EC no. 2.7.1.1); IDH 1, 2, isocitrate dehydrogenase (EC no. 1.1.1.42); MDH 2, malate dehydrogenase (EC no. 1.1.1.37); ME, malic enzyme (EC no. 1.1.1.40); PGM, phosphoglucomutase (EC no. 2.7.5.1).

[b] N, no detectable enzymatic activity.

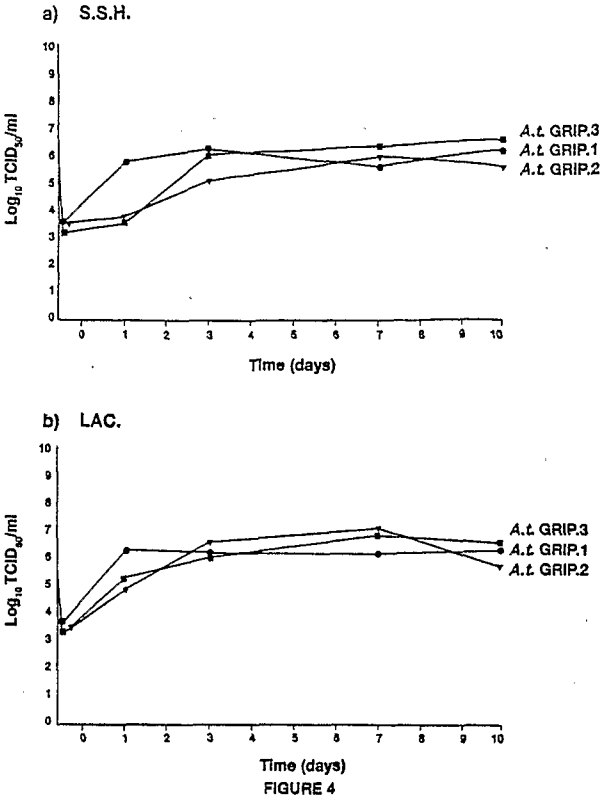

Fig. 4. a, Growth curves of snowshoe hare virus (SSH) in the 3 cell lines from *Aedes triseriatus*. b, Growth curves of La Crosse virus (LAC) in the 3 cell lines from *Aedes triseriatus*.

Cytotoxicity Tests with Toxins from *Bacillus thuringiensis* serovar. *israelensis*. After 24 h of contact, soluble *B. thuringiensis* serovar. *israelensis* toxins caused rapid "rounding up" of *Ae. aegypti* ATC 10 and *A.t.* GRIP-1, 2, and 3 cells, followed by swelling and cell lysis (Fig. 1D). The $TCTD_{50}$s obtained were $10^{3.64}$, $10^{4.31}$, $10^{3.82}$, and $10^{3.95}$/ml, respectively, corresponding to 1.03 μg, 0.17 μg, 0.68 μg, and 0.56 μg/ml of proteins as estimated by Bio-Rad protein assay on the sterile supernatant and taking into account dilution factors. With the control blank formulated, there was a low $TCTD_{50}$ on each cell line of $10^{2.37}$/ml corresponding to 19.2 μg/ml of proteins.

Discussion

Although many cell lines have been established from various species of mosquitoes, before our work only one was derived from neonate tissues of *Ae. triseriatus* (Rowley et al. 1984). We established 3 cell lines from the GRIP colony of *Ae. triseriatus*, 2 cell lines from neonate larvae and 1 from embryonated eggs at ⅔ of embryogenesis (*A.t.* GRIP-1). *A.t.* GRIP-1 is the fastest growing line and may be cultivated in a FCS concentration as low as 5%. This cell line seems to be different from that of Rowley et al. (1984), described as slow growing, and from *A.t.* GRIP-2 and 3, which also are slow-growing cells. There also are morphological and karyotypical differences between the *A.t.* GRIP-1 cell line and *A.t.* GRIP-2 and 3.

Isozyme analysis confirmed these differences. The EST 6 and GPI enzymes distinguished the *A.t.* GRIP-1 cell line from the 2 others, whereas the IDH 2, ME, and PGM enzymes showed different motility patterns for each of the 3 cell lines. The isozyme analysis of cell lines was done in conjunction with a study to discriminate among subspecies or geographical strains of field-collected *Ae. triseriatus* mosquitoes. This explains why so many enzymatic systems were used instead of the 4 usual ones (namely GPI, IDH, ME, and PGM) adequate for distinguishing cell lines of insects (Tabachnik and Knudson 1980). In our study, we found genetic heterozygosity in natural populations of mosquitoes from the Trois-Rivières area that agreed with the results of Mathews and Craig (1980), and our laboratory colony has retained some of this heterozygosity (data not shown). Thus, it is not surprising that heterozygosity was also retained in our cell lines.

LAC and SSH viruses replicated in our 3 *Ae. triseriatus* cell lines. The virus titers (after a decrease caused by discarding the inoculum and washing cells) increased to levels approximately equal or superior to the inoculum titers. Rowley et al. (1984) obtained a higher titer with LAC virus in their *Ae. triseriatus* cell line that increased from $10^{4.5}$ PFU/ml to 10^{8} PFU/ml on day 9. However, they were unable to demonstrate the replication of trivittatus virus in the same cell line. This probably was caused by the fact that they apparently neither discarded the nonadsorbed virus nor washed the cell monolayer. This procedure is useful to distinguish between a low replication rate and persistence of the virus. In our study, there was clear replication of LAC and SSH viruses in the *Ae. triseriatus* cell lines. As suggested by Rowley et al. (1984), such cell lines can be used to isolate viruses from field-collected mosquitoes and for the evaluation of vector competence. Therefore, the 3 cell lines reported in this paper will have further importance in evaluating the capacity of *Ae. triseriatus* from new habitats such as old tires to transmit arboviruses such as dengue.

Our 3 cell lines were 1.5- to 6-fold more sensitive to the solubilized toxins of *B. thuringiensis* serovar. *israelensis* than the *Ae. aegypti* ATC 10 cell line under our experimental conditions. The most sensitive of our cell lines was the *A.t.* GRIP-1 cell line. The cytotoxicity effects observed with these cell lines are similar to those already described in the literature (Thomas and Ellar 1983). These *Ae. triseriatus* cell lines may be useful in the detection of *B. thuringiensis* serovar. *israelensis* toxins in laboratory or field samples, especially the environmental detection of *B. thuringiensis* serovar. *israelensis* persistence where the remaining doses are sublethal for the standard assay on *Ae. aegypti*.

These cell lines may be useful for the study of other entomopathogens of mosquitoes.

Acknowledgments

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