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Detection of Rocio Virus-specific Antigenic Polypeptides in Virus-infected Cells and Culture Fluid by Western Blotting Method

Tiyo SAKURAI*, Keiko BUNDO and Akira IGARASHI

Department of Virology, Institute for Tropical Medicine, Nagasaki University, Nagasaki 852, Japan

Abstract: Rocio virus, a member of flaviviruses, was grown in cultured *Aedes albopictus*, clone C6/36, cells at 28°C, or BHK21, and Vero cells at 37°C. Over 10^8 PFU/ml of virus production was obtained in C6/36 cells after 36 hours of infection, while the virus titers in Vero and BHK21 cells were 10^8 and 10^7 PFU/ml, respectively. Virus infectivity and ELISA antigen titers in the infected C6/36 cell homogenate did no exceed that in the infected fluid, however, the ELISA titers were higher in the infected cell homogenates than in the fluids in the case of BHK21 and Vero cells. Virus infected C6/36 cells and fluids were analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by the Western blotting to reveal antigenic and virus-specific polypeptides. Under nonreducing conditions, major antigenic component in the infected fluid was 55K dalton polypeptide with 7 minor components of 170K, 140K, 115K, 100K, 90K, 52K, and 23K daltons. While, there were 4 major (105K, 83K, 60K, and 56K daltons) and 5 minor (170K, 140K, 115K, 24K, and 23K daltons) polypeptides in the infected C6/36 cells. Some of these polypeptides (105K and 60K) began to appear at 12 hours and others were fully observed at 24-36 hours after infection in C6/36 cells at 28°C. The 170K, 60K, 56K, 24K, and 23K polypeptides in the infected cells disappeared or markedly reduced when the specimens were treated under reducing condition, so were the polypeptides in the infected fluid. On the other hand, 140K and 83K polypeptides in the infected cells appeared to be heat sensitive. The 55K and 52K polypeptides in the infected fluid were cross-reactive to several flaviviruses by the Western blotting method, and could probably be the envelope glycoproteins of the virus.

Key words: Rocio virus, Antigenic polypeptides, Western blotting, Growth in cultured cells, Cross-reactions.

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*Present address: Servico de Virologia, Instituto Adolfo Lutz. Av. Dr. Arnaldo, 355, 01246-Sao Paulo, SP, Brasil

INTRODUCTION

Rocio virus is one of the latest discovered flaviviruses, isolated from the central nervous system of a patient who died during an outbreak of epidemic encephalitis in several coastal counties of Sao Paulo State, Brasil, 1975. Antigenic relationship to other flaviviruses including Japanese encephalitis (JE) virus was evidenced by serological tests (Lopes *et al.*, 1978a). Field studies in the epidemic area indicated that Rocio virus is an arthropod-borne virus, however, its natural history remained unknown (Lopes *et al.*, 1978b). Although the virus was isolated from a single wild bird, *Zonotrichia capensis*, and a pool of *Psorophora ferox* mosquitoes (Lopes *et al.*, 1981), the role of this mosquito as a vector of Rocio virus was questioned by laboratory experiments (Mitchell *et al.*, 1981). Morphological studies on the infected suckling mouse brains (SMB) by electron microscope showed 43nm diameter particles and organelles of the infected cells with similar appearance to those in other flavivirus-infected cells, such as JE, or Murray Valley encephalitis (MVE) viruses (Tanaka *et al.*, 1983). However, there have been no definite reports on the structural polypeptides of Rocio virus or virus-specific polypeptides in the infected cells.

In this report, we describe the growth of Rocio virus in cultured mosquito and vertebrate cells and detection of antigenic polypeptides in the infected fluid and cell homogenate as revealed by the Western blotting method, together with the cross-reactivity of possible envelope glycoproteins with other flaviviruses.

MATERIALS AND METHODS

Viruses: Rocio virus (strain SPH34675) at the second passage in SMB was supplied by the Secao de Virus Transmitidos por Artropodes, Instituto Adolfo Lutz, Sao Paulo, Brasil. JE virus, Nakayama strain, West Nile (WN), MVE, St. Louis encephalitis (SLE), dengue type 2 New Guinea B strain (D2), and Russian spring-summer encephalitis (RSSE) viruses have been kept in this Department. The viruses were grown once in *A. albopictus*, clone C6/36, cells at 28°C to make seed viruses, except RSSE which was grown in BHK21 cells at 37°C.

Cells: *A. albopictus* clone C6/36 cells (Igarashi 1978) were grown at 28°C with cell growth medium of 10 % heat-inactivated fetal calf serum and 0.2 mM each of nonessential amino acids in Eagle's medium (Eagle 1959). BHK21 and Vero cells were grown at 37°C with the same medium as C6/36 cells.

Virus infection and growth curve experiment: Monolayer cultures of C6/36, Vero, or BHK21 cells in 2-ounce bottles were inoculated with 0.2 ml/bottle of seed virus with more than 10^8 PFU/ml. After 2 hours of adsorption, residual virus was removed and the cells were washed twice with phosphate-buffered saline, pH 7.4 (PBS). The cells were covered with 5 ml/bottle of the maintenance medium (cell growth medium from which

serum concentration was reduced to 2 %) and incubated either at 28°C for C6/36 cells or at 37°C for Vero and BHK21 cells. Infected fluids were harvested every 12 hours and the cells were scraped with 2.5 ml/bottle of PBS and homogenized in glass homogenizer. Infectivity and antigenic titer in these specimens were assayed as described be'ow.

Infectivity assasy of the virus: Infected fluid and cell homogenate were serially diluted with 10-fold steps with 0.2 % egg albumin in PBS. Medium was removed from BHK21 cells grown on 24-well plates and 0.1 ml of the diluted specimen was inoculated to each well. After 2 hours of adsorption at 37°C, the cells were covered by 1 ml of 1.5 % methylcellulose in the maintenance medium. After 7 days of incubation at 37°C in humidified 5 % CO_2 atmosphere, the overlay medium was removed and the cells were fixed with cold methanol for 30 min and stained with 0.1 % Trypan Blue to reveal plaques (Hashimoto *et al.*, 1971). The infectivity was expressed as plaque-forming units (PFU) per ml.

Determination of antigen titer by the sandwich ELISA: The principle is as described by Voller *et al.* (1976). Gamma globuin fraction was prepared from high-titered patients' sera of dengue hemorrhagic fever by ammonium sulfate fractionation. A part of the gamma globulin was used as the catching antibody and the rest was conjugated with horseradish peroxidase by Nakane and Kawaoi's method (1974) to prepare detecting antibody. Serial 2-fold dilution of a standard positive antigen with known endpoint titer and a fixed dilution of test specimens were distributed on U-shaped 96-well microplate precoated with the catching antibody. After incubation and washing with PBS-Tween, the plate was reacted with detecting antibody, followed by the washing and peroxidase reaction using *o*-phenylene diamine and hydrogen peroxide as substrates. Optical density of the colored product was measured by Micro ELISA autoreader MR580 (Dynatech, USA). The titer of test specimen was estimated by comparing the color density of each specimen with those by the serial dilution of standard positive antigen with known endpoint titer.

SDS-PAGE and Western blotting: The methods were slightly modified from those described by Towbin *et al.* (1979), Burnette (1981), and Naser and Miltenburger (1983). Specimens of infected fluid or cell homogenate were solubilized either under reducing condition (1 % SDS and 1 % 2-mercaptoethanol) or under nonreducing condition (1 % SDS and 0.5mg/ml of iodoacetamide) in 0.125 M Tris-HCl, pH6.8 (Durbin and Stollar 1984), either by heating at 100°C for 1 min or kept at room temperature for 10-20 min before electrophoresis by 10% polyacrylamide slab gels with discontinuous buffer system (Laemmli, 1970). After the run, separated polypeptides were electro-transferred to nitrocellulose membrane. The membrane was inactivated by incubation with 3 % casein for 45 min, followed by the antivirus mouse serum or immune ascitic fluid (IAF) diluted 1:300 to 1:1000 in PBS. After 3 hours of incubation at 37°C and washing with PBS, the membrane was reacted with peroxidase-conjugated anti-mouse IgG goat IgG at 1:1000 dilution in PBS at 37°C for 2 hours. The membrane was washed with PBS and peroxidase reaction was performed with 25μg/ml of *o*-dianisidine and 0.01% of hydrogen

peroxide in PBS at room temperature until positive bands became distinct against background. Molecular weights of polypeptides were estimated by comparing their mobilities with those of the molecular weight markers (Pharmacia, Sweden) which were run in a parallel slot and stained with Coomassie Brilliant Blue.

Antisera: Hyperimmune mouse IAF against Rocio virus and a Brazilian isolate of SLE virus, strain SPAn11916, were prepared by 4 immunizations of infected SMB homogenate and were supplied by Secao de Virus Transmitidos por Artropodos, Instituto Adolfo Lutz. Hyperimmune mouse sera agasint JE, WN, MVE, SLE, D2, and RSSE viruses have been kept in this Institute after prepared by 5 immunizations of infected SMB homogenates. Peroxidase-conjugated anti-mouse IgG goat IgG was the product of Cappel Laboratories, USA.

Reagents: Horseradish peroxidase, type VI, was obtained from Sigma Chemicals Co. USA. Iodoacetamide, acryamide, bisacrylamide, *o*-phenylene diamine and *o*-dianisidine were the products of Wako Pure Chemicals Co. Osaka.

RESULTS

Growth of Rocio virus in cultured mosquito and vertebrate cells

Seed of Rocio virus (over 10^8 PFU/ml) was inoculated to bottle cultures of C6/36, Vero, and BHK21 cells and specimens were harvested at various times after the infection to assay virus infectivity and antigen titer as described in the Materials and Methods. The results in Fig. 1 showed that the virus grew well in all the 3 lines of cultured cells, although the growth rate in C6/36 cells was somewhat slower than in BHK21 or Vero cells. However, higher virus infectivity (over 10^8 PFU/ml) was obtained in C6/36 cells than in Vero (10^8 PFU/ml) and BHK21 cells (10^7 PFU/ml). The virus infectivity in the fluid was higher than that in the cell homogenate. In C6/36 cells, the virus ELISA antigen became detectable at 24 hours both in the infected fluid and cell homogenate and gradually increased its titer in the fluid during the observation period up to 96 hours after infection, however, the titer of ELISA antigen in the cell homogenate was less than in the fluid and leveled off after 36 hours and began to decline after 72 hours of infection. On the other hand, virus antigen was under detectable levels in the infected culture fluid of BHK21 or Vero cells, although it was detected in the infected cell homogenates after 12 hours of infection.

Antigenic polypeptides in the infected fluid and cell homogenate as revealed by Western blotting method

Specimens of infected C6/36 cell culture fluid and cell homogenate were analyzed by SDS-PAGE followed by the Western blotting as described in the Materials and Methods. The results in Fig. 2 showed that some of the antigenic polypeptides in the infected cells (105K and 60K daltons) which reacted with anti-Rocio IAF began to appear at 12 hours

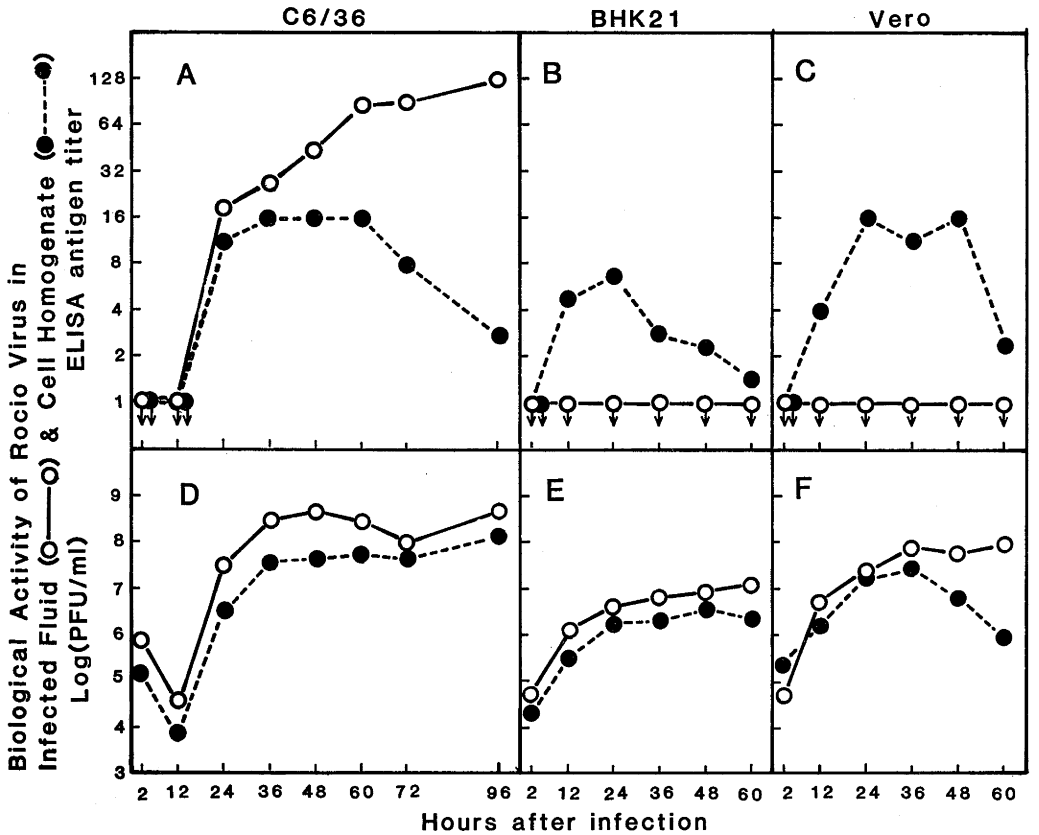

Fig. 1. Production of infective virus and ELISA antigen of Rocio virus in infected mosquito and vertebrate cells. Rocio virus was inoculated to bottle cultures of C6/36 (Panels A, D), BHK21 (Panels, B, E), and Vero (Panels C, F) cells, and infectivity (Panels D, E, F) and ELISA antigen (Panels A, B, C) titers in the infected fluid (○——○) and cell homogenate (●·····●) were measured as described in the Materials and Methods and Text.

after infection and their staining intensity became almost maximum 24 hours after infection. After 24-36 hours of infection, 2 more major (83K, and 56K) and 5 minor (170K, 140K, 115K, 24K, and 23K daltons) antigenic polypeptide in the infected cells were also observed. The major antigenic polypeptide of 55K daltons in the infected fluid began to appear at 24 hours after infection, while other components of 170K, 140K, 115K, 100K, 90K, 52K, and 23K daltons were visible in the infected fluid after 36 hours of infection. Specimens obtained from C6/36 cells 96 hours after infection were treated at 100°C for 1 min or at room temperature under reducing or nonreducing conditions before SDS-PAGE and Western blotting and the result is shown in Fig.3. The reactivity of several polypeptides in the infected cells (170K, 60K, 56K, 24K, and 23K) and those in the infected fluid

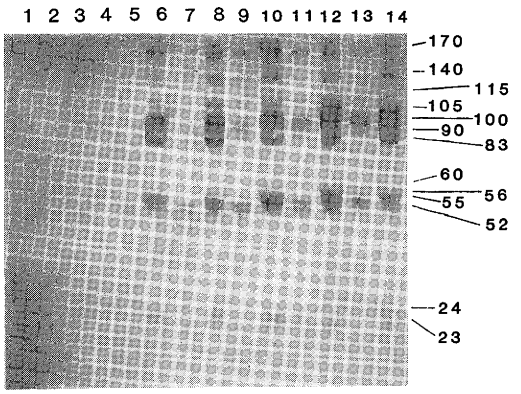

Fig. 2. Rocio virus-specific polypeptides in infected C6/36 cells as revealed by the Western blotting. *A. albopictus*, C6/36 cell were infected with Rocio virus as described in the Materials and Methods. Infected culture fluid (lanes 1, 3, 5, 7, 9, 11, 13) and cell homogenate (lanes 2, 4, 6, 8, 10, 12, 14) were prepared at various times after infection. Sampling time was: lanes 1, 2: 0 hour, lanes 3, 4: 12 hours, lanes 5, 6: 24 hours, lanes 7, 8: 36 hours, lanes 9, 10: 48 hours, lanes 11, 12: 60 hours, lanes 13, 14: 72 hours after infection and incubated at 28°C. Specimens were solubilized under nonreducing condition at room temperature before SDS-PAGE and Western blotting using anti-Rocio IAF. Figures on the right side of the picture represent estimated molecular weights for each band.

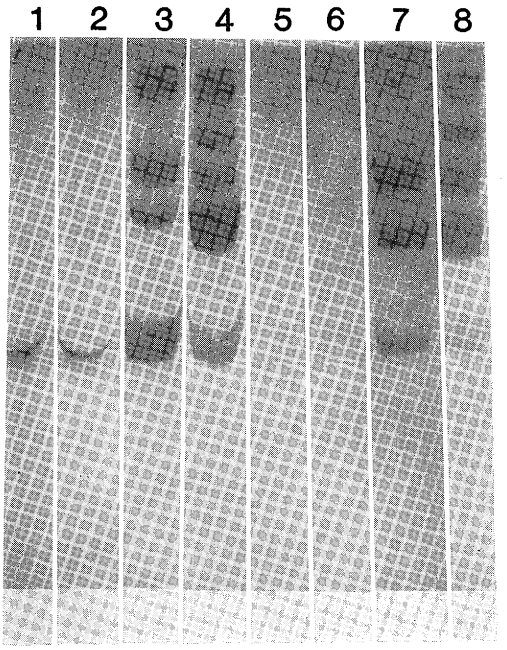

Fig. 3. Rocio virus-specific polypeptides detected by the Western blotting using various methods of sample preparation. Rocio virus-infected fluid (lanes 1, 2, 5, 6) and cell homogenate (lanes 3, 4, 7, 8) were prepared from C6/36 cells 96 hours after infection and specimens were solubilized under nonreducing condition (lanes 1, 2, 3, 4) or under reducing condition (lanes 5, 6, 7, 8), either by heating at 100°C (lanes 1, 3, 5, 7) or by keeping at room temperature (lanes 2, 4, 6, 8), before SDS-PAGE and Western blotting using anti-Rocio IAF.

against anti-Rocio IAF was markedly reduced or disappeared when the specimens were treated under reducing conditions either at room temperature or at 100°C, indicating the importance of disulfide bonds to maintain antigenicity of these components. On the other hand, polypeptides of 140K, and 83K daltons in the infected cells disappeard when the specimen was heated at 100°C for 1 min either under reducing or nonreducing condition, indicating that the antigenicity of these components were heat-labile.

Cross-reactions of antigenic polypeptides of Rocio virus and other flaviviruses in the infected fluids as revealed by the Western blotting method

Infected culture fluids were harvested from C6/36 cells 5-7 days after infection with

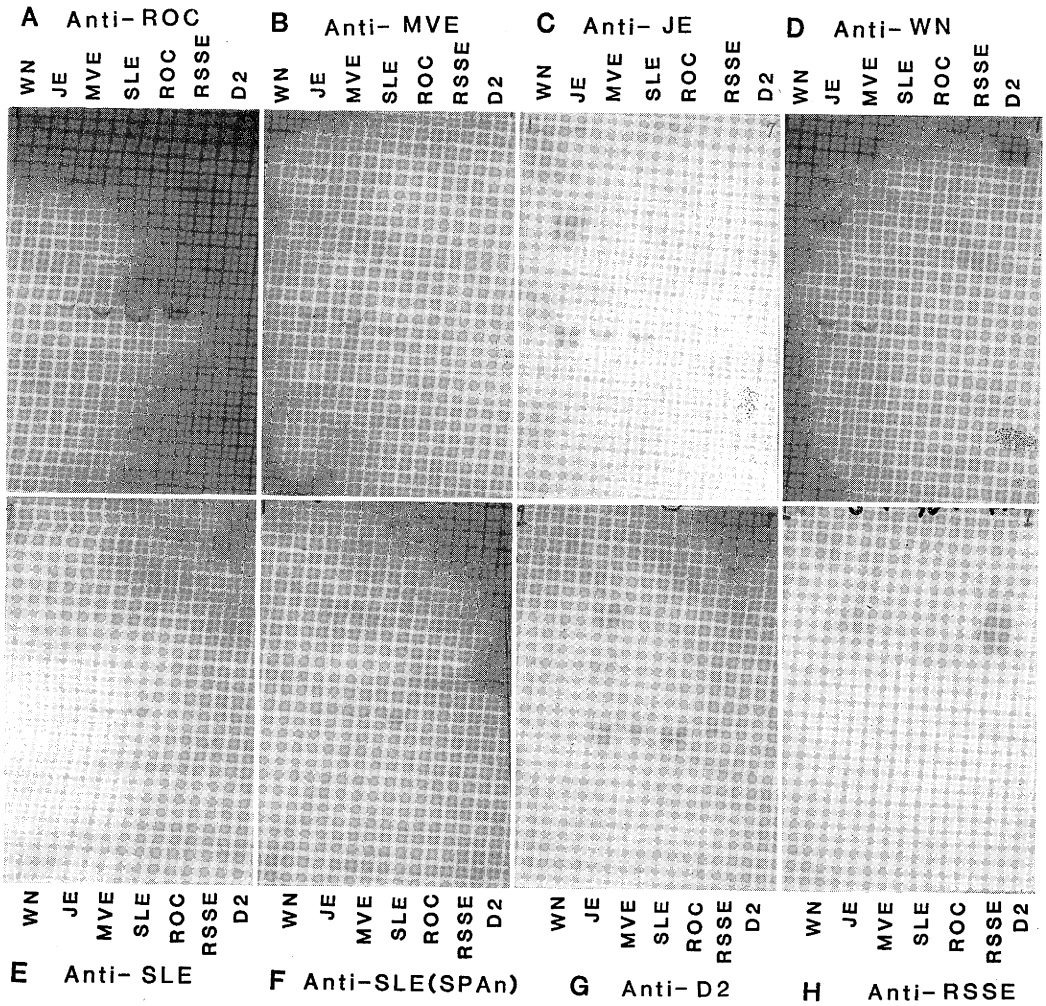

Fig. 4. Cross-reactions between Rocio virus and several flaviviruses as revealed by the Western blotting of infected culture fluid. Infected fluids were prepared from C6/36 cells infected with Rocio (ROC), WN, JE, MVE, SLE, and D2 viruses 5-7 days after incubation at 28°C, and from BHK21 cells infected with RSSE virus 3 days after incubation at 37°C. Specimens were solubilized under nonreducing condition at room temperature before SDS-PAGE and Western blotting using anti-Rocio IAF (Panel A), anti-MVE (Panel B), anti-JE (Pane C), anti-WN (Panel D), anti-SLE (Pane E), anti-SLE (SPAn strain: Panel F), anti-D2 (Panel G), and anti-RSSE (Panel H) mouse sera, respectively.

various flaviviruses, such as JE, WN, MVE, SLE, and D2 viruses. RSSE virus was grown in BHK21 cells at 37°C. These infected fluids, together with C6/36 cell culture fluid obtained at 96 hours after Rocio virus infection, were treated under nonreducing condition at room temperature before SDS-PAGE and Western blotting using anti-Rocio, anti-JE, anti-WN, anti-MVE, anti-SLE, anti-SLE (SPAn11916), anti-D2 and anti-RSSE virus sera or IAF. Fig. 4, Panels A through H, show the results of the experiments. Rocio virus antigen reacted most with the homologous anti-Rocio IAF, followed by anti-MVE and anti-D2 sera. Lower grades of reactions were observed with anti-WN, anti-SLE (SPAn11916), anti-SLE, and anti-JE sera or IAF. The homologous anti-Rocio IAF reacted with both 55K and 52K components (as described in the previous section), so were with anti-MVE, anti-WN, and anti-SLE(SPAn11916), however, only a single band (probabaly 52K component) reacted with anti-D2, anti-SLE, or anti-JE sera. On the other hand, anti-Rocio IAF reacted with a single component of JE, and MVE virus infected fluid, and less with a single component of WN or RSSE virus infected fluid. Anti-MVE, anti-WN, anti-SLE, anti-SLE (SPAn11916), and anti-D2 appeared to detect 2 components with close apparent molecular weights for Rocio, MVE, and JE; JE, and Rocio; SLE; JE, and Rocio; RSSE, and MVE virus infected fluids, respectively. On the other hand, anti-JE serum reacted with only one of these 2 components, except that of 140K dalton component in JE virus infected fluid. The 140K dalton component was also observed in MVE virus infected fluid with anti-D2 serum, and in RSSE virus infected fluid with the homologous anti-RSSE serum. Whlie, 170K dalton component in MVE and D2 virus infected fluids reacted with anti-WN serum. The identity of these apaprently high-molecular weight components with those observed in Rocio virus-infected C6/36 cells remains to be investigated.

DISCUSSIONS

The assumption that Rocio virus is a mosquito-borne arbovirus (Lopes *et al.*, 1981) was supported by the present results showing its growth to high titer in cultured *A. albopictus* C6/36 mosquito cells. The higher infectivity titer and even higher ELISA antigen titer were obtained in C6/36 cells than in Vero or BHK21 cells. The results might be due to the lower incubation temperature of C6/36 cells compared with those for mammalian cells resulting in the better maintenance of virus infectivity and antigenicity. However, the result might also be due to the intrinsic characteristics of C6/36 cells to produce larger amount of mosquito-borne arboviruses, showing that C6/36 is the best cell system for the production of these viruses, incuding Rocio virus. The reason why less amount of ELISA antigen was detected in the infected fluid than in infecten C6/36 cell homogenates, especially at late stage of infection, is unknown, although higher reaction was obtained with infected cells than infected fluid after Western blotting. There may be

some inhibitory factor(s) produced in the cell homogenate at late stage of infection and interfered the binding of virus antigen to catching antibody, such factor (s) could be separated from antigenic components or inactivated after SDS-PAGE. Another possibility could be that the antigenic components in the cell homogenate at late stage of infection were not exposed by simple homogenization but could be soubilized after SDS treatment. Also it could be conceivable that the Rocio virus ELISA antigens became monovalent at late stage of infection and could not be detected by the sandwich ELISA, although they could react in the Western blotting. On the other hand, Rocio virus ELISA antigen was detected better in infected Vero or BHK21 cell homogenate than with infected culture fluid. Therefore, such inhibitory factor(s) or masking were less operating in these mammalian cells compared with C6/36 mosquito cells.

Apparent molecular weights estimated for various antigenic components observed in Rocio virus-infected C6/36 cell culture fluid or cell homogenate were compared with those reported for flavivirus-specified proteins (Westaway, 1973). The 55K and 52K dalton components in the infected fluid and probably also the 56K dalton component in the infected cells could represent envelope glycoproteins, because of their molecular weight similarity as well as the reactivity of the 55K and 52K components with other flavivirus antisera. Sensitivity of their antigenicity toward reducing reagent like 2-mercaptoethanol was observed, indicating the importance of disulfide bonds to maintain their antigenicity, although their apparent molecular weight did not change significantly either under reducing or nonreducing condition. Such sensitivity of antigenicity to 2-mercaptoethanol treatment was also observed with several other polypeptides (170K, 140K, 115K, 23K, both in the infected cells and fluid; 60K, 24K in infected cells; and 100K, 90K in the infected fluid). On the other hand, 140K and 60K dalton polypeptides appeared to lose their antigenicity by heating at 100°C for 1 min. From their apparent molecular weights, 105K in the infected cells and 100K in the infected fluid may correspond to NV-5, while 83K in the infected cells and 90K in the infected fluid may correspond to NV-4, and 23-24K polypeptides to NV-2½, respectively (Westaway, 1973). However, these assignments should wait further analysis, because present data were mostly obtained by the Western blotting method under nonreducing conditions and previous data by other investigators were obtained by radiolabeling under reducing conditions. This precaution is especially required for NV-2½, because this polypeptide was never reported in the infected fluid although it was reported in the flavivirus infected cells. The components wtih higher molecular weight over 115K daltons may represent dimers, trimers or tetramere of other lower molecular weight components, because of the milder conditions of sample preparation used in this study. However, some of them could be independent entities because of their different heat stability and sensitivity to 2-mercaptoethanol treatment and also different time course of the appearance after infection.

By classical serology, Lopes *et al.* (1978a) observed the strongest cross-reaction of Rocio virus with SLE, Ilheus, JE, and MVE viruses among flaviviruses. The present

data generally agree with their results, however, the closest relation of Rocio virus was observed to MVE by the Western blotting and the relation to WN, SLE, and JE was less and the relatedness to D2 and RSSE appeared to be further less. These results is compatible with the classification of Rocio virus as a member of JE-WN-MVE-SLE subgroup of flaviviruses (Shope, 1980), although Porterfield (1980) listed it under Uganda S subgroup. Apparent doublet nature of probable envelope glycoproteins (55K and 52K) as shown by present study requires further examinations because all the other studies on the envelope protein of flaviviruses were performed under reducing conditions using radiolabeled specimen. However, there may be 2 closely related envelope glycoproteins with slightly different molecular configurations under nonreducing conditions, which could be reduced to a single component under reducing conditions. It would be especially interesing to study whether each of these components reacts specifically to different flavivirus antisera, as suggested from some of our results.

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Western blotting 法による Rocio ウイルス感染細胞および培養液内の ウイルス特異的ポリペプチド抗原の検出

Tiyo SAKURAI, 分藤桂子, 五十嵐 章 (長崎大学熱帯医学研究所ウイルス学部門)

フラビウイルスの一つである Rocio ウイルスを 28°C のヒトスジシマカ培養細胞クローン C6/36 細胞, および37°Cの BHK21 と Vero 細胞で増殖させた. C6/36 細胞では感染後36時間で 10^8PFU/ml 以上のウイルス産生が見られたが, Vero 細胞と BHK21 細胞でのウイルス産生は各々 10^8PFU/ml と 10^7PFU/ml であった. C6/36 細胞ではウイルス感染価と ELISA 抗原価は共に感染細胞乳剤よりも感染培養液中で高かったが, BHK21 と Vero 細胞では細胞乳剤中の ELISA 抗原価の方が感染培養液中の値よりも高かった. ウイルス感染 C6/36 細胞培養液と細胞乳剤とを SDS-ポリアクリルアミドゲル電気泳動後, Western blotting によってウイルス特異的な抗原性を有するポリペプチドを検出した. 還元剤を加えない条件下で試料を作成した場合, 感染培養液中の主なポリペプチドは分子量 55K ダルトンであり, 他に分子量 170K, 140K, 115K, 100K, 90K, 52K, および 23K ダルトンの7種のポリペプチドが少量ずつ検出さ

た．一方，感染細胞内には，分子量 105K，83K，60K，56K ダルトンの４つのペプチドが多量に検出された他，分子量 170K，140K，115K，24K，23K ダルトンの５つのポリペプチドが少量づつ検出された．これらのポリペプチドのうち 105K と 60K のものは感染後12時間目より出現しはじめ，他のポリペプチドも感染後24～36時間では充分に検出されるようになった．還元状態で試料を作成すると感染細胞内の分子量 170K，60K，56K，24K，23K のポリペプチドおよび感染培養液中のポリペプチドは検出されなくなるか量的に検出されにくくなる．それに対して，感染細胞内の分子量 140K と 83K のポリペプチドは易熱性であるらしい．感染培養液中の分子量 55K と 52K のポリペプチドは Western Blotting 法で他のフラビウイルスと交叉反応を示し，恐らくウイルス外被膜の糖タンパクであろうと思われる．

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