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RAPID COMMUNICATION

Light-Independent Inactivation of Dengue-2 Virus by Carboxyfullerene C3 Isomer

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Carboxyfullerene (C60) is known as a photosensitizer for virus inactivation. Its regioisomer with C3 symmetry, named the C3 isomer, could also inactivate the dengue-2 virus without light when the dose of C3 isomer was increased to 40 μM, indicating the possible involvement of a light-independent mechanism. Further analysis showed that the C3 isomer blocked viral replication at the attachment and penetration stages, suggesting that a direct interaction between the C3 isomer and the virion is required for inactivation. The C3 isomer with a bipolar structure showed better lipid interaction and dengue-2 virus suppression than D3, another isomer that contains evenly distributed hydrophilic side chains. Moreover, the C3 isomer selectively inactivated enveloped viruses (viz., dengue-2 virus and Japanese encephalitis virus) instead of nonenveloped viruses (viz., enterovirus 71 and coxsackievirus B3). Collectively, these findings support the hypothesis that C3 isomer suppression of enveloped viruses is effected through its hydrophobic interaction with the viral lipid envelope. Our report, which demonstrates the light-dependent and -independent mechanisms of C60 on viral inactivation, will aid in the development of novel anti-viral agents for use against enveloped viruses. © 2000 Academic Press

Key Words: C60; dengue-2 virus; enveloped virus; HuH-7.

Introduction. Carboxyfullerene (C60) and carboxyl clusters with more than 60 carbon atoms are commonly referred to as fullerenes (*1*). Native C60 is hydrophobic and soluble only in organic solvents. Dugan *et al.* added functional groups to C60 (three malonic acid groups per molecule) to enhance its water solubility (*2*). The C3 isomer, a C60 derivative, has a bipolar structure with all hydrophilic moieties on one side and the hydrophobic portion on the other side of the fullerene cage (Fig. 3A). The C3 isomer showed better lipid interaction than D3, another isomer with its hydrophilic moieties around the equator (*3, 4*) (Fig. 3A). C60 is an excellent electron acceptor and can be used as a free radical scavenger to block the apoptotic signal triggered by transforming growth factor-β (*4*). C60 can also inhibit *Escherichia coli*-induced meningitis (*5*). Moreover, C60, also known as a photosensitizer, can photoinactivate Semliki Forest virus (SFV, *Togaviridae*) and vesicular stomatitis virus (VSV, *Rhabdoviridae*) by generating singlet oxygen following the absorption of light (*6–8*).

Dengue viruses (serotype 1 to 4) belonging to *Flaviviridae* are single-stranded RNA viruses. Patients with dengue virus infection showed various clinical symptoms, from mild fever to life-threatening hemorrhagic fever and/or shock syndrome (DHF/DSS) (*9*). The serum of patients with dengue fever and DHF also showed elevated levels of transaminase AST (*10*). Dengue viral antigen has been detected in the hepatocytes and dengue viruses in the liver biopsies of DHF patients (*11, 12*). Recently, a mouse model also demonstrated the pathogenic role of the liver during dengue virus infection. All together, these indicate that the liver is a target organ of dengue virus infection (*13*). It is, therefore, important to use liver cell lines as a model for studying dengue virus.

In this study, we evaluated the potency of the C3 isomer in inhibiting dengue-2 virus infection in human hepatoma cells, HuH-7, and explored the mechanism of its action against dengue-2 virus. We concluded that a mechanism other than photoinactivation is possibly involved.

Results. Light-independent inactivation of dengue-2 virus by C3 isomer. Because C60 was able to inactivate SFV or VSV through a photodynamic reaction (*7*), we evaluated its effect on the dengue-2 virus. The synthesis of dengue-2 virus negative-stranded (−) RNA (representing virus replication) in the infected HuH-7 cells was detected at 36 h p.i. by slot blot analysis (*14*). The C3 isomer at a concentration of 10 μM inhibited dengue-2 virus replication when illuminated by a 20-W white light bulb for 1 h (Fig. 1A; illuminated). The C3 isomer can also completely suppress viral replication in the absence of illumination (Fig. 1A; unilluminated) or in total darkness (Fig. 1A; dark), but at a concentration of 40 μM.

Singlet oxygen produced during the photodynamic reaction with C60 is responsible for the virucidal effects; it can be removed by NaN_3, a singlet-oxygen scavenger (*6,*

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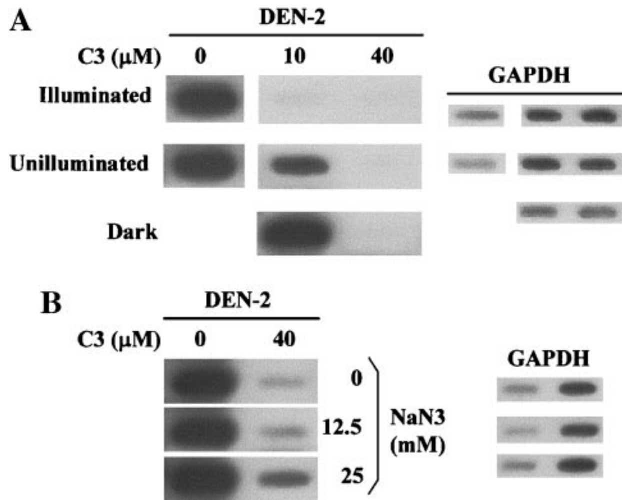

FIG. 1. The C3 isomer without illumination can inactivate dengue-2 virus. The inactivation is only partially blocked by NaN_3. A: The C3 isomer (10 or 40 μM) combined with dengue-2 virus (10 m.o.i.) was used to infect HuH-7 cells. After virus adsorption, the virus–C3 mixtures were removed and the cells were washed three times with PBS. The total cellular RNA was harvested for viral (−) RNA detection by slot blotting at 36 h p.i. Illuminated: The virus–C3 mixtures were added to the 6-well tray and placed on ice. The mixtures were then illuminated by a 20-W white light bulb (20 cm away) for 1 h before being added to the culture. Unilluminated: The virus–C3 mixtures were placed on ice for 1 h without illumination. Dark: The virus–C3 mixtures were covered by aluminum foil and placed on ice for 1 h. All of the viral infections were created in the dark. B: NaN_3 (12.5 or 25 mM) combined with dengue-2 virus was used to infect HuH-7 cells in the presence or absence of the C3 isomer (40 μM). After virus adsorption at 37°C for 2 h, the cells were washed three times with PBS. At 36 h p.i., the total cellular RNA was extracted for viral (−) RNA detection by slot blotting. DEN-2, dengue-2 virus; GAPDH, RNA loading control.

7). We studied the effect of NaN_3 on C3-isomer-induced inactivation of dengue-2 virus in HuH-7 cells. NaN_3 (12.5 or 25 mM) treatment did not affect dengue-2 virus replication (Fig. 1B) and only partially prevented the inhibitory effect of the C3 isomer on dengue-2 virus replication (Fig. 1B). We used concentrations higher than 25 mM, and the results were the same as those described above (data not shown). The involvement of reactive oxygen species in viral inactivation by the C3 isomer was excluded by using a strong free radical remover, pyrrolidine dithiocarbamate (data not shown). Collectively, these experiments show that the C3 isomer may utilize a light-independent mechanism to inactivate dengue viruses.

C3 Isomer Suppresses Dengue-2 Virus Replication at the Adsorption Stage. To demonstrate the light-independent mechanism, we initially determined the stage of dengue-2 virus infection at which the C3 isomer could inactivate the virus. The C3 isomer was added at the preadsorption, "1," attachment, "2," penetration, "3," or postadsorption "4," stage of dengue-2 virus infection in HuH-7 cells (Fig. 2A) (*15*). Since 40 μM of the C3 isomer could inactivate the virus effectively without illumination and in total darkness (Fig. 1A), we conducted the following experiments at that concentration in the absence of illumination to further explore the light-independent mechanism. Figure 2B shows that dengue-2 virus (−) RNA became undetectable after the C3 isomer was added at either the attachment or the penetration stage of the viral invasion, indicating that coexistence of the C3 isomer and the virion is required for viral inactivation.

C60 with Bipolar Structure Shows Better Inhibition of Dengue-2 Virus. The C3 isomer showed strong interaction with lipid bilayers because of its bipolar structure (*3, 4*) (Fig. 3A). We therefore speculate that the mechanism utilized by the C3 isomer may be through interaction with the viral lipid envelope to inactivate the dengue-2 virus. To confirm this hypothesis, we used another isomer of C60, D3, which showed less affinity for the membrane than did C3. We mixed C3 and D3 isomers at different doses (0, 10, 20, and 40 μM) with dengue-2 virus (10 m.o.i.) and then used them to infect HuH-7 cells. After virus adsorption, we removed the virus mixtures and washed the cells with PBS three times. Viral (−) RNA synthesis was detected by slot blotting at 36 h p.i. Figures 3B and 3C show that the C3 isomer could inhibit virus infection at the adsorption stage in a dose-dependent pattern and was more effective than D3. Plaque-

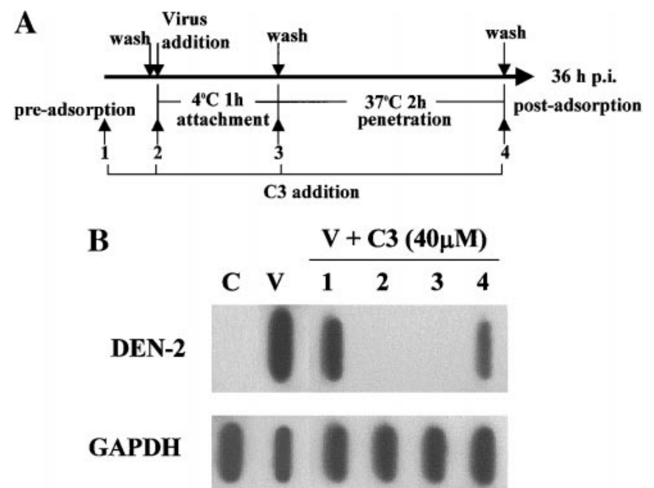

FIG. 2. The C3 isomer suppresses dengue-2 virus replication at the adsorption stage. A: The schematic procedure for C3 isomer treatment. Preadsorption stage: One hour before dengue-2 virus was added to the cells. Attachment stage: Dengue-2 virus was added to HuH-7 cells and allowed to adsorb the cells for 1 h at 4°C. Penetration stage: The cultures were then washed twice with PBS and incubated at 37°C for 2 h with medium containing 2% FBS. Postadsorption stage: Finally, the cultures were washed again and replaced with medium containing 10% FBS and then cultured at 37°C. B: The C3 isomer (40 μM) was added at different points indicated by arrows (1, 2, 3, and 4). After C3 isomer treatment, the cells were washed as indicated. At 36 h p.i., the total cellular RNA was extracted for viral (−) RNA detection by slot blotting. C, mock infection; V, dengue-2 virus infection; V + C3, dengue-2 virus combined with C3 isomer; 1, C3 isomer was added 1 h before viral adsorption; 2, C3 isomer was added at the attachment stage; 3, C3 isomer was added at the penetration stage; 4, C3 isomer was added after the adsorption stage.

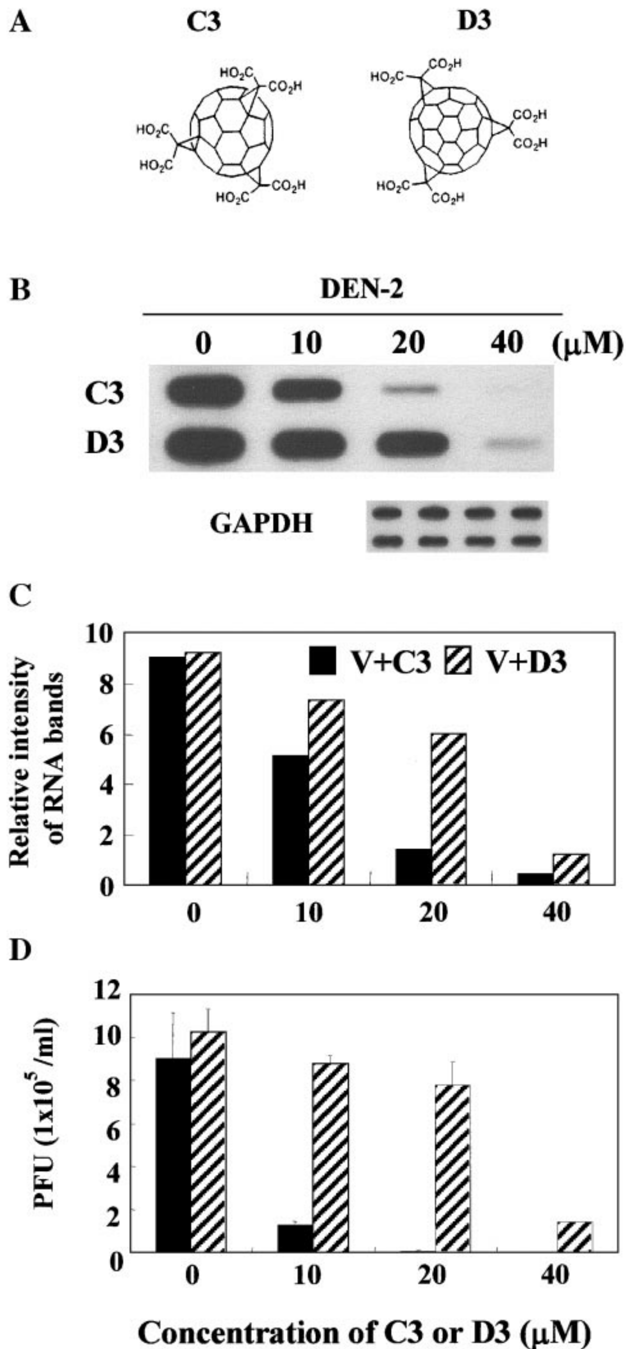

FIG. 3. The C3 isomer shows better inhibitory effect on dengue-2 virus than the D3 isomer. The molecular structures of the C3 and D3 isomers are illustrated in (A) (*3*). Different dosages of the C3 and D3 isomers (0, 10, 20, and 40 μM) combined with dengue-2 virus were used to infect HuH-7 cells. After virus adsorption, the cells were washed with PBS three times to avoid viral remnants. At 36 h p.i., the culture supernatant was harvested for plaque assay (D), and the total cellular RNA was extracted for viral (−) RNA detection (B). The RNA band intensity was quantified by a densitometer (Vilber Lourmat, France) (C). V + C3, dengue-2 virus combined with C3 isomer; V + D3, dengue-2 virus combined with D3 isomer.

assay analysis of the virion released in the culture supernatant was consistent with the result of slot blotting (Fig. 3D).

C3 Isomer Preferably Suppresses Enveloped Viruses. To further verify that the C3 isomer uses lipid interaction for viral inactivation, we compared the inhibitory effect of the C3 isomer on enveloped and nonenveloped viruses. Dengue-2 virus and Japanese encephalitis virus (JEV) are members of the *Flaviviridae* family with envelope, while enterovirus 71 (EV71) and coxsackievirus B3 are members of the *Picornaviridae* family without envelope. We mixed the C3 isomer at various doses with each of the four viruses and used the mixture to infect the cells. We observed the plaque formation 3 to 5 days later. Our data show that the C3 isomer effectively inhibited the plaque formation of the enveloped viruses but not of the nonenveloped viruses (Fig. 4). This indicates that the C3 isomer may bind with the lipid envelope to inactivate the virus.

C3 Isomer Prevents the Increase of Aminotransferase Activity by Dengue-2 Virus Infection in HuH-7 Cells. We analyzed the cellular response while C60 was used for viral inactivation. Dengue-2 virus infection of liver cell lines could cause an increase in extracellular aminotransferase AST, an indicator of liver injury, which was consistent with the increase of AST in patients with dengue fever (*14*). We therefore measured the extracellular AST level of HuH-7 cells after inoculation with the dengue-2 virus (with or without the C3 isomer) at 36 h p.i. Figure 5 shows that the C3 isomer per se did not increase the AST level at a dose of 40 μM. Furthermore, when it was used in combination with dengue-2 virus, it prevented an increase of AST in HuH-7 cells. Consistently, the C3 isomer could also prevent morphological changes of dengue-2 virus-infected HuH-7 cells (data not shown).

Discussion. In this report, we show that both light-dependent and -independent mechanisms are involved

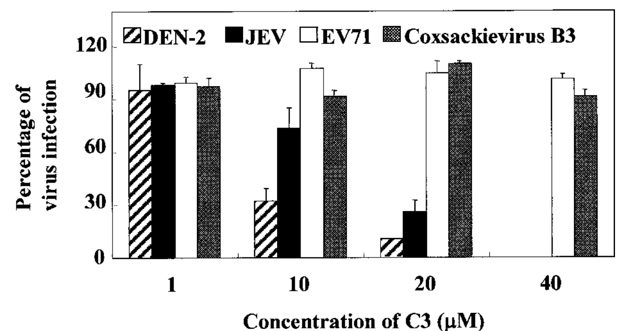

FIG. 4. The C3 isomer preferably inactivates the enveloped viruses. The cells in 35-mm tissue culture dishes were inoculated with 10^{-3} m.o.i. of viruses. BHK21 cells were used for dengue-2 virus and JEV infection. Vero cells were used for EV71 and coxsackievirus B3 infection. Different doses of the C3 isomer (1, 10, 20, and 40 μM) were mixed with the virus solutions for infection. After adsorption at 37°C for 2 h, the virus mixtures were removed and the cells were overlaid with 0.5% MC for further incubation until plaque formation (5 days for dengue-2 virus and 3 days for the other viruses). The percentage of virus infection was calculated by dividing the plaque number of virus infection with C3 isomer ($P^{(V+C3)}$) by the plaque number of virus infection without C3 isomer (P^{V}).

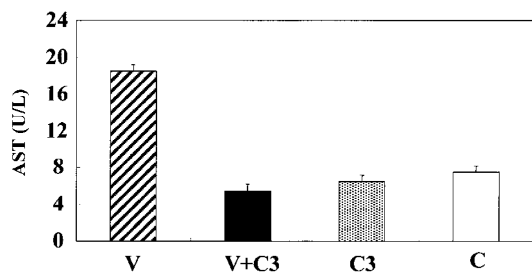

FIG. 5. The C3 isomer prevents an increase in AST level by dengue-2 virus infection in HuH-7 cells. HuH-7 cells were infected with dengue-2 virus (10 m.o.i.) and the culture supernatants were harvested for AST analysis at 36 h p.i. V, dengue-2 virus infection; V + C3, dengue-2 virus combined with C3 isomer (40 μM); C3, C3 isomer (40 μM); C, mock infection.

in the inactivation of dengue viruses by the C3 isomer. Further analysis finds that in the light-independent mechanism, the C3 isomer inactivated the virus through hydrophobic interaction with the viral lipid envelope. Although photodynamic inactivation of VSV and SFV by C60 has been reported (*6, 7*), this is the first paper to report that C60 could specifically inhibit enveloped viruses through hydrophobic interaction with the lipid envelope.

Methylene blue, the dye most widely used to generate singlet oxygen, can photodynamically inactivate the viruses (*16*). Similarly, C60 acting as a photosensitizer can be excited into a short-lived singlet state following the absorption of light; it then generates singlet oxygen in an aqueous system (*6–8*). Singlet oxygen may inactivate the viruses by damaging viral protein or nucleic acid. We also show that inactivation of the dengue virus by the C3 isomer at a lower concentration (10 μM) was very effective in the presence of illumination. Without illumination, however, C60 was also able to suppress the virus when the dose of C3 isomer was increased from 10 to 40 μM, indicating the involvement of a light-independent mechanism, which may complement the inactivation effect generated by singlet oxygen.

While analyzing what the mechanism might be, we learned that the C3 isomer prevented virus replication predominantly at the adsorption stage. Once inside the cell, C3 could no longer inhibit virus replication, indicating that the inactivation needs direct interaction between the C3 isomer and the intact virion. Although C60 can act as an antioxidant inside the cell to suppress apoptosis (*2–4*), the C3 isomer seems unable to exert any inhibitory effect on dengue-2 virus replication once the virus is inside the cell.

C3 and D3 isomers reacted similarly to illumination, but they showed different affinities for the lipid membrane because of the polarity difference between their structures. Our data indicate that the hydrophobic interaction of the C3 isomer with the viral lipid envelope is probably the light-independent mechanism that causes viral inactivation. It is well known that enveloped viruses can be inactivated through hydrophobic interaction with lipid solvents like ether and chloroform (*17*). The hydrophobic region of the C3 isomer may serve as a lipid solvent able to specifically inhibit the enveloped virus. Our hypothesis is further supported by treatment of the C3 isomer with dimethyl sulfoxide, which dissolved the hydrophobic region of the C3 isomer and prevented its interaction with viruses as well as its viral inactivation effect (data not shown). The interaction between the C3 isomer and the viral lipid envelope may cause two possible underlying mechanisms for light-independent inactivation: (1) inhibition of viral-cell fusion; (2) inactivation of viral protein anchoring on the envelope.

Although C60 can be used as a photosensitizer, the photodynamic reaction is also cytotoxic (*18*). For this reason, photo illumination of C60 can be used only to enhance viral inactivation in a cell-free system. Our study shows, however, that the concentration of the C3 isomer used to inactivate enveloped viruses without illumination was not toxic to the host cells. Our finding that C60 has no cytotoxic effect on the cells when manipulated without light is important for clinical treatment. In conclusion, the finding that C60 uses two mechanisms (light-dependent and light-independent) to inactivate enveloped viruses will broaden the application of C60 in antiviral study.

Materials and Methods. Cell lines and viruses. HuH-7 (human hepatoma cell line), BHK-21 (baby hamster kidney cell line), and Vero (African green monkey kidney cell line) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibcol BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Trace, Australia), penicillin (200 unit/ml), and streptomycin (100 μg/ml) at 37°C in a 5% CO_2 incubator. Dengue-2 virus (strain PL0146 isolated from a patient in Taiwan) and JEV were prepared from C6/36 cells. EV71 (strain 4643 isolated from a patient in Taiwan) (*19*) and coxsackievirus B3 (strain H0297 isolated from a patient in Taiwan) (*20*) were amplified using Vero cells.

Chemicals. Two hexacarboxylic acid derivatives of C60 $[C(COOH)_2]_3$ with C3 or D3 symmetry were synthesized and purified following the method of Lamparth and Hirsch and colleagues (*3, 21*). The purity of these compounds was verified by NMR and ultraviolet/visible spectral analyses. Sodium azide (NaN_3) was purchased from Sigma (St. Louis, MO).

Plaque Assay. Plaque assay was performed as previously reported (*14*). For the C3 isomer study, the cells (3×10^5/well) were plated onto a 6-well plate overnight and then infected with 10^{-3} m.o.i. of viruses with or without the C3 isomer. After virus adsorption, the cells were washed three times with PBS and overlaid with DMEM containing 2% FBS and 0.5% methyl cellulose (MC) (Sigma). While the plaques formed, the cells were fixed and stained with crystal violet solution (1% crystal violet, 0.64% NaCl, and 2% formalin).

Primers and Probes. A 419-bp DNA fragment of dengue-2 virus was amplified by RT-PCR using the primers AD3 (3412–3428; 5′-CTGATTTCCATCCCGTA-3′) and AD4 (3009–3028; 5′-GATATGGGTTATTGGATGGA-3′). The PCR DNA was purified for asymmetry PCR labeling in a reaction solution of 100 μM dATP, dGTP, and dTTP, 1× reaction buffer (Kevin Science Technology, Brea, CA), 1 unit of Biothermal DNA polymerase (Kevin Science Technology), 10 μCi of [α-^{32}P]dCTP (Amersham, Buckinghamshire, UK), 1 μM of AD4 primer, and 50 ng of DNA. The reaction was then performed in a PCR thermal cycler (Perkin–Elmer) under the following conditions: 2 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 50°C, 50 s at 72°C, and, finally, 7 min at 72°C (*14*). A *Pst*I-digested 1250-bp DNA fragment of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from plasmid pIBI30GAPDH was labeled with [α-^{32}P]dCTP using a megaprime DNA labeling system (Amersham) (*14*).

Slot Blotting. Total cellular RNA (10 μg) was depolarized with 1 M glyoxal and 10 mM sodium phosphate, and then the mixture was transferred to a Hybond-N transfer membrane (Amersham) by the Bio-Dot SF blotter (Bio-Rad, NY). The blot was further hybridized with 2×10^6 cpm/ml of [α-^{32}P]dCTP-labeled probes and exposed to Kodak X-OMAT AR film. The RNA band intensity was quantified by a gel scanner with densitometer function (Vilber Lourmat, France) (*14*).

Analysis of Aminotransferase Activity. The aminotransferase activities in culture medium were measured using a routine automatic analyzer (Hitachi 747, Japan) in the clinical analysis laboratory of National Cheng Kung University Hospital (*14*).

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