

**BIOCHEMISTRY, BIOPHYSICS,  
AND MOLECULAR BIOLOGY**

## **Genetic Antiviral Program against Hepatitis C Virus**

**I. G. Sivov\*, E. I. Samokhvalov\*\*, and V. A. Knyazhev\*\*\***

Presented by Academician R.V. Petrov March 12, 2003

Received March 18, 2003

The technology “virus against virus” was first realized in 1997 by two research groups from Yell University (the United States) [4] and Federal Research Center for Virus Diseases of Animals in Tubingen (Germany) [3] using HIV as a model. In this study, the above-mentioned technology was realized on the model of hepatitis C virus (HCV).

The method of selective elimination of cells infected with HCV and HCV RNA, using antiviral genetic program (AGP), first described in 2001 [2, 6, 7], allows avoiding the stage of immunological recognition of infected cells with CD8<sup>+</sup> lymphocytes and uses RNA-dependent RNA polymerase HCV-specific activity for the activation of genetically programmed elimination. The mechanism of elimination is triggered by HCV RNA polymerase, which recognizes the 3'- and 5'-untranslated terminal regions (UTRs) of AGP and synthesizes on the antisense sequence a complimentary sense copy that programs the formation of the subunit A of diphtheria toxin (DT\_A). The synthesis of sense RNA copy for DT\_A and its subsequent translation results in the expression of highly-toxic enzymatic activity in infected cell.

This sequence (hereafter called AGP) can be delivered using noninfectious RNA-containing HCV-like particles (the so-called HCV bellerophonts), whose defective genomes contain AGP. The defective-genome cDNA, obtained using an RNA template of infectious HCV genotype 1B (accession number 156973), contained the 5'- and 3'-UTRs and a fragment of ORF encoding the morphogenetic program of HCV (proteins C, E1, E2, and p7), i.e., was truncated. An essential element of cDNA obtained was the presence of a unique *HindIII* site immediately after ORF, in which an AGP

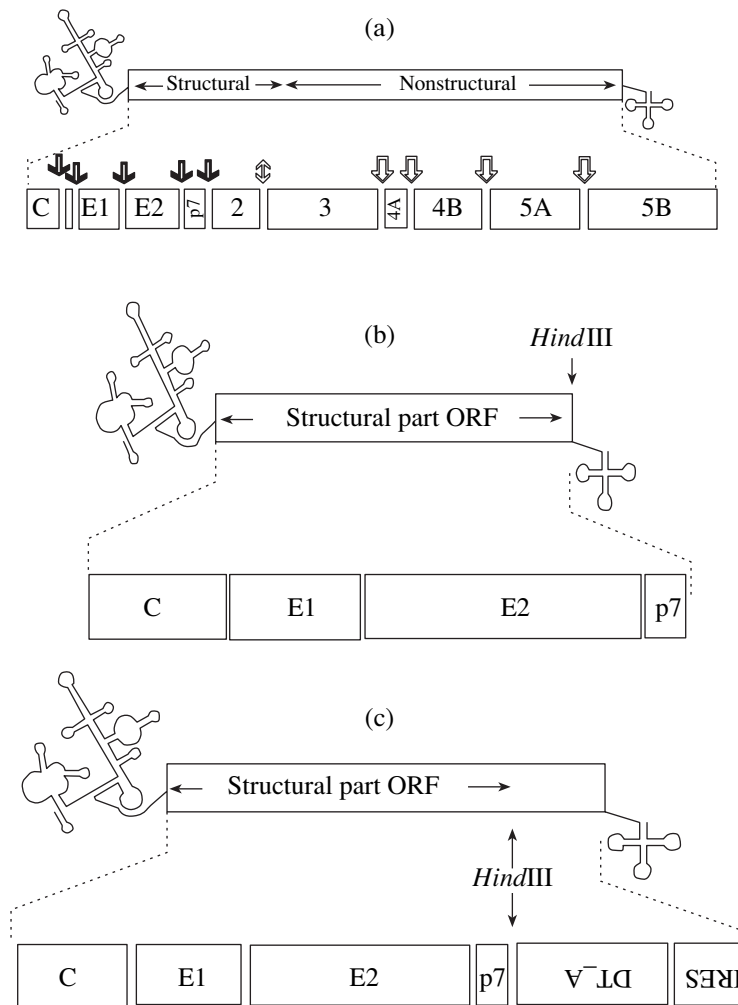
DNA fragment could be inserted (Fig. 1). Figure 1 shows the structures of genomes and their cDNAs: infectious HCV (Fig. 1a), truncated HCV-like genome (Fig. 1b), and HCV bellerophont (Fig. 1c). The structure shown in Fig. 1b was truncated by elimination of part of ORF, encoding nonstructural proteins. As a result, the *HindIII* site occurred at the boundary between the ORF and 3'-UTR. HCV bellerophont was obtained by inserting AGP into the *HindIII* site of the truncated HCV-like genome in the antisense orientation.

The ability of the truncated AGP-containing HCV-like genome to form HCV bellerophonts and exhibit anti-HCV activity was corroborated in two series of experiments. In the first series, HCV bellerophonts were obtained. The only way to obtain HCV bellerophonts was cotransfection of cells with two plasmids, one of which contained a truncated AGP-containing HCV-like genome and the other served for accumulation of excessive amount of the virion proteins. The strategy of the experiment on constructing HCV bellerophonts reduced to selection of such molar ratio between the two types of plasmid DNA, at which the formation of RNA-containing noninfectious HCV-like particles was the most effective. The results of these experiments are shown in Fig. 2. It is seen that, in the preparations containing HCV bellerophonts, the ratio between total protein content (white columns) and AGP RNA (black columns) was almost 100-fold greater than that predicted for HCV. This indicates that these preparations contained at least 97–99% of the aggregates of RNA-free HCV-specific protein. Indeed, according to the electron-microscopic data (Figs. 2b, 2c), the percentage of HCV bellerophonts among the defective RNA–protein aggregates was not more than 0.5%. Nevertheless, taking into account the titer of aggregates (about 10<sup>9</sup>/ml), the titer of HCV bellerophonts could be taken 5 × 10<sup>6</sup>/ml. This value was confirmed by RT-PCR, using AGP-transducing RNA-containing bacteriophage MS2 as a standard [1].

\* Bioeffect Research Institute (Federal State Unitary Enterprise), Moscow, Russia

\*\* Ivanovsky Research Institute of Virology, Russian Academy of Medical Sciences, ul. Gamalei 16, Moscow, 123098 Russia

\*\*\* Sechenov Medical Academy, ul. Bol'shaya Pirogovskaya 2-6, Moscow, 119435 Russia



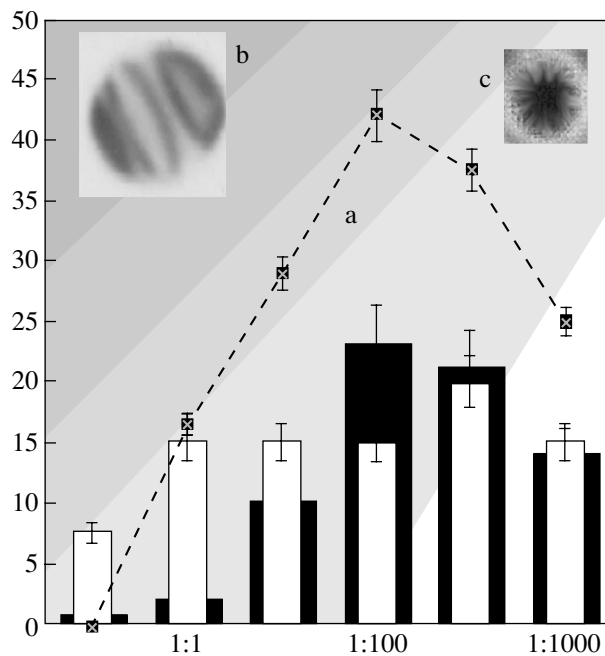
**Fig. 1.** Scheme of (a) infectious HCV genome, (b) truncated HCV-like genome, and (c) HCV bellerophont genome. Terminal sequences are shown in the form of two dimeric structures. Polyproteins, fragmented to individual proteins, whose amino acid sequences were encoded by ORFs of genomic RNAs, are shown under the genomes. The precursor was cleaved at protease-sensitive sites (shown with arrows in part a). Black and white arrows show the sites of hydrolysis of the polyprotein with cell and viral proteases, respectively.

In the second series of experiments, we estimated the anti-HCV potential of the plasmid *pHCVbell*, which encodes HCV bellerophonts. For this purpose, HepG2 hepatocarcinoma cells were treated with a mixture of calcium-phosphate precipitate of *pHCVbell* DNA and HCV RNA (genotype 1A), isolated from blood of patients with hepatitis C. The results of these experiments are shown in Fig. 3. Figure 3a shows intact culture of HepG2 cells, transfected with HCV RNA and *pHCVbell* DNA (Figs. 3b and 3c). Addition of 10  $\mu$ M ApU, an inhibitor of toxic activity, decreased the lethal effect (Fig. 3c). As seen from Fig. 3b, 90% of the cell population died on the day 3 after the treatment with the mixture of calcium-phosphate precipitate of *pHCVbell*

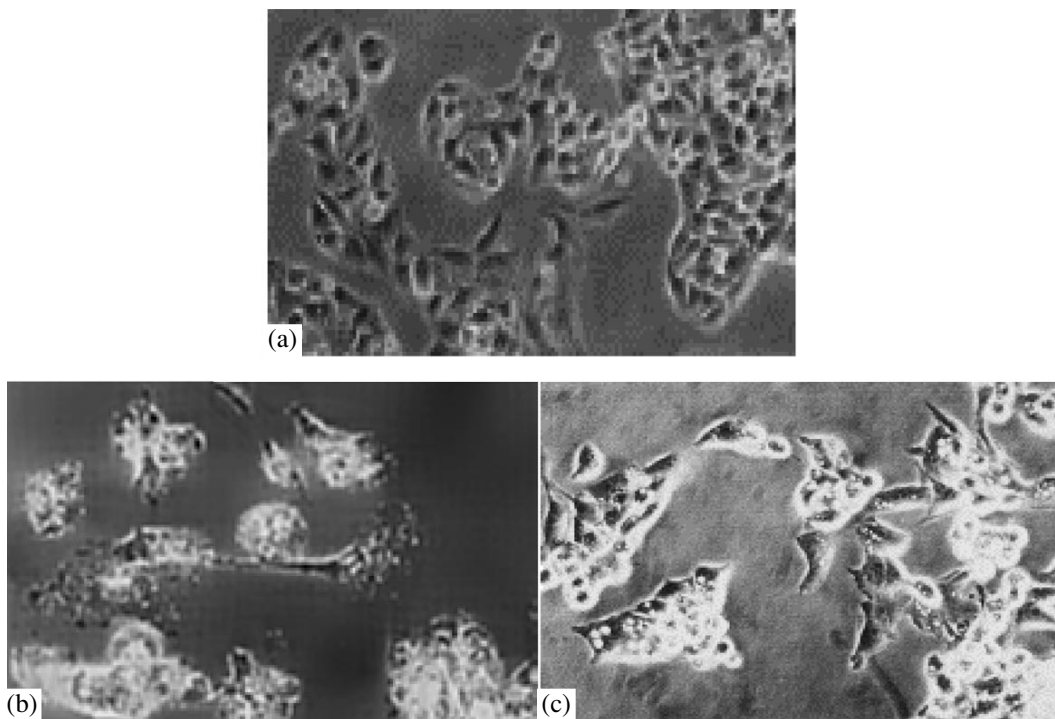
DNA and HCV RNA. Addition to the reaction medium of the dinucleotide ApU, an inhibitor of enzymatic activity of the subunit A [5], decreased the number of dead cells in three times (Fig. 3b).

Three days after the transfection of HepG2 human hepatocarcinoma cells with RNA isolated from blood of patients with hepatitis C ( $10^8$  genome-eqv/ml, genotype 1A) and simultaneous addition of HCV bellerophonts (one particle per cell), the number of dead cells was 95–97%. Addition of the inhibitor ApU to the reaction medium decreased the lethal effect of HCV bellerophonts in three times (data not shown).

In the case of penetration of HCV bellerophonts into all cells potentially sensitive to HCV, lethal effect caused by them will be observed only in the cells



**Fig. 2.** (a) Dependence of the occurrence frequency (expressed in hundredth parts of percent) of HCV-like RNA-containing virions, HCV bellerophonts, on the molar ratio between DNA of *pHCVbell* and helper plasmid (from 1:1 to 1:1000, respectively) in calcium-phosphate precipitates (DNA concentration, 1.2  $\mu\text{g}/\text{ml}$ ), shown with dashed line connecting the experimental values. Black columns show the results of determination of the RNA titer by RT-PCR [2]; white columns, protein titer by immunoassay (using a commercial preparation of HCV-specific serum). (b) Morphology of the aggregate of HCV-specific protein. (c) Morphology of HCV bellerophont.



**Fig. 3.** Intact HepG2 cells (a) and HepG2 cells transfected with the *pHCVbell* plasmid and HCV RNA in the absence (b) and presence (c) of the inhibitor ApU (the method see in [2]).

infected with HCV, irrespective of the genotype of persisting HCV.

#### ACKNOWLEDGMENTS

We are grateful to Ya.I. Alekseev (AO Sintol) for sequencing the DNA constructs. This study was supported by the Ministry of Industry, Science, and Technology of the Russian federation (state contract no. 43.106.11.0027).

#### REFERENCES

1. Sivov, I.G., Knyazhev, V.A., and Sergienko, V.I., *Mol. Gene. Mikrobiol. Virusol.*, 2002, no. 3, pp. 43–47.
2. Sivov, I.G., Sobolev, A.Yu., Samokhvalov, E.I., *et al.*, *Dokl. Akad. Nauk*, 2001, vol. 377, no. 6, pp. 834–837.
3. Mebatsion, T., Finke, S., Weiland, F., *et al.*, *Cell*, 1997, vol. 90, no. 5, pp. 841–847.
4. Schnell, M.J., Johnson, J.E., Buonocore, L., *et al.*, *Cell*, 1997, vol. 90, no. 5, pp. 849–857.
5. Zucker, D.R., Murphy, J.R., and Pappenheimer, A.M., *Mol. Immunol.*, 1984, vol. 21, no. 4, pp. 795–800.
6. Knyazhev, V.A., Sergienko, V.I., Sivov, I.G., *et al.*, RF Patent 2159285, *Byull. FIPS*, 2000, vol. 10, no. 7, p. 27.
7. Knyazhev, V.A., Sergienko, V.I., Sivov, I.G., *et al.*, RF Patent 2158139, *Byull. FIPS*, 2000, vol. 10, no. 7, p. 27.