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Effect of Polyadenylic·polyuridylic Acid on Cellular Responses of Peripheral Blood Mononuclear Cells from Patients with Chronic Active Hepatitis B

Won Ho Kim, Ki Baik Hahm, Sang Jin Park, Jin Kyung Kang, In Suh Park, Heung Jai Choi, Jeon Soo Shin[1] and Jung Koo Youn[1]

We have investigated in vitro proliferative responses of peripheral blood mononuclear cells and productions of interferon-γ and soluble interleukin-2 receptors by these cells from 6 patients with chronic active hepatitis B immediately before and 24 hours after a single intravenous injection of 100 mg of polyadenylic.polyuridylic acid. Cell proliferations were assessed by the technique of tritiated-thymidine incorporation and productions of interferon-γ and soluble interleukin-2 receptors were measured by enzyme-linked immunosorbent assay. The administration of polyadenylic.polyuridylic acid to the patients has resulted in significant increases of in vitro proliferations of their peripheral blood mononuclear cells as well as productions of interferon-γ by these cells. However, in vitro productions of soluble interleukin-2 receptors were not changed significantly. These results suggest that the enhanced cellular responses by polyadenylic.polyuridylic acid might be due to the increased sensitivity rather than the increased expression of cellular interleukin-2 receptor.

Key Words: Polyadenylic.polyuridylic acid, chronic active hepatitis B, cytokine

Abnormalities in cell-mediated immunity have been suggested in patients with chronic hepatitis B virus (HBV) infection, among which diminished proliferative responses of lymphocytes have been well documented (Hanson *et al.* 1984; Saxena *et al.* 1985, Lisker-Melman *et al.* 1988). In addition, decreased productions of interferon (IFN)-γ (Fuji *et al.* 1987; Lisker-Melman *et al.* 1988) and interleukin (IL)-2 (Saxena *et al.* 1985; Anastassakos *et al.* 1988) by peripheral blood mononuclear cells (PBMC) and increased serum levels of soluble IL-2 receptors (sIL-2R) have been reported (Yamaguchi *et al.* 1988; Lai *et al.* 1989; Müller *et al.* 1989). However, increased productions of IL-2 have also been reported during the acute exacerbation stage of chronic hepatitis (Onji *et al.* 1988), suggesting that immune reactions are not consistent during the course of the disease. Effects of various immune modulators and antiviral agents, such as corticosteroid, adenine arabinoside, IL-2 and IFNs, on these altered immune responses have been extensively studied with variable results (Hanson *et al.* 1986; Fuji *et al.* 1987; Lisker-Melman *et al.* 1988; Yamaguchi *et al.* 1988; Scully *et al.* 1990; Mutchnick *et al.* 1991; Leung *et al.* 1992).

Polyadenylic.polyuridylic acid [poly(A).poly(U)] is a non-toxic biological response modifier consisting of twin-chain RNA polymers, polyadenylic and polyuridylic acids, forming a synthetic helical double-stranded complex of polyribonucleotides (Hakoshima *et al.* 1981).

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Department of Internal Medicine and Department of Microbiology[1], Yonsei University, College of Medicine, Seoul, Korea

Address reprint requests to W H Kim, M.D., Department of Internal Medicine, Yonsei University, College of Medicine, C.P.O. Box 8044, Seoul, Korea, 120-752

This complex is endowed with a variety of immunopharmacological activities on both humoral (Hovanessian *et al.* 1988) and cellular components (Johnson, 1979) of the immune response and exhibits antitumor effects on animal tumors (Lacour *et al.* 1972; Youn *et al.* 1982) as well as resectable human breast (Lacour *et al.* 1988) and gastric cancers (Youn *et al.* 1990). The mechanisms of antitumor action of poly(A).poly(U) have been discussed with respect to various biological effects, such as enhancements of phagocytic activity of macrophages (Petrequin and Johnson, 1984) and cytotoxic activity of natural killer cells (Youn *et al.* 1983; Kim *et al.* 1992), inductions of IFN (Odean *et al.* 1990) and other cytokines (Lee *et al.* 1990; Pignol *et al.* 1991). Recently, we have reported a significant clinical efficacy of poly(A).poly(U) in patients with chronic active hepatitis B (CAH B) (Hahm *et al.* 1992). In order to study the underlying mechanism of the complex in this disease, we have investigated *in vitro* proliferative responses of PBMC and productions of IFN-γ and sIL-2R by PBMC before and 24 hours after an intravenous administration of 100 mg of poly(A).poly(U) in 6 patients with CAH B.

MATERIALS AND METHODS

Subjects

Six patients with CAH B were selected for this study. Liver biopsy performed at least within 1 year of the study showed CAH in all patients. They were men with ages ranging from 32~48 years. Their seropositivities for HBsAg and HBeAg as well as for HBV-DNA were checked for at least 1 year and serum alanine aminotransferase (ALT) levels were elevated on more than 3 determinations at least 1 month interval prior to the study. None of these patients abused intravenous drugs or alcohol and they had not received corticosteroid or IFN therapy at least within 1 year of the study. Informed consents were obtained from all patients.

Six healthy medical staff members of our department were selected as controls.

Poly(A).poly(U)

Poly(A).poly(U) was prepared from adenine diphosphate and uridine diphosphate by Dr. A. M. Michelson (Institut de Biologie-Physicochimie, Paris 6ème). The respective monomers were polymerized with polynucleotide phosphorylase and purified polynucleotides were mixed in a 1:1 ratio to prepare the complex. The melting temperature of the material was 61℃ in 0.15M NaCl solution and the thermal hyperchromicity (20~70℃) at 260 nm was 52%. Melting was cooperative and 100% reversible on cooling to 20℃. The sedimentation value of the complex, $S_{20,w}$, was 10.07.

For injection, 100 mg was dissolved in 50 ml of sterile 0.15M NaCl solution, filtered through 0.22 μm Millipore filters and infused intravenously.

Measurement of *in vitro* proliferative responses of PBMC

Heparinized venous bloods were obtained immediately before and 24 hours after the injection of poly(A).poly(U). PBMC were separated by Ficoll-Hypaque (density=1.077 g/ml; Pharmacia, Piscataway, NJ, U.S.A.) density gradient centrifugation. The cells were washed three times with RPMI 1640 medium (Hazleton, Lenexa, KS, U.S.A.) and suspended at a concentration of 1×10^6 cells/ml in complete culture media which consisted of RPMI 1640 medium supplemented with 10 mM HEPES [4-(2-hydroxy-ethyl)-l-piperazine ethane, sulfonic acid] buffer, 10% heat-inactivated fetal calf serum (Gibco Laboratories, Grand Island, NY, U.S.A.), 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin.

For the assay of *in vitro* proliferative responses of PBMC, 2×10^5 cells in 200 μl of complete culture media were seeded in 96-well round-bottomed microtiter plates (Corning, Corning, NY, U.S.A.) and cultured with or without 5 μg/ml of concanavalin A (con A; Sigma Chemical, St Louis, MO, U.S.A.) for 72 hours at 37℃ in a humidified 5% CO_2 incubator. They were pulsed with 1 μCi of ^{3}H-thymidine (specific activity=6.7 Ci/mmol; New England Nuclear, Boston, MA, U.S.A.) for each well at 18 hours before harvest. The cells were harvested on glassfiber filter papers using a cell harvestor (Skatron Inc., Stering, VA, U.S.A.), and ^{3}H-thymidine incorporations were measured by beta scintillation counter

(Beckman, LS 5000TA, Palo Alto, CA, U.S.A.). All experiments were performed in triplicate. Results were given as counts per minute (cpm) and the stimulation index was calculated as follows:

$$\text{Stimulation index} = \frac{^3\text{H-thymidine uptake of PBMC stimulated with con A}}{^3\text{H-thymidine uptake of unstimulated PBMC}}$$

Measurement of *in vitro* productions of IFN-γ and sIL-2R

PBMC at a concentration of 1×10^6 cells/ml were cultured at 37℃ in a humidified 5% CO_2 incubator with or without 10 μg/ml of phytohemagglutinin (PHA; Sigma Chemicals, St Louis, MO, U.S.A.) in 24-well plates with complete culture media. Supernatants were harvested after 24 hours and stored at −70℃ until assay. Enzyme-linked immunosorbent assay (ELISA) kits were used to measure human IFN-γ (Genzyme, Cambridge, MA, U.S.A.) and sIL-2R (T Cell Science, Cambridge, MA, U.S.A.) according to the instruction manuals. In brief, culture supernatants were added to the bottoms of wells of 96-well polystylene microtiter plates on which specific monoclonal antibodies against human IFN-γ or sIL-2R were previously absorbed. Then, for sIL-2R assay, horse-radish peroxidase-conjugated anti-IL-2R antibody was immediately added to each well and incubated for 3 hours at room temperature. For IFN-γ assay, goat anti-human IFN-γ polyclonal antibody was added and incubated for 2 hours. After washing, anti-goat-biotin was added and incubated for another 45 minutes and then streptovidin-peroxidase was added. Finally, for both assay, a substrate solution (*O*-phenylenediamine) was added to each well to form a colored product. The reaction was terminated by the addition of 2.5N H_2SO_4, and the absorbance was measured at 490 nm.

Statistical analysis

The data were expressed as mean±SEM. The Mann-Whitney U test and the Kolmogorov-Smirnov two sample test were used for comparing the stimulation index between healthy controls and patients with CAH B, and the Wilcoxon signed-ranks test was used for comparing proliferation of PBMC as well as productions of IFN-γ and sIL-2R by these cells before and after an intravenous administration of poly(A).poly(U) in patients with CAH B.

RESULTS

In vitro proliferative responses of PBMC

The mean ^{3}H-thymidine incorporations of PBMC, collected from patients 24 hours after an intravenous administration of poly(A).poly(U), was significantly higher (81,051±4,053 cpm) than that of PBMC collected from the same patients before the administration (39,829±11,410 cpm) ($p<0.05$) when cultured without con A. Similarly, the mean ^{3}H-thymidine incorporations of PBMC cultured in the presence of 5 μg/ml of con A was also significantly increased by poly(A).poly(U) in these patients (97,526±18,001 cpm before and 178,288 ±21,380 cpm after the injection) ($p<0.05$) (Fig. 1).

The stimulation index by con A was signif-

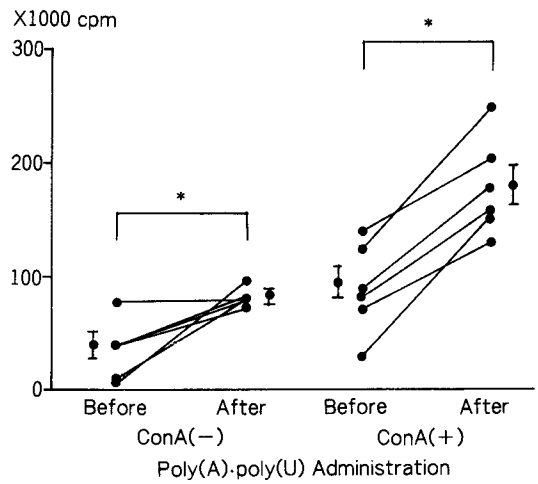

Fig. 1. *Proliferations of PBMC from patients with CAH B before and 24 hours after an intravenous administration of 100 mg of poly(A).poly(U). PBMC were cultured for 72 hours with or without 5 μg/ml of con A. Cell proliferations were determined by the technique of tritiated-thymidine incorporation. *: $p<0.05$*

Table 1. Stimulation index[a] by con A of PBMC from patients with CAH B and healthy controls before and 24 hours after the administration of poly(A).poly(U)

	Administration of Poly(A).poly(U)[b]	
	Before	After 24 Hours
Healthy Controls	26.72±7.19 *	29.56±3.91 *
Patients with CAH B	3.41±7.69	2.23±3.10

*: $p < 0.05$

[a]: Stimulation index was calculated by dividing ^{3}H-thymidine incorporations of PBMC in the presence of 5 μg/ml of con A with that in the absence of con A following *in vitro* culture for 72 hours.

[b]: Poly(A).poly(U), 100 mg, was intravenously injected.

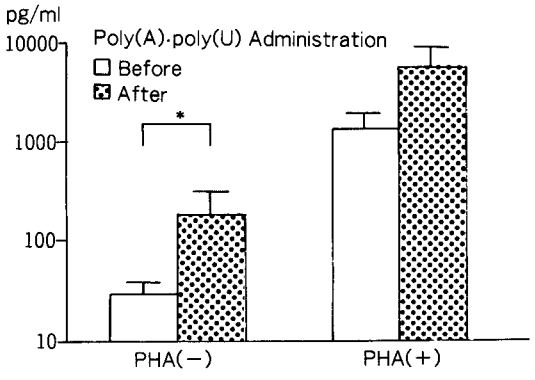

Fig. 2. *In vitro productions of IFN-γ by PBMC from patients with CAH B before and 24 hours after an intravenous administration of 100 mg of poly(A).poly(U). PBMC were cultured for 24 hours with or without 10 μg/ml of PHA. The levels of IFN-γ in culture supernatants were measured by ELISA. *: $p<0.05$*

icantly lower in patients with CAH B (3.41±0.77) as compared to that in healthy controls (26.72±7.19) ($p<0.05$). However, such indices were not significantly changed after the administration of poly(A).poly(U) in these patients as well as in healthy controls (Table 1).

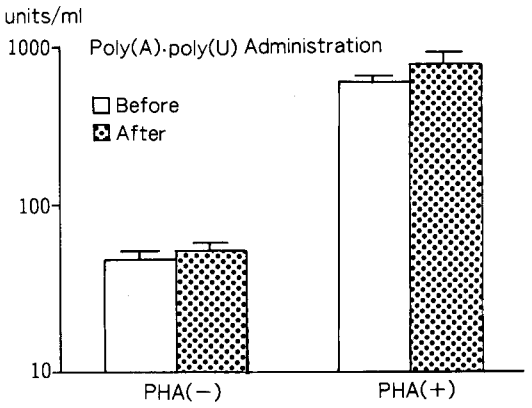

Fig. 3. *In vitro productions of sIL-2R by PBMC from patients with CAH B before and 24 hours after an intravenous administration of 100 mg of poly(A).poly(U). PBMC were cultured for 24 hours with or without 10 μg/ml of PHA. The levels of sIL-2R in culture supernatants were measured by ELISA.*

In vitro productions of IFN-γ and sIL-2R

Productions of IFN-γ by PBMC from patients with CAH B were significantly increased (6-fold) after an intravenous administration of poly(A).poly(U) (31.5±8.7 pg/ml before and 189.0±132.3 pg/ml after the injection) ($p<0.05$) when cultured without PHA. When PBMC were cultured with 10 μg/ml of PHA, productions of IFN-γ were also increased 4-fold after an intravenous admini-stration of poly(A).poly(U) (1,300.7±566.1 pg/ml before and 5,411.9±3,150.5 pg/ml after the injection), though the difference was not statistically significant ($p=0.24$) (Fig. 2).

Productions of sIL-2R by these PBMC were not significantly changed by poly(A).poly(U) injection when cultured with or without PHA (49.8±5.0 units/ml before and 55.3±8.4 units/ml after the injection when cultured without PHA, and 604.1±52.0 units/ml before and 773.7±141.2 units/ml after the injection when cultured with 10 μg/ml of PHA) (Fig. 3).

DISCUSSION

Continuous replication of the HBV in pa-

tients with chronic HBV carrier is thought to be related with the progression of the disease from chronic hepatitis to cirrhosis (Hoofnagle, 1983) and hepatocellular carcinoma (Beasley *et al.* 1981), whereas loss of active viral replication in such patients is usually associated with a decrease in infectivity and a reduction in necroinflammatory activity of liver tissue. For these reasons, treatments of chronic HBV infection with antiviral agents, such as adenine arabinoside, adenine arabinoside monophosphate, and acyclovir, have been tried alone or in combination, but the results have been unsatisfactory (Bassendine *et al.* 1981; Hoofnagle *et al.* 1984; Alexander and Williams, 1986).

The HBV itself is known to be noncytopathic (Edgington and Chisari, 1975) and the severity of liver damage in chronic HBV infection is related to the intensity of cell-mediated immune reactions toward infected hepatocytes (Mondelli *et al.* 1982). However, trials for the treatment of chronic HBV infection using various immune suppressors or modifiers, such as corticosteroid, 6-mercaptopurine, azathioprine, transfer factor, OK-432, IL-2, levamisole and thymosin, have been inconclusive (Jain *et al.* 1977; Yoshioka *et al.* 1979; Chadwick *et al.* 1980; Hanson *et al.* 1986; Kakumu *et al.* 1988; Mutchnick *et al.* 1991). The IFNs, which have both direct antiviral and immunomodulating effects (Kirschner, 1984), have been widely used to treat chronic hepatitis B even in combinations with other agents, but the response rates, especially in Asian countries, have not been high enough to become a proven therapy for this disease (Lok *et al.* 1989; Perrilo *et al.* 1990). In addition, side effects and the cost of IFNs limit its usefulness (Renault and Hoofnagle, 1989).

In patients with chronic liver disease, abnormalities in immunity, especially in cell-mediated immunity, have been demonstrated. Among these, the diminished proliferative responses of PBMC to mitogenic stimulation have been well documented (Hanson *et al.* 1984; Saxena *et al.* 1985, Lisker-Melman *et al.* 1988). Our results also showed that the stimulation index was significantly lower in patients with CAH B compared with that in healthy controls.

The activity and production of IL-2, which stimulates cytotoxic T cells to increase in number and maturity, have been reported to be decreased in patients with chronic HBV infection (Saxena *et al.* 1985; Anastassakos *et al.* 1987; Anastassakos *et al.* 1988). Furthermore, this impaired IL-2 activity has also been claimed to be associated with high levels of HBV replication (Civeira *et al.* 1987). However, Onji *et al.* (1988) reported that T cells in peripheral blood from the patients with chronic hepatitis produced more IL-2 during an exacerbation stage than in remission.

The functional significance of sIL-2R, which are smaller than the membrane bound form (Rubin *et al.* 1985), remains to be defined, but its ability to bind free IL-2 with a low affinity has been reported (Müller *et al.* 1989). For this reason, it is speculated that sIL-2R play a role in regulating IL-2-dependent processes including lymphocyte proliferations and T cell-dependent immune mechanisms which are known to be defective in patients with chronic HBV infection. The release of sIL-2R is thought to be parallel with cellular IL-2R expression (Leung *et al.* 1992), thus it can be used to monitor the T cell activity. Furthermore, serum levels of sIL-2R have been used as a serological marker for inflammatory activity in hepatitis, due to its significant correlation with the serum ALT level (Leung *et al.* 1992) and histologic findings (Yamaguchi *et al.* 1988). Elevated serum levels of sIL-2R have been reported in patients with acute and chronic hepatitis, particularly during an acute exacerbation (Yamaguchi *et al.* 1988; Lai *et al.* 1989; Müller *et al.* 1989), and these elevations could be the result of its increased productions or diminished clearances (Müller *et al.* 1989). The increased levels of sIL-2R might remove circulating IL-2, and thus result in decreasing serum levels of IL-2, which is a usual finding in chronic HBV infection. In addition, the presence of sIL-2R might be the reason why exogenous IL-2 fail to correct the decreased *in vitro* proliferative responses to mitogen in chronic hepatitis B (Anastassakos *et al.* 1988; Müller *et al.* 1989).

A unique feature of poly(A).poly(U) action is its lack of mitogenicity *in vitro* (Lederman and Johnson, 1979). However, it remarkablely enhances lymphocyte proliferations in the

presence of coexisting antigenic stimulation, even of threshold doses (Friedman *et al.* 1969; Lee *et al.* 1990). Thus, it has been thought that the primary cellular targets of poly(A).poly(U) might be macrophages and monocytes rather than the lymphocytes themselves (Friedman *et al.* 1969), even this complex have been known to act on a variety of cell types including immunocompetent (Johnson, 1979) as well as non-competent cells (Kim *et al.* 1975). Recently, Lee *et al.* (1990) demonstrated that *in vivo* administration of poly(A).poly(U) remarkablely enhanced *in vitro* proliferations of mouse thymus and spleen cells as well as the production of IL-1 by macrophages and IL-2 by spleen cells. They suggested that this enhanced cellular responsiveness may be due to the potentiation not only by these cytokines endogenously produced but also by the increased antigen recognition of thymus-derived cells activated through the adjuvant effect of poly(A).poly(U). In our study, ^{3}H-thymidine incorporations of PBMC with or without mitogenic stimulation significantly increased after an intravenous administration of poly(A).poly(U) into the patients but the stimulation indices were not increased. Poly(A).poly(U) is a well known IFN-inducer, particularly of IFN-γ, in mouse models (Lee *et al.* 1992). Our results also showed that the productions of IFN-γ were increased significantly after a single administration of poly(A).poly(U) irrespective of mitogenic stimulation. It is well known that IFN has antiproliferative properties in addition to immune modulating and antiviral action. However, IFN-γ, in contrast to IFN-α, are known to increase proliferative responses of lymphocytes in patients with chronic hepatitis B, thus the effects on lymphocytes have been thought to be distinct (Lisker-Melman *et al.* 1988). The mechanism of action of poly(A).poly(U) to enhance the lymphocyte proliferations might be related to the increased sensitivity of IL-2R to IL-2 rather than an increased expression of IL-2R, because the levels of sIL-2R in culture supernatants were not significantly increased after an intravenous administration of poly(A).poly(U) in our study. This concept is further supported by the evidence that the decreased proliferative responses of lymphocytes in patients with chronic hepatitis might be due to the impaired sensitivity of lymphoid cells to IL-2 (Saxena *et al.* 1985), because it could not be corrected by exogenous IL-1 and IL-2 (Anastassakos *et al.* 1987).

From our results, it could be concluded that an intravenous administration of 100 mg of poly(A).poly(U) in patients with CAH B significantly increased *in vitro* proliferations of PBMC as well as productions of IFN-γ by PBMC without increased stimulation indices. These enhanced cellular responses might be due to an increased sensitivity of IL-2R to IL-2 rather than the increased expression of IL-2R. Further studies on the effects of poly(A).poly(U) on cellular IL-2R expression and serum levels of sIL-2R are expected to confirm this hypothesis.

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