Effect of Heptaminol AMP Amidate, a New Nucleotide Derivative, on In Vitro Humoral Immunity

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Accepted February 12, 1988

Abstract—Heptaminol AMP amidate (HAA), a newly developed derivative of 5'-AMP, was found to potentiate the in vitro primary humoral immune response against T cell-dependent antigen, sheep red blood cells, when HAA was present in the early phase of spleen cell culture. Such a potentiating effect was not found against T cell-independent antigens such as lipopolysaccharide (LPS), trinitrophenylated (TNP)-LPS and TNP-Ficoll. The pattern of HAA-mediated immunopotentiation was similar to that of dibutyryl cyclic AMP. When HAA was added to the culture simultaneously with theophylline and imidazole, the immunopotentiating effect of HAA was further augmented and suppressed, respectively. The present results suggested that HAA-mediated immunopotentiation might be in some way related to the intracellular level of cyclic nucleotides in the early phase of culture.

Heptaminol AMP amidate (HAA), N-(1,5-dimethyl-5-hydroxy) hexyladenosine-5'-phosphoramidate, was prepared by condensing 5'-AMP with heptaminol, a cardiotonic, following the method of Chambers and Moffatt (1). In the previous paper (2), it was found that HAA potentiated the humoral immune response in both the normal mice and spontaneously hypertensive rats. The present study was carried out to clarify the mechanism of HAA-mediated immunopotentiation in the in vitro spleen cell culture. In this study, T cell-dependent (TD) antigen, sheep red blood cells (SRBC), was used to examine whether HAA induced its effect through T cells. On the other hand, T cell-independent (TI) antigens such as lipopolysaccharide (LPS), trinitrophenylated (TNP)-LPS and TNP-Ficoll were used to investigate whether HAA can cause the immunopotentiation directly through B cells.

Furthermore, heptaminol, a constituent of HAA, was reported to increase the cyclic AMP (cAMP) level in canine heart preparation partially by increasing the activity of adenylate cyclase and partially by inhibiting phosphodiesterase activity (3). This observation indicates the possibility that HAA may influence the level of cyclic nucleotides in the spleen cell. Dibutyryl cyclic AMP (DbcAMP), adenosine, and other agents which raise intracellular cAMP level were reported to potentiate the in vitro immune response only when they were present in the early phase of cell culture (4). The present study was designed to find out the effects of HAA when it was present for certain lengths of time in the early phase of spleen cell culture.

Moreover, it was also investigated whether 5'-AMP and heptaminol can affect immune response by either individual or concomitant presence in the culture. A possible mechanism of the potentiation of the immune response by HAA has also been discussed.

† Part of this paper was presented at the 60th General Meeting of the Japanese Pharmacological Society, held in Chiba, Japan, March 29–April 1, 1987.
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**Materials and Methods**

**Animals and Materials:** Male BALB/c mice (Charles River Inc., Japan), 8–10 weeks of age, were used. The mice were housed under a 12-hr light and dark cycle. The following 4 types of antigens were used in the experiments: SRBC from Nihon Bio-Material Center Co. (Japan), LPS (Salmonella typhosa 0901) from Difco Laboratories (U.S.A.), TNP-LPS from List Biological Laboratories, Inc. (U.S.A.) and TNP-Ficoll from Bioresearch (U.S.A.). HAA, DbcAMP, adenosine, heptaminol and 5'-AMP were kindly given to us by Yamasa Shoyu Co., Ltd. (Japan). Other reagents used in the experiments were as follows: Eagle’s minimum essential medium (MEM), RPMI 1640 medium and PBS (Nissui Pharmaceutical, Japan), fetal bovine serum (FBS) (Lot No. 30051) and agar (Gibco), guinea pig complement (Denka Seiken, Japan), dextran (Pharmacia), trinitrobenzene sulfonic acid, glycyglycine, trypan blue, theophylline and imidazole (Wako Pure Chemical, Japan), 2-mercaptoethanol (2-ME) (Nakarai Chemicals, Japan).

**Culture System and Assay of Antibody Forming Cells:** Mice spleen cells were cultured (0.5×10^7 cells/well) according to the method of Mishell and Dutton (5), except that 2-ME (1×10^{-5} M) was added to the culture medium. Concentrations of the antigens were: SRBC (2×10^6 cells/well), LPS (200 μg/well), TNP-LPS (2 μg/well), TNP-Ficoll (20 ng/well). All cultures were carried out in quadruplicate in a 24-well multidish culture plate (Nunc, Denmark).

HAA was added at the initiation of the culture with 4 different antigens, and the splenocytes were pulsed for certain lengths of time ranging from 3 to 24 hr (TD antigen: 12–24 hr, TI antigens: 3–12 hr). Then the cells were washed 3 times with MEM and were recultured with different antigens for the following durations: 96 hr for SRBC, 48 hr for LPS, and 72 hr for TNP-LPS and TNP-Ficoll. When the cells were recultured, no correction was made for possible cytotoxicity due to the effects of drugs in the pulse phase.

In some cases, cell cultures were done in the presence of HAA for the above mentioned respective durations with 4 different antigens without any early pulse, and these cultures have been indicated as a continuous culture in the Results and Discussion.

In vitro humoral immune response was evaluated by IgM plaque forming cells (PFC) assay according to the method of Jerne and Nordin (6). The indicator SRBC for LPS, TNP-LPS and TNP-Ficoll response were lightly coupled with trinitrophenyl following the method of Rittenberg and Pratt (7). Cell recovery has been expressed on the basis of the number of cells, cultured in the pulse phase. The statistical analyses were performed using Student’s t-test.

**Results**

**Early Pulse Effects of HAA on the In Vitro Humoral Immunity:** As depicted in Fig. 1A, in a continuous culture of 96 hr against SRBC, HAA at 10^{-3} M concentration decreased the PFC/culture value to 60% of the control value. However, when SRBC-stimulated spleen cells were pulsed with HAA for 12, 18 and 24 hr and then recultured for 96 hr with SRBC only, dose-dependent potentiation of PFC responses were found. HAA (10^{-3} M) was found to cause about a 3-fold potentiation in both the 12 and 18 hr pulse periods. Particularly in the 18 hr pulse period, HAA caused a linear dose-dependent potentiation of PFC responses. From these results, an 18 hr pulse was considered to be the most effective condition for HAA-mediated immunopotentiation against SRBC.

As illustrated in Fig. 1B, in a continuous culture of 48 hr against polyclonal LPS, HAA caused 57% immunosuppression at 10^{-3} M concentration. When LPS-sensitized spleen cells were pulsed with HAA for 12, 18 and 24 hr, although such inhibition was not found, there was no potentiation of PEC responses. As has been depicted in Fig. 2A and 2B, HAA at a concentration of 10^{-3} M caused 40% and 54% suppression of the immune response in 72 hr continuous culture against TNP-LPS and TNP-Ficoll, respectively. Early pulsing of the splenocytes for 3, 6 or 12 hr with HAA did not cause such immunosuppression. However, such early pulsing with HAA was not found to be effective against either of the TI antigens. When TI antigens-sensitized spleen cells were
pulsed with HAA for more than 12 hr and then recultured, it was noted that the recovery of cells decreased substantially (data not shown) and PFC response was not at a detectable level.

Comparison of the effects of HAA, 5'-AMP and heptaminol on immune response in vitro: As has been shown in Fig. 3, heptaminol and 5'-AMP, constituents of HAA, were added separately and concomitantly to the cultures during the 18 hr pulse phase. There was no potentiation in anti-SRBC PFC response in any of the cultures. On the contrary, HAA caused dose-dependent potentiation of the anti-SRBC PFC response.

Comparison of the effects of HAA,
DbcAMP and adenosine on immune response in vitro: As illustrated in Fig. 4, under the 18 hr pulse condition, activity of HAA was compared with that of DbcAMP, which enters into the cells and mimics the effect of intracellularly formed cAMP. Furthermore, the activity of HAA was compared with that of adenosine, which increases the cAMP level by interacting with a receptor on the lymphocyte surface (8). A significant (P<0.05) and more than 2.5 times increase of PFC response was found for 10^{-3} M HAA. At the concentration of 10^{-3} M, DbcAMP and adenosine significantly potentiated the response by 3 times (P<0.05) and 1.7 times (P<0.001) respectively, as compared to the corresponding control (Fig. 4B). In addition to the similar effects of HAA and DbcAMP on the PFC response, it was found that both agents also decreased the number of recovered cells (Fig. 4A).

Effect of theophylline and imidazole on the immunopotentiating activity of HAA: Theophylline, a phosphodiesterase inhibitor was added in 10^{-6} to 10^{-4} M concentrations to spleen cell cultures simultaneously with 10^{-4} M HAA (Fig. 5). The cultures were done under 18 hr pulse condition. Addition of only theophylline (10^{-5} M) to the cultures for an 18 hr pulse period did not magnify the in vitro anti-SRBC PFC response. HAA at the concentration of 10^{-4} M induced a moderate (148%) potentiation of the anti-SRBC PFC response, which was further augmented dose-dependently by three different concentrations of theophylline.

Imidazole, which reduces cAMP level by activating phosphodiesterase activity, was added in 10^{-8} to 10^{-6} M concentrations to the cultures simultaneously with 10^{-3} M HAA.
The experiment was done under 18 hr pulse condition. Addition of only $10^{-7}$ M imidazole to the cultures did not exert any significant effect on anti-SRBC PFC response. In this experiment, immunopotentiation mediated by $10^{-3}$ M HAA was inhibited significantly by 3 different doses of imidazole.

Discussion

In the previous paper, it was reported that HAA, which was produced from 5'-AMP and heptaminol, caused maximum immunopotentiation in normal mice and spontaneously hypertensive rats, when orally administered at the dose of 10 mg/kg (2). The present in vitro study was undertaken to find out the mechanism of HAA-mediated immunopotentiation, i.e., whether HAA mediates its effect by directly activating B cells or through T cells, which is expressed by B cells. Furthermore, heptaminol, a constituent of HAA was reported to increase cAMP level in canine heart preparation (3). HAA also contains adenosine in the structure, which increases the cAMP level in lymphocytes (8). These evidences suggested that HAA-mediated immunopotentiation may be related to the intracellular cAMP level. It was one of the objectives of the present in vitro study to clarify the relationship between HAA-mediated activity and intracellular cAMP. It was also attempted to verify the necessity for phosphoramidate conjugation of 5'-AMP and heptaminol to generate an immunopotentiating compound.

From the results of the present study, it is clear that HAA caused potentiation of in vitro IgM PFC response, when TD antigen SRBC-sensitized cells were pulsed with HAA during the early phase of culture. It was found that an 18 hr pulse with HAA was the most effective condition, and this potentiation of humoral immune response was against TD antigen SRBC only. These data suggest that HAA mediates its function through the participation of both T and B lymphocytes.

Moreover, in the present investigation, it was also found that the nature of HAA-mediated potentiation of in vitro PFC response was similar to that of DbcAMP with respect to the requirement of an early pulsing. Immunopotentiating effects of DbcAMP and
adenosine under the early pulse condition, as were found in the present study, are in agreement with the reports of Teh and Paetaku (4) and Bruchiel and Melmon (9), and the functions of these two compounds are mediated by an elevated level of cAMP in the lymphocytes. The similarity in the condition for in vitro immunopotentiating activity of HAA with that of DbcAMP and adenosine indicates a possibility that the activity of HAA may also be related in some way to the elevation of intracellular cyclic nucleotides level.

Furthermore, in a continuous culture, HAA did not potentiate the anti-SRBC PFC response at the concentrations of $10^{-5}$ M and $10^{-4}$ M, and it inhibited the immune response to 60% of the control value at $10^{-3}$ M concentration. The similar type of response was reported for DbcAMP and other cAMP active compounds in continuous culture (9). It was reported that inhibition of immune response by cAMP active compounds in continuous culture may arise from an inhibition of T helper cell activity after the early hours (as late as 48 hr after antigen stimulation) (10). Therefore, it might be possible that the inductions caused by HAA at the concentrations of $10^{-5}$ M and $10^{-4}$ M at the early phase was abrogated by the moderate inhibition of T helper cell activity in the late phase of continuous culture, and thus there was almost no change in immune response. On the other hand, it might be possible that a severe T helper cell inhibiting activity in the late phase of continuous culture by HAA at the higher concentration of $10^{-3}$ M caused the suppression of the immune response.

To clarify the relationship of the activity of HAA with cAMP, we further investigated the effect of theophylline and imidazole on the HAA mediated immunopotentiation. The doses for theophylline and imidazole were carefully selected in accordance with other observations (8, 11, 12). It was recognized that HAA-mediated immunopotentiation was magnified further by theophylline and suppressed by imidazole dose-dependently. Theophylline and imidazole may modify HAA-mediated immunopotentiation by exerting their known modulating effect on phosphodiesterase activities. Ishizuka et al. (11) reported that poly A : poly U-mediated immunopotentiation by elevated cAMP level was also further augmented by theophylline both in vivo and in vitro.

The results of experiments with theophylline and imidazole strongly suggest that HAA-mediated immunopotentiation is closely related to an elevated level of cAMP. The role of cAMP in the control of immune response is yet to be determined precisely. It was reported that the inhibitory effects of histamine, $\beta$-catecholamines, prostaglandins and cholera enterotoxin on humoral immunity were related with cAMP (13, 14). On the other hand, there were evidences that cAMP triggered activation of B cells (10) and also caused a suppressive effect on suppressor T cells (15). Several steps in lymphocytes differentiation and maturation were also reported to be enhanced by cAMP (16–22). HAA induced immunopotentiation might be possible by influencing some of these phases of immune responses through an elevated cAMP level. The effect of HAA on cyclic nucleotides level in the cell and on the possible influence of HAA on a specific cell population are under investigation in our laboratory.

Furthermore, it was also found in the present study (Fig. 3) that neither 5'-AMP nor heptaminol but only the phosphoramidate conjugation form of these two compounds, which generated HAA, was capable of inducing the observed immunopotentiation. Moreover, in the previous in vivo experiment, there was no cytotoxicity of HAA at the dose of 10 mg/kg, which was required for the maximum immunopotentiation (2). The decrease in the number of recovered cells as observed in the present in vitro studies may be attributed to a higher concentration of HAA in the culture. From the observed potentiating effect of HAA on humoral immune response in the present in vitro studies and also in the previous in vivo studies, it is suggested that HAA may have possible use as a potential immunopotentiator in the future.

Acknowledgment: We are grateful to Yamasa Shoyu Co., Ltd. (Japan) for providing us with HAA and to Miss H.W. Lim for her technical assistance. This work was partly supported by a Grant from The Fujisawa Foundation, Japan.

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