Effects of Heptaminol AMP Amidate (HAA) on Cyclic AMP Level and Mitogen-Induced Proliferation of Murine Spleen Cells

Koichi UENO, Anutosh Ranjan SAHA, Haruo KITAGAWA and Tetsuo SATOH

Laboratory of Biochemical Pharmacology and Biotoxicology, Department of Drug Evaluation and Safety, Faculty of Pharmaceutical Sciences, Chiba University, 1-33 Yayoi-cho, Chiba 260, Japan

Accepted December 7, 1989

Abstract—Heptaminol AMP amidate (HAA), a nucleotide derivative, was found to elevate the intracellular cyclic AMP (cAMP) level of cultured mice spleen cells in a time- and dose-dependent manner. Theophylline and imidazole, when added to the spleen cell culture simultaneously with HAA, respectively caused a further rise and a fall of the cAMP level increased by HAA alone. When comparatively higher doses of the T cell mitogen concanavalin A (Con A) were used in the culture, Con A-induced cell proliferation was mildly inhibited in the culture of spleen cells pooled from HAA administered mice in comparison to the culture of spleen cells pooled from saline treated mice. On the other hand, when another T cell mitogen phytohemagglutinin P (PHA) was used in different concentrations in the culture, there was a trend of enhanced cell proliferation in the culture of spleen cells pooled from HAA administered mice in comparison to the responses in the culture of spleen cells pooled from saline treated mice. The present results supported the previous findings that HAA-mediated immunopotentiation was closely related with a cAMP level elevating property of HAA, and the compound also enhanced the function of helper T cells.

Heptaminol AMP amidate (HAA), N-(1,5-dimethyl-5-hydroxy) hexyladenosine 5'-phosphoramidate, was prepared from 5'AMP and heptaminol following the method of Chambers and Moffatt (1). HAA was found to potentiate the primary humoral immune response in both the normal mice and immunosuppressive spontaneously hypertensive rats (SHR) due to decreased T cell function (2). In another study, it was found that HAA possessed specific suppressive and potentiating effects on suppressor T (Ts) and helper T (Th) cells, respectively (3). Furthermore, it was reported that HAA potentiated the in vitro primary humoral immune response only against T cell-dependent (TD) antigen, sheep red blood cell (SRBC), when antigen-sensitized spleen cells were pulsed with HAA during the early phase of culture (4). The conditions and pattern of HAA-mediated in vitro immunopotentiation were found to be similar to those of dibutyryl cAMP (DbcAMP) and adenosine, which are known to raise the intracellular cAMP level. In the same study, it was also found that HAA-mediated immunopotentiation was significantly magnified further by theophylline and suppressed by imidazole. It was considered that theophylline and imidazole might modify the HAA-mediated immunopotentiation through changing the cAMP level increased by HAA, which would be possible through their known modulating effects on phosphodiesterase activities. These evidences indicated that HAA might have the ability to raise the intracellular cAMP level.

The present study was undertaken to elucidate the effect of HAA on the intracellular
cAMP level of cultured splenocytes. The mitogen-induced proliferation of spleen cells pooled from HAA administered mice was also investigated.

Materials and Methods

Animals: Male BALB/c mice (Charles River Inc., Japan), 8–10 weeks of age, were used in the experiments. The mice were housed under a 12 hr light and dark cycle.

Materials: HAA was kindly given to us by Yamasa Shoyu Co., Ltd. (Japan). Other reagents used in this study were as follows: Eagle’s minimum essential medium (MEM), phosphate-buffered saline and RPMI 1640 medium (Nissui Pharmaceutical Co., Ltd., Japan); fetal bovine serum (Gibco Laboratories, U.S.A.); trypan blue and 2-mercaptoethanol (2-ME) (Nacalai Tesque, Ltd., Japan); theophylline and imidazole (Wako Pure Chemical Industries, Ltd., Japan); and cAMP [125I] radioimmunoassay kit (Dupont Company, U.S.A.). Concanavalin A (Con A) and phytohemagglutinin P (PHA) were purchased from Seikagaku Kogyo Co., Ltd. (Japan) and Difco Laboratories (U.S.A.), respectively. [Methyl-3H]-thymidine was purchased from Amersham International plc (England).

Determination of intracellular level of cAMP: Mouse spleen cells (5×10^6 cells/well) were cultured in triplicate in the presence of HAA following the conditions used by Mishell and Dutton (5), except that 2-ME (1×10^-5 M) was added to the culture medium. In these experiments, no antigen was added to the culture. After completion of the culture period, cells were pooled and suspended in MEM. The cells were counted, and then cAMP was extracted from 5×10^6 spleen cells following the method of Goffstein et al. (6). The cAMP level was determined by using a commercial radioimmunoassay kit. All data have been expressed as the percentage of the control determined from the average values obtained from duplicate assay.

Determination of mitogen-induced spleen cell proliferation: HAA was administered orally to BALB/c mice (4 mice per group) at a dose of 10 mg/kg for 4 consecutive days. Then the spleen cells were withdrawn and pooled. These spleen cells were cultured with three different concentrations of two mitogens, Con A and PHA. The number of spleen cells in the culture was 1×10^8 cells/ml, according to the culture condition used by Anderson et al. (7). All cultures were done in a total volume of 0.2 ml in quadruplicate in a 96-well microculture plate (Corning Glass Works, U.S.A.), at 37°C in a 5% CO2 atmosphere for 48 hr. For the in vitro determination of mitogen-induced cell proliferation, [3H]-thymidine (0.2 μCi/well) was added at the last 18 hr to the cultures. The cultured cells were harvested on glass fiber filter paper with a multiple cell harvester (Labo Science Co., Ltd., Japan). The uptake of [3H]-thymidine was determined by counting the radioactivity on the filter paper which was extracted with Clear-sol (Nacalai Tesque, Ltd., Japan).

Results

Time- and dose-dependent elevation of intracellular cAMP level by HAA: As has been shown in Fig. 1A, HAA at the concentration of 10^-3 M caused a time-dependent elevation of the cAMP level of cultured spleen cells. The cAMP level increased rapidly in the first few hours, to a maximum of 3.2 times elevation in 3 hr, which then declined gradually and returned to the normal level within 24 hr. In Fig. 1B, the dose-dependent effect of HAA is depicted. In the 3 hr culture, a maximum of 2.8 times elevation in the cAMP level was caused by HAA at a concentration of 10^-3 M.

Theophylline and imidazole modified the effect of HAA on the cAMP level: Theophylline, a phosphodiesterase inhibitor, was added at 10^-6 to 10^-4 M concentrations simultaneously with 10^-4 M HAA to spleen cell cultures (Fig. 2). As a 3 hr culture was found to be the most effective condition for HAA-mediated elevation of intracellular cAMP level, in this experiment, the culture was done for the same length of time. Addition of only theophylline (10^-5 M) and only HAA (10^-4 M) to the spleen cell cultures caused 1.2 and 1.9 times elevation of the intracellular cAMP level, respectively. The results showed that simultaneous addition of theophylline at 10^-6, 10^-5 and 10^-4 M concentrations with HAA (10^-4 M) to the cultures caused around 2–3 times further elevation of the increased cAMP level mediated by only HAA (10^-4 M). Imidazole, which reduces the cAMP level
Fig. 1. Time- and dose-dependent elevation of intracellular cAMP level caused by HAA in the cultured splenocytes. Mice spleen cells (5×10^6 cells/well) were cultured, (A) in the presence of HAA at 10^-3 M for different lengths of time and (B) in the presence of different concentrations of HAA (10^-6 M–10^-3 M) for 3 hr. The cAMP concentration was determined by a radioimmunoassay kit. Each point represents the percent of control determined from the average value obtained from duplicate assays.

Fig. 2. The cAMP level increased by HAA in splenocytes was further elevated by theophylline, when it was simultaneously added with HAA to the culture of 3 hr duration. Results represent the percent of the control determined from the average values obtained from duplicate assays.

by activating phosphodiesterase activity, was added at 10^-8 to 10^-6 M concentrations to spleen cell cultures simultaneously with 10^-3 M HAA (Fig. 3). The experiment was done under the 3 hr culture condition. Addition of only 10^-7 M imidazole to the culture decreased the intracellular cAMP level to 85% of the control culture. Addition of only HAA at 10^-3 M concentration to the culture caused

Fig. 3. The cAMP level increased by HAA in splenocytes was decreased by imidazole, when it was simultaneously added with HAA to the culture of 3 hr duration. Results represent the percent of the control determined from the average values obtained from duplicate assays.
2.8 times elevation of the intracellular cAMP level. The simultaneous addition of imidazole at 10^{-8}, 10^{-7} and 10^{-6} M concentrations with HAA (10^{-3} M) to the cultures caused this HAA-mediated increased cAMP level to decrease half to one fourth.

**In vitro mitogenic responses in HAA-pre-treated mice spleen cells:** Figure 4A shows the Con A-induced mitogenic responses in spleen cells pooled from saline and HAA administered mice. At the lowest concentration of Con A (0.4 μg/ml), there was almost no difference in proliferative responses between the spleen cells pooled from saline and HAA administered mice. At the higher concentrations of Con A, there was a trend of mild inhibition of the proliferative responses in cells pooled from HAA administered mice. At the Con A concentration of 1.0 and 2.5 μg/ml, the proliferative responses in cells pooled from HAA administered mice were 14 and 17% lower than that of the saline control group, respectively.

On the other hand, as depicted in Fig. 4B, at different concentrations of another T cell mitogen, PHA, a trend of mild proliferation enhancement was found in cells pooled from HAA administered mice in comparison to the responses in cells pooled from the saline control group. At the higher concentrations of PHA (2.5 and 6.25 μg/ml), the proliferation was 10–12% higher in the cells pooled from HAA administered mice than that of the saline control group.

**Discussion**

It was revealed from the previous in vivo study that both single and 4 consecutive days oral administration of HAA at a dose of 10 mg/kg caused the maximum immunopotentiation against TD antigen SRBC in normal mice (2). In the same in vivo study, it was found that chronic administration of HAA (10 mg/kg) to immunosuppressive SHR rats significantly potentiated the immune response. These effects were accompanied by an increase of serum antibody titer.

The results of another study showed that the conditions and pattern of HAA-mediated in vitro immunopotentiation were similar to those of two cAMP-elevating agents, DbcAMP and adenosine (4). It was also found that theophylline and imidazole significantly modified the HAA-mediated in vitro immunopotentiation. These findings strongly suggested that HAA-mediated immunopotentiation was closely related to a possible cAMP elevating property of HAA.

Moreover, heptaminol, a constituent of HAA was reported to increase the level of cAMP in canine heart preparation, partially through activating adenylate cyclase and partially by an inhibiting effect on phosphodiesterase activity (8). HAA also contained adenosine in its structure, which has been reported to elevate the cAMP level in lymphocytes by acting on a surface receptor (9). These two facts also suggested that HAA might possess a cAMP elevating property.
These observations have been confirmed by the findings of the present study that HAA caused time- and dose-dependent elevation of the cAMP level of cultured splenocytes.

From the present study, it was also found that theophylline and imidazole caused the rise and fall of cAMP level increased by HAA, respectively. In almost the same pattern, theophylline and imidazole modified the HAA-mediated elevation of cAMP level, which eventually influenced the HAA-mediated immunopotentiation. The doses of HAA, theophylline and imidazole used in this study were selected in accordance with the previous in vitro study and other studies (4, 10, 11).

Furthermore, it was reported that cAMP elevating compounds showed suppressive effects on the in vitro mitogenic responses when added directly to the culture (12–14). In the previous investigation, it was found that both HAA and DbcAMP decreased the number of recovered cells from the cultures at the same concentration that showed in vitro immunopotentiating activity (4). It was speculated that a possible antiproliferative activity of HAA like other cAMP elevating agents might arise at least partially from its cytotoxic behavior in the culture. To avoid this possibility, mitogenic responsiveness of spleen cells pooled from HAA administered mice were studied instead of adding HAA directly to the 48 hr culture used in these experiments. HAA was orally administered to the normal mice at the dose of 10 mg/kg for 4 consecutive days, because the same treatment was found to cause the maximum in vivo immunopotentiation (2). At the higher concentrations of the T cell mitogen Con A (1.0 and 2.5 μg/ml), a trend of mild inhibition of the mitogenic responses was found in the cultures of spleen cells pooled from HAA administered mice, when compared with the responses in the cultures of spleen cells pooled from saline treated mice. This difference was not found at lower concentrations of Con A (0.4 μg/ml). On the other hand, when another T cell mitogen, PHA, was added at different concentrations to the cultures, a trend of mild enhancement in proliferation of spleen cells pooled from HAA administered mice was observed, as compared to that in the control group. Renoux and Renoux also reported that a difference in response to PHA and Con A was observed in the spleen cells pooled from immunosuppressed mice, which were administered with another immunopotentiating agent, diethylthiocarbamate (15).

It may be worthy to mention here that Con A also caused generation of Ts cells in doses higher than that required to cause the mitogenic response (16). Rich and Pierce reported that a mitogenic dose of Con A, as low as 0.75 μg/ml, also suppressed the humoral immune response, possibly by inducing Ts cells, when added at the time of initiation of the spleen cell culture (17). Therefore, it might be possible that mild inhibition of in vitro proliferation of spleen cells pooled from HAA administered mice in response to the addition of higher concentrations of Con A to the culture reflected the reduction of Con A-mediated generation of Ts cells. This is supported by the findings of a previous study that HAA inhibited the induction of Ts cells and enhanced the function of Th cells both in vivo and in vitro (3).

Acknowledgments: We are grateful to Yamasa Shoyu Co., Ltd., Japan, for providing us with HAA and express our sincere thanks to Miss H.W. Lim for her cooperation in the experimental work.

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