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IMMUNOSUPPRESSIVE EFFECTS OF ADAMANTOYL CYTARABINE—I

INHIBITION OF HEMAGGLUTININ FORMATION AND GRAFT VERSUS HOST REACTIONS IN MICE

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Abstract—The immunosuppressive effects of adamantoyl cytarabine [1-β-D-arabinofuranosylcytosine-5′-(1-adamantanecarboxylate); AdOCA] have been examined and compared with the parent compound, cytarabine, in two mouse immunologic systems: the hemagglutinin response to sheep erythrocytes, and the graft versus host reaction. Previous reports by the authors have shown that cytarabine effects were of short duration and that multiple daily injections were necessary for maximum immunosuppression.

In contrast, AdOCA demonstrated long-lasting immunosuppressive effects after one injection. Data accumulated in other experiments clearly demonstrate the greater immunosuppressive activity of AdOCA compared with cytarabine. This was true irrespective of the basis of comparison (single injections, daily injections, milligram or mole). Further, AdOCA immunosuppression was observed before, during, and after antigenic challenge, whereas cytarabine was shown previously to be most immunosuppressive 2 days after antigenic challenge.

THE IMMUNOSUPPRESSIVE effects of cytarabine (*ara*-cytidine, cytosine arabinoside) have been described in many immunologic systems and species,[1–11] including man.[12] The conclusions that are salient to the present communication were that cytarabine effects were of short duration, and that multiple daily injections were necessary for maximum immunosuppression.[8,9]

We wish now to report on a cytarabine derivative, adamantoyl cytarabine* (AdOCA), first synthesized in our laboratories by Dr. P. F. Wiley, which differs from cytarabine in that AdOCA possesses greater immunosuppressive activity than cytarabine on a milligram or mole basis, and also possesses long-lasting immunosuppressive activity after one injection. The structures of AdOCA and cytarabine are shown in Fig. 1. The synthesis and chemical characteristics of AdOCA (P. F. Wiley and R. C. Kelly, in preparation) and the effects of AdOCA on a mouse leukemia system (G. L. Neil, in preparation) will be discussed in separate communications.

The immunosuppressive effects of AdOCA and cytarabine have been compared in two mouse immunologic systems: hemagglutinin responses to sheep erythrocytes, and the graft versus host reaction. The former represents a model for humoral antibody

* 1-β-1D-Arabinofuranosylcytosine-5′-(1-adamantanecarboxylate).

responses, while the latter presumably represents a model for cell-mediated immune responses.[13,14]

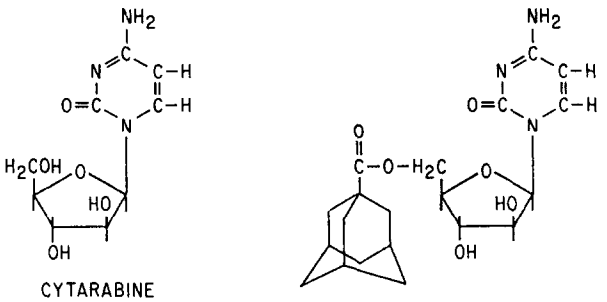

FIG. 1. Structural configuration of cytarabine and of adamantoyl cytarabine.

METHODS AND MATERIALS

Preparation of AdOCA suspensions. AdOCA (mol. wt. = 405), generously supplied by Drs. P. F. Wiley and R. C. Kelly, was suspended in 0·25% methycellulose, 25 cps, using a motor-driven Teflon pestle in a glass homogenizer. Cytarabine (mol. wt. = 243) was dissolved in 0·15 M NaCl.

Graft versus host reactions (GVHR). Parental spleen donor cell suspensions were prepared from male, 20–30 g, C57Bl mice. The hosts were male, 20–22 g, (C57Bl × $C_3H)F_1$, Cumberland View Farms, Clinton, Tenn.). Previous studies[9] have shown that GVHR-induced splenomegaly development in the F_1 mice was most consistent 8 days after injection of 10^9 parental spleen cells. The syngeneic controls consisted of injecting F_1 spleen cells into F_1 hosts. The results, expressed as ratios of milligrams of spleen wt. to grams of body wt. represent the average of five individual mice $\pm$ 1 standard deviation.

Determination of hemagglutinin (HAg) titers. The determination of HAg antibodies in ICR/Upjohn mice, formed in response to sheep erythrocytes, was performed on doubling dilutions of mouse serum by previously described techniques.[8] The HAg titers are expressed as the reciprocal of $\log_2$ of the highest serum dilution possessing HAg activity. The titers given represent the average of five individual mouse sera $\pm$ 1 mean deviation.

RESULTS

Comparison of AdOCA and cytarabine in the GVHR. The results presented in Table 1 show that AdOCA was more immunosuppressive than cytarabine in inhibiting the GVHR-induced splenomegaly, irrespective of whether the comparison was made on a milligram or mole basis. For instance, when both compounds were injected at 24 mg/kg for 5 days, the inhibition by AdOCA (96 per cent, 60 μmoles/kg) was far greater than that of cytarabine (24 per cent, 100 μmoles/kg). Similar results were obtained when the compounds were injected on days 1, 3 and 5 after initiation of the GVHR.

TABLE 1. COMPARISON OF AdOCA AND CYTARABINE IN INHIBITION OF THE GRAFT VERSUS HOST REACTION

Compound	Day of injection	Dose, i.p. mg/kg(μmoles/kg)	mg spleen/ g body wt.*	Inhibition %
Normal GVHR			8·44 ± 1·81	none
Controls			3·79 ± 0·42	100
AdOCA	1–5	40 (100)	2·77 ± 0·29	100†
Cytarabine	1–5	40 (167)	4·07 ± 0·65	93
AdOCA	1,3,5	40 (100)	3·79 ± 0·32	100
Cytarabine	1,3,5	40 (167)	6·07 ± 0·84	50
AdOCA	1–5	24 (60)	3·94 ± 0·33	96
Cytarabine	1–5	24 (100)	7·31 ± 1·12	24
AdOCA	1,3,5	24 (60)	4·55 ± 0·48	83
Cytarabine	1,3,5	24 (100)	7·24 ± 0·86	25

*Average of five mice ± 1 standard deviation.
†This dose of AdOCA was toxic and was responsible for the decrease in spleen weight below that of the controls.

When AdOCA was examined under conditions which had been shown previously to be most immunosuppressive for cytarabine,[9] i.e. injections on days 2, 3, and 4, no differences were noted between the two compounds, except at the lower dose (Table 2). However, the results of subsequent experiments demonstrated that AdCOA was at least as effective (or more effective) when given in a single large injection rather than in daily injections. Thus, one injection of 200 mg AdOCA/kg on either day 1,

TABLE 2. COMPARISON OF AdOCA AND CYTARABINE IN INHIBITION OF THE GRAFT VERSUS HOST REACTION

Compound	Dose, i.p.; days 2–4, mg/kg/day(μmoles/kg/day)	Mg spleen g body wt.*	% Inhibition
Normal GVHR		7·52 ± 0·36	none
Controls		3·42 ± 0·16	100
AdOCA	100 (250)	3·33 ± 0·13	100
Cytarabine	60 (250)	3·68 ± 0·12	94
AdOCA	50 (125)	4·47 ± 0·15	74
Cytarabine	30 (125)	4·38 ± 0·21	77
AdOCA	25 (62)	6·15 ± 0·18	33
Cytarabine	15 (62)	7·43 ± 0·29	2

*Average of five mice ± 1 standard deviation.

day 2 or day 3, resulted in complete suppression of the GVHR (Table 3). A single injection on day 4 also resulted in essentially complete suppression. Therefore, in contradistinction to the previous results with cytarabine,[9] the day of injection of AdOCA to obtain complete suppression of the GVHR-induced splenomegaly was not critical. It should be noted that this dose of AdOCA must have had a direct effect on the spleen, independent of inhibition af the GVHR-induced splenomegaly, since the spleens from GVHR-mice injected with AdCOA on day 1 or day 2 were significantly smaller ($P<0·01$ and 0·05 respectively) than the controls. When lower doses were used in subsequent experiments, no direct effects were observed.

TABLE 3. EFFECT OF A SINGLE AdOCA INJECTION ON THE GRAFT VERSUS HOST REACTION

Day of AdOCA injection, i.p. (200 mg/kg)	mg spleen/ g body wt.*	% Inhibition
Normal GVHR	9·20 ± 0·28	none
Controls	3·13 ± 0·13	100
1	2·40 ± 0·22	100
2	2·52 ± 0·19	100
3	2·87 ± 0·16	100
4	3·54 ± 0·19	93

*Average of five mice ± 1 standard deviation.

Effect of AdOCA and cytarabine on the primary HAg response. Table 4 shows that by increasing the dose and the length of AdOCA injections, very marked immunosuppression was obtained. Injections of 20 mg AdOCA/kg/day on days 0–8 severely suppressed the HAg response, even when the titers were measured 21 days later (3·6 vs. control 12·8). The suppression was less at 10 mg AdOCA/kg, but the titer was still significantly different from the controls.

TABLE 4. EFFECT OF DAILY AdOCA INJECTIONS ON THE PRIMARY HEMAGGLUTININ RESPONSE

Compound	Hemagglutinin titer*	
	Day 10	Day 21
Controls, i.p., vehicle		
Days 0–8	10·4 ± 0·4	12·8 ± 0·6
AdOCA, i.p., 20 mg/kg		
Days 0–4	5·4 ± 0·7	9·0 ± 0·8
Days 0–8	4·0 ± 1·2	3·6 ± 1·5
AdOCA, i.p., 10 mg/kg		
Days 0–4	7·0 ± 0·8	11·0 ± 0·4
Days 0–8	7·0 ± 0·8	10·0 ± 0·4

*The titers represent the average of five mice ± 1 mean deviation.

AdOCA and cytarabine were then compared using the dosage schedule which was shown above to be effective for AdOCA immunosuppression. The results in Table 5 demonstrate quite clearly the greater immunosuppressive properties of AdOCA on a milligram basis. In all three parameters measured, i.e. days 10 and 21 of the primary response and day 10 of the subsequent secondary response, AdOCA was more immunosuppressive than cytarabine. The difference between cytarabine and AdOCA is accentuated further if the comparison is made on a mole basis, since AdOCA has a higher molecular weight (405 vs. 243).

However, the most important characteristic which distinguished AdOCA from cytarabine was found when the two compounds were compared using single injections Previous studies[8] and unpublished data (G. D. Gray *et al.*) have shown a consistent

TABLE 5. COMPARISON OF DAILY AdOCA AND CYTARABINE INJECTIONS ON THE PRIMARY AND SUBSEQUENT SECONDARY HEMAGGLUTININ RESPONSE

Compound	Hemagglutinin titer* Primary response Day 10	Primary response Day 21	Secondary response Day 10
Control, vehicle only	11·8 ± 0·6	11·8 ± 0·6	13·8 ± 0·6
AdOCA, i.p., 20 mg/kg	1·0 ± 1·0	2·8 ± 1·6	9·2 ± 1·6
Cytarabine, i.p., 20 mg/kg	5·6 ± 1·1	9·2 ± 0·6	12·0 ± 0·8

*All mice were injected on days 0–8 with either compound or vehicle. The mice were rechallenged with antigen only, 1 month later, and the titers were measured 10 days later. The titers represent the average of five mice ± 1 mean deviation.

lack of immunosuppressive activity by cytarabine when single injections were employed, even at very large doses (1 g/kg). Single injections of AdOCA, however, resulted in marked immunosuppression (Table 6). Approximately 20 per cent of the mice injected once with AdOCA had no detectable antibody 10 days after immunization.

The results in Table 6 are also in accord with the GVHR experiments and demonstrate, in contrast to previous studies with cytarabine,[5,8,9] that the timing of AdOCA injections to exert maximum immunosuppression was not critical. Subsequent experiments with differents lots of AdOCA have not confirmed the apparent biphasic nature of immunosuppression seen in Table 6 (day $1>2>3>-1>0$), but the maximum immunosuppressive activity has always been obtained with AdOCA injections 1 day after sheep erythrocyte challenge.

TABLE 6. EFFECT OF A SINGLE AdOCA INJECTION ON THE PRIMARY HEMAGGLUTININ RESPONSE

Day of AdOCA injection, i.p. (200 mg/kg)	Hemagglutinin titer* Day 10 Expt. 1	Expt. 2
None, controls	10·2 ± 1·0	11·2 ± 1·0
−1	7·0 ± 0·4	4·2 ± 0·4
0	9.5 ± 1·0	6·4 ± 1·1
1	2·8 ± 1·3	1·2 ± 1·3
2	4·3 ± 1·3	1·8 ± 1·7
3	5·2 ± 0·6	3·6 ± 1·5

*The titers represent the average of five mice ± 1 mean deviation.

DISCUSSION

The reasons for the greater and more prolonged activity of the cytarabine adamantoate derivative can only be speculative at this time. Since AdOCA was almost totally insoluble, part of the prolonged activity may be due simply to the time required for solubilization *in vivo*. Further, it is not known whether AdOCA must be hydrolyzed

in vivo to adamantane-1-carboxylic acid and cytarabine (and the latter accounts for all the activity), or whether AdOCA *per se* possesses immunosuppressive activity. Since AdOCA is very insoluble, experiments *in vitro* designed to test this point are not feasible. Attempts in this laboratory to produce immunosuppression with the potential hydrolysis product of AdOCA, i.e. adamantane-1-carboxylic acid, either alone or in combination with cytarabine, have been unsuccessful even when both were used at twice (40 mg/kg/day) the effective AdOCA dose. Morever, immunosuppression has not been attained with other non-nucleoside adamantane derivatives, although other investigators have reported that amantadine (1-amino-adamantane), under certain conditions, could influence immunologic reactions.[15,16]

AdOCA might possess unique tissue distribution characteristics which could be of importance, since Camiener and Smith[17] have shown that the kidney of the mouse is primarily responsible for the deamination of cytarabine to the inactive product, uracil arabinoside. Thus, localization in selected sites, e.g. lipid depots, may reduce inactivation by the kidney deaminase.

Related to this, and perhaps of greater importance, is the observation that, while adenosine-5′-adamantoate does not prevent the deamination of adenosine, it itself is not deaminated by adenosine deaminase.[18] The possibility exists, therefore, that part of the greater activity of AdOCA is due to its resistance to deamination. Studies to test these possibilities will depend on the availability of radioactively labeled AdOCA.

This report tends to substantiate the extensive investigations of Gerzon and Kau,[18] Gerzon *et al.*,[19] and Rapala *et al.*[20] These investigators have described the interesting effects of adamantoylation on tolbutamide,[19] anabolic steriods[20] and nucleosides.[18] The proposal was made by Gerzon and Kau[18] that the primary effect of adamantoylation was to influence the precision with which biologically active compounds fit into receptor sites, rather than to influence the absorption, distribution or metabolism of the agent. Unfortunately, no evidence was obtained in the current experiments to support or refute any of the suggestions, and thus, the determination of the mechanism of enhancement of biological activity by adamantoylation of nucleosides (in this case, of cytarabine) remains an interesting challenge.

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