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INVESTIGATIONS INTO THE *IN VITRO* AND *IN VIVO* IMMUNOSUPPRESSANT ACTIVITY OF *N*-ACETYL-3-SULPHONAMOYL-L-ALANINE METHYL ESTER, A POTENTIAL L-ASPARAGINE ANTIMETABOLITE

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Abstract—*N*-Acetyl-3-sulphonamoyl-L-alanine methyl ester (Asame) has been previously reported to inhibit mitogen-induced DNA synthesis by human lymphocytes *in vitro* and to exhibit immunosuppressant activity in monkeys *in vivo* without overt signs of toxicity. We have confirmed and extended the *in vitro* studies by demonstrating that Asame inhibits concanavalin A-induced DNA synthesis and blast cell formation by mouse spleen lymphocytes. However, a requirement for L-asparagine by cultured lymphocytes could not be demonstrated and the inhibitory effects of Asame were not reversed by L-asparagine. Thus, the *in vitro* immunosuppressant activity of Asame could not be attributed to interference with L-asparagine metabolism. Furthermore, no *in vivo* immunosuppressant activity could be demonstrated for Asame in mice. These results cast doubt on the concept that L-asparagine antimetabolites have potential as immunosuppressants.

In 1975, Visser *et al.* [1] reported that a sulphonamide analogue of asparagine, *N*-acetyl-3-sulphonamoyl-L-alanine methyl ester (Asame), inhibited mitogen-induced proliferation of human lymphocytes *in vitro* and delayed renal allograft rejection in monkeys. This compound had been shown previously to compete with L-asparagine in several systems (guinea pig serum asparaginase, asparagine-dependent *Neurospora crassa*) but to have no toxicity for normal chick fibroblasts [2]. Since no obvious toxic effects were seen in the treated monkeys, it was suggested that this compound was a novel immunosuppressant, possibly acting as an L-asparagine antimetabolite, which did not have the serious side-effects of conventional immunosuppressants [1]. The concept that the activity of Asame was due to its acting as an L-asparagine antimetabolite was strengthened by the literature reports that asparaginase obtained from various sources was immunosuppressant in experimental animals [3–6]. Additional apparently confirmatory evidence came from a number of studies in which asparaginase added to lymphocyte cultures *in vitro* inhibited their proliferative response to mitogens and antigens [7–10]. These results appeared to indicate that lymphocyte function was L-asparagine dependent and its removal by asparaginase or antagonism by Asame could lead to immunosuppression.

We have investigated these claims using *in vitro* and *in vivo* techniques in mice and failed to demonstrate a role for L-asparagine in the immune response or *in vivo* immunosuppressant activity for Asame.

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† Hepes = 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

METHODS

Lymphocyte proliferation in vitro

Spleens were removed from male 6 to 8 week-old MFI or CFW mice and teased apart in RPMI 1640 medium containing 10 per cent heat inactivated fetal calf serum (Flow Laboratories, Irvine, Scotland), 20 mM Hepes† (pH 7.4) (Hopkins Williams, Romford, Kent, U.K.) and 200 μg/ml gentamycin (Nicholas Laboratories, Slough, Berks, U.K.).

The spleen capsules were discarded, and single cell suspensions were prepared, centrifuged (800 r.p.m. × 5 min) and resuspended in medium. The cells were incubated at 10^6 cells/ml in 200 μl aliquots in 96 well round-bottomed microtitre plates (Nunc, Roskilde, Denmark) with 5 μg/ml of concanavalin A (Sigma Chemical Co., Poole, Dorset, U.K.) at 37° in a humidified air atmosphere. Ten microliters of 50 μCi/ml [^{3}H]thymidine ([*methyl*-^{3}H]thymidine, Cat. No. TRA120, Radiochemical Centre, Amersham, Bucks, U.K.) were added to each well after 54 hr and the incubation was continued up to 72 hr. To terminate the incubation, the contents of the wells were washed onto glass fibre filters with distilled water using an automatic harvester (Flow Laboratories). The radioactivity on the filter paper was counted using a conventional liquid scintillation counter system. The concentration of drug required to inhibit thymidine incorporation by 50 per cent (I_{50}) was estimated by interpolation on a graph of c.p.m. against concentration.

In some experiments, 5×10^6 mouse spleen lymphocytes were cultured in plastic bijoux bottles containing the above medium, supplemented with 10^{-5}M 2-mercaptoethanol and 5 μg/ml of concanavalin A. After 3 days, the size distribution of the cell nuclei was measured using a Coulter Counter

model ZB with P64 size distribution analyzer and XY recorder (Coulter Electronics, Harpenden, Herts, U.K.). The orifice diameter was 100 μm and the cell membranes were lysed with saponin (Zaponin, Coulter Electronics) prior to analysis.

Determination of cell viability

At various times after setting up the lymphocyte cultures for [^{3}H]thymidine incorporation studies, 100-μl aliquots were removed from the wells after mixing and added to 20 μl of 0.4 per cent trypan blue. Counts of stained and unstained cells were carried out and viability was determined.

Lymphocyte proliferation in vivo *in mice*

The effect of Asame on lymphocyte proliferation *in vivo* in mice was studied as described by Mackaness *et al.* [11]. This involves determining the effect of the drug on [^{3}H]thymidine incorporation into the left popliteal lymph node 3 days after stimulation of lymph node proliferation by injection of sheep red blood cells into the left hind foot pad.

Groups of five to ten male MFI or CFW mice, 6- to 8-weeks-old, were sensitized by injection of 10^8 washed sheep red blood cells (preserved in Alsever solution, Tissue Culture Services, Slough) subcutaneously into the left hind foot pad. Three days later [^{3}H]thymidine (1 μCi/g body wt) was injected intravenously. After 30 min, the left popliteal lymph node was removed, homogenized in ice-cold 5 per cent trichloroacetic acid (TCA), washed three times in cold 5 per cent TCA, and the pellet, obtained after the last wash, was hydrolyzed in 1.2 ml of 5 per cent TCA for 1 hr at 90°.

After centrifugation of the hydrolysate, an aliquot of the supernatant fraction was added to scintillation fluid (NE 260, Nuclear Enterprises Ltd., Sighthill, Edinburgh, Scotland) and counted in a liquid scintillation counter. The d.p.m./node were calculated with appropriate quenching correction. The degree of stimulation was estimated by comparing the d.p.m. in the nodes on the injected and non-injected sides in control animals. Asame, dissolved in saline, was administered subcutaneously.

Antibody production in mice

The effects of Asame on antibody production in mice, in response to an intraperitoneal injection of 2×10^8 sheep red blood cells, were studied by measuring hemagglutinating antibody levels in sera collected at various days after immunization in groups of five to ten mice. The drug, dissolved in saline, was administered by various routes. The sera were titrated using doubling dilutions in microtitre plates. Results are expressed as titres, i.e. $-\log_2$ of the highest dilution causing agglutination.

Materials

Asame and L-2-amino-3-sulphonamoyl propionic acid were prepared by the published methods [2]. L-2-Acetamido-3-sulphonamoyl propionic acid was prepared from the amino acid by direct acetylation, and methyl *L*-2-amino-3-sulphonamoyl propionate was prepared from methyl L-2-benzyloxycarbonyl-amino-3-sulphamoyl propionate [12] by catalytic hydrogenolysis. All other reagents were obtained from the sources mentioned under Methods or from usual laboratory suppliers.

RESULTS

In vitro *studies*

The inhibitory activity of Asame *in vitro* was confirmed on many occasions (Fig. 1). Although inhibition was always obtained, the potency of the compound varied from experiment to experiment, e.g. I_{50} in Figs. 1–3 was 80, 35 and 50 μM. No single factor could be identified as being responsible for this variation, so all experiments were designed to be controlled internally and no comparisons were made between experiments performed on different occasions. Usually in this test, the drug is present throughout the 3-day culture period and so has the opportunity to act at any of the many stages of the lymphocyte response. By adding the drug at different times after initiation of the culture, it is possible to

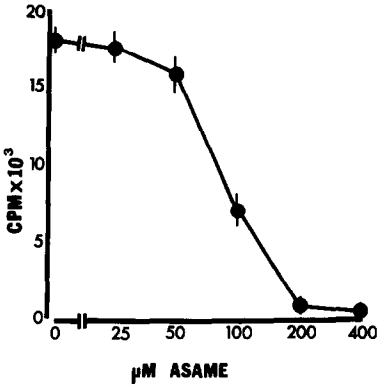

Fig. 1. Effect of Asame on lymphocyte proliferation *in vitro*. Asame was added to the cultured lymphocytes at $t = 0$, and the effect on thymidine incorporation was assessed at 72 hr of culture.

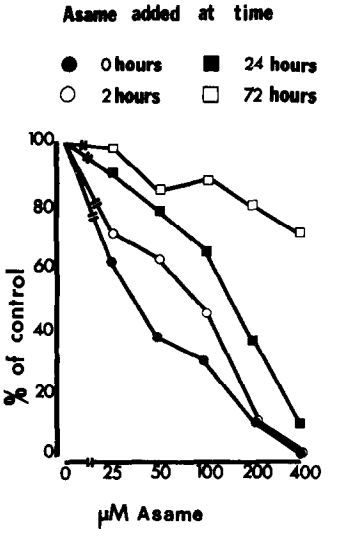

Fig. 2. Time dependence of the *in vitro* action of Asame. Asame was added to cultured lymphocytes at various times after the initiation of the culture, and the effect on thymidine incorporation was assessed at 72 hr of culture.

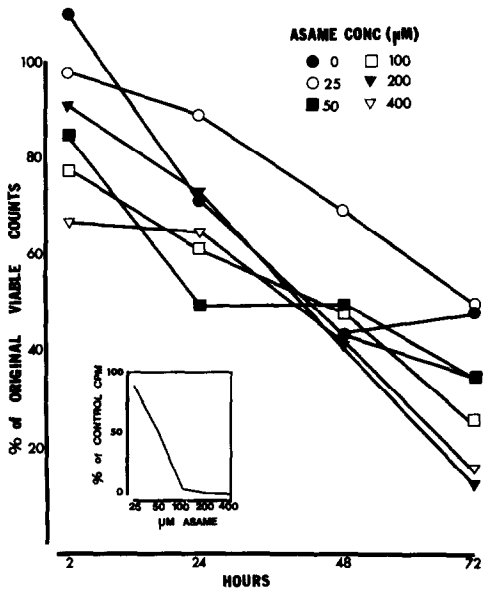

Fig. 3. Effect of Asame on lymphocyte viability *in vitro*. Asame was added to the cultured lymphocytes at $t = 0$, and cell viability was assessed at various times using the trypan blue exclusion technique. The inset shows the activity of the compound in inhibiting thymidine incorporation in the same experiment.

learn something about its possible mode of action. As can be seen from Fig. 2, delaying the addition of Asame for up to 2 hr had no effect on its inhibitory activity. This indicates that the drug does not act by interfering with the interaction between the concanavalin A and the cell membrane, an event which is complete well within this time [13]. When addition of drug was delayed for 24 hr, there was a slight fall in activity, but when the drug was added at 48 hr, i.e. only during the period of [^{3}H]thymidine labeling, activity was virtually abolished. The data suggest that either the Asame-sensitive events occur between 24 and 48 hr after initiation of the culture or that the drug requires in excess of 24 hr to develop its effect. Direct interference with [^{3}H]thymidine uptake or utilization can be excluded as a mode of action since Asame was inactive when present only during the labeling period.

The free amino acid (L-2-amino-3-sulphonamoyl propionic acid) and the partially protected derivatives (methyl-L-2-amino-3-sulphonamoyl propionic acid, L-2-acetamido-3-sulphonamoyl propionic acid) were inactive at concentrations up to 200 μM.

Effect of Asame on cell viability in vitro. The viability of concanavalin A-stimulated lymphocytes exposed to Asame was monitored by trypan blue exclusion in an experiment in which the drug was present throughout the culture period, and viability was assessed at 2, 24, 48 and 72 hr. The total number of cells in control cultures (i.e. viable plus non-viable) fell by 26 per cent over the 72 hr period, presumably due to lysis of a proportion of the dead cells. As a result, cell debris accumulated, making cell counting more difficult. Figure 3 shows the number of viable cells per culture at the various times. Asame caused a roughly dose-related decrease in the numbers of viable cells, particularly at 72 hr. However, as can be seen from the inset in Fig. 3, the inhibition of [^{3}H]thymidine incorporation by Asame was much more profound than the effect on viability, suggesting that the drug has a more subtle effect on lymphocyte function than just non-specific cytotoxicity. However, due to the limitations of dye exclusion methods of assessing viability [14], no further conclusions can be drawn from these results.

Effect of L-asparagine on the inhibitory effect of Asame in vitro. The usual lymphocyte culture medium (RPMI 1640) contained 50 mg/l of L-asparagine. Addition of L-asparagine to 500 mg/l did not shift the dose–response curve to Asame at all. Furthermore, lymphocyte transformation occurred equally well in either RPMI 1640 or Eagle's MEM (which contained no L-asparagine), even when the serum used had been dialyzed to remove L-asparagine. However, the omission of L-glutamine from Eagle's MEM containing 10 per cent dialyzed fetal calf serum virtually abolished the response to concanavalin A (15.8 ± 2.1 per cent of control, mean ± S.E.M. of five replicates). This response could be restored by the addition of 300 mg/l L-glutamine (100 ± 8.8 per cent of control) but not of 50 mg/l L-asparagine (21.6 ± 3.4 per cent of control). The combination of L-glutamine and L-asparagine (92.0 ± 11.0 per cent of control) was not better than L-glutamine alone. The concentrations of L-glutamine and L-asparagine used correspond to those in complete RPMI 1640. These results suggested that the mode of action of Asame cannot be due to interference in the metabolism of exogenous L-asparagine despite the fact that it was possible to suppress lymphocyte transformation by addition of *Escherichia coli* asparaginase (Fig. 4).

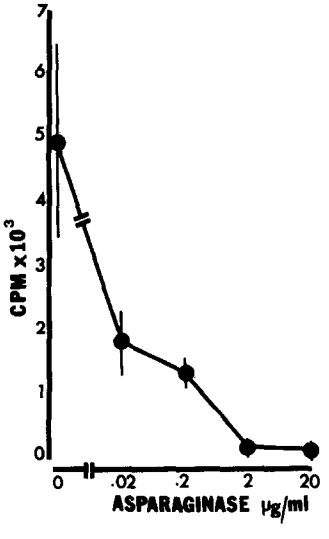

Fig. 4. Effect of *E. coli* asparaginase on lymphocyte proliferation *in vitro*. Asparaginase was added to the cultured lymphocytes at $t = 0$, and the effect on thymidine incorporation was assessed at 72 hr. The medium (RPMI 1640) contained 50 mg/l asparagine.

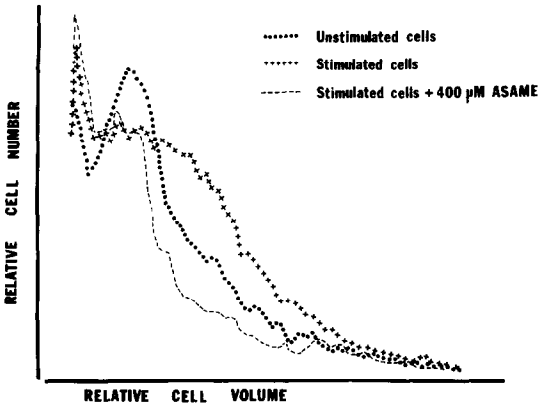

Fig. 5. Effect of Asame on blast transformation *in vitro*. Cultures were carried out in 5 ml volumes of medium supplemented with 5×10^{-5} M 2-mercaptoethanol. Asame was added at $t = 0$, and the size distribution of the cell nuclei was assessed at 72 hr.

Table 1. Effects of Asame on antibody production *in vivo*

	Treatment	Titre* of antibody
Exp. A	Controls	7.16 ± 0.70 (19)
	200 mg/kg, i.v.†	8.00 ± 0.68 (6)
	400 mg/kg, i.v.†	8.33 ± 0.21 (6)
	800 mg/kg, i.v.†	8.00 ± 0.82 (6)
	800 mg/kg, s.c.†	8.80 ± 0.20 (5)
Exp. B	Controls	5.36 ± 0.49 (11)
	100 mg/kg, i.v.‡	5.00 ± 0.56 (8)

* The titre of the sera is $-\log_2$ of the highest dilution causing agglutination of sheep red blood cells. Results are means ± S.E.M. The number of animals per group is given in parentheses.

† A daily dose was given on days 0, 1, 2 and 3. Sera were collected on Day 4. Day 0 was the day of sensitization with 10^8 SRBC, i.p.

‡ A daily dose was given on days −1, 0, 2 and 3. Sera were collected on day 4. Day 0 was the day of sensitization with 10^8 SRBC, i.p.

Effect of Asame on blast transformation in vitro. In order to exclude the possibility that the observed inhibition of thymidine incorporation was due to some interference with the indicator system and was unrelated to inhibition of lymphocyte proliferation, the effect of the drug on blast cell formation was studied.

In the method used, the numbers of blast cells were determined by analyzing electronically the volume of lymphocyte nuclei (blast cells, having large nuclei, shift the size distribution curve to the right).

As shown in Fig. 5, Asame produced an inhibition of the number of blast cells formed in response to concavalin A stimulation, which proved to be dose-related. This result indicated that the drug was inhibiting lymphocyte proliferation.

In vivo *studies*

Effect of Asame on antibody production in vivo. Despite the use of very high doses of Asame by various routes, no inhibition of antibody production was observed (Table 1). In our laboratories, this system has proved sensitive to a broad range of conventional immunosuppressants.

Effect of Asame on lymphocyte proliferation in vivo. Asame administered at 200 mg/kg subcutaneously on days 0, 1, 2 and 3 failed to inhibit DNA synthesis in stimulated popliteal lymph nodes (controls 8770 ± 1116 d.p.m./node, Asame-treated 8634 ± 682 d.p.m./node, means ± S.E.M., nine animals/group). In the control animals, DNA synthesis in the stimulated (left) popliteal lymph node was 16.7-fold greater than in the non-stimulated (right) popliteal lymph node. No signs of toxicity were seen in any of the animals despite the high doses employed. In our laboratories, this test has proved sensitive to a broad range of conventional immunosuppressants.

DISCUSSION

The reported *in vitro* immunosuppressant activity of Asame [1] has been confirmed in these studies. The potency of the compound in our mouse lymphocyte system was similar to that observed by the original authors [1] using human lymphocytes, thus indicating the absence of species specificity.

However, we have been unable to confirm the suggestion that this activity could be due to antagonism of L-asparagine since a 10-fold increase in medium L-asparagine levels did not modify the effect of Asame and, furthermore, lymphocyte [^{3}H]thymidine incorporation is normal in the complete absence of L-asparagine from the medium.

The well-established *in vitro* and *in vivo* immunosuppressant activity of certain asparaginase preparations is apparently at variance with our data [4]. However, it has been shown that many asparaginase preparations also contain glutaminase activity [15,16]. Since L-glutamine is an absolute requirement for lymphocyte function, depletion of L-glutamine probably explains the actions of these enzyme preparations on the immune system. Furthermore, a glutaminase-free asparaginase preparation has been shown to be devoid of *in vitro* immunosuppressant activity [17]. Thus, it now seems clear that the *in vitro* biological activity of Asame against lymphocytes is unrelated to L-asparagine metabolism.

These *in vitro* effects of Asame are seen at relatively high concentrations, i.e. 5×10^{-5} to 10^{-4} M (10–100 μg/ml). One explanation for the lack of *in vivo* activity could be that these relatively high levels of the drug cannot be achieved in the target tissues. Alternatively, the compound could be metabolized so rapidly that it fails to reach the target tissues in its intact form. The lack of activity *in vitro* of the deprotected forms of the drug supports this suggestion since the protecting groups are probably biologically labile. These results provide no encouragement for further studies on Asame as an immunosuppressant.

The data also cast doubt on the concept that L-asparagine antimetabolites have potential as immunosuppressants since these functions of the immune response which we have examined proved to be independent of asparagine. On the other hand, the

demonstration that L-asparagine is not required by normal lymphocytes plus the observation that some lymphocytic leukemias do require L-asparagine [18, 19] suggests that some L-asparagine antimetabolites [20] may prove to have anti-leukemic properties and be devoid of immunosuppressant activity.

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