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Quantitative and Qualitative Effects of *m*-AMSA (Amsacrine) on Cellular Immune Components*

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Abstract—*The recently developed acridine derivative* 4′-[(9-*acridinyl*)*amino*] *methanesulphon*-m-*anisidine* (m-*AMSA*) *has become one of the preferred agents in the management of acute leukaemia but little is known of its effects on cellular immune components. We evaluated the qualitative and quantitative effects of* m-*AMSA on immune cell numbers and function, during both the leucopenic and myelorestorative phases, following intermittent high-dose therapy. Cell-mediated and inflammatory responses were depressed in the treated animals during the leucopenic phase but restoration of immune capability paralleled the recovery of circulating leucocyte numbers. Additionally,* m-*AMSA displayed unexpected immunomodulatory features: thymus-dependent antibody and delayed-type hypersensitivity responses were actually increased, suggesting the potential of* m-*AMSA as an immunoregulator in clinical and experimental studies.*

INTRODUCTION

THE RECENTLY developed acridine derivative 4′-[(9-acridinyl)amino] methanesulphon-*m*-anisidine (*m*-AMSA) is commonly used in the management of acute leukaemia [1]. To date, there is little information available on the effects of the drug on cellular immune components and resistance to infection. Agents used in the chemotherapy of malignant diseases are known to be immunosuppressive and, in some cases, have also been shown to possess immunostimulating properties [2–6]. According to a recent report [7], the enhancement of cell-mediated immune responses may in fact be a common biological phenomenon associated with a variety of anti-cancer drugs. These agents vary in their effect on cellular components of the immune system. Cell-cycle-specific drugs delete both T- and B-cells, whereas cell-cycle non-specific drugs such as cyclophosphamide may selectively delete immune components [8]. In the case of *m*-AMSA, there is laboratory evidence for cell-cycle-specific activity [9].

It is generally accepted that immune components complement the effect of chemotherapeutic agents in the management of malignant disease and, in view of this relationship, we set out to evaluate the qualitative and quantitative effects of *m*-AMSA on immune cell numbers and function. These effects were studied in the leucopenic and recovery phases following high-dose intermittent therapy. The experiments demonstrated a number of unexpected effects of *m*-AMSA on immune capability that we had not anticipated. Although thymus-independent antibody response was depressed, thymus-dependent antibody response was enhanced by *m*-AMSA and the activity of the RES was also enhanced. Delayed-type hypersensitivity was depressed, as expected, if *m*-AMSA was given before sensitization but enhanced if the dose followed sensitization.

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MATERIALS AND METHODS

Animals

Male and female rats (200–230 g) from an inbred Dark Agouti (DA) strain or an F1 DA × AS_2 cross were used in these experiments.

Dosage of m-*AMSA*

The pharmaceutical form of the drug, amsacrine (Laboratoires Substantia, Courbevois Cédex, France—via Parke, Davis & Coy, Auckland, N.Z.), is supplied as two solutions which are mixed to provide 5 mg/ml *m*-AMSA in 10% v/v *N,N*-dimethylacetamide and 0.03 M L-lactic acid. The solutions were mixed immediately prior to use. Pilot experiments were carried out to

establish the maximum tolerable dose schedule. *m*-AMSA was administered i.p. at doses of 12.5, 8.0 and 7.0 mg/kg on days 0, 4 and 8 respectively. In some short-term experiments only the first two doses were given. These schedules paralleled the short-term, high-dose regimen favoured in clinical management.

Cyclophosphamide

Doses of 37.5 mg/kg were administered i.p. as indicated in the individual protocols. This well-characterized drug has similar effects on circulating leucocytes and splenic cell numbers as *m*-AMSA. It was therefore studied as a reference drug in parallel with *m*-AMSA.

In vitro *analysis of the mitogenic response to Con A*

RPMI-1640 (Gibco Diagnostics, Madison, WI) was used in all the experiments. Glass-distilled water, deionized using Milli Q apparatus (Millipore Corp., Bedford, MA), was used to reconstitute the medium. Each litre of culture medium contained 70 ml of 2.8% sodium bicarbonate and 100 μl of both penicillin and streptomycin. Human serum was heat-inactivated and added to the medium to give a final concentration of 5% serum in RPMI. This preparation formed the RPMI-1640 tissue culture medium. Single-cell splenic lymphocyte suspensions were prepared as previously described [10]. Triplicate cultures of splenic lymphocytes were established in a round-bottomed tissue culture plate (Linbro No. 76-013-05, Flow Laboratories Inc., Hamden, CT) using 5×10^3 lymphocytes in 190 μl per well. A 0.5-mg/ml solution of concanavalin A (Sigma Chemical Co. Cat. No. 2010, St Louis, MI; Con A) was prepared in PBS and 4.0 μl was added to each well. Cultures were held for 48, 72 and 96 hr at 37°C in a humid atmosphere of 10% CO_2 in air. To each culture 0.25 μCi of [^{3}H]-thymidine (Radiochemical Centre, Amersham, U.K.) was added 18 hr before harvesting. The amount of [^{3}H]-thymidine incorporated into cellular DNA was determined after terminating the culture by the addition of distilled water and filtration of each culture onto filter pads using a 'Titertek' multiple cell harvester (Flow Laboratories Inc.). The filters were then transferred to glass vials for ^{3}H-counting in a Beckman Model B liquid scintillation spectrometer (Beckman Instruments, Fullerton, CA).

Host-vs-graft response

The popliteal lymph node weight method for determining the host-vs-graft activity of rat lymphoid tissue was carried out as described by Ford *et al.* [11]. The F1 progeny of a cross between the Ag-B disparate rat strains DA and AS_2 were used. Splenic lymphocytes were obtained from the F1 progeny when aged 4–6 weeks and 7.5×10^6 cells were injected subcutaneously into the left and right rear footpads of two groups of DA parental animals (control and *m*-AMSA treated). A third group was challenged in one footpad with 7.5×10^6 syngeneic cells; these animals served as negative controls for the experiment. Four days later the recipient animals were killed and the popliteal lymph nodes were removed and weighed after the adherent connective tissue had been removed.

Delayed-type hypersensitivity

Two groups of DA rats (control and *m*-AMSA-treated) were sensitized intradermally with 50 μg keyhole limpet haemocyanin (KLH) (Calbiochem, San Diego, CA) in 0.2 ml Freund's complete adjuvant (Commonwealth Serum Laboratories, Melbourne, Australia). Eight days later, the animals were challenged in their hind footpads with 20 μg KLH in 20 μl saline. The resultant reaction was quantitated by measuring the footpad swelling which was maximal 24–48 hr after challenge.

Immune response to Escherichia coli

The strain of *E. coli* 075 used in this experiment was the same as that used in our previous studies [10, 12, 13]. A killed antigen suspension was prepared by growing *E. coli* in nutrient broth to a concentration of 1×10^9 bacteria/ml. The culture was then steamed for 2 hr, washed twice in PBS and resuspended in its original volume of saline. In each trial three groups of animals (control, *m*-AMSA-treated and cyclophosphamide-treated) were immunized with 1×10^9 killed *E. coli* injected i.m. A passive haemagglutination technique was used to determine antibody levels to *E. coli*. SRBC were sensitized with activated endotoxin prepared from Boivin-type endotoxin which has been extracted with trichloracetic acid from a culture of *E. coli* 075 [14]. To activate the endotoxin a solution of 0.12 mg/ml in 0.02 M NaOH was heated for 5 min in a boiling water bath. After cooling, the pH was adjusted to 7 and an equal volume of double-strength PBS was added. SRBC were sensitized by mixing equal volumes of 10% SRBC and activated endotoxin solution and incubating the suspension of cells at 37°C for 2 hr. They were then washed three times in PBS. Sensitization of the SRBC was confirmed by carrying out passive haemagglutination tests with an anti-*E. coli* 075 serum that had been standardized against bacterial antigen. Titres were expressed as the reciprocal of the highest dilution showing gross agglutination.

Immune response to sheep red blood cells

SRBC, anticoagulated with Alsever's solution (Laboratory Services, Auckland, N.Z.), were washed once in citrate saline and three times in phosphate-buffered saline (PBS). A 5% solution was made in PBS and adjusted to contain 1×10^9 SRBC/ml. In each trial three groups of animals (control, *m*-AMSA-treated and cyclophosphamide-treated) were challenged with an i.p. injection of 1×10^9 SRBC in saline. Serum antibody responses to SRBC were measured by a direct haemagglutination assay using a semi-automated microtitre apparatus fitted with 50-μl diluters (Model 360-1 Minidiluter, Cooke Laboratory Products, Alexandria, VA). Doubling dilutions of serum in complement fixation buffer were prepared in duplicate and an equal volume of 0.05% SRBC was added. The plates were sealed and incubated for 1 hr at 37°C before being held overnight at 4°C. Titres were expressed as the reciprocal of the highest dilution showing gross agglutination.

Mobilization of cells into implanted sponges

Cylindrical sponges (2.5 × 1 cm, 40 ± 1 mg) were cut from polyurethane absorbent foam packing (ICI Tasman Ltd., Upper Hutt, N.Z.), washed in distilled water and sterilized by autoclaving in saline. Before implanting, the fluid was removed by squeezing the sponge in a sterile plastic syringe. An incision was then made in the dorsal midline of each rat, the facia retracted by blunt dissection and a sponge implanted each side of the midline in the subcutaneous space. Care was taken to observe strict aseptic conditions at all times and the wound was treated with an antibiotic spray after suturing to reduce the risk of infection. Cells were harvested from the sponges 24 hr after implantation by rinsing the cells out with Hanks' basic salt solution. Total and differential leucocyte counts were then carried out and the number of cells present were expressed as the total per sponge.

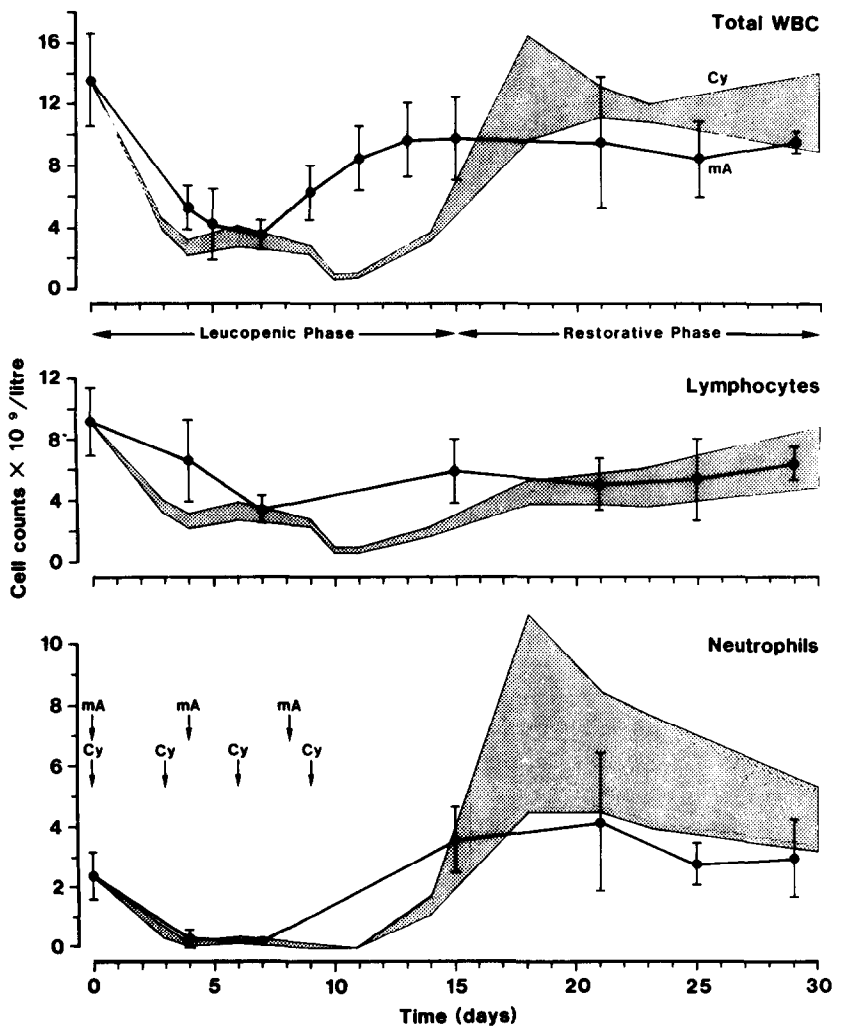


Fig. 1. *Peripheral blood leucocyte numbers in animals treated with* m-*AMSA (mA). Three doses of* 12.5, 8.0 *and* 7.0 mg/kg *were administered on days* 0, 4 *and* 8 *respectively. The reference drug cyclophosphamide (Cy) was administered at* 37.5 mg/kg *on days* 1, 3, 6 *and* 9. *(Shaded area = range of Cy effect, range = S.D.,* n = 28).

Measurement of phagocytosis

The *in vivo* catabolic and phagocytic capacities of mononuclear phagocytic cells of the reticular–endothelial system were assessed using ^{125}I-labelled microaggregated human serum albumin. In this assay the phagocytic rate is calculated by measuring the clearance of the aggregate from the blood, while an assessement of the catabolic activity is derived by monitoring the appearance of free ^{125}I in the vascular compartment. This aggregate was prepared by the method of Iio *et al.* [15] and labelled using the lactoperoxidase method. The phagocytic rate was also measured using Iio's method. Calculation of the catabolic rate was based on the model proposed by Bouveng *et al.* [16]. The details of this method have been previously described [17].

Statistical analysis

All results, except antibody titres, were subjected to the Wilcoxon rank sum test to assess the significance between groups.

RESULTS

Effect of m-*AMSA on peripheral blood leucocyte numbers*

Administration of the first two doses of *m*-AMSA caused the total leucocyte to fall to 4 × 10^9/l. Neutrophil and lymphocyte numbers were similarly affected. Restoration of circulating leucocyte numbers occurred despite a third dose of *m*-AMSA given to extend the depression (Fig. 1). In contrast, depression of leucocyte numbers was maintained by the continued administration of cyclophosphamide.

Host immune status in the leucopenic phase (0–15 days)

(*a*) *T-lymphocyte mitogenic responsiveness* in vitro. Animals were killed on day 7 of the experiment and the splenic lymphocyte numbers counted. Either *m*-AMSA or cyclophosphamide administration led to a marked reduction in spleen cell numbers (43 and 35% of normal in the *m*-AMSA- and cyclophosphamide-treated groups respectively). *m*-AMSA also induced a significant qualitative change in the remaining lymphoid cells: when the T-lymphocyte-specific mitogenic (Con A) responses of splenic lymphocytes from *m*-AMSA-treated animals were compared with the control group, the peak response was reduced to 66% of normal ($P = 0.01$, Fig. 2).

(*b*) *Host-vs-graft reaction.* This procedure evaluated the cell-mediated immune capability *in vivo* 7 days after commencement of treatment with *m*-AMSA and cyclophosphamide. Animals treated with *m*-AMSA retained 79% of their capability to respond to allogeneic lymphocytes.

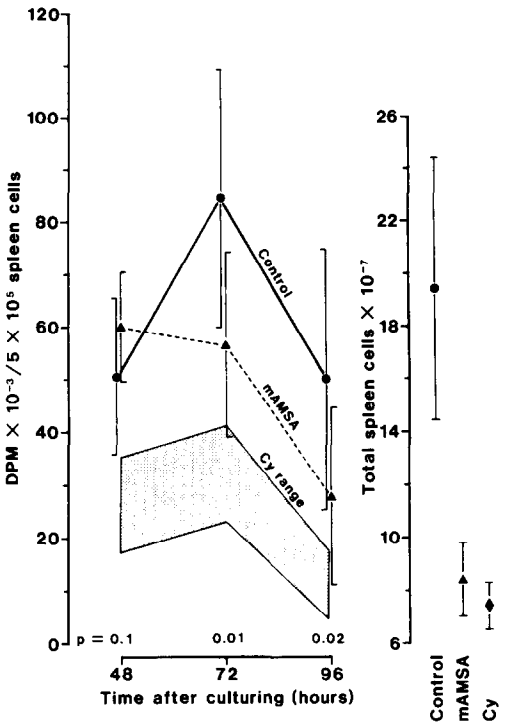


Fig. 2. T-lymphocyte mitogenic responsiveness in vitro *during the leucopenic phase. Two doses of* m-*AMSA and three doses of cyclophosphamide were administered commencing* 7 days *before the spleens were removed for culture.* [^{3}H]-*thymidine incorporated by splenic lymphocytes was measured* 48, 72 *and* 96 hr *after culture initiation and the levels of* ^{3}H *expressed as disintegrations per minute (dpm). (Range =* S.D. n = 10).

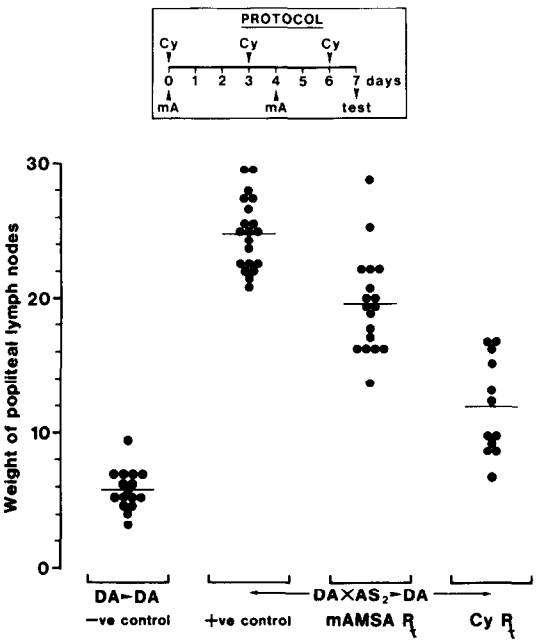


Fig. 3. Host-vs-graft reaction during the leucopenic phase in m-*AMSA-treated rats. Splenic lymphocytes from the F1 progeny of a cross between disparate rat strains (DA × AS$_2$) and from the syngeneic strain (DA) were injected into the hind footpad of other DA animals. Four days later the lymphoid response was measured as the weight of the popliteal lymph nodes. The significant difference between all groups was* P = 0.0001.

In contrast, cyclophosphamide induced a greater reduction of T-cell function and host responsiveness declined to 44% of normal (Fig. 3).

(*c*) *Delayed-type hypersensitivity (DTH) reaction.* Analysis of T-lymphocyte function by the DTH response involves both antigen sensitization and elicitation phases. Animals treated with *m*-AMSA before the sensitization phase showed a small reduction in responsiveness ($P = 0.08$). However, when *m*-AMSA administration commenced after sensitization of the host to KLH, a marked enhancement ($P = 0.02$) of host responsiveness was found (Fig. 4).

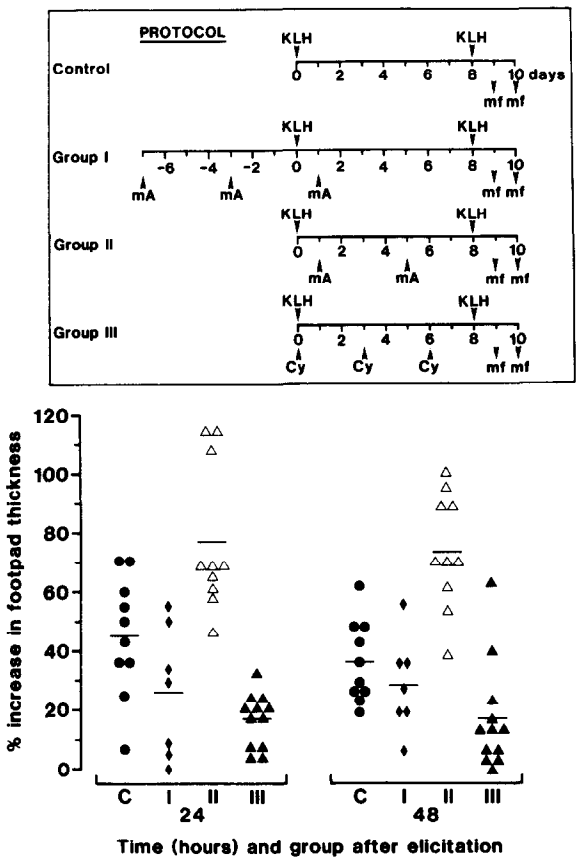
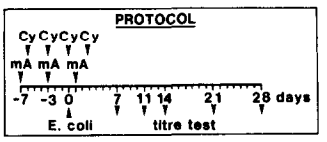


Fig. 4. Effect of m-*AMSA on keyhole limpet haemocyanin (KLH)-induced delayed-type hypersensitivity during the leucopenic phase. KLH was injected into the footpads of control animals (C), those treated with* m-*AMSA (mA) before sensitization (I), those treated with* m-*AMSA before elicitation (II) and those treated with cyclophosphamide (Cy) before elicitation (III). The response was determined by measuring the footpads (mf). Significant differences existed between the control group and all other groups* ($P < 0.08$) *except group I at* 48 hr ($P = 0.04$).

(*d*) *B-lymphocyte response to* E. coli *and SRBC.* *m*-AMSA administration ablated the host response to a challenge with heat-killed *E. coli* 075. A slight increase in antibody above baseline levels was observed after 7 days but this represented only 3% of the response of the control group (Fig. 5). In contrast, the antibody response of animals sensitized and challenged with SRBC was enhanced following *m*-AMSA treatment; although the peak response in treated animals was delayed by 4 days, antibody levels were consistently higher than control animals over the following 17 days (Fig. 6). Cyclophosphamide



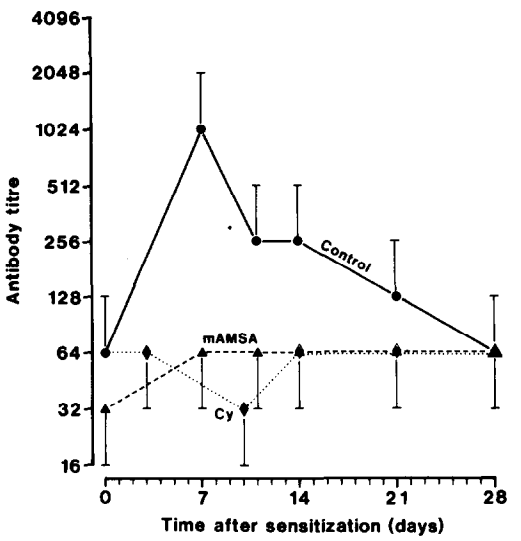
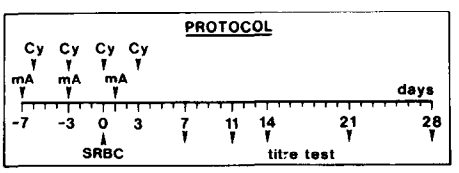


Fig. 5. Antibody response to killed E. coli *during the leucopenic phase following three doses of* m-*AMSA (mA) or four doses of cyclophosphamide (Cy). (Range = S.D.,* n = 10).



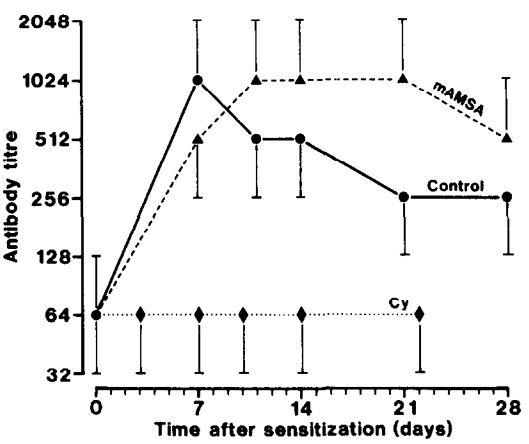


Fig. 6. Antibody response to sheep red blood cells (SRBC) during the leucopenic phase following three doses of m-*AMSA (mA) or four doses of cyclophosphamide (Cy). (Range = S.D.,* n = 10).

treated animals failed to respond to *E. coli* or SRBC.

(*e*) *Neutrophil mobilization.* *m*-AMSA administration effectively blocked the ability of neutrophils to respond to an inflammatory stimulus. In control animals an average of 11.1×10^6 neutrophils were found in subcutaneously implanted sponges removed after 24 hr, whereas in the *m*-AMSA-treated group only 1.6×10^6 cells could be found (10% of normal). Similar effects were found in the cyclophosphamide treated group (Fig. 7).

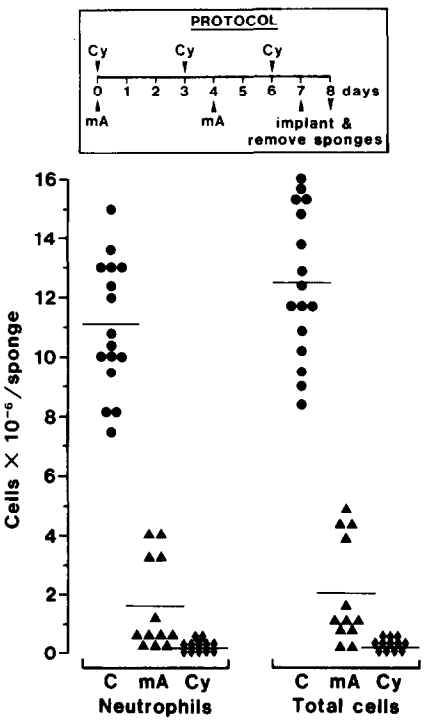


Fig. 7. Effect of m-*AMSA on neutrophil mobilization into sponges implanted subcutaneously for* 24 hr *during the leucopenic phase. The significant difference between controls and treated groups for both total cells and neutrophils was* P = 0.0001. *Between treated groups the significance was* P = 0.004.

(*f*) *Reticulo-endothelial system function.* Clearance of micro-aggregated human albumin by the RES and catabolism of the agent was enhanced in animals treated with *m*-AMSA ($P = 0.05$ in both cases). Cyclophosphamide did not have a significant effect on the RES function (Fig. 8).

Host immune status in the restorative phase (18–36 days)

(*a*) *T-lymphocyte function.* The mitogenic responsiveness of splenic lymphocytes was examined 25 days after the initial dose of *m*-AMSA was administered. Although splenic lymphocyte numbers in *m*-AMSA-treated animals

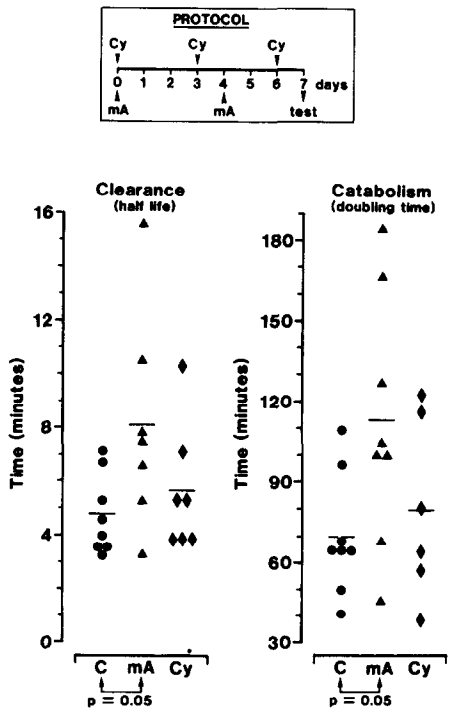


Fig. 8. Phagocytic function of the reticulo-endothelial system during the leucopenic phase. Clearance and catabolism rates for micro-aggregated human serum albumin were measured in control (C), m-*AMSA-treated (mA) and cyclophosphamide-treated (Cy) groups. There was no significant difference between the control and Cy-treated groups.*

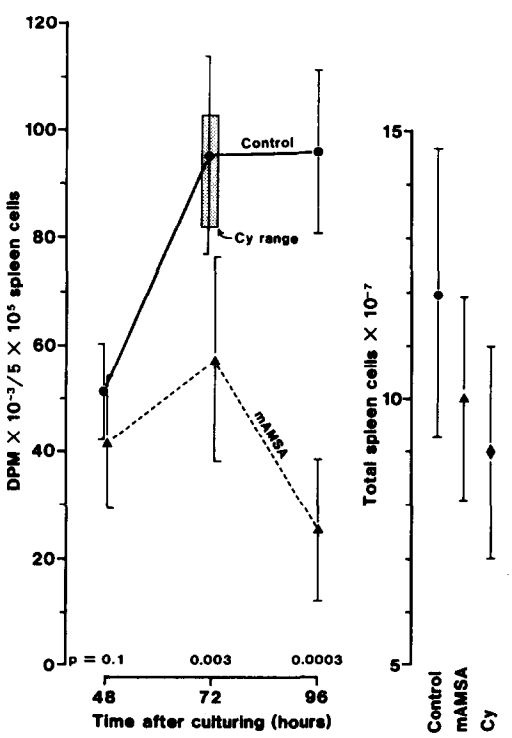


Fig. 9. T-lymphocyte mitogenic responsiveness in vitro *during the restorative phase. Three doses of* m-*AMSA (mA) and four doses of cyclophosphamide (Cy) were administered to the respective groups, commencing* 25 days *before removal of the spleens for culture. (Shaded area = range of Cy effect, ranges = S.D.,* n = 10).

had returned to 83% of normal, the peak response of their lymphocytes to Con A was still markedly depressed ($P = 0.003$). The splenic lymphocyte numbers in cyclophosphamide-treated animals had returned to 75% of normal and the response to Con A was unimpaired (Fig. 9).

(*b*) *Host-vs-graft reaction.* The response of *m*-AMSA-treated animals to a challenge of allogeneic lymphocytes 28 days after the first drug dose was not significantly different from that of the positive control animals (Fig. 10).

(*c*) *DTH reaction.* KLH-induced hypersensitivity was still significantly reduced ($P = 0.004$) when compared with the control group 25 days after the first *m*-AMSA dose (Fig. 11).

(*d*) *B-lymphocyte function.* Antibody responses to *E. coli* and SRBC by *m*-AMSA-treated animals were not different from controls in the recovery phase. Peak titres and the profile of the response were similar in both groups (Figs 12 and 13).

(*e*) *Neutrophil mobilization.* This had returned to normal in *m*-AMSA-treated animals, although mobilization in Cy-treated animals was still significantly reduced ($P = 0.02$) (Fig. 14).

DISCUSSION

Anti-neoplastic chemotherapy has emerged as a preferred treatment in the management of malignancy. Despite the widespread use of cancer chemotherapeutic agents, there have been few attempts to assess systematically the effects of individual agents on the immune system. This is surprising in view of the probable importance of the immune system in the recovery from malignant disease. The acridine derivative amsacrine (*m*-AMSA) is now a front-line agent in the treatment of leukaemia [18, 19] but few investigations into its effect on cellular components of the immune system have been reported [20]. We used a clinically relevant, short-term, high-dose protocol that reduced circulating leucocyte numbers and was expected to depress immune competence. Our objective was to differentiate the cytoreductive effects of *m*-AMSA from any qualitative effects that it might have on cellular immune components. Qualitative and quantitative assessments of immune competence were carried out at the point of maximum depression of circulating leucocytes and during the recovery phase when peripheral blood leucocyte numbers had returned to normal. Cyclophosphamide (Cy) was used as a reference drug at a dose that had a similar effect to *m*-AMSA on circulating leucocytes and splenic lymphocyte numbers.

The experiments have demonstrated a number of unexpected effects of *m*-AMSA on host immune

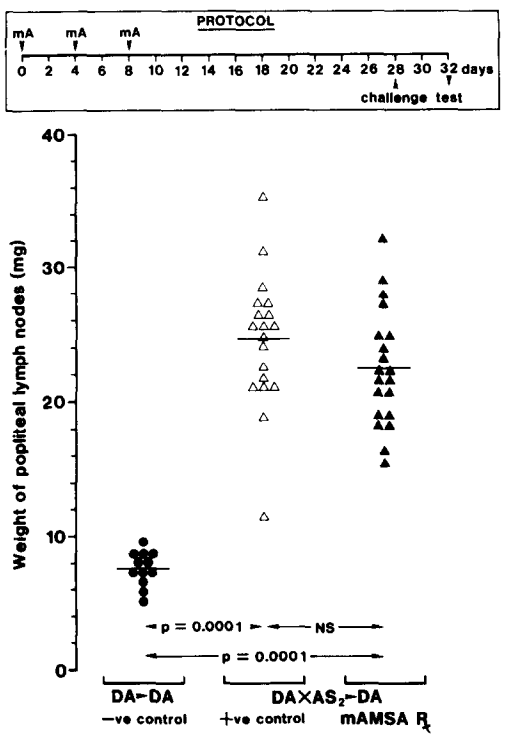


Fig. 10. *Effect of* m-*AMSA (mA) on the host-vs-graft reaction during the restorative phase. Splenic lymphocytes from the F1 progeny of a cross between disparate rat strains (DA × AS₂) and from the syngeneic strain (DA) were injected into the hind footpads of other DA animals. Lymphoid response was assessed by measuring the weight of popliteal lymph nodes.*

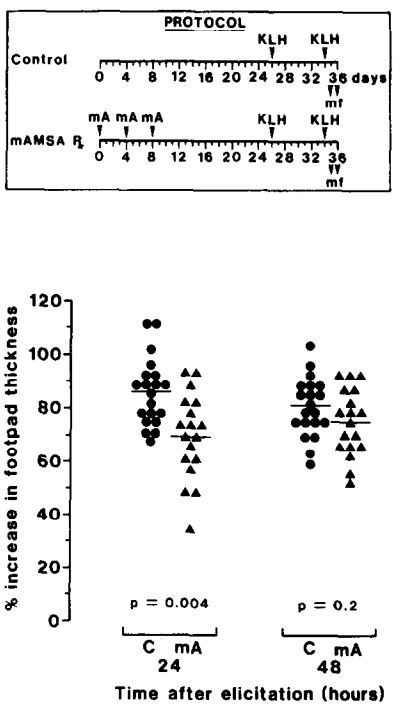


Fig. 11. *Keyhole limpet haemocyanin (KLH)-induced delayed-type hypersensitivity reaction in* m-*AMSA-treated animals during the restorative phase. The response was determined by measuring the footpads (mf)* 24 *and* 48 hr *after elicitation in control (C) and* m-*AMSA (mA)-treated animals.*

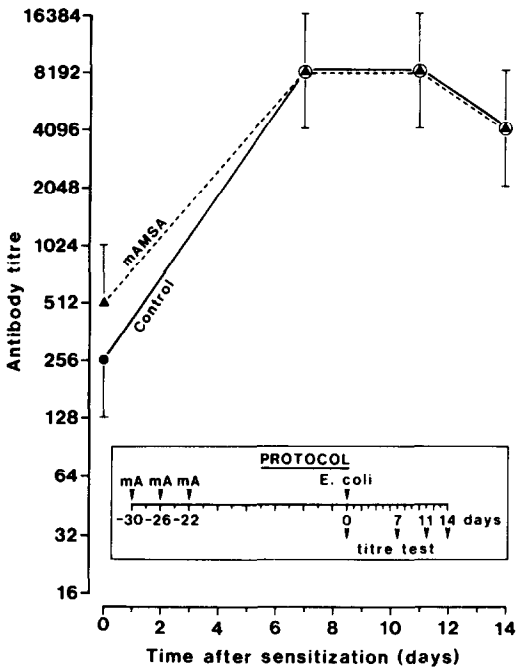


Fig. 12. *Antibody response to killed* E. coli *in* m-*AMSA-treated rats during the restorative phase. (Range* = S.D., n = 10).

capability that could not have been anticipated. In the leucopenic phase both the Con A response and the host-vs-graft reaction were depressed but to a comparatively modest degree compared with the responses in Cy-treated animals with similarly depressed leucocyte numbers. Delayed-type hypersensitivity (DTH) responses were depressed when *m*-AMSA was given before the sensitization step but there was a marked enchancement of the DTH reaction when the administration of *m*-AMSA was delayed until after sensitization. The mitogenic responsiveness of lymphocytes, and the number of splenic lymphocytes, from *m*-AMSA-treated animals was still markedly reduced even in the recovery phase, although other T-cell functions had returned to normal. *m*-AMSA administration also had a suppressive effect on the host antibody response to *E. coli*. In contrast, the response to SRBC was actually enhanced but animals challenged during the recovery phase responded normally to SRBC and *E. coli*. After *m*-AMSA administration phagocytic cell function was also variable: *in vivo* analysis of neutrophil function in *m*-AMSA-treated animals showed that these cells failed to respond to an inflammatory stimulus, whereas the clearance of micro-aggregated human albumin by the RES and catabolism of the agents was enhanced. At the doses used the intermittent administration of both *m*-AMSA and Cy led to a comparable reduction in circulating leucocyte numbers over a 7-day period. There was, however, an interesting difference in the ability of the two drugs to maintain the level of cytoreduction. Administration of Cy continued to depress counts but leucocyte counts returned to normal in *m*-AMSA-treated animals despite continued dosage. If the anti-neoplastic activity is maintained during the

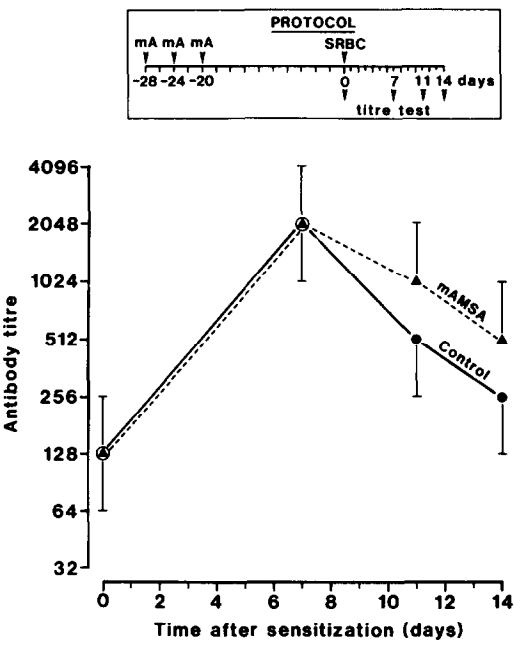


Fig. 13. *Antibody response to sheep red blood cells (SRBC) in* m-*AMSA-treated animals during the restorative phase. (Range* = S.D., n = 10).

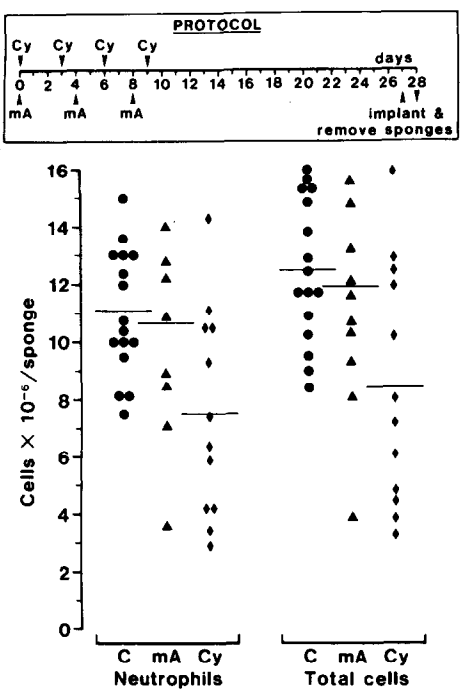


Fig. 14. *Effect of* m-*AMSA (mA) and cyclophosphamide (Cy) on neutrophil mobilization into sponges implanted subcutaneously for* 24 hr *during the restorative phase. There were significant differences between control and Cy-treated groups* (P = 0.02) *but none between the control and mA-treated groups* (P = 0.5).

restorative phase; the observation could be helpful in designing protocols for the use of *m*-AMSA.

One hope at the beginning of this investigation was that *m*-AMSA might display novel immunomodulatory features that have been a characteristic of some chemotherapeutic substances. We used the approach suggested by Addison and Babbage [21] to determine the effect of therapeutic agents on lymphocyte subpopulations. They compared the effects of several agents on the antibody response to thymus-dependent and independent antigens *in vivo* and were able to distinguish three different patterns of immune suppression. These differences could be interpreted in terms of the effects of the agents on T- and B-lymphocytes. In the present experiments we have shown that, while the response of the *m*-AMSA-treated host to a thymus-independent antigen (*E. coli*) was ablated, the response to a thymus-dependent antigen (SRBC) was actually enhanced to supranormal levels. In attempting to interpret these results, we have drawn on the studies of Addison and Babbage [21], who used the responses to X-rays, a non-selective immunosuppressive method which affected both T- and B-lymphocytes equally. The effect of *m*-AMSA on the immune response to T-lymphocyte-dependent and -independent antigens was the exact reverse of their findings for X-rays but closely resembled their findings with the anti-tumour agent mitoclomine. In both cases the thymus-independent responses were more depressed that the thymus-dependent responses. One explanation could be that the drug is selective for B-lymphocytes and that we used a dose which depressed B-cell activity but left the T-cell population relatively unaffected. In this situation the antibody response could be maintained, or even enhanced, by increased T-cell amplification of the remaining B-cell activity. Alternatively, B-lymphocytes involved in thymus-dependent and -independent responses may vary in their sensitivity to mitoclomine and *m*-AMSA. Irrespective of the explanation, the results are of considerable interest in that they have shown that *m*-AMSA could be used in an immunomodulatory capacity to regulate the immune response. The potential of *m*-AMSA for use in manipulating the immune response for therapeutic and experimental purposes is currently under consideration.

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