Europ. J. Cancer Vol. 16, pp. 53–58
© Pergamon Press Ltd. 1980. Printed in Great Britain

0014-2964/80/0101-0053 $02.00/0

# Effect of Cytotoxic Agents on Suppressor Cells in Mice*

A. ANACLERIO, G. CONTI, G. GOGGI, M. C. HONORATI, A. RUGGERI, M. L. MORAS and F. SPREAFICO

*Istituto di Ricerche Farmacologiche 'Mario Negri' Via Eritrea, 62–20157 Milan, Italy*

**Abstract**—*The cytotoxic drugs cyclophosphamide (Cy), azathioprine (AZA), adriamycin (AM) and daunomycin (DM) significantly reduce suppressive activity induced by overloading mice with high doses of sheep red cells (SRBC). Transfer of splenocytes from drug treated animals to syngeneic hosts, immunized with $10^8$ SRBC, results in larger numbers of antibody-producing cells than after transfer of spleen cells from suppressor controls. Single doses of Cy, AZA or AM are followed by a dose-related reduction of transferable suppressive activity, but multiple doses of DM are needed to obtain comparable effects. Reduction in suppressive activity also depends on the timing of drug administration, AM being effective at all times of donor treatment tested, whereas Cy, AZA and DM are only effective when given simultaneously with or after antigen overloading.*

## INTRODUCTION

SUPPRESSOR cells are now recognized as playing a critical role in control of immune responses [1]. There is also evidence that inhibition or activation of these cells might be a factor in a number of important pathological conditions such as neoplasms [2] and autoimmune diseases [3–5]. More precisely suppressor cells, identified as T-lymphocytes [6–8] or cells of the monocyte–macrophage series [9–11], have been found in tumor bearing animals and in patients [12] and preliminary indications are that inactivation or stimulation of suppressor T-cells can retard or augment tumor growth in mice [13, 14].

So far little information exists on the sensitivity of suppressor cells to immunomodulatory agents, although it could be useful in designing more rational approaches to pharmacological control of this cell population in the above pathology. Experiments were therefore made in mice to evaluate the effects of a series of widely used cytotoxic agents on *in vivo* generation of suppressive activity elicited by the injection of high doses of SRBC. It has been demonstrated that in this system suppressive activity is mediated by T-cells [15, 16].

Accepted 5 June 1979.

*This work was supported by Contracts NIH NCI-CM 53826 and Euratom 221-761 BIOI.

## MATERIALS AND METHODS

### *Animals*

(BALB/c × DBA/2)$F_1$ mice (hereafter referred to as $CDF_1$) 8–10 weeks old, were obtained from Charles River Italia, Calco, Italy, and used throughout.

### *Drugs*

AM and DM, obtained from Farmitalia, Milan, Italy, and Cy, a gift of Dr. H. J. Wood (NCI, NIH, Bethesda, Maryland), were dissolved immediately before use in sterile saline and injected i.v. (AM and DM) or i.p. (Cy). AZA (Wellcome Research Lab., Beckenham, U.K.) was resuspended in 0.3% hydroxypropylcellulose and injected i.p. All drugs were given in a volume of 0.01 ml/g body weight and controls received equal amounts of vehicle.

### *Test of suppressive activity*

Suppressive activity was induced *in vivo* following the method described by Weitzman *et al.* [15]. Briefly, $10^{10}$ washed SRBC, resuspended in 0.5 ml of sterile saline, were injected i.v. in $CDF_1$ mice on day 0. Five days later the animals were killed, spleen cells were obtained aseptically and $3.5 \times 10^7$ splenocytes in 0.5 ml of sterile BME (Gibco Bio-Cult, Glasgow, Scotland) were transferred i.v.

to syngeneic hosts, previous experiments having shown this cell number to be optimal in transferring maximal suppression. Three hours later animals were injected i.p. with $10^8$ SRBC and the primary humoral response was evaluated 4 days later, counting the number of plaque-forming cells per spleen (PFC/spleen) by the method of Jerne and Nordin [17]. In some experiments, as controls, $CDF_1$ mice were injected either with $10^8$ SRBC i.p., or with the same number of spleen cells from normal syngeneic donors and 3 hr later with $10^8$ SRBC. Because no significant differences were observed between these two groups only data regarding the first group of controls will be reported.

*Statistical analysis*

Data are presented as the geometrical mean ±S.E. of six animals per group and were analyzed by Duncan's new multiple range test.

## RESULTS

In a first series of experiments the effect of single doses of Cy, AZA, AM or DM on the magnitude of transferable suppressive activity was evaluated by injecting donor mice with $10^{10}$ SRBC and, 2 days later, with different drug doses. As shown in Fig. 1A, single doses of Cy or AZA induced dose-dependent reductions of transferable suppressive capacity as assessed by numbers of PFC/spleen, highly effective doses being 50 mg/kg Cy and 200 mg/kg AZA. Lower drug doses were followed by significant inhibition (Cy 25 mg/kg) or a reduction of borderline significance (AZA, 100 mg/kg) of suppressive activity.

Similarly, dose-related effects (Fig. 1B) were obtained by transfer of spleen cells from animals treated with 5 or 10 mg/kg of AM, 10 mg/kg being the optimal dose. Conversely, the transfer of splenocytes from mice given single doses of DM was followed by no significant changes in suppressive activity (2.5 and

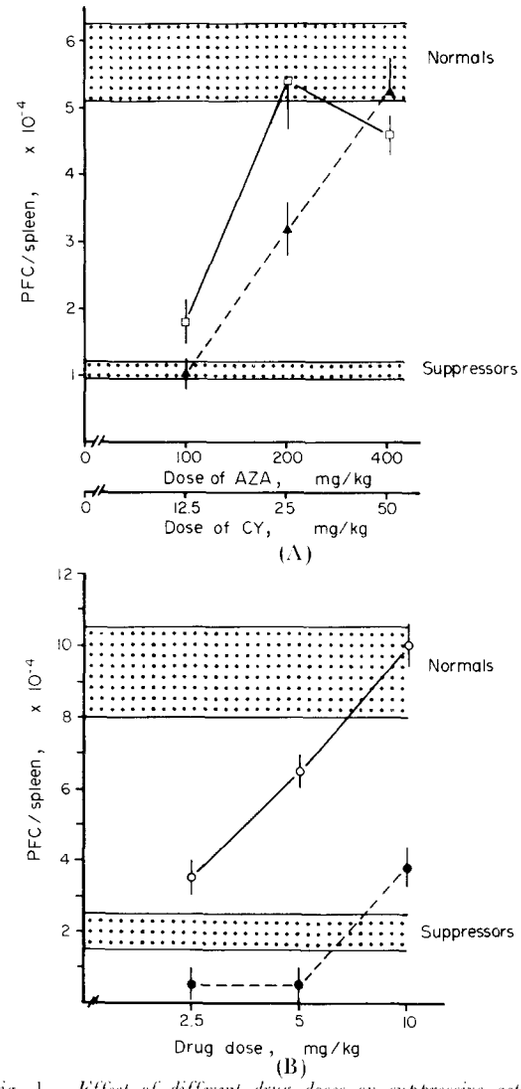


*Fig. 1. Effect of different drug doses on suppressive activity elicited by overloading $CDF_1$ mice with $10^{10}$ SRBC on* day 0. *Drugs were given on* day 2 *and* $3.5 \times 10^7$ *splenocytes were transferred to syngeneic hosts on* day 5. *Hosts were sensitized with* $10^8$ *SRBC on same day and primary humoral response was evaluated* 4 days *later.* (A) *Effect of Cy* (▲——▲) *and AZA* (□ ····· □). (B) *Effect of AM* (○ ····· ○) *and DM* (●– – – ●).

*Table 1. Effect of multiple doses of DM on suppressive activity**

| Exp. group | Days of treatment | PFC/spleen |
|---|---|---|
| Controls | — | 76,883 ± 6380 |
| Suppressors | — | 21,699 ± 1324† |
| DM-treated suppressors | +2 | 35,416 ± 3084‡ |
| DM-treated suppressors | 0, +1, +2 | 57,566 ± 6064§ |

*Suppressor cells elicited by injection of $10^{10}$ SRBC in $CDF_1$ mice on day 0. DM, given on day 2 (10 mg/kg) or on days 0–2 (5 mg/kg). Suppressor splenocytes transferred on day 5. Other details as reported in Fig. 1.

†$P < 0.01$ vs controls.

‡$P < 0.05$ vs suppressors.

§$P < 0.01$ vs suppressors.

5 mg/kg) or by a modest but significant reduction (10 mg/kg). However, as shown in Table 1, repeated doses of DM (5 mg/kg on days 0–2) clearly decreased transferable suppressive activity.

In subsequent experiments donor mice received single drug doses before, simultaneously with or after treatment with $10^{10}$ SRBC. As shown in Fig. 2A the greatest inhibition of suppressive activity was induced by administration of Cy or AZA, 50 and 200 mg/kg respectively, 2 days after overloading with SRBC; drug injections on the same day as SRBC administration or 2 days before had only a slight effect (Cy) or no effect at all (AZA). Similar treatment with AM or DM (Fig. 2B) resulted in significant inhibition of suppressive activity at all times of drug injection (AM) or in a slight or no effect at all

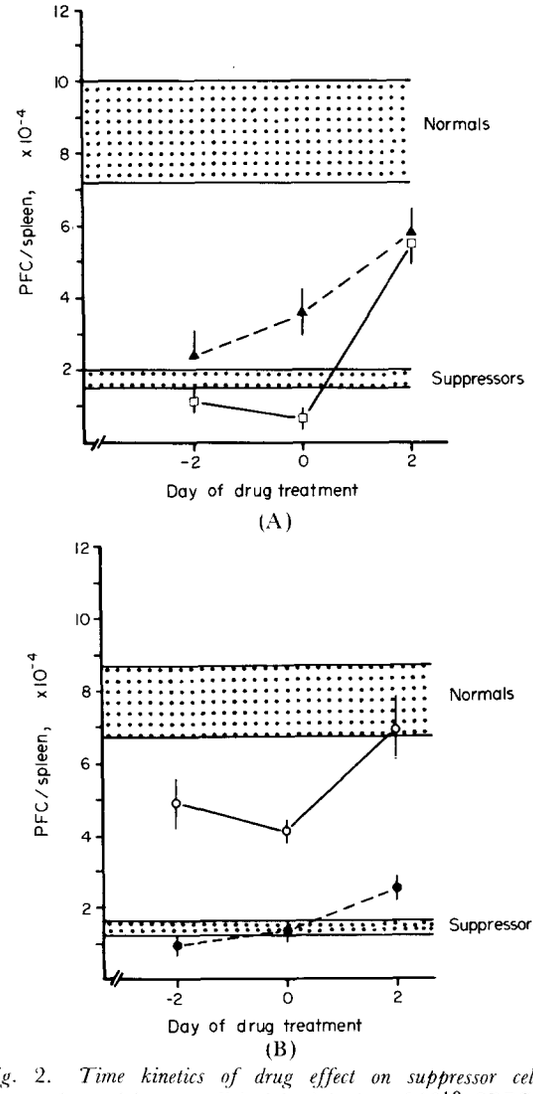


*Fig. 2. Time kinetics of drug effect on suppressor cells. Suppressive activity was elicited by injection of $10^{10}$ SRBC in $CDF_1$ mice on* day 0. *Drugs were given on* day −2, 0 *or* 2. *Suppressive activity was evaluated as described in Fig.* 1. (A) *Effect of Cy and AZA.* (B) *Effect of AM and DM. Symbols as in Fig.* 1.

(DM). In order to evaluate whether the reduction of suppressive activity observed after drug treatment depended on the number of cells transferred, $3.5 \times 10^7$ splenocytes from Cy or AM treated donors (50 and 10 mg/kg respectively, 2 days after $10^{10}$ SRBC) were transferred to syngeneic hosts. As shown in Fig. 3, injection of $3.5–7 \times 10^7$ spleen cells

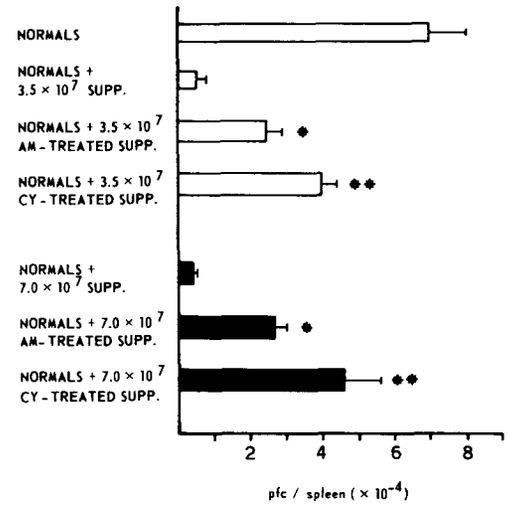


*Fig. 3. Effect of transfer of* $3.5–7 \times 10^7$ *suppressor cells from untreated or Cy or AM treated donors on primary humoral response to SRBC. Experimental details as reported in previous Figures.*

from suppressor controls resulted in high and comparable suppressive activity; this effect was significantly reduced by treatments with Cy or AM even when a double dose of suppressive splenocytes was transferred to syngeneic hosts.

## DISCUSSION

The suppressive activity obtained by the administration of supraoptimal doses of SRBC in mice has been shown to depend mainly on the activation of suppressor T-cells [15, 16]. The data reported here, showing that administration of the cytotoxic drugs, Cy, AZA, AM and DM may be followed by reduced suppressive activity, leads to the conclusion that these agents may impair T-dependent suppressive activity. However, since it is still possible that other cells may participate in induction of this suppressive activity, an effect of these drugs on other cell subpopulations cannot be completely dismissed.

An effect of Cy on suppressor T-cells has already been reported in a number of experimental conditions in which inhibition of precursors or mature suppressor T-cells has

been described [18–22]. Our data confirm those recently obtained by Whisler and Stobo [23] in a model similar to that employed here, and show that the effect of Cy is maximal during suppressor cell stimulation, i.e., when Cy is given after SRBC overloading. This result, suggesting that precursors of suppressor T-cells for humoral response are not affected by Cy, is at variance with Cy-sensitivity shown by precursors of suppressor T-cells for delayed hypersensitivity to SRBC [20, 21]. Analogous results have been obtained with AZA, which is effective in these conditions only when given after antigen. Although no data are available on the effect of this drug, or of AM and DM, on suppressor T-cells, these compounds are known to inhibit T-cell dependent responses [24]. Unlike Cy and AZA, treatment with AM results in the inhibition of suppressive activity even when the drug is given before priming donor mice with $10^{10}$ SRBC; this could imply that AM inhibits precursors of suppressor T-cells. However, we cannot exclude that AM, which persists in the spleen at low levels for a relatively long time [25], is still present at a high enough concentration when the supraoptimal dose of SRBC is given, inhibiting the early phases of suppressor cell stimulation.

In view of the available evidence that DM inhibits immune responses significantly more than its analogue AM [26], no clear explanation yet exists why DM is less effective than AM on suppressive activity. The marked reduction of secondary humoral responses and allograft rejection following treatments with DM [26, 27] does not support a general resistance of T-cells to this agent. However T-suppressor cells may reveal less sensitivity to DM than other T-cell subclasses, as already shown with other immunodepressive interventions. Different sensitivity to Cy and radiations between suppressor T-cells for humoral and delayed hypersensitivity or between suppressor and helper T-cells has recently been reported [16, 23], and also between suppressor and cytolytic T-cell precursors [22]. Furthermore, the different capacities of AM and DM to impair macrophage-mediated cytolytic activity against tumor cells [28] indicates that other lymphoid cell subpopulations can show different sensitivity to these analogues and that the difference observed here is not a peculiarity of T-cells.

Although the role of suppressor cells during tumor growth has not yet been elucidated, these results lead to the suggestive hypothesis that the greater inhibition by AM of suppressor T-cells, combined with the reduced impairment of macrophage antitumoral effect already mentioned, could play some role in the greater antineoplastic efficacy of AM than DM.

Finally, results presented here and previous reports showing that T-cell dependent suppressive activity can be augmented by Levamisole or Freund's adjuvant [29, 30] and reduced by treatment with *Corynebacterium parvum* or BCG [31], agents known on the other hand to activate macrophage-like suppressor cells [32, 33] suggest that pharmacological control of these cell subpopulations can be achieved.

## REFERENCES

1. G. Möller (Editor), Suppressor T-lymphocytes. *Transplant. Rev.* 26 (1975).
2. S. Broder, L. Muul and T. A. Waldmann, Suppressor cells in neoplastic disease. *J. nat. Cancer Inst.* **61,** 5 (1978).
3. N. L. Gerber, J. A. Hardin, T. M. Chused and A. D. Steinberg, Loss with age in NZB/W mice of thymic suppressor cells in the graft-vs-host reaction. *J. Immunol.* **113,** 1618 (1974).
4. R. S. Krakauer, T. A. Waldmann and W. Strober, Loss of suppressor T-cells in adult NZB/NZW mice. *J. exp. Med.* **144,** 662 (1976).
5. N. I. Abdou, A. Sagawa, E. Pascual, J. Hebert and S. Sadeghee, Suppressor T-cell abnormality in idiopathic systemic Lupus erythematosus. *Clin. Immunol. Immunopathol.* **6,** 192 (1976).
6. A. J. Treves, C. Carnaud, N. Trainin, M. Feldman and I. R. Cohen, Enhancing T-lymphocytes from tumor-bearing mice suppress host resistance to a syngeneic tumor. *Europ. J. Immunol.* **4,** 722 (1974).
7. S. Fujimoto, M. I. Greene and A. H. Sehon, Regulation of the immune response to tumor antigens. I. Immunosuppressor cells in tumor-bearing hosts. *J. Immunol.* **116,** 791 (1976).

8. K. D. Elgert and W. L. Farrar, Suppressor cell activity in tumor-bearing mice. I. Dualistic inhibition by suppressor T-lymphocytes and macrophages. *J. Immunol.* **120,** 1345 (1978).
9. H. Kirchner, T. M. Chused, R. B. Herberman, H. T. Holden and D. H. Lavrin, Evidence of suppressor cell activity in spleens of mice bearing primary tumors induced by Moloney sarcoma virus. *J. exp. Med.* **139,** 1473 (1974).
10. B. C. Veit and J. D. Feldman, Altered lymphocyte functions in rats bearing syngeneic Moloney sarcoma tumors. I. Mitogen responses, mixed lymphocyte reactions (MLR) and mixed lymphocyte-tumor reactions (MLTR). *J. Immunol.* **117,** 646 (1976).
11. A. Anaclerio, M. L. Moras, G. Conti, G. Mistrello and F. Spreafico, Suppression of mitogen responses and graft-vs-host reaction by splenocytes from mice bearing Lewis lung carcinoma. *J. nat. Cancer Inst.* **61,** 471 (1978).
12. M. Zembala, B. Mytar, T. Popiela and G. L. Asherson, Depressed *in vitro* peripheral blood lymphocyte response to mitogens in cancer patients: the role of suppressor cells. *Int. J. Cancer* **19,** 605 (1977).
13. M. I. Greene, M. E. Dorf, M. Pierres and B. Benacerraf, Reduction of syngeneic tumor growth by an anti-I-J-antiserum. *Proc. nat. Acad. Sci. (Wash.)* **74,** 5118 (1977).
14. M. Glaser, Adjuvant-induced thymus-derived suppressor cells of cell-mediated tumor immunity. *Nature (Lond.)* **275,** 654 (1978).
15. S. Weitzman, F. W. Shen and H. Cantor, Maintenance of hyporesponsiveness to antigen by a distinct subclass of T-lymphocytes. *J. Immunol.* **117,** 2209 (1976).
16. K. Yamaguchi and S. Kishimoto, Distinction between suppressors of the delayed-type hypersensitivity and the humoral response to sheep erythrocytes. *Immunology* **35,** 721 (1978).
17. N. K. Jerne and A. A. Nordin, Plaque formation in agar by single antibody-producing cells. *Science* **140,** 405 (1963).
18. M. Röllinghoff, A. Starzinski-Powitz, K. Pfizenmaier and H. Wagner, Cyclophosphamide-sensitive T-lymphocytes suppress the *in vivo* generation of antigen-specific cytotoxic T-lymphocytes. *J. exp. Med.* **145,** 455 (1977).
19. N. Chiorazzi, D. A. Fox and D. H. Katz, Hapten-specific IgE antibody responses in mice. VII. Conversion of IgE 'non-responder' strains to IgE 'responders' by elimination of suppressor T-cell activity. *J. Immunol.* **118,** 48 (1977).
20. A. Mitsuoka, M. Baba and S. Morikawa, Enhancement of delayed hypersensitivity by depletion of suppressor T-cells with cyclophosphamide in mice. *Nature (Lond.)* **262,** 77 (1976).
21. H. K. Gill and F. Y. Liew, Regulation of delayed-type hypersensitivity. III. Effect of cyclophosphamide on the suppressor cells for delayed-type hypersensitivity to sheep erythrocytes in mice. *Europ. J. Immunol.* **8,** 172 (1978).
22. R. M. Ferguson and R. L. Simmons, Differential cyclophosphamide sensitivity of suppressor and cytotoxic cell precursors. *Transplantation* **25,** 36 (1978).
23. R. L. Whisler and J. D. Stobo, Suppression of humoral and delayed hypersensitivity responses by distinct T-cell subpopulations. *J. Immunol.* **121,** 539 (1978).
24. F. Spreafico and A. Anaclerio, Immunosuppressive agents. In *Immunopharmacology.* (Edited by J. W. Hadden, R. G. Coffey and F. Spreafico) p. 245. Plenum Press, New York (1977).
25. M. A. Pacciarini, B. Barbieri, T. Colombo, M. Broggini, S. Garattini and M. G. Donelli, Distribution and antitumor activity of adriamycin given by a high-dose and a repeated low-dose schedule to mice. *Cancer Treat. Rep.* **62,** 791 (1978).
26. A. Vecchi, A. Mantovani, A. Tagliabue and F. Spreafico, A characterization of the immunosuppressive activity of adriamycin and daunomycin on humoral antibody production and tumor allograft rejection. *Cancer Res.* **36,** 1222 (1976).
27. A. Mantovani, A. Vecchi, A. Tagliabue and F. Spreafico, The effects of adriamycin and daunomycin on antitumoral immune effector mechanisms in an allogeneic system. *Europ. J. Cancer* **12,** 371 (1976).

28. A. MANTOVANI, *In vitro* and *in vivo* cytotoxicity of adriamycin and daunomycin for murine macrophages. *Cancer Res.* **37,** 815 (1977).
29. D. SAMPSON and A. LUI, The effect of levamisole on cell-mediated immunity and suppressor cell function. *Cancer Res.* **36,** 952 (1976).
30. C. L. REINISCH, N. A. GLEINER and S. F. SCHLOSSMAN, Adjuvant regulation of T-cell function. *J. Immunol.* **116,** 710 (1976).
31. A. ANACLERIO, G. CONTI, M. L. MORAS, A. MACONI and F. SPREAFICO, Effect of immunomodulators on thymocyte suppressive activity in mice. *Europ. J. Cancer* **14,** 829 (1978).
32. H. KIRCHNER, M. GLASER and R. B. HERBERMAN, Suppression of cell-mediated tumor immunity by *Corynebacterium parvum*. *Nature* (*Lond.*) **257,** 396 (1975).
33. G. R. KLIMPEL and C. S. HENNEY, BCG-induced suppressor cells. I. Demonstration of a macrophage-like suppressor cell that inhibits cytotoxic T-cell generation *in vitro*. *J. Immunol.* **120,** 563 (1978).