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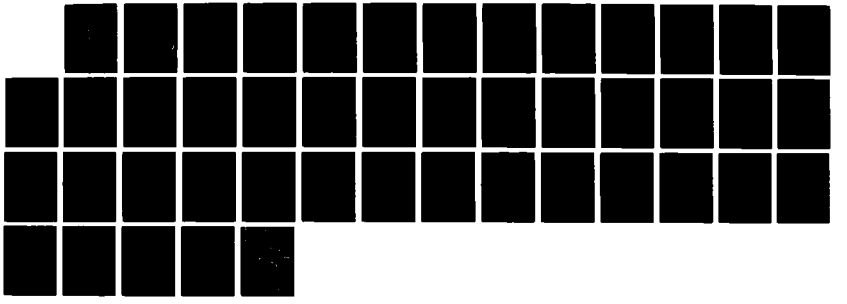

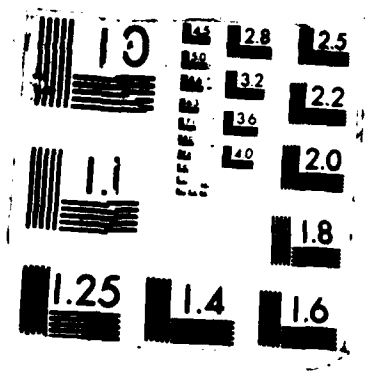
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EFFECTS OF ANTIPARASITE CHEMOTHERAPEUTIC AGENTS ON IMMUNE FUNCTIONS

FINAL REPORT

Abdul Ghaffar, Ph.D

May 1, 1984

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FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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INTRODUCTION

Immunological reactions are generated, sustained, mediated, and regulated by a large array of cells and molecules. Although capable of performing individual functions, these are usually integrated in the body's defense system of networks, interactions, and cascade, thereby leading to antagonistic or protagonistic cellular and molecular interplays. Components of the immune system and their interactions are often modified by exogenous influences such as chemotherapy administered for a variety of reasons. These modifications may cause suppression or augmentation of one or more of the functional moieties of the defense system. Examples of such immunomodulations are numerous. Most pertinent from the point of view of antiparasitic chemotherapy are effects of quinine and its derivatives, niridazole and levamisole which alter different functions of the immune system (1-3).

Some drugs are known to be totally immunosuppressive while others may be discretely or selectively inhibitory of certain specialized immunologic reactions. Drugs which are not immunosuppressive or which affect the immune mechanism in a limited way, i.e., without compromising immunologic competence compatible with resistance to infection, are obviously more desirable than drugs which severely compromise resistance. With this in mind, it was deemed necessary to test newly developed agents with antiparasitic potential for their effects on the immune system. Due to limited resources, only a few very basic tests were conducted. These included humoral immune response assayed by antibody plaque forming cell (PFC) technique, delayed hypersensitivity (a function of T cells), and the reticuloendothelial system (RES) function (test for competence of phagocytic cells).

Immunotoxic agents often exert their effect by depletion of certain populations of lymphocytes. For example, immunosuppression with cyclophosphamide (Cy) is associated with a selective depletion of B lymphocytes (4). By contrast, depletion of macrophages contributes to the immunosuppressive effect of carageenan (5). Preferential depletion of T lymphocytes by some agents also causes immunosuppression (6).

Therefore, enumeration of T and B lymphocytes can provide, especially in man where in vivo assays are not convenient, at least one means of assessing the immunosuppressive potential of a chemotherapeutic agent. Peripheral blood lymphocytes from volunteers receiving an antimalarial agent were, therefore, analyzed for their T and B cell contents.

A RESUME OF WORK ACCOMPLISHED

During the period of the contract, from February 1979 to May 1983, 11 agents were provided by Dr. Brian G. Schuster of the Department of Pharmacology, Division of Experimental Therapeutics, Walter Reed Army Institute of Medical Research (WRAIR). These agents were tested in vivo, in mice, for their effects on PFC response, splenic cellularity, RES function, and induction of DH reaction. The drugs were tested at two different doses, normally prescribed by WRAIR. Generally, the higher dose was 80% of LD10 over 8 days and the lower dose was 20% of the higher dose. A list of agents tested in the above assays and their doses have been incorporated in Table 1. The time of administrations of the drug and schedule of tests are summarized in Table 2.

In addition to studies in mice, 35 samples of peripheral blood lymphocytes from normal donors or donors treated with different doses of WR 171669 were analyzed for T and B cell contents. Furthermore, throughout the investigations, Cy was studied as a reference agent for immunosuppression and studies on the mechanism and target of action of this agent were conducted.

The detailed methodology used in these studies has been described in Appendix A.

Results of these studies were provided to the USAMRDC in the form of annual reports submitted in August 1979, August 1980, August 1981, and August 1982. Summaries from these reports have been attached to this report as Appendix B

Some of the data were presented at the workshop on "Assessment of the Chemical Regulation of Immunity in Veterinary Medicine," organized by the Food and Drug Administration Bureau of Veterinary Medicine and will be published in the proceedings. A copy of the article is submitted as Appendix C. Data from Cy studies have been published in the International Journal of Immunopharmacology. A reprint of this article is attached as Appendix D.

The period between August 1982 and May 1983 was devoted to the preparation of reports on selected individual drugs for submission to the FDA as requested by the WRAIR and preparation of manuscripts for publication.

TABLE 1

LIST OF DRUGS STUDIED AND DOSAGE TESTED

Drugs Studies	Dosage Tested (mg/kg) Low	High
WR 1544	25	100
WR 2975	25	100
WR 2976.5	40	160
WR 2976.HCl	50	200
WR 99210	12.8	64
WR 122455	16	80
WR 142490	10	40
WR 171669*	250	1,000
WR 172435	10	50
WR 180409	10	40
WR 228258	160	800
Cyclophosphamide+	100	N.T.

*Also tested in human subjects

+Studies for mechanism of action

TABLE 2

EXPERIMENTAL DESIGN - PRELIMINARY ASSAYS (BALB/c MALES 8-12 WEEKS OF AGE)

Group	Number	Treatment	Drug Dose	Day of Treatment	Day of Immunization	Type of Assay	Day of Test
1	8	Diluent (Control)	0.2 ml	-1	0	PFC	5
2	8	Drug A	Dose 1	-1	0	PFC	5
3	8	Drug A	Dose 2	-1	0	PFC	5
4	8	Diluent	0.2 ml	+1	0	PFC	5
5	8	Drug A	Dose 1	+1	0	PFC	5
6	8	Drug A	Dose 2	+1	0	PFC	2
7	8	Diluent (Control)	0.2 ml	-1	0	DHS	6
8	8	Drug A	Dose 1	-1	0	DHS	6
9	8	Drug A	Dose 2	-1	0	DHS	6
10	8	Diluent (Control)	0.2 ml	+1	0	DHS	6
11	8	Drug A	Dose 1	+1	0	DHS	6
12	8	Drug A	Dose 2	+1	0	DHS	6
13	8	Diluent (Control)	0.2 ml	-2	None	Carbon Clearance	0
14	8	Drug A	Dose 1	-2	None	"	0
15	8	Drug A	Dose 2	-2	None	"	0

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2. Webster, L.T., Butterworth, A.E., Mahmoud, A.A.F., Mngola, E.N. and Warren, K.S. (1975). Suppression of delayed hypersensitivity in schistosome-infected patients by niridazole. New Eng. J. Med. 292:1144.

3. Sampson, D. (1978). Immunopotentiation and tumor inhibition with levamisole. Cancer Treat. Rep. 62:1623.

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APPENDIX A

METHODOLOGY

Plaque-forming cell (PFC) assay. [12] Five days following i.p. immunization of mice with sheep red blood cells (SRBC), spleens were excised and gently disrupted by means of a glass/Teflon tissue homogenizer in Hank's balanced salt solution containing 0.1% bovine serum albumin (BSA-HBSS). The cells were washed once and resuspended in 4 mL of the same medium. The tubes were allowed to stand for 60 sec to permit clumps and tissue debris to settle. A 1 mL aliquot of singly-dispersed cells was removed from the top of the suspension and transferred to another tube. The number of nucleated cells per mL of this suspension was enumerated and adjusted to a concentration of 2-3 x 10^6 nucleated cells per mL. One volume (0.1 mL) of this suspension was mixed with one volume of 10% SRBC suspension, one volume of medium and one volume of 1:2 dilution of complement. Aliquots (0.1 mL) of the mixture were placed in chambers prepared by assembling two 3" x 1" microscopic slides by means of 5 mm wide double-faced adhesive tape. The chambers were sealed along the edges with molten wax and incubated at 37°C in a humidified incubator for 40 min, after which the number of plaques was enumerated. This technique allowed the assay of the IgM PFC contents of the splenic suspension. For the detection of IgG PFC, the splenic cell suspension was mixed with equal volumes of 10% SRBC, rabbit anti-mouse IgG serum at an optimal dilution (previously determined) and complement. The mixture was placed in chambers and incubated as above. Plaques observed in these chambers were a mixture of IgM and IgG, and the latter were derived by the deduction of the IgM PFC calculated from plaques observed in chambers containing no antiserum against mouse IgG. From the number of plaques per chamber the number of PFC per 10^6 nucleated splenocytes and also PFC per spleen were calculated.

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1. Cunningham and Szenberg. Immunology 14:599, 1968.
2. Dresser and Wortis. In Handbook of Experimental Immunology (D. M. Weir, ed.), p. 1054. Blackwell Scientific Publications, Oxford, 1967.

Delayed hypersensitivity (DH) reaction.3 Mice were injected s.c. with $5x10^6$ SRBC in 0.1 Ml volume on the dorsal side of the neck. Six days later they were challenged with 1 x 10^8 SRBC in 20μL volume i.d. in the pinnea of the ear; 24 hr later μCi of ^{125}IUdR in 0.2 mL volume was injected i.v. via the tail vein. Both ears were excised 18 hr later at the base of the pinnea and the radioactivity incorporated in each ear was measured in a gamma spectrometer. The ratio of radioactivity incorporated in injected and non-injected ears was calculated and taken an index of the DH reaction. This method has been reported as a reliable procedure for assaying the DH reaction to a number of agents and we have confirmed the validity of the technique in our own laboratory.

Phagocytic function of the reticuloendothelial system (RES)2. SRBC were washed three times in saline and to 1 mL of packed SRBC was added 100 μCi of ^{51}Cr (but always less than 5 μg Na_2CrO_4). The cells were mixed and incubated for 30 min at room temperature and then washed three times in saline. Washed cells were suspended at a final concentration of 10% (v/v) in saline and 0.2 mL of this suspension (4 x 10^8 SRBC) was injected i.v. via the tail vein and 20 μL of blood was collected immediately (zero time sample) from the retro-orbital sinus using a heparinized capillary (hematocrit) tube. The same volumes of samples were collected similarly at 2,5, 10 and 15 min intervals from the time of original bleeding. All samples were placed in test tubes and the radioactivity was counted in a gamma the relative concentration of SRBC in circulation at a particular time. The cpm were converted to log cpm and plotted against time, and the slope of the best-fitting straight line for the plot was calculated; it represented the phagocytic index K. Since the concentration of particles in circulation, and hence the K value, is dependent on the total body weight of the mouse, its liver and spleen weights, a corrected phagocytic index α was calculated as follows:

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3. Vadas et al. Int. Arch. Allergy Appl. Immunol. 49:670, 1975.
4. Sljiviv and Warr. In Manual of Macrophage Methodology (H.B. Herscowitz) et al., eds.), p. 447. Marcel Dekker, New York, 1981.

$$\alpha = \frac{w}{l + s} \sqrt[3]{K}$$

where w is whole body weight, l is liver weight and s is spleen weight.

Localization of particulate material in liver and spleen. Spleen and liver were removed intact and weighed. The radioactivity associated with each organ (proportionate with their SRBC content) was measured in a gamma counter and the radioactivity was calculated per gram of the organ weight.

Presentation of data. Numbers of spleen cells have been presented as geometric means. PFC per 10^6 splenocytes and per spleen have been recorded as $\log_{10}$ mean together with one standard error. Anti-log of $\log_{10}$ mean values are recorded in parentheses. DH reaction has been reported as the mean of relative ear reaction together with the standard error of the mean. The relative ear reaction was calculated as follows:

$$\frac{^{125}\text{IUdR incorporation (cpm) in challenged ear}}{^{125}\text{IUdR incorporation (cpm) in control ear}}$$

Data on phagocytic function have been expressed as the mean of K or α values within limits of one standard error. The uptake of SRBC in spleen and liver is expressed as the means of cpm per gram of tissue within one standard error of the mean.

Statistical significance of the data was evaluated by the standard two-tailed Student's t-test with correction for small groups and expressed as p values. P values greater than 0.05 were considered not significant.

Preparation of human lymphocytes (method recommended to Walter Reed Army Institute of Research):

1. Collect 20 mL heparinized blood. Spin at 18 x g (300 rpm) in a bench centrifuge for 15 min. Remove platelet-rich (top 3/4 volume) plasma and discard, leaving about 1/4 of the supernatant in the tube.

2. Spin remainder at 600 x g for 5 min.

3. Discard all but 1 mL of plasma. Harvest this volume of plasma, the buffy coat and 1 mL of red cells. Mix the harvested cells and layer on top of lymphocyte separation medium (LSM; Litton Bionetics). A 2 mL blood sample can be layered on 3 mL LSM.

4. Centrifuge at 400 x g for 20 min. The resultant tube should look like this:

5. Pipet plasma off down to 1/16 inch from the lymphocyte band and discard.

6. Collect all the contents of the lymphocyte band. Dilute with medium (2 mL of LSM-lymphocytes + 3 mL medium). Spin at 600 x g for 5 min. Decant the supernatant. Resuspend cells in RPMI-1640 complete medium (containing 20% fetal calf serum, 100 U/mL penicillin, 100 μg/mL streptomycin and 2mM L-glutamine).

7. Wash the cells 3X in 1 mL complete medium, resuspend, count the cell concentration and adjust at 12-15 x 10^6 cells/mL.

Cryopreservation of human lymphocytes (method recommended to Walter Reed Army Institute of Research):

1. Divide the lymphocyte suspension into two aliquots in labeled screwtop polypropylene 3 mL tubes and chill to 4°C.

2. Cool the freezing chamber to 4°C.

3. Add an equal amount of cryopectant (20 mL DMSO + 80 mL complete medium) to the cell suspension.

4. Place samples in the cryopreservation chamber and start cooling at 1° per min to -40°C, then rapidly cool to -100°C.

5. Samples must be stored in liquid nitrogen and transported on dry ice.

Determination of the viability of cells in human samples. Samples prepared, frozen and shipped by the Walter Reed Army Institute for Research were removed from the liquid nitrogen and thawed in a 37°C water bath on the day of test. As soon as the specimen was thawed, it was diluted 10:1 with RPMI-1640 medium containing 20% fetal calf serum. The dilution was conducted at room temperature by adding the medium drop-by-drop and with continuous gentle mixing (swirling) of the sample. The cells were washed and resuspended in 1 mL medium. A small aliquot was mixed with an equal volume of 0.1% trypan blue and the viable cells were counted on the basis of dye exclusion.

Enumeration of human T lymphocytes. Sheep erythrocytes (SRBC) obtained from Flow Laboratories as heparinized blood were washed and suspended in RPMI-1640 medium at a concentration of 1%. Neuraminidase (Sigma) was diluted to 1 U/mL and 0.4 mL of this solution was added to and mixed with 10 mL of 1% SRBC and incubated at 35°C for 35 min. The neuraminidase-treated SRBC were washed 3 times and resuspended in the medium at a concentration of 0.5%. Lymphocytes were adjusted to a concentration of 5×10^6 cells/mL and mixed with an equal volume (0.1 mL) of the neuraminidase-treated SRBC suspension. The mixture was incubated at 37°C for 5 min, centrifuged at 400 g for 5 min, and the tubes were placed in an ice bath for 45 min. The pellet was gently resuspended and rosettes were counted in a sample of at least 200 cells. Lymphocytes with 3 or more bound SRBC were considered a rosette.

Enumeration of human B lymphocytes. Lymphocytes were suspended at 5 x 10^6 cells/mL in Hepes-buffered RPMI-1640 medium containing 0.02% sodium azide. One-tenth mL of lymphocyte suspension was mixed with 0.1 mL at aggregated normal rabbit IgG and incubated on ice for 10 min. One-tenth mL of FITC-labeled rabbit anti-human immunoglobulin (at a previously determined optimal dilution) was added to the tube and the incubation of 4°C was continued for another 30 min. The cells were washed three times, resuspended in 0.2 mL azide-containing medium, and the fluorescent cells were counted.

APPENDIX B

SUMMARY OF ANNUAL REPORTS 1979-1982

(Report of August, 1979)

SUMMARY

Five agents, namely chloroquine phosphate (WR 1544), primaquine phosphate (WR 2975), quinine sulphate (WR 2976-S), quinine dihydrochloride monohydrate (WR 2976-HCl), and 1-1,3-dichloro-6-trifluoromethyl-9-phenanthryl-3-di-(n-butyl)aminopropanol hydrochloride (DTPA) (WR 171669), were studied for their effect on humoral response to sheep erythrocytes (SRBC), as measured by the plaque-forming cell (PFC) contents of spleens, delayed hypersensitivity (DH) reaction to SRBC, and phagocytic activity in mice (see Table 1).

Humoral PFC responses were not affected by WR 1544 or WR 2975. Quinine sulphate (WR 2976-S) and quinine dihydrochloride monohydrate (WR 2976-HCl) suppressed PFC responses when injected one day before, but enhanced these responses when injected one day after the antigen. The DH reactions were not affected significantly by any of the drugs except WR 1544 at the higher dose (100 mg/kg) injected one day post antigen. Phagocytic activity was depressed by the high dose (100 mg/kg) of WR 1544, WR 2975, both low and high doses (40 and 160 mg.kg) of WR 2976-S and by the high dose of compound WR 171669. Deviations from normal controls in these experiments, wherever they occurred, although statistically significant, were never of a tremendously great magnitude and their biological significance might be questionable. Furthermore, the effects of higher doses of these drugs should be considered with caution because these doses were the highest that the majority of treated mice could tolerate and survive (see Table 2).

(Report of August, 1980)

SUMMARY

Immunotoxic effects of four agents, WR 171669, WR 142490, WR 172435, and WR 180409, were studied.

WR 171699, at maximal sublethal dose, caused a slight increase in the antibody plaque-forming cell (PFC) response, and marginal suppression in the delayed hypersensitivity (DH) reaction and in vivo phagocytic function.

WR 142490 caused slight reduction in the splenic cell content at the higher dose given before or after the antigen. It did not significantly affect antibody PFC responses at any dosage or timing. The DH reaction was suppressed by this agent given before or after the antigen but the statistical significance was demonstrable only in the group receiving a high dose of the drug before the antigen. WR 142490 injected at 10 mg/kg but not 40 mg/kg caused a significant augmentation of the phagocytic function.

WR 172435 caused a significant reduction in IgG response in only one instance: in mice treated with 10 mg/kg of the drug one day after antigen. Since this reduction was of a very modest magnitude and a higher dose of the drug failed to exert a similar effect, the biological significance of this effect is questionable. No statistically significant change in DH reaction was noted in mice treated with this agent; however, it caused significant augmentation of RE function. The augmentation in K value was significant in mice receiving both the high and low doses of the drug but the increase in α value was significant only in mice receiving the higher dose of the drug.

WR 180409, at the higher dose, caused a small but significant reduction in the cellularity of the spleen. Given at the higher dose before antigen it caused significant suppression of the IgG response but when given after the antigen it caused a slight augmentation. DH reactions were not at all altered by this drug. Only the lower dose of this agent caused slight augmentation of RE function, as reflected by the K value but not the α value.

In conclusion, none of these drugs had a dramatic effect on any of the immune parameters measured.

(Report of August, 1981)

SUMMARY

The effect of Cyclophosphamide (Cy) on helper T lymphocytes using an adoptive transfer approach in athymic nude mice was investigated. The results indicated that Cy, at a dose (100 mg/kg) which virtually abolished anti-sheep crythrocyte (SRBC) antibody plaque forming cell (PFC) response of Balb/c mice, did not alter significantly the capacity of their splenic T cells to restore the anti-SRBC PFC response of nude mice. This resistance of T helper cells was observed in unimmunized mice and in mice injected with SRBC two days prior to Cy administration. It has been concluded that both resting and antigen stimulated T helper cells responsible for reconstituting anti-SRBC response of nude mice are resistant to Cy.

(Report of August, 1982)

SUMMARY

Immunotoxic effects of three agents were tested in the animal model for the screening. These agents were: WR 99,210,WR 122,455, and WR 228,258.

WR 99,210 caused a significant enhancement in the phagocytic function (α value), and severe depression in the antibody PFC response when given before antigen, and reduction in the liver and spleen sizes. WR 122,455 and WR 228,258 had similar effects, although with somewhat less severity. Thus, the reduction in the PFC responses and reduction in splenic and hepatic sizes were less pronounced. WR 122,455 was relatively more effective in stimulating phagocytic function whereas WR 228,258 caused no significant alteration in the phagocytic function. None of the agents seemed to alter significantly the delayed hypersensitivity reaction.

In addition, T and B lymphocyte contents in the peripheral blood of human volunteers treated with WR 171,669 (single dose of 750 to 2,000 mg) were enumerated. These studies evaluated 24 individuals before and 2 days after the treatment: half of these volunteers received various doses of the drug and the other half received a placebo. No significant differences were noticed in the proportions of T and B cells in the peripheral blood of these volunteers when samples from placebo and drug-treated groups on Day 0 and Day 2 (pre- and post-treatment) were compared.

APPENDIX C

PUBLICATIONS SUPPORTED

BY

THIS CONTRACT

Chemical Regulation of Immunity in Veterinary Medicine, pages 249-257

IMMUNOMODULATION BY NOVEL ANTIMALARIAL CHEMOTHERAPEUTIC AGENTS

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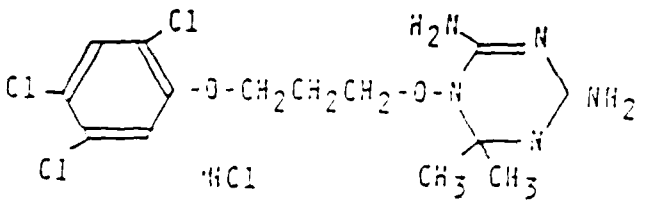
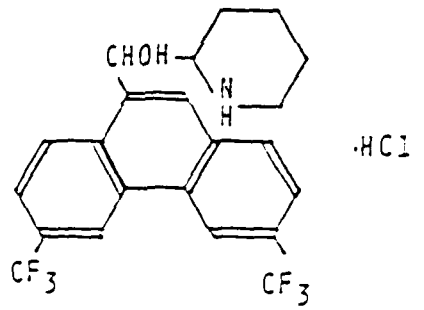
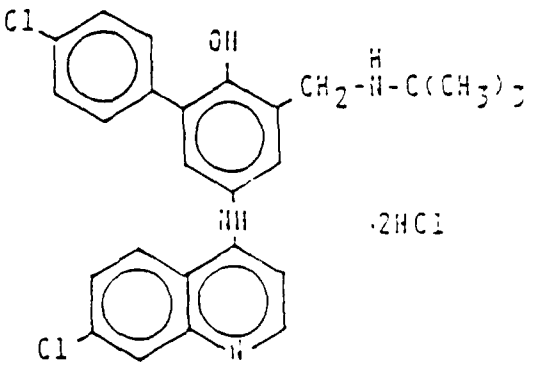
Chemotherapeutic agents, used in the treatment of bacterial, mycotic, viral, protozoan and helminthic infections and malignant diseases often modify the immune function of the host. For example, tetracyclines inhibit chemotaxis and phagocytosis (Martin, Warr, Couch, Yeager, Knight 1974 Forsgren, Schmeling, Quie 1974), and decrease the bactericidal activity (Forsgren, Gnarpe 1973). Another antibiotic, rifampin, causes suppression of various immune functions (Bassi, DiBerardino, Arioli, Silvestri, Cherie 1973 Pogo 1972 Mukerjee, Schuldt, Kasik 1973). Antifungal agent amphotericin B modifies the immune system in quite the opposite way; it stimulates the reticuloendothelial function and enhances specific immune responses (Thomas, Medoff Kobayashi 1973). Antiviral agents which affect cell metabolism and/or cell replication are often immunosuppressive (Stevens, Jordan, Waddell, Merigan 1973). Other antiviral agents which exert their effect by the induction of interferon also modify the host's immune functions (Regelson Munson 1970), probably via the action of interferon which can, under differing conditions, suppress or augment immune functions (Johnson, Baron 1977). Two of the antihelminthic agents, niridazole and levamisole have opposite immunomodulating effects; while the former is strongly immunosuppressive (Mahmoud, Mandel, Warren, Webster 1975 Webster Butterworth, Mahmoud, Mngola, Warren 1975) the latter causes both immunosuppression and immunopotentiation (Renoux Renoux 1973 Rosenthal, Trabert, Meuler 1976 Sampson 1978). Antimalarial agent, chloroquin, also modifies functions of phagocytic cells and other components of the host's immune machinery (Ohkuma, Poole 1981). The immunosuppressive



WR 99,210 : Substituted Diamino Triazine



WR 122,455 : Phenanthrene Methanol



WR 228,258 : Amodiaquine Analogue

Figure 1. Chemical structures of antimalarial agents studied

properties of anticancer agents are well documented (Sigel Gnaffar, Paul, Lichter, Wellham, McCumber, Huggins 1982). It is, therefore, apparent that agents which are used to reduce the burden of infection can also reduce the resistance of the host to the same or other infections. With this point in view, we tested a number of newly developed antimalarial agents for their effects on three basic components of the immune system: humoral immune response, delayed cutaneous reaction and reticuloendothelial system (RES) function. Although numerous agents were tested, only three of these seemed to have altered noticeably the above listed functions and have been reported here.

These studies were conducted in male Balb/c mice of 8-10 weeks of age. Sheep erythrocytes (SRBC) were used as the antigen. Mice were immunized intraperitoneally (i p) with 0.2 ml of 2.5% (v/v) SRBC suspension (1 x 10^8 erythrocytes) for humoral response, or subcutaneously (s c) with 0.1 ml of 0.25% (5 x 10^6) SRBC for delayed hypersensitivity (DH) test. Humoral response was measured 5 days after immunization by the antibody plaque forming cell (PFC) assay described earlier (Cunningham, Szenberg 1968 Dresser Wortis 1967). Delayed hypersensitivity was measured, following intradermal challenge of immunized mice with 0.02 ml of 25% (1 x 10^8) SRBC in the pinnea of the ear six days after s.c. immunization, by the radioisotopic technique described by Vadas, Miller, Gamble Whitelaw (1975). The RES function was evaluated by the measurement of the clearance of ^{51}Cr- labeled SRBC from the circulation and its localization in the spleen and the liver by the method described by Sijivic, Warr (1981).

Among the drugs tested were a substituted triazine (WR 99,210), a phenanthrene methanol (WR 122,455) and an amino-diaquine analog (WR 228,258). These compounds were developed and provided by the Division of Experimental Therapeutics, Walter Reed Army Medical Center, Washington, DC. The structural formulae of the compounds have been shown in Figure 1. All agents were injected i p as a suspension in 0.4 ml of a solvent containing 0.2% (v/v) methylcellulose and 0.4% (v/v) Tween-80 in saline. The higher doses of the drugs were arrived at on the basis of their LD(10) over a period of seven days. The lower dose represented 20% of the higher dose. Control animals received solvent alone, which was shown earlier to have no significant effect on any of the three parameters of the immune function studied. Treatment

with solvent or drug was applied one day before or one day after immunization for humoral response or DH reaction and two days before test for the RES function.

Results of several experiments on effects of the three drugs on anti-SRBC antibody PFC responses have been summarized in Table 1. It is apparent from data that WR 99,210 injected one day before antigen significantly suppressed both IgM and IgG responses (p 0.001-0.005). The drug was suppressive at both lower and higher doses and the suppression was significant at both PFC per spleen and PFC per 10^6 splenocytes (data not presented) levels. The drug, when injected after antigen, was markedly less suppressive and the suppression was not always consistent. In fact, the suppression was statistically significant only in the IgM response at the PFC per spleen level. The drug caused a dose-dependent reduction in the number of nucleated cells in the spleen. This cytoreductive effect of the drug was more prominant in mice injected with the drug after antigen (observed four days after treatment), than in those injected before antigen (observed six days after treatment), which may reflect a beginning of a recovery of mice from the drug treatment.

TABLE 1
Effect of Antimalarial Drugs on Anti-SRBC Antibody Responses.

		Percent of Control Response[2]			
		Pre Ag		Post Ag	
Treatment[1]	Dose mg/kg	IgM	IgG	IgM	IgG
WR 99,210	12.8	22.1[3]	8.4[3]	62.7[3]	100.4
	64.0	9.1[3]	1.7[3]	54.4[3]	52.7
WR 122,455	16.0	66.5	73.3	41.0[3]	60.6
	80.0	16.3[3]	5.7[3]	57.3	46.7
WR 228,258	160.0	59.3[3]	37.8[3]	111.6	76.8
	800.0	52.4[3]	16.1[3]	100.6	74.8

1. Drugs injected 1 day before (PreAg) or 1 day after (PostAg) immunization with $1x10^8$ SRBC. PFC measured 5 days later.
2. $100 \times \frac{\text{geometric mean of response in drug-treated mice}}{\text{geometric mean of response in control mice}}$
   Data derived from 2-3 experiments with 10-20 mice per group.
3. Significantly different from controls: P values less than 0.05 by the Student's t test.

WR 122,455 also caused suppression of anti-SRBC antibody responses although the magnitude of suppression was not as great as that observed with WR 99,210. When administered before antigen, this drug was not significantly suppressive at the lower dose. At the higher dose, however, it did cause a severe suppression of both IgM and IgG responses. When administered after antigen, it caused only a modest suppression which was statistically significant only for IgM response at both PFC per 10^6 splenocytes and PFC per spleen levels. Oddly, only the lower dose of the drug caused significant suppression after antigen. Other reductions in the PFC response by this agent were statistically not significant. Like WR 99,210, WR 122,455 also caused a reduction in the cellularity of the spleen which was more prominant four days after treatment than six days after.

TABLE 2

Effect of Antimalarial Agents on the In vivo Phagocytic Function of Mice

Drug[1]	Phagocytic Index (α values)[2]		
	Solvent	Low Dose	High Dose
WR 99,210	6.06 ± 0.12	6.69 ± 0.27	6.98 ± 0.30[3]
WR 122,455	6.06 ± 0.12	6.93 ± 0.20[3]	6.59 ± 0.21[3]
WR 228,258	6.06 ± 0.12	6.36 ± 0.18	6.03 ± 0.29

1. Drugs were injected i p 2 days before the test for phagocytic function which was measured by monitoring the clearance rate of ^{51}Cr-SRBC from the circulation. See Table 1 for the details of dosage.
2. α values are clearance rates corrected for the spleen, liver and body weights of the mouse. Each value represents arithmetic mean ± 1 s.e. of 7-10 observations (mice) per group.
3. Values are significantly ($P < 0.02$) different from control values.

The suppression caused by WR 228,258 was also moderate and was statistically significant only in animals receiving the drug before immunization. Both IgM and IgG responses were affected, although the IgG response seemed more vulnerable. Except for IgM PFC per 10^6 splenocytes in the high dose treatment group, the reductions were statistically significant at both PFC per 10^6 splenocytes and PFC per spleen levels. No reduction in the IgM response and no significant reduction in the IgG response was observed in animals receiving the drug after immunization. Once again, there was a significant reduction in the cellular content of spleens in mice treated with this drug similar to that observed in animals treated with the other two agents.

Both WR 99,210 and WR 122,455 caused some stimulation of the RE system as reflected in modest but consistent increase in the rate of clearance of ^{51}Cr-SRBC from circulation, recorded in Table 2. The alpha values represent phagocytic indices corrected for alterations in liver and spleen sizes (Sljivic, Warr 1981). The statistical significance of this augmentation was apparent only in animals receiving the higher dose of WR 99,210 or the lower does of WR 122,455. No significant alteration in the RES function of mice receiving WR 228,258 was observed. Concurrent with the augmentation of the phagocytic index, there was an increased localization of ^{51}CR-SRBC in the spleen and liver of mice injected with either doses of WR 99,210 (Table 3). WR 122,455, at both doses, caused an increased SRBC localization in the liver but not in the spleen. WR 228,258 did not significantly affect the hepatic uptake of ^{51}CR-SRBC; it actually caused a significant reduction in the splenic uptake at the higher dose of the drug (Table 3).

No significant alteration in the DH reaction of mice was produced by any of the three drugs described above (data not presented).

Results described above demonstrate that certain antimalarial agents can modify immune responses. As to what is the mechanism of action of these agents, is not understood. The fact that they produced immunosuppression when injected before antigen but were rarely immunosuppressive when administered after antigen would indicate that they were active at a stage early in the immune response, i.e., at the level of macrophage function, or precursors of T helper or B lymphocytes, which all interact in the presence of antigen to

produce a humoral immune response (Sigel, Ghaffar, McCumber Huggins 1982). Since the RE function of the treated animals

TABLE 3

Effect of Antimalarial Agents on the Splenic and Hepatic Uptake of ^{51}CR-SRBC in Mice.

Treatment[1]		Organ Uptake[2]	
Drug	Dose (mg/kg)	Spleen	Liver
Solvent		60.1 ± 3.8	50.3 ± 1.4
WR 92,210	12.8	89.1 ± 5.2[3]	63.8 ± 1.5[3]
	64.0	71.6 ± 3.3[3]	61.9 ± 2.1[3]
WR 122,455	16.0	51.5 ± 4.1	63.0 ± 3.7[3]
	80.0	66.8 ± 5.1	82.3 ± 2.8[3]
WR 228,258	160.0	64.3 ± 3.3	59.1 ± 2.9[3]
	800.0	46.1 ± 4.0[3]	67.3 ± 3.0[3]

1. See Table 2 for details of treatment.
2. Arithmetic mean of C.P.M. (counts per minute) per mg wet weight ± 1 s.e. in the organ 30 minutes after i v inoculation of ^{51}Cr-SRBC.
3. Values significantly ($P < 0.05$) different from controls (injected with solvent).

was generally unaltered and sometimes enhanced, the phagocytic function of splenic macrophages and Kupffer cells must be spared by the drugs. Whether there was an impairment in antigen processing/presenting ability of macrophages in drug-treated mice remains unknown. The suppressive effects of the drugs might also result from other mechanisms, such as induction of suppressor cells or other inhibitors (e.g., interferon or suppressor factors: Sigel, Ghaffar, McCumber Huggins 1982). The cytoreductive effects of these agents in the spleen are consistent with leukopenia produced by these agents in other animals (personal communication: Dr. B. G. Schuster, Department of Pharmacology, Division of Experimental Therapeutics, Walter Reed Army Medical

Center, Washington, DC). The exact nature of the population depleted is not currently known and is a topic of future investigations. What these studies do warrant is a need for surveillance for immunotoxic effects of newly developed agents with potential therapeutic applications in animals and man.

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SELECTIVE ACTION OF ALKYLATING AGENTS AGAINST CELLS PARTICIPATING IN SUPPRESSION OF ANTIBODY RESPONSES*

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Abstract—The effect of several alkylating agents on the induction and expression of specific suppressor cell activity induced by supraoptimal immunization (SOI) with (4×10^9) SRBC was studied. Splenocytes taken 8–28 days after SOI and transferred to normal syngeneic recipients together with optimal dose of (3×10^8) SRBC caused 70–90% suppression. By contrast splenocytes harvested 2 days after SOI did not exert a significant suppressive effect. Treatment of donor mice with 30 mg/kg BCNU, CCNU, or MeCCNU, 8 mg/kg Melphalan or as much as 200 mg/kg Cy 2 days before SOI uniformly had no effect on the subsequent development of suppressor cells. By contrast, different drugs had diverse effects when injected after SOI: both BCNU and Cy injected 2 days post SOI alleviated suppression, whereas CCNU, MeCCNU and Melphalan injected 2 days post SOI were without effect. Another diversity between BCNU and Cy was noticed when the administration of drugs was delayed further. While Cy alleviated suppression 12 days post SOI, BCNU was ineffective at this time. Dose response and time course studies revealed that the effect of Cy was most severe when injected 2 days post SOI and gradually diminished with the passage of time after SOI. These results have been discussed in the light of the current concepts of multiple cell participation in the induction and expression of suppressor cell function.

There is general agreement that immune suppressor functions involve different types of cells including different subsets of T cells (Cantor & Gershon, 1979). It is therefore necessary to recognize the cells and to define their functions and properties in order to better understand the various interactive effects associated with immunoregulation. Thus far much emphasis has been placed on certain membrane markers (Cantor, Shen & Boyse, 1976). While surface markers are proving useful in distinguishing different subsets of T cells involved in the genesis and function of suppressor pathways, other means of distinguishing and selectively eliminating subsets of cells involved in the induction of suppressor functions remain desirable. One such tool is the use of immunosuppressive agents which under certain conditions are selective in affecting immune functions ascribed to different subsets of lymphocytes (Sigel, Ghaffar, Paul, Lichter, Wellham, McCumber & Huggins, 1982*b*).

A number of alkylating agents which are used as chemotherapeutic drugs in malignancy have also been shown to be immunosuppressive (Makinodan, Santos & Quinn, 1970; Ghaffar, Lichter, Wellham & Sigel, 1978; Berenbaum, 1979). Some of these are stronger immunosuppressants than others (Ghaffar *et al.*, 1978). Among these cyclophosphamide has been most extensively studied. This agent has been shown to induce a state of unresponsiveness when injected shortly after a large dose of antigen (Aisenberg, 1967; Dietrich & Dukor, 1967; Playfair, 1969; Miller & Mitchell, 1970) which has been attributed to induction of suppressor cells (Ramshaw, Bretscher & Parish, 1977). Very few studies have explored the effect of these agents on suppressor cells. Cy, which has attracted most attention from this point of view

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Abbreviations used: BCCNU: 1,3-Bis (2-chloroethyl)-1-nitrosourea, CCNU: 1-(2-chloroethyl-3-cyclohexyl)-1-nitrosourea, Cy: cyclophosphamide, HBSS: Hanks' balanced salt solution, MeCCNU: 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea, Mel: melphalan, PFC: plaque-forming cells, SOI: supraoptimal immunization, SRBC: sheep red blood cell, TH: T helper cell, TS: T suppressor cell, TSe: T suppressor effector cells, TSi: T suppressor inducer cells, TSp: T suppressor precursor cells.

has produced conflicting results. According to some authors, this drug itself induced suppressor cells (Milton, Carpenter & Addison, 1976; Marbrook & Baguley, 1976; Poupon, Kolb & Lespinats, 1977; Neta, Winkelstein, Salvin & Mendelow, 1977; Bonavida, 1977) while according to others it would eliminate suppressor cells (Polak & Turk, 1974; Sy, Miller & Claman, 1977; Polak & Rinck, 1977; Duclos, Galanud & Devinsky, 1977; Schwartz, Askenase & Gershon, 1978). In some studies, the depletion of suppressor cells was achieved prior to immunization (Sy *et al.*, 1977; Polak & Rinck, 1977; Duclos *et al.*, 1977; Schwartz *et al.*, 1978) whereas in others it was realized after immunization (Polak & Turk, 1974).

In this report we have examined the effect of several alkylating agents of known immunosuppressive potential on suppressor cells which are effective in a secondary host. Results clearly reveal a heterogeneity among these immunosuppressive agents relative to their effect on suppressor cells. The diversity in their effect is further accentuated by the time of administration of these drugs in relation to the time of induction and maturation of suppressor cells.

EXPERIMENTAL PROCEDURES

Mice and immunizations

(C57/Bl × DBA2) F_1 (BDF_1) mice aged between 8 and 10 weeks were used in all experiments. Optimal immunization consisted of a single i.v. injection with 3×10^8 SRBC. SOI for the induction of suppressor cells was achieved by i.p. injection of 4×10^9 (0.4 ml) SRBC.

Induction of suppressor cells and adoptive transfer

Suppressor cells were induced by SOI and their expression was tested in adoptively transferred hosts (Whisler & Stobo, 1976). Adoptive transfer was performed by using splenocytes dispersed with a glass homogenizer, washed, and suspended in HBSS. The donors of these cells will be referred to as SOI (supraoptimally immunized) mice. Unless otherwise stated, the transfers were made 14 days after antigen using 40×10^6 cells in 0.4 ml HBSS i.v. via the tail vein. The recipients were immunized at the same time with 3×10^8 SRBC.

Treatment with drugs

Drugs were dissolved in an appropriate solvent and the required dose was injected i.p. in 0.2 ml volume. With the exception of cyclophosphamide, all drugs were used as a single dose which was known to be sublethal and immunosuppressive. These were 30 mg/kg for BCNU, CCNU, and MeCCNU and 8 mg/kg for Mel. Cy was used at 200, 100, 50, 25, 10 and 5 mg/kg. Control mice received equal volumes of solvents for the respective drugs which were 10% DMSO for BCNU, CCNU, and MeCCNU, N/20 NaOH for Mel. and saline for Cy.

PFC assays

Splenocytes were prepared as above and cell concentrations were appropriately adjusted (between 3 and 5×10^6/ml). Antibody PFC were detected by the modified Jerne technique as described by Cunningham & Szenberg (1968). IgG responses were measured by the use of developing anti-IgG serum (Miles Laboratories, Elkhart, Ind.) as described by Dresser & Greaves (1973).

Presentation of data

Geometric means of PFC data on individual spleen with limits of one standard error from the mean have been presented. Statistical analysis of the data was performed using Student's *t*-test for unpaired data and P values of 0.05 or less were considered significant.

RESULTS

Kinetics of induction of suppressor cells

First series of experiments were designed to determine the time course of the appearance and persistence of suppressor cells following SOI. Splenocytes from mice were transferred at various intervals after SOI and tested for the expression of suppressor cells in the secondary host. The results obtained with assays of IgM and IgG PFC are given in Fig. 1. It can be seen that expression of suppressor activity, measured by adoptive transfer, was a relatively slow process. The maximum depression of the immune re-

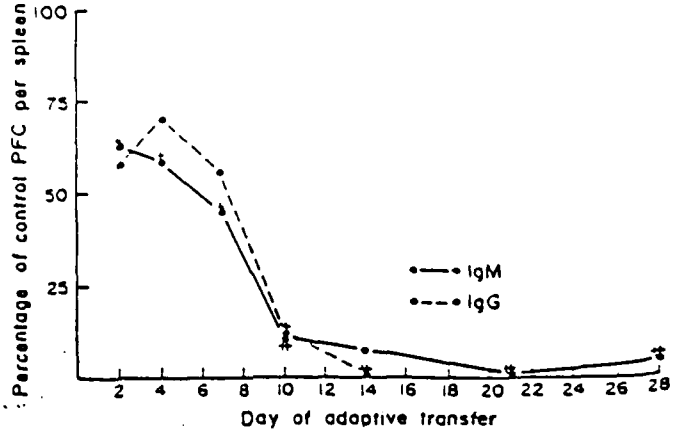

Fig. 1. Generation of suppressor cells by supraoptimal doses of SRBC. Splenocytes from normal or SOI mice were harvested at different intervals and injected i.v. into syngeneic mice. Recipients were simultaneously immunized with 3×10^8 SRBC and their responses were measured 4 days later. + = $P<0.05$; + + = $P<0.001$.

sponse in the recipients occurred with cells taken from donors between 10 and 28 days after SOI. Cells taken at 2–4 days caused relatively little suppression in the recipients. The cells responsible for the transfer of suppression were susceptible to treatment with anti-Thy plus complement (data not presented). It is well documented that by day 4 the IgM PFC is at its peak and it can be concluded, therefore, that TH cells for antibody production mature and become functional considerably earlier than TS cells or the cells which are capable of transferring suppression.

Effect of alkylating agents injected before SOI on nonactivated precursors of suppressor cells

Groups of mice were treated with saline or alkylating agents two days prior to SOI. Fourteen days later splenocytes from these mice as well as mice which received no SOI were transferred to untreated mice and the recipients were immunized on the same day with the optimal dose of SRBC; their responses were measured four days later. Data summarized in Table 1 indicate that recipients of splenocytes from non-immunized but drug-treated mice gave responses comparable to those obtained in recipients of splenocytes from untreated donors. In contrast, recipients injected with splenocytes from SOI mice showed 80 to over 90% suppression of PFC responses. This suppression was not alleviated nor significantly altered by treatment of donors with any of the alkylating agents injected in doses shown in Table 1 two days prior to SOI. Thus it appears that nonactivated precursors of TS cells were not susceptible to these agents.

Effect of alkylating agents injected two days after SOI on the generation of suppressor cells

Donors were injected either with saline or given SOI and two days later were subdivided into groups which were treated with various alkylating agents or with saline. Adoptive transfer experiments were made 14 days after immunization. The recipient animals were immunized at the same time with 3×10^8 SRBC. As shown in Table 2, Cy 100 mg/kg and BCNU 30 mg/kg relieved the immune suppression, restoring the responses of the recipients to almost the level of those which received splenocytes from donors that were not given SOI. Thus Cy and BCNU but not the other alkylating agents could prevent the emergence of suppressor cells when these drugs were administered 2 days after activation by

Table 1. Effect of alkylating agents on nonactivated precursors of suppressor cells

Drugs* tested	PFC response per 10^6 splenocytes†			
	Non SOI control		SOI‡	
	Solvent	Drug	Solvent	Drug
Cy	2.964±0.043 (921)	2.947±0.04 (885)	1.851±0.113 (71)	2.054±0.096 (113)
BCNU	3.114±0.132 (1393)	2.854±0.076 (683)	2.514±0.082 (327)	2.471±0.084 (295)
CCNU	3.006±0.048 (1014)	2.947±0.042 (885)	2.210±0.113 (166)	2.054±0.029 (113)
MeCCNU	2.832±0.055 (679)	2.716±0.067 (521)	2.281±0.075 (191)	2.356±0.061 (227)
Mel.	2.909±0.036 (812)	2.748±0.052 (560)	1.921±0.176 (83)	1.845±0.094 (70)

* 100 mg/kg Cy, 30 mg/kg BCNU, CCNU, or MeCCNU, or 8 mg/kg Mel. injected 2 days before SOI; splenocytes transferred together with 3×10^8 SRBC to normal recipients and their responses measured 4 days later.

† $\log_{10}$ mean±1 s.e.; numbers in parentheses are geometric mean of at least 5 observations.

‡ All SOI groups were significantly different from Non SOI controls ($P<0.001$) and drug and SOI were different from drug alone ($P<0.001$ for all except BCNU which was <0.02).

Table 2. Effect of alkylating agents on emergence of suppressor cells when administered 2 days after SOI

Drugs* tested	PFC per 10^6 nucleated splenocytes†			
	Non SOI control		SOI‡	
	Solvent	Drug	Solvent	Drug
Cy	2.996±0.076 (990)	2.980±0.033 (955)	1.840±0.098 (69)	2.805±0.082 (638)
BCNU	2.742±0.116 (552)	2.772±0.092 (592)	1.832±0.091 (68)	2.630±0.051 (426)
CCNU	3.256±0.029 (1804)	3.035±0.112 (1804)	1.783±0.131 (61)	1.850±0.086 (71)
MeCCNU	2.832±0.055 (679)	2.921±0.035 (834)	2.281±0.075 (191)	2.109±0.142 (129)
Mel.	2.963±0.136 (919)	2.881±0.044 (760)	2.035±0.113 (108)	1.858±0.133 (72)

* 100 mg/kg Cy, 30 mg/kg BCNU, CCNU, or MeCCNU, or 8 mg/kg Mel. injected 2 days after SOI; splenocytes transferred together with 3×10^3 SRBC to normal recipients and their responses measured 4 days later.

† *log_{10} mean*±1 s.e.; numbers in parentheses are geometric mean of at least 5 observations.

‡ groups treated with SOI and Cy or BCNU were statistically higher ($P<0.001$) from those treated with SOI solvent but no different from groups treated with drug alone; groups treated with SOI and CCNU, MeCCNU or Mel. were significantly lower ($P<0.001$) than those treated with drug alone and no different from groups treated with SOI and solvent.

Table 3. Susceptibility of suppressor cells to treatment with cyclophosphamide

Donor treatment*	PFC per† 10^6	*P* values	PFC per† spleen	*P* values
(1) None	2.964±0.043 (921)		5.266±0.114 (184,662)	
(2) SRBC	1.851±0.113 (71)	<0.001 (1)	3.820±0.125 (6,605)	<0.001 (1)
(3) Cy	2.958±0.053 (907)	n.s. (1,4)	4.970±0.093 (93,286)	n.s. (1,4)
(4) Cy + SRBC	2.862±0.030 (727)	<0.001 (2)	4.857±0.062 (71,956)	<0.001 (2)

* 100 mg/kg injected 12 days after 4×10^9 SRBC; splenocytes harvested 14 days after SRBC injection and transferred into normal recipients. Figures in parentheses are group numbers.

† log_{10} mean±1 s.e. Figures in parentheses are geometric mean of 9–10 observations.

‡ Figures in parentheses indicate groups compared; n.s. = not significant.

Table 4. Long-term persistance of suppressor cells and their sensitivity to cyclophosphamide

Donor treatment* Group	SRBC	Cy	PFC per† 10^6	*P* values‡	PFC per† spleen	*P* values
(1)	None	None	2.990±0.154 (977)		4.950±0.067 (89,033)	
(2)	None	Day + 2	2.956±0.062 (903)	n.s. (1)	5.017±0.062 (104,030)	n.s. (1)
(3)	None	Day + 26	3.028±0.049 (1,067)	n.s. (1)	5.036±0.057 (108,713)	n.s. (1)
(4)	4×10^9	None	2.351±0.110 (244)	<0.001 (1)	4.343±0.114 (22,053)	<0.001 (1)
(5)	4×10^9	Day + 2	2.958±0.054 (907)	n.s. (1,2) <0.001 (4)	4.965±0.045 (92,342)	n.s. (1,2) <0.001 (4)
(6)	4×10^9	Day + 26	2.652±0.029 (449)	<0.001 (1,3,5)	4.710±0.037 (51,264)	0.01 (1) <0.001 (3,5)

* 100 mg/kg Cy injected 2 or 26 days after SRBC; splenocytes harvested 28 days after SRBC injection and transferred into normal recipients. Figures in parentheses are group numbers.

† $\log_{10}$ mean±1 S.E. Figures in parentheses are geometric mean of 9–10 observations.

‡ Figures in parentheses indicate groups compared; n.s. = not significant.

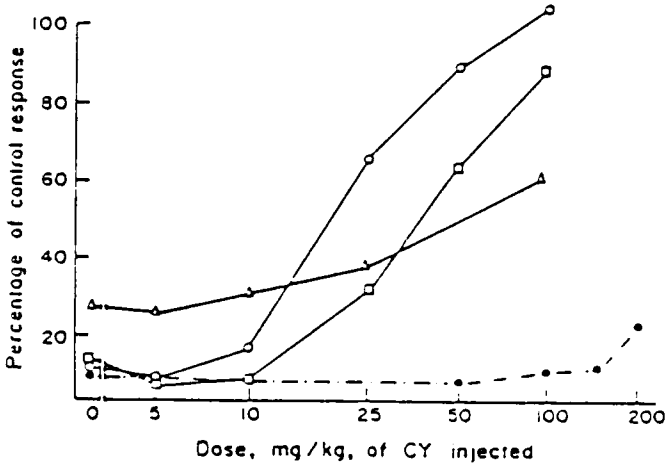

Fig. 2. Sensitivity of suppressor cells and their precursors to different doses of Cy injected at different times in relation to the inducing antigen. Closed circles represent donors injected with Cy 2 days before and open circles, squares, and triangles represent those injected 2, 12 and 26 days, respectively, after SOI. Per cent suppression was calculated as follows:

$$100 \times \frac{\text{PFC/spleen in recipients of SOI splenocytes}}{\text{PFC/spleen in recipients of control splenocytes}}.$$

Whenever SOI donors were injected with Cy, the respective control donors also received the same treatment.

supraoptimal dose of antigen. Since at this time suppresor cell activity in SOI donor spleens was largely undetectable (Fig. 1), the results signify that activated precursors of suppressor cells or activated inducers of TS or immature TS cells are susceptible to Cy and BCNU.

Effects of alkylating agents on mature suppressor cells

Since splenocytes from SOI mice expressed full suppressor activity over a long time span—10–28 days—it was of interest to compare their susceptibility to Cy and BCNU in relation to time after induction. BCNU administered on day 12 or day 26 did not relieve suppression. Thus the only time BCNU was effective in removing the suppressive activity was when given two days after SOI. The other alkylating agents had no effect when given at the later times just as they had no effect 2 days after SOI. Cy on the other hand was found to be effective at various stages following SOI. A comparison of the effects of 100 mg/kg of Cy administered on day 12 or 26 to SOI donors is given in Tables 3 and 4. It can be seen that at 12 days the suppressor cells were fully susceptible to this dose, on par with the activated precursors or immature suppressor cells treated on

day 2 (Table 3 compared to Table 2). By 26 days post SOI, the suppressor cells, while still susceptible, demonstrated a degree of resistance to Cy (Table 4). Thus, there appears to be a gradient of susceptibility of suppressor cells to Cy, with the early mature cells being somewhat more susceptible than the late mature cells. This observation was confirmed by experiments with graded doses of Cy.

Relative sensitivity of suppressor cells and their precursors to Cy

In order to examine the relative sensitivity of suppressor cells and their precursors to Cy, varying doses of the drug were injected into SOI mice 2 days before, or 2, 12 or 26 days after SOI. From the data summarized in Fig. 2, it is obvious that Cy in doses ranging from 5 to 200 mg/kg failed to affect resting precursors of suppressor cells. It is noteworthy that 200 mg/kg was the highest sublethal dose that could be injected into these mice, and a higher dose of 300 mg/kg caused 100% mortality over a period of 14 days. In contrast, when Cy was injected 2 days after SOI, as little as 25 mg/kg alleviated suppressor cell activity. The sensitivity of suppressor cells to Cy appeared to diminish as these cells matured. Thus in order to achieve a comparable diminution of the suppressor cell effect at days 12 and 26, it was necessary to administer doses of 50 and 100 mg/kg of Cy, respectively.

DISCUSSION

Data reported here would confirm that SOI, with SRBC, induced suppressor cells which were sensitive to anti-Thy serum and complement (Whisler & Stobo, 1976). The current knowledge of effector mechanisms in the induction of suppressor cells implicates three sub-populations of T cells; namely suppressor cell inducers (TSi) which act on suppressor cell precursors (TSp) and lead to differentiation of the latter to suppressor cell effectors (TSe) (Cantor & Gershon, 1979; Germain & Benacerraf, 1981; Sigel, Ghaffar, McCumber & Higgins, 1982*a*). In the light of the kinetics of induction of transferable suppressor activity in our experiments we assume that TSe dominated after 14 days of SOI whereas prior to this time there were variable ratios of TSi and TSe (the latter increased with the passage of time after SOI). It is also indicated by our results that 2 days after SOI there were no TSe. Our unpublished data indicate that at this time there was a population of cells which upon adoptive transfer did not suppress response to SRBC injected simultaneously but if allowed to mature in the recipient for 10–12 days they did suppress the anti-SRBC response. We assume that there were TSi which interact, in the host, with TSp to generate TSe. Prior to SOI, all mice would be expected to have non-activated TSi, and also precursors of TSe (i.e. TSp).

We tested the effects of several alkylating agents on the induction and expression of suppressor cells using the cell transfer model which differentiates between precursors, inducers and effectors. The choice of times of treatment was dictated by several considerations. There are reports in the literature which indicate that resting precursors of suppressor cells are susceptible to Cy (Debre, Waltenbaugh, Dorf & Benacerraf, 1976; Zembala & Asherson, 1976; Sy *et al.*, 1977; Schwartz *et al.*, 1978; Cantor, McVay-Boudreau, Hugenberger, Naidorf, Shen & Gerson, 1978). On the other hand, there are also reports that Cy may actually induce suppressor cells (Milton *et al.*, 1976; Marbrook & Baguley, 1976; Poupon *et al.*, 1977; Neta *et al.*, 1977; Bonavida, 1977; L'Age-Stehr & Diamantstein, 1978). Presently, no information is available on the effect of other alkylating agents on suppressor cells.

The data indicate that none of the alkylating agents injected 2 days prior to SOI prevented the induction of TSe capable of exerting suppression in the secondary host. When injected 2 days following SOI, both Cy and BCNU, but not the other drugs, caused a dramatic reduction in the capability of donor splenocytes to induce suppression in the secondary host. The two active agents also manifested a disparity in their effect on suppressor cell induction when administered at later intervals (days 12 and 26 post SOI).

The lack of effect of Cy injected 2 days before SOI on the induction of suppressor cells, at first glance, would seem to contradict findings of others who demonstrated that Cy eliminated suppressor cells (Polak & Turk, 1974; Zembala & Asherson, 1976; Polak & Rinck, 1977; Duclos *et al.*, 1977; Schwartz *et al.*, 1978). However, this contradiction is easily reconciled by the nature of the experimental model. It is more than likely that, in our experiments, TSp cells may also have been eliminated by pretreatment with Cy as was the case in the experiments of others (Duclos *et al.*, 1977; Cantor *et al.*, 1978), but the design of our experiment did not allow for the testing of this possibility as the assays were performed by adoptive transfer to normal hosts endowed with functional TSp cells. By the same token our model does not reveal whether TSp are sensitive to BCNU, CCNU, MeCCNU and Mel. Furthermore, our results concern humoral immune responses whereas some of the contrasting results referred to above concern delayed hypersensitivity reactions (Polak & Turk, 1974; Zembala & Asherson, 1976; Polak & Rinck, 1977).

It is clear that Cy and BCNU but not CCNU,

MeCCNU and Mel. when injected 2 days after SOI severely curtailed the expression of suppressor activity when transferred on day 14. Since at the time of the drug administration there were no effectors of suppression (TSe) cells, it is reasonable to assume that the abolition of suppressor function was due to elimination or inactivation of inducer cells (the host contains a full component of TSp). The lack of effectiveness of CCNU, MeCCNU and Mel. should not be construed as a sign of the innocuous nature of these agents as they severely suppress the antibody responses to SRBC and other antigens (Ghaffar *et al.*, 1978; Berenbaum *et al.*, 1979).

BCNU and Cy showed differential effectiveness in that they both affected suppressor activity when given 2 days post SOI, but only Cy was effective on day 12. This would suggest that TSe are more sensitive to Cy than to BCNU. Furthermore, the data on dose related sensitivity of suppressor cell activity to Cy would indicate that, with passing of time after SOI as the suppressor cells differentiated and matured, their sensitivity to this drug gradually diminished.

In conclusion, equally potent immunosuppressive agents can have diverse effects on the induction and expression of suppressor cells and their action may depend on the time when they are administered in relation to antigen. Cy injected at different times in relation to a large dose of antigen may influence the induction of suppressor cell activity in a variable manner probably due to its differential effect on various cellular components involved in the induction of this function. We tentatively postulate that, while TSp are sensitive (Cantor *et al.*, 1978), TSi, prior to contact with antigens, are resistant to Cy; they may become sensitive following activation with antigen. TSe, on the other hand, might be relatively resistant to Cy. Our results also indicate, contrary to the previously published view, that specific suppression of antibody response by high antigen dose and Cy combination is not due to the induction of suppressor cells (Ramshaw *et al.*, 1977). Further studies are in progress to identify the definitive nature of cells affected by Cy administered at various times in relation to antigen.

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