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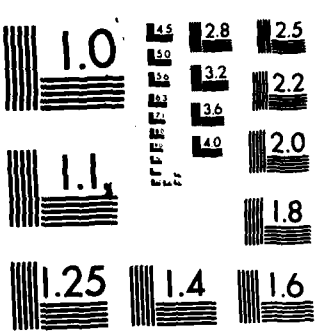

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Annual Report on

EFFECT OF ANTIPARASITE CHEMOTHERAPEUTIC AGENTS ON IMMUNE REACTIONS

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INTRODUCTION

No drugs were submitted by the U. S. Army Medical Research and Development Command for immunotoxic evaluation between January and July, 1981. Three drugs have been received recently (July 7, 1981) and are currently undergoing evaluation. A report on these will be submitted shortly.

In the past PI has used cyclophosphamide as a positive immunosuppressive agent. Due to its potent suppressive effects in some systems (1), lack of effect in other systems (2), and dramatic augmentation in some other systems (3), the PI has extended studies on the mode and target of action of this agents.

Cyclophosphamide is one of the family of alkylating agents with severe immunosuppressive properties. Interestingly, before the chemotherapeutic anti-cancer potential of alkylating agents were realized, immunosuppressive effects of certain members of the alkylating agents were evident. Thus, soon after the introduction of mustard gas as a chemical warfare agent, its destructive effects on bone marrow and other lymphoid cells were discovered. First, comprehensive studies on the immunosuppressive effect of an alkylating agent (dichloroethyl sulphide: a sulphur mustard) were conducted by Hecktoeen and Corper (4). Since then several alkylating agents have received attention for their immunosuppressive actions but cyclophosphamide has been by far the focus of most investigations. It has been shown to suppress the primary and secondary humoral responses to various antigens (5,6,) prolong homograft survival (7), inhibit delayed hypersensitivity reactions (8,9), be anti-proliferative in mitogen and antigen induced blastogenesis (10-12) and to depress migration inhibition factor production, antibody dependent cellular cytotoxicity as well as mitogen induced cytotoxicity (10).

In recent years, it has become apparent that the immune response is a product of interactions among different cellular and humoral components and the final magnitude of a response is dependent on the balance between positive and negative regulatory events in the circuits (13,14). Any alterations in the components of these regulatory circuits will alter the levels of immune responses. We have, therefore, been studying the target of action of cyclophosphamide to determine if this agent causes suppression by affecting macrophages, helper T-lymphocytes, B-lymphocytes or suppressor cells. In this report, we are presenting our recent observations indicating that a population of T-helper (TH) lymphocytes essential to reconstitute the immune response of athymic nude mice is resistant to cyclophosphamide. Moreover, macrophages as indicated by the clearance of particulate material from circulation, are also resistant. Our preliminary conclusion is that Cy exerts its suppressive effect on anti-SRBC antibody response by altering the B-cells function.

Mice: Male BALB/c (+/+) or congenic athymic nude (BALB/c nu/nu) mice weighing 20-25 grams were used in all experiments. All mice were maintained according to the "Guide for the Care and Use of Laboratory Animals" (1972) prepared by the Committee on Revision of the Guide for Laboratory Animal Facilities and Care, Institute of Laboratory Animal resources; National Research Council (DHEW Publ. No. NIH-73-23).

Antigen and Immunization: Sheep erythrocytes (SRBC) suspended in normal saline were used for immunization. Mice received 0.2 ml of 2.5% ($1x10^8$) SRBC by the i.p. route for studies involving humoral response.

Humoral Responses: Humoral IgM and IgG responses against SRBC were measured by assaying individual spleens for the hemolytic PFC contents by the method described by Cunningham and Szenberg (15).

Four days following i.p. or i.v. immunization of mice with 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 allow 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 a 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.

The above 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 (16). From the number of plaques per chamber was calculated the number of PFC per 10^6 nucleated splenocytes and also PFC per spleen.

Phagocytic Function of the Reticuloendothelial System (RES). Clearance of SRBC labeled with ^{51}Cr (chromium isotope) was used as a criteria for the RES function (17,18). 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 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 70 µl of blood was collected immediately (zero time sample) from the retroorbital 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 scintillation spectrometer. The radioactivity in the sample represented the relative concentration of SRBC in circulation at a particular time. The cpm were converted to $\log_{10}$ 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 weight, a corrected phagocytic index,

α, was calculated as follows:

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

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

Presentation of Data. PFC per spleen have been recorded as $\log_{10}$ mean together with one standard error. Anti-log of $\log_{10}$ mean PFC are recorded in parentheses. RE function has been expressed as the arithmetic mean of phagocytic index, 100 x K values or α values corrected for variations in the whole body weight and liver and spleen weight of individual mice within the limit one standard error.

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.

RESULTS

Effect of Cy on TH-cells: The effect of Cy on helper T-cells was measured using an adoptive transfer system. BALB/c (+/+) mice served as donors of spleen cells to reconstitute the response of BALB/c (nu/nu) mice. Since nude mice only lack T-cells and can be reconstituted by the injection of T-cells (19), any alteration in the ability of splenocytes of donor BALB/c (+/+) following Cy treatment would reflect an alteration in the helper T-cell population.

TABLE 1.

Effect of Cy on the Ability of Splenocytes to Transfer Immunocompetence in Nude Mice

Donor (BALB/c) Treatment[1]	IgM PFC per spleen[2]	
	Nude recipients	Balb/c donors
Saline	5.049 ± 0.075 (111,856)	5.416 ± 0.048 (260,439)
Cy	5.079 ± 0.071 (119,946)	4.337 ± 0.128 (21,751)
No spleen transfer[3] control (nude)	3.646 ± 0.100 (4,430)	

1. Balb/c mice were injected with 100 mg/kg Cy 2 days before their splenocytes were harvested and transferred to nude mice with 1 x 10^8 SRBC. PFC assays were performed on individual nude spleens 4 days later. Saline and Cy treated donors were also immunized and tested at the same time as the nude recipient. Cy treated donors responded significantly ($P < 0.001$) lower than saline treated donors, but the recipient of spleen from the two groups were not significantly different.

2. Log_{10} mean ± 1 s.e. from individual observations from 5 different experiments with a total of 20-23 mice per group. Figures in parenthesis represent geometric (anti-log) means.

3. Nude mice which received no splenocytes but were immunized with SRBC.

The results of several experiments have been summarized in Table 1. It is clear that nude mice give very poor response to SRBC (line 3) unless they are injected with splenocytes from normal congenic BALB/c mice (line 1, column 1). Data in line 2 clearly indicates that treatment of donor with Cy did not reduce their ability to restore the immune response of nude mice. By comparing lines 1 and 2 of the second column it can be easily seen that Cy drastically suppressed the response of donor animals. Since nude mice only lack T-cells, the data indicate that Cy did not deplete helper T-cells in donor animals. Further proof of this was obtained in the following experiments. All protocals for this experiment were the same except two groups of nude mice received splenocyte from Cy treated animals. In one group, splenocytes were treated with anti-thy and anti-Lyl antiserum and complement to remove TH cells whereas in the other group splenocytes were treated with complement alone. It is clearly shown in Table 2 that when splenocytes from Cy treated mice were incubated with complement alone, they were fully competent in restoring the response of nude mice. By contrast when splenocytes were treated with anti-thy, anti-Lyl, serum and complement, they could not restore the response of nude mice. Thus, it can be concluded that it is a population of Cy resistant T-cells which reconstitute nude mice to respond to SRBC.

TABLE 2.

Splenocytes of Cy Treated Mice Which Transfer Immunocompetence in Nude Mice are T-cells

In vivo Donor (BALB/c) D Treatment[1]	In vitro[2] Splenocyte Treatment	IgM PFC response[3]	
		Nude Recipients	BALB/c donors
Saline	None	5.235 ± 0.078 (171,738)	5.375 ± 0.073 (236,797)
Cy 100 mg/kg	C	5.108 ± 0.083 (128,093)	3.318 ± 0.145 (2081)
Cy 100 mg/kg	Anti-thy, Anti-Ly, +C	3.407 ± 0.158 (2,552)	
	No splenocytes[4]	3.469 ± 0.506 (2,945)	

1. BALB/c mice were injected wih 100 mg/kg Cy 2 days before their splenocytes were harvested and transferred to nude mice with 1 x 10^8 SRBC. PFC assays were performed on nude spleen 4 days after immunization. Cy treated were also immunized and tested at the same time as the nude recipients. Cy treated donors responded significantly ($P<0.001$) lower than saline controls but recipients responded similarly ($P>0.05$) unless spleens were treated with anti-thy and anti-Lyl serum and C ($P<0.001$) compared to group treated with C alone.

2. Splenocytes from Cy-treated mice was incubated *in vitro* with anti-thy serum and anti-Ly1 serum and complement (C) to remove T-cells or C alone before transfer.

3. Log_{10} mean ± 1 s.e. of PFC per spleen for groups containing a minimum of 5 mice. Figures in parenthesis are geometric means.

4. Nude mice which received no splenocytes but were immunized with SRBC.

A number of agents are more active in suppressing immune responses when injected after antigen than when given before (1,20). This has been proposed to be due to their greater toxicity against dividing cells. To test if TH-cells proliferating in response to antigen became sensitive to Cy, donor mice were immunized 2 days before treatment with Cy. Specifically, BALB/c mice were injected i.p. with saline or 1×10^8 SRBC. Two days later they were treated with saline or 100 mg/kg Cy. Splenic cells were harvested two days thereafter and injected i.v. into nude mice together with 1×10^8 SRBC. Control nude mice received SRBC alone. At the time of cell transfer, groups of donor mice were also immunized with 1×10^8 SRBC and their responses measured 4 days later. These groups provided controls indicating the effect of Cy on donors' immune response.

TABLE 3.

Effect of Cy on helper T-lymphocytes after exposure to SRBC

Donor (BALB/c) Treatment[1]	PFC per spleen[2] Nude Recipients		BALB/c donors	
	IgM	IgG	IgM	IgG
1×10^8 SRBC	4.721 ± 0.134 (52,640)	4.165 ± 0.186 (14,622)	5.270 ± 0.109 (186,241)	Not Tested (NT)
1×10^8 SRBC	4.598 ± 0.167 (39,637)	4.291 ± 0.134 (19,564)	3.042 ± 0.222 (1,100)	
No spleen transfer[3]	2.629 ± 0.268 (426)	NT		

1. BALB/c mice were injected with 1×10^8 SRBC on day 0 and saline or (100 mg/kg) Cy on day 2. 2 days later, their splenocytes were transferred into nude mice together with 1×10^8 SRBC. PFC assay were performed on recipients 4 days after transfer. Saline or Cy treated donors were also tested for their response at the time of cell transfer. Cy treated donors responded significantly lower ($P<0.001$) that saline control but receipts responded similarly ($P>0.05$).

2. Log_{10} mean ± 1 s.e. from individual observations from 2 experiments with a total of 5-10 mice in each group. Figures in paranthesis represent geometric (anti-log) means.

3. Nude mice which did not receive splenocytes but were immunized with SRBC.

It is apparent from Table 3 that nude mice restored with donor lymphocytes from mice given Cy 2 days after SRBC responded similar to those restored with splenocytes from mice receiving SRBC only. It is obvious from last two columns of the table that the response of donor mice was drastically reduced and the response measured in nude mice could not be the residual antibody producing cells from donors.

Effect of Cy on the RES function: The suppression of anti-SRBC response by Cy could be due to a malfunction of phagocytic function. A number of agents which deplete macrophages also cause suppression of immune responses (21,22). The effect of Cy on the *in vivo* phagocytic function of mice was measured in BALB/c mice. Mice were injected with 100 mg/kg Cy and two days later their *in vivo* phagocytic function was assayed by monitoring the clearance rate of SRBC. Results have been summarized in Table 4 and clearly indicate that cyclophosphamide did not affect the phagocytic function of mice.

TABLE 4.

Effect of Cy on *In Vivo* Clearance of Particulate Material

Treatment[1]	Phagocytic Index[2]	
	K x 100	α^3
Saline	10.22 ± 0.55	6.52 ± 0.34
Cy	11.05 ± 0.97	6.95 ± 0.17

1. 100 mg/kg Cy injected i.p. two days before the measurement of the RE function.

2. Mean ± 1 s.e. for groups of mice containing a minimum of 5 mice.

3. Values for Saline and Cy-treated groups were not significantly different ($P>0.05$).

DISCUSSION

Cyclophosphamide has been shown to suppress primary response to both thymus dependent and thymus independent antigens ~~(20)~~. Under certain conditions, it also suppresses secondary responses ~~(12)~~. Studies reported here were designed to determine whether the suppression of anti-SRBC response by Cy was due to a lesion in macrophage function, B-cells or helper T-cells. Data from Berenbaum would indicate that B-lymphocytes which respond to thymus independent antigen, lipopolysaccharide

are depleted by Cy. However, recent data point to the existence of several B-cell populations (23,24) and in view of the selectivity of Cy for certain T-cell populations (12,25), it remains possible that B-cells responding to T-dependent antigen might be spared and the suppression of anti-SRBC response occurs via the depletion of helper T-cells. This was certainly our first impression, since Cy injected before antigen spared the response to another thymus independent antigen pneoumococcal polysaccharide (26).

There have been conflicting data on the target of action of Cy in the suppression of anti-SRBC responses. Schwarze has suggested that suppression of anti-SRBC response by Cy is entirely due to its effect on helper T-cells (27). By contrast, Shand has provided data by using microsonially activated Cy in vitro that both TH and B-cells are damaged by Cy, although there was some preferential damage to B-cells when a higher concentration of Cy was used (28). Both studies cited above are subject to criticisms. The former one utilized mice which were first treated with phytohaemagglutinin and were at the height of proliferative response when Cy was administered and the antigen was injected at a time when the response of normal mice injected with Cy is usually recovering (20,30). Shand's data were obtained from in vitro experiments in which B- and TH cells were in direct contact with microsomally active Cy (28). In vivo, lymphoid cell may not encounter such doses of active components of Cy and hence may be affected differently. We therefore utilized nude mice as a model which have all cellular components needed for an anti-SRBC response except T-lymphocytes (19). Our assumption was that if Cy damaged the helper cells in the donor mice, nude mice receiving cells from these mice will not respond. The results recorded here clearly indicate that cells from Cy treated animals are as compentent in restoring the response of nude mice as cells from normal mice, and these cells are thymus derived. Furthermore, these cells remain resistant to Cy even after exposure to the antigen when cells might be in a proliferating phase. Our data suggest that the suppression of anti-SRBC antibody responses may be via the depletion of B-lymphocytes, a conclusion which is in agreement with previous observations indicating that B-lymphocytes are relatively more sensitive to Cy than T-lymphocytes (31,32).

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