

IN VITRO EFFECTS OF
4-HYDROPEROXYCYCLOPHOSPHAMIDE ON HUMAN
IMMUNOREGULATORY T SUBSET FUNCTION*

I. Selective Effects on Lymphocyte
Function in T-B Cell Collaboration

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The alkylating agent cyclophosphamide (CYP)¹ may suppress or enhance both B and T cell-mediated immune responses *in vivo* but requires microsomal enzyme activation for *in vitro* efficacy. 4-hydroperoxycyclophosphamide (4-HC) is a synthetic compound spontaneously hydrolyzed in aqueous solution to 4-hydroxycyclophosphamide, the initial metabolite formed by microsomal activation, and which subsequently yields the several active metabolites formed *in vivo*. It has been demonstrated that CYP activated by liver microsomes *in vitro* as well as 4-HC will mimic the immunosuppressive or immunoenhancing effects observed in the intact organism (1-4).

By using 4-HC as a probe of immunoregulatory T subset function *in vitro*, Diamantstein et al. (4) recently demonstrated in a murine system that the enhancement of the cellular immune response to sheep erythrocytes by the compound is a result of selective inactivation of suppressor T cells or their precursors at low concentrations (3). These data provide support for the hypothesis that the enhancement of cellular responses by CYP administered before antigenic challenge *in vivo* (5-9) is a result of selective elimination of precursors of suppressor T cells. Diamantstein et al. (2, 4) have further demonstrated that the murine inducer T cell subset mediating humoral immunity is relatively more sensitive to lower concentrations of 4-HC than are differentiated suppressor T cells for humoral responses or effector T cells mediating delayed-type hypersensitivity.

This selective property of differential concentrations of 4-HC has been employed in the studies described here to investigate functional immunoregulatory subsets of human T cells involved in a pokeweed mitogen (PWM)-driven polyclonal immuno-

* Supported by grants CA-27691, CA-5834, CA-21071, CA-15142, CA-13038, and CA-25438 from the U. S. Public Health Service.

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¹ Abbreviations used in this paper: AET, 2-aminoethylisothiuronium bromide; C, complement; Con A, concanavalin A; CYP, cyclophosphamide; E⁺, E⁻, E-rosette-positive and -negative, respectively; FCS, fetal calf serum; [³H]Tdr, tritiated thymidine; 4-HC, 4-hydroperoxycyclophosphamide; PHA, phytohemagglutinin; PWM, pokeweed mitogen; RFC, rosette-forming cells; SRBC, sheep erythrocytes.

globulin (Ig) secretion assay. In this report, data are presented that demonstrate that low concentrations of 4-HC selectively inhibit the differentiation of presuppressors to suppressor-effector cells. T cell inducers were found to be sensitive to moderate concentrations, whereas suppressor effector function is resistant to all but the highest in vitro levels of 4-HC. The 4-HC-sensitive presuppressor is further shown to be of the OKT4⁺,OKT8⁻ phenotype. The effects of 4-HC on suppressor precursors occur not only at concentrations that are cytotoxic, but also at levels that do not result in measurable DNA cross-linking or decreased blastogenesis. In addition, T cell-regulated B cell differentiation is reversibly blocked by treating B cells with low concentrations of 4-HC but may be restored if treated B cells are cocultured with T cells depleted of mitogen-inducible suppressor activity, suggesting that B cell subpopulations are also differentially susceptible to CYP.

Materials and Methods

Isolation of Lymphocyte Populations. Peripheral leukocyte populations were obtained from buffy coats separated by gravity sedimentation of 500 ml of citrated blood from volunteer donors. Phagocytic mononuclear cells were depleted by diluting each buffy coat 1:2 with medium containing carbonyl iron (1 mg/ml) in 50-ml aliquots and incubating the mixture for 60 min with gentle continuous agitation. Each leukocyte population was then layered in 10-ml aliquots on 3-ml Ficoll-Hypaque density gradients in 15-ml siliconized glass tubes and centrifuged 40 min at 400 *g*. The interface layer was resuspended in RPMI 1640 (Grand Island Biological Co., Grand Island, N. Y.) and washed twice. The viability of the purified lymphocyte populations was routinely >99% and monocyte contamination was always <2% by nonspecific esterase staining and latex-bead ingestion.

E-rosette-positive (E⁺) and -negative (E⁻) lymphocyte populations were obtained by rosetting with sheep erythrocytes (SRBC) that had been treated with 2-aminoethylisothiuronium bromide (AET) according to the method of Kaplan and Clark (10). AET-treated sheep erythrocytes were washed twice in RPMI 1640 containing 10% fetal calf serum (FCS) and antibiotics and mixed with lymphocyte suspensions at a ratio of 50 SRBC:1 lymphocyte in 15-ml conical, siliconized glass tubes. These mixtures were centrifuged for 8 min at 200 *g* and incubated 18 h at 4°C. The pellets were gently resuspended, rosette-forming cells (RFC) were enumerated, and the E⁺ population sedimented on Ficoll-Hypaque at 400 *g* for 40 min. Sheep erythrocytes were removed by brief hypotonic lysis and the pellets resuspended in 3% saline to isotonicity. E⁺ and E⁻ populations (for brevity, the E⁻ population, which always contained >70% surface Ig⁺ [sIg⁺] B cells and ~10–15% null cells, is subsequently referred to as B cells) were washed twice in supplemented RPMI 1640 and kept at 4°C. Purity of these and other populations was determined by enumerating sIg⁺ cells with rabbit anti-human F(ab')₂-coupled bovine erythrocytes (Grand Island Biological Co.) according to the method of Gold and Fudenberg (11) as previously described (12).

Isolation of T Cell Subsets by Complement-mediated Lysis with the Monoclonal Antibodies OKT4 and OKT8. The monoclonal antibodies OKT3, OKT4, and OKT8 used in these studies were generously donated by Dr. Gideon Goldstein of Ortho Pharmaceutical Corp., Raritan, N. J. OKT4 monoclonal antibody reacts with a subset that comprises 60% of peripheral T cells, proliferates in response to soluble antigen, and contains the inducer T subpopulation for PWM-driven, immunoglobulin-secretion assays (13). A second monoclonal antibody, OKT8, reacts with 30–35% of peripheral T cells and is described as defining the cytotoxic/suppressor subset in functional assays (14). The unfractionated T cell populations were resuspended at 50 × 10⁶ cells/ml in RPMI 1640 containing 5% FCS. 0.2 ml of the appropriate monoclonal antibody at a dilution of 1:50 was added to 0.2 ml of the T cell suspension, and the cells were then incubated for 45 min at room temperature. After incubation, rabbit complement (C) was added at a final dilution of 1:10 and incubation was continued for 1 h at 37°C in a humid atmosphere. This entire procedure was repeated twice to thoroughly deplete the antibody-binding cells of the designated T cell subset. FACS-II (B-D FACS Systems, Mountain View, Calif.) analysis of the resulting T cell populations showed that the OKT4-treated population contained >94%

OKT3⁺ cells, >90% OKT8⁺ cells, and <4% OKT4⁺ cells. The OKT8-treated population contained >90% OKT3⁺ cells, >90% OKT4⁺ cells, and <4% OKT8⁺ cells. Because of these results, the notation OKT4⁺ is used to designate the T subset remaining after treatment with OKT8 plus C and OKT8⁺ is used for the reciprocal population remaining after treatment with OKT4 plus C.

4-HC. 4-HC was synthesized in the laboratory of Michael Colvin at Johns Hopkins Oncology Center, Baltimore, Md. 4-HC is a relatively unstable compound which spontaneously hydrolyzes in aqueous solution to 4-hydroxycyclophosphamide, the compound formed by liver microsomal activation of cyclophosphamide. 4-HC and 4-hydroxycyclophosphamide have been previously shown to have similar cytotoxic activity and cause comparable cross-linking of DNA at equimolar concentrations (M. Colvin, unpublished data). 4-HC was maintained as a desiccated powder at -70°C and prepared in a stock solution of RPMI 1640 medium with 5% FCS within 30 s of lymphocyte exposure. Cell suspensions were added immediately to obtain the appropriate final dilution of 4-HC and further incubated 1 h at 37°C in a humidified 5% CO₂ atmosphere. After 4-HC exposure, the cell suspensions were washed three times and prepared in appropriate dilutions for functional assays. No immediate cytotoxic effects of 4-HC were noted after drug exposure over the entire range of concentrations from 10⁻⁴ to 100 nmol/ml with viability of all treated and washed cell populations being uniformly >99% by trypan blue dye exclusion.

Coculture Methods. IgG produced by isolated subpopulations and in coculture experiments was quantitated by a radioimmunoassay technique as previously described (15). Lymphocyte populations were adjusted to a final concentration of 2 × 10⁶ cells/ml in RPMI 1640 supplemented with glutamine, 0.1 ng/ml gentamicin, and previously screened, heat-inactivated 10% FCS. The cell suspensions were incubated in flat-bottomed microtiter plates (Linbro Chemical Co., Hamden, Conn.) for 7 d at 37°C in a humidified 5% CO₂ atmosphere. In coculture experiments, ratios of B/T cells were maintained at 1:2, and T cell subpopulations and putative suppressor populations were recombined in equal T/T cell ratios unless otherwise noted. The total well volume was 350 μl. In some experiments, T cells were irradiated with 2,000 rad x-irradiation from a General Electric Maxitron 250 x-ray machine using 0.25-mm Cu and 1-mm Al filters. Proliferative responses were determined on day 3 of culture after a 6-h pulse with 2 μCi/well of tritiated thymidine (³H]Tdr) (sp act 6.7 Ci/mmol) and subsequent harvesting of the entire contents of the well for liquid scintillation counting. Affinity-column-purified human IgG or IgM, previously shown to be specific by gel double diffusion and immunoelectrophoresis, was labeled with 1 mCi of ¹²⁵I using a chloramine T method. The dilution of anti-IgG or IgM that bound 60% of a suitable amount of labeled immunoglobulin was determined. Anti-IgG and anti-IgM were prepared by multiple rabbit immunizations with purified protein followed by affinity-column purification of the antibody. Antigen-antibody dilution curves were constructed for an antigen range of 1-30 ng and down to a 1:8,000 dilution of antiserum. The amount of goat anti-rabbit Ig added to precipitate the rabbit Ig was twice the equivalence amount. From 10 to 100 μl of culture supernatant or standard was added to 20 μl of anti-IgG for 1 h at 37°C in 10 × 75-mm plastic Falcon tubes (Falcon Labware, Div. Becton, Dickinson & Co., Oxnard, Calif.) after which 10 μl of goat antirabbit Ig was added and the mixture incubated for 1 h at 37°C. After overnight incubation at 4°C, 1.0 ml of 0.01 M Tris, pH 7.0-7.4, was added to each tube. The tubes were centrifuged at 4°C and 2,000 g for 20 min after which the supernatants were discarded and the pellet counted. Standards were prepared in 0.01 M Tris with 1% bovine serum albumin. The binding of labeled Ig could be >90% specifically inhibited by cold Ig.

Concanavalin A (Con A)-Induced Suppression of Ig Secretion. T lymphocyte populations or T cell subsets from normal donors were activated in the presence of 25 μg/ml Con A (Calbiochem-Behring Corp., San Diego, Calif.) for 48 h in RPMI 1640 supplemented with 10% FCS, 200 mM L-glutamine, antibiotics, and 25 mM Hepes buffer at a concentration of 4 × 10⁶ cells/ml. Untreated control cells were cultured in an identical fashion without Con A. After activation, cultured T cells were washed three times, adjusted to a concentration of 2 × 10⁶ cells/ml, and incubated in standard culture media for 20 min in the presence of 40 μg/ml mitomycin C (Bristol Laboratories, Syracuse, N. Y.). These activated T cells and subsets were subsequently washed four times, readjusted to 2 × 10⁶ cells/ml, and added to normal allogeneic T and B cell

cocultures to determine Con A-induced suppression of Ig secretion. In preliminary experiments, 0.3 M α -methyl-D-mannoside (Sigma Chemical Co., St. Louis, Mo.) was included in the wash, but was found not to influence the results significantly and reported experiments were performed in the absence of α -methyl-D-mannoside. The percent suppression of total Ig secretion was calculated according to the following formula:

$$\text{Percent suppression} = \frac{(\text{Ig secretion by B + T + cultured T cells}) - (\text{Ig secretion by B + T + Con A-cultured T cells})}{\text{Ig secretion by B + T + cultured T cells}} \times 100,$$

where B + T cell cocultures were performed using normal autologous cell populations and allogeneic cultured T cell controls. Total cocultured cell numbers in the Con A suppressor assays consisted of 2×10^5 cells of each T cell population.

Results

Inhibition of T Cell Blastogenic Responses by 4-HC. To obtain a dose-response curve for cytotoxicity of 4-HC on T cells, the effects of a 1 h in vitro incubation of the compound with purified normal T cells (>96% E RFC⁺) were determined by trypan blue dye exclusion as well as functional impairment of DNA synthesis by blastogenesis in response to phytohemagglutinin (PHA), Con A, and PWM. 4-HC was diluted in standard culture media and isolated T cells were exposed to final concentrations of between 10^{-4} and 100 nmol/ml for 1 h. In no instance was immediate cytotoxicity demonstrable by trypan blue dye exclusion after the 1-h incubation and three washes. Drug-induced cytotoxicity as a result of DNA cross-linking was, however, evident at 72 h by decreased [³H]TdR incorporation in response to mitogenic stimulation. As shown in Fig. 1, purified T cells demonstrated decreased blastogenesis to all three mitogens after exposure to a concentration of ~30 nmol/ml 4-HC with complete loss of DNA synthetic capacity at drug levels >80 nmol/ml. The drug concentration that results in initial DNA cross-linking in lymphoid cell populations after in vitro exposure to 4-HC has been previously shown to be ≥ 20 nmol/ml (M. Colvin, unpublished data). It should be noted that there is neither an immediate nor a 72-h cytotoxic effect of 4-HC concentrations <1 nmol/ml, although significant effects on mitogen-

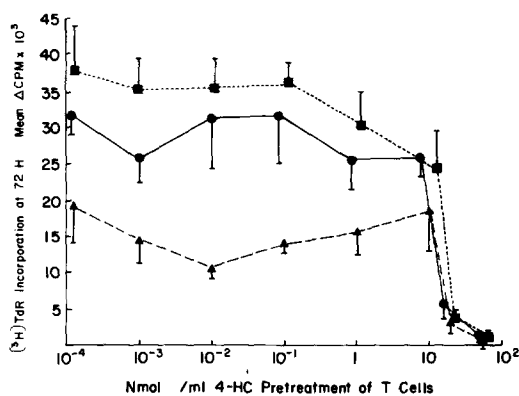


FIG. 1. Dose-response curve of mitogen responsiveness of 4-HC-pretreated T cells. T cells were incubated for 1 h with the indicated concentration of 4-HC, washed, cultured 72 h in the presence of mitogen, and [³H]TdR incorporation after a 6-h pulse was determined. (---) Con A, 25 μ g/ml (control counts per minute above background [Δ cpm]: 18,859 \pm 3,189 SEM); (—) HA-17, 0.5 μ g/ml (control Δ cpm: 31,921 \pm 1,817 SEM); and (---) PWM, 1:200 (control Δ cpm: 38,693 \pm 5,853 SEM).

induced T suppressor activity were demonstrable at these very low levels as described below. Treated cell populations were also examined for viability after incubation by trypan blue dye exclusion when the cells were harvested for functional assays. No significant effects of 4-HC on T or B cell recovery or viability were apparent when cells were exposed to concentrations of <10 nmol/ml and subsequently cultured for up to 7 d, although cell yields were reduced by as much as 70% after treatment at concentrations >40 nmol/ml. The concentration of 20 nmol/ml was empirically selected for its maximum effects on Con A-induced suppression without resulting in a >10% decrement in cell yields after 48 h.

Augmentation of Ig Secretion by Pretreatment of T Cells with 4-HC. To compare the relative sensitivity of suppressor and inducer T cells in the Ig secretion assay, T cells were pretreated with graded concentration of 4-HC, washed, and then cocultured with autologous B cells for 7 d. The data obtained for three to four separate experiments are shown in Table I. The in vitro production of IgG and IgM by cocultured B and 4-HC-pretreated T cells in the absence of monocytes (<2%) was compared with baseline secretion by untreated populations and with production after 2,000-rad x-irradiation of T cells. Ig secretion after the 7-d incubation period is expressed as ng Ig/ml, which is equivalent in this culture system to ng/3 × 10⁵ total lymphocytes or ng/1 × 10⁵ cultured B cells. As previously described (16), 2,000-rad x-irradiation of cocultured T cells at a 2:1 T/B cell ratio results in an ~50% increment in Ig secretion, although this augmentation is not seen at lower T/B cell ratios, probably because inducer function is also partially radiosensitive (17). 4-HC pretreatment was found to have a parallel effect on Ig secretion, although at lower concentrations (<10 nmol/ml), the augmentation obtained was approximately 30%, whereas at a concentration of 20 nmol/ml, Ig secretion was increased by nearly 50% for both IgG and IgM. Treatment of T cells with concentrations of 60 or 100 nmol/ml resulted in a sharp decline in Ig secretion to levels obtained with isolated B cells alone. These

TABLE I
Effect of 4-HC Pretreatment of T Cells on Subsequent IgG and IgM Secretion by Autologous B Cells in Coculture

Composition of coculture	IgG secretion	IgM secretion
	<i>ng/ml per 7 d</i>	
B + T + PWM control	1,397 ± 162	2,009 ± 332
B + pretreated T + PWM	Percent of control	Percent of control
B + T _x *	152.3 ± 10.5	153.4 ± 6.9
B + T _{10⁻⁴} ‡	107.3 ± 9.9	104.4 ± 7.4
B + T _{10⁻³}	118.8 ± 3.2	114.6 ± 6.3
B + T _{10⁻²}	135.1 ± 3.8	127.8 ± 1.9
B + T _{10⁻¹}	130.8 ± 7.4	121.2 ± 9.7
B + T ₁	133.3 ± 7.9	123.6 ± 1.8
B + T ₂₀	143.4 ± 5.1	148.5 ± 5.3
B + T ₆₀	42.8 ± 5.3	41.1 ± 1.5
B + T ₁₀₀	11.5 ± 4.3	5.9 ± 3.2

* x, 2,000-rad x-irradiation. Data presented as the mean of three to four experiments ± SEM.

‡ Subscripts represent 4-HC concentrations in nmol/ml.

DNA cross-linking occurs at concentrations ≥20 nmol/ml.

data therefore suggest an initial selective effect of 4-HC on mitogen-induced suppressor T cell function at very low concentrations followed by concomitant elimination of inducer activity at levels that yield maximum DNA cross-linking (30–60 nmol/ml) and thus abrogation of both T cell functions. The possibility of carry-over of 4-HC or its metabolites with subsequent leakage during incubation at higher concentrations was excluded by coculturing T cells treated with 100 nmol/ml 4-HC with untreated T and B cells, which resulted in no decrease in Ig synthesis.

Effect of 4-HC on Con A-inducible T Cell Suppression. To determine the effects of 4-HC on Con A-inducible suppressor function, T cells were incubated 48 h in the presence of 25 μ g/ml of Con A after exposure to graded concentrations of 4-HC and assayed for suppression of IgG and IgM secretion by untreated allogeneic B cells. The baseline Ig secretion selected as the 100% control level for these four experiments was obtained by coculturing allogeneic T cells that had been preincubated in standard media alone and were mitomycin C-treated after harvesting. As shown in Table II, T cells incubated in the presence of 25 μ g/ml Con A suppressed IgG secretion by ~85% and IgM secretion by 90%. Pre-exposure of the Con A-cultured T cells to concentrations of 4-HC ranging from 10^{-2} nmol/ml to 20 nmol/ml resulted in cell yields after 48 h that were within $\pm 10\%$ of control cultures and were $>90\%$ viable by trypan blue dye exclusion. These drug-exposed, Con A-activated T cells were, however, completely devoid of suppressor activity for either IgG or IgM synthesis, even when exposed to lower 4-HC concentrations, and despite normal blastogenic responses to Con A after exposure (Fig. 1). In several experiments, there was marked augmentation of both IgG and IgM synthesis when 4-HC and Con A-treated T cells were added to B + T cell cocultures, suggesting that the treated cell populations were enriched for inducer activity that further augmented baseline Ig secretion by providing a more favorable inducer T:B cell effector ratio.

Because allogeneic T cells did not demonstrate suppressor function in this assay

TABLE II
Effect of 4-HC on Con-A-inducible T Cell Suppression of Ig Synthesis

T suppressor cells added to B + T + PWM coculture	IgG secretion	IgM secretion
T cells cultured 48 h with media alone	1,750 \pm 462 ng/ml per 7 d (100%)	2,450 \pm 599 ng/ml per 7 d (100%)
T cells incubated 48 h with 25 μ g/ml Con A	15.5 \pm 1.3%*	10.8 \pm 3.3%*
T cells treated with 4-HC before Con A activation		
10 ⁻² nmol/ml	100.6 \pm 6.4*	134.1 \pm 7.8
10 ⁻¹ nmol/ml	107.4 \pm 7.6	117.6 \pm 4.9
1 nmol/ml	105.9 \pm 4.6	140.9 \pm 14.1
10 nmol/ml	135.8 \pm 4.6	99.2 \pm 6.7
20 nmol/ml	170.0 \pm 14.5	185.7 \pm 4.9

Data expressed as means of four experiments \pm SEM. T cells were pretreated with the designated concentrations of 4-HC for 1 h at 37°C, washed three times, and incubated 48 h in the presence of 25 μ g/ml Con A.

* Percentage of control \pm SEM.

unless activated by mitogens, the 4-HC metabolite was also used to determine whether CYP exerts its anti-suppressor activity before the induction of suppression, on the mitogen-induced suppressor-effector cell, or both. Table III presents data obtained in three experiments in which T cells were activated in the presence of 25 $\mu\text{g/ml}$ Con A and examined for suppression of IgG or IgM synthesis using allogeneic T + B cell cocultures. Suppressor activity was completely abrogated by pretreatment of T cells with 10 nmol/ml 4-HC before Con A activation. If Con A-activated cells were drug-treated either at 10 or 80 nmol/ml after the 48-h Con A incubation and before mitomycin C treatment, absolutely no reduction in suppressor activity was observed. To eliminate the possibility that synergism or competition between mitomycin C and 4-HC might result in inactivation of potential suppressors in the 4-HC pretreated group, similar experiments were performed excluding the mitomycin C treatment. Again, drug-induced loss of T suppression was virtually 100%. In aggregate, then, these data provide evidence that 4-HC is capable of blocking mitogen-induced T suppression at concentrations well below those required to block DNA-synthetic activity. The abrogation of suppression appears to occur by preventing the maturation of presuppressors to suppressor-effectors either directly, or by blockade of suppressor-inducer subset function. Further, mature suppressor-effector function is resistant to concentrations of 4-HC (80 nmol/ml) that fully block inducer T cell function (Table I) in this assay.

Sensitivity of B Cell Function to 4-HC. The immunosuppression caused by in vivo CYP administration in animals was originally observed as a decrease in serum Ig levels with a concomitant loss of primary follicles in spleen and lymph nodes (18). The effects of CYP on the immune system were, therefore, attributed to selective cytotoxic activity against B cells, whereas its effects on delayed hypersensitivity were discovered at a later date. Because the in vitro Ig secretion assay provides the opportunity to examine both T and B cell sensitivity to 4-HC, Ig secretion after exposure of the B cell population to 4-HC was also examined. As shown in Table IV for a total of four

TABLE III
Effect of 4-HC on Con A-induced T Suppressor Function Before and After Mitogen Activation

Suppressor population added to B + T cocultures	IgG secretion <i>ng/ml per 7 d</i>	Percent suppression	IgM secretion <i>ng/ml per 7 d</i>	Percent suppression
None	1,823 \pm 708	—	2,235 \pm 569	—
T cells activated 48 h with 25 $\mu\text{g/ml}$ Con A	756 \pm 195	59	270 \pm 68	88%
T cells pretreated with 10 nmol/ml 4-HC before Con A activation	1,810 \pm 423	1	3,115 \pm 738	0%
T cells treated with 4-HC after Con A activation*				
10 nmol/ml	436 \pm 163	76	340 \pm 181	85%
80 nmol/ml	347 \pm 148	81	553 \pm 202	75%

Data presented as the mean of three experiments \pm SEM.

* Data obtained with T populations incubated with both 4-HC and mitomycin C after Con A activation. Identical results were obtained in similar experiments omitting mitomycin C treatment to exclude a synergistic or competitive effect of the two drugs (see text).

TABLE IV
Effect of 4-HC Pretreatment of B Cells on Subsequent Secretion of IgG and IgM in Culture

Composition of coculture	IgG secretion	Percent of control	IgM secretion	Percent of control
	<i>ng/ml per 7 d</i>		<i>ng/ml per 7 d</i>	
B + T control	1,342 ± 332	100%	2,194 ± 305	100
B + T _x *	2,418 ± 311	167.8%	3,088 ± 713	140.7
B + T ₂₀	2,422 ± 510	168.0%	3,184 ± 582	145.1
B ₂₀ + T	512 ± 147	35.5%	204 ± 80	9.3
B ₂₀ + xT	2,330 ± 516	161.6%	2,428 ± 296	110.7
B ₂₀ + T ₂₀	2,068 ± 421	143.4%	3,006 ± 415	137.0

* x, 2,000-rad x-irradiation. Means of four to six experiments ± SEM.

‡ Subscripts represent 4-HC concentrations in nmol/ml.

to six experiments, treatment of B cells with 20 nmol/ml 4-HC results in a nearly complete loss of polyclonal Ig secretion on day 7. An unexpected result was obtained, however, when the same population of drug-treated B cells from each experiment was recombined with either an irradiated (2,000 rad) or drug-treated (20 nmol/ml) autologous T cell population. In either case, Ig secretion was restored to normal levels with some augmentation in total Ig secretion above baseline T + B cell cocultures. Thus, these data demonstrate that the augmentation of B cell effector function obtained with low concentrations of 4-HC is not simply a result of the effects of the drug on suppressor T cell function alone, but may result from an alteration in the normal balance between regulatory and effector cell function in both the T and B cell populations. They further suggest that treated B cells are injured or modified by 4-HC pretreatment in some manner that results in an increased sensitivity to T suppressor function. Whether this represents a selective kill of a B cell subpopulation, thus effectively altering the suppressor/effector ratios in coculture, or is the result of increased sensitivity of the response of an individual B cell to suppression remains to be determined.

Sensitivity of Isolated T Subset Function to 4-HC. Because T suppressor function in the polyclonal Ig secretion assay was clearly sensitive to pretreatment with extremely low concentrations of 4-HC, and because the data suggested that the mechanism of action of CYP at these concentrations is to prevent the differentiation of presuppressors to suppressor-effector cells, further experiments were designed to define the phenotype of the 4-HC-sensitive suppressor-precursor cell using the OKT4 and OKT8 monoclonal antibodies. To investigate the effects of 4-HC on T cell subsets defined by the monoclonal antibodies OKT4 and OKT8, complement-mediated lysis of total T cells with a 1:50 dilution of each antibody was employed to enrich for the reciprocal subset. After a double lysis and washing, each cell population was labeled with OKT3, OKT4, and OKT8, indirectly labeled with fluoresceinated goat anti-mouse Ig, and analyzed on the FACS-II. Lysis of the specific subset in each experiment was found to be >98% complete with >90% of the total residual T population staining positive for the reciprocal antibody. Fig. 2 illustrates the capacity of the total T population and each of the enriched subsets to collaborate with B cells in the secretion of IgG and IgM. As described above, either 2,000-rad x-irradiation or low-dose 4-HC pretreatment of total T cells resulted in a 40–50% augmentation in Ig secretion when pretreated T cells were cultured with autologous B cells. The OKT4⁺ subset was also

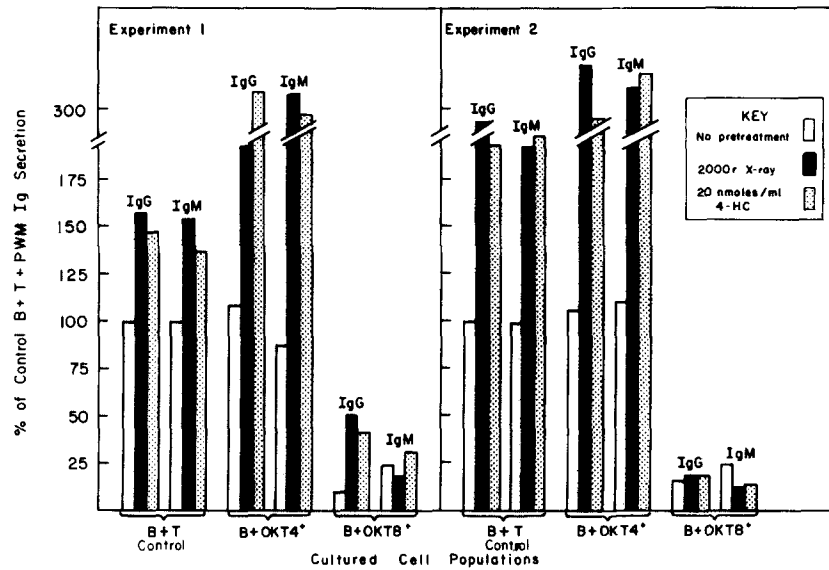


FIG. 2. Ability of T cells and OKT subsets to collaborate with autologous B cells after 2,000-rad x-irradiation or 20 nmol/ml 4-HC pretreatment. Data are presented as percent of control B + untreated T cocultures in the presence of PWM for two representative experiments. Mean control IgG secretion in these experiments was $1,293 \pm 123$ ng/ml and for IgM, $1,981 \pm 209$ ng/ml. Cocultures were performed at a 1:2 B:T cell ratio.

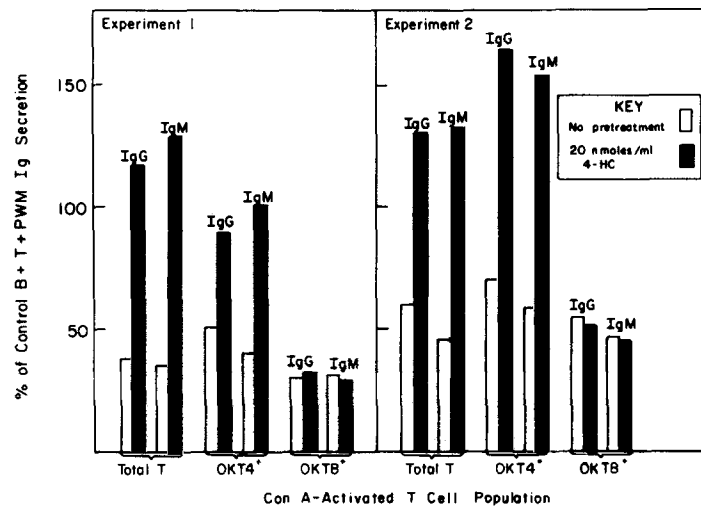


FIG. 3. Ability of 4-HC pretreatment to block Con A-induced suppressor function of T cells and OKT subsets. T cells or subsets were activated with $25 \mu\text{g/ml}$ Con A with and without 4-HC pretreatment and assayed for suppressor activity on allogeneic T + B cocultures. Data are presented as percent of control B + T cocultures in the presence of PWM for two representative experiments. Mean baseline IgG secretion in these experiments was $1,304 \pm 178$ ng/ml and for IgM, $2,108 \pm 243$ ng/ml. Con A-activated T cells were added on day 0 at equal T/T cell ratios.

fully able to induce Ig secretion, whereas the OKT8⁺ subset failed to help autologous B cells secrete Ig. After x-ray or drug treatment, OKT4⁺ cells responded as do total T populations with, in these two experiments, a several-fold augmentation in Ig secretion by cocultured B cells. Only a weak or nonexistent augmentation could be elicited by drug or x-ray treatment of the OKT8⁺ subset. These results suggested that the OKT4⁺ subset contains both an inducer and a presuppressor population, whereas OKT8⁺ cells represent a differentiated suppressor population because they have been previously shown to actively suppress ongoing T-B collaboration but require the presence of inducer cells for suppressor-effector function (17). Both subsets were therefore examined for their ability to suppress Ig secretion after activation with Con A and after preexposure to 4-HC. As shown in Fig. 3, OKT4⁺ cells, although not initially suppressive of Ig secretion (17), are fully capable of generating Con A-induced suppressor activity, which is equivalent in magnitude to that of unfractionated T cells. Pretreatment with 4-HC at the concentration of 20 nmol/ml completely prevents the generation of suppressor effectors within the OKT4⁺ subset and frequently results in an augmentation of inducer activity when drug-treated, Con A-activated T cells are added to B + T cell cocultures. In contrast, the OKT8⁺ subset retains suppressor activity after Con A activation, which is unaffected by preexposure of this subset to 4-HC or, apparently, by mitogen activation. These results provide strong support for the hypothesis initially proposed by Thomas et al. (17, 19) that the presuppressor is contained within the OKT4⁺ subset.

Discussion

It is well established that CYP, in addition to its immunosuppressive properties, enhances humoral as well as cellular immune responses to a variety of antigens in vivo (1-9). Although CYP-induced potentiation of cellular responses was originally ascribed to the elimination of B cells (20), Askenase et al. (7) demonstrated that doses of CYP too low to affect antibody production were nonetheless immunoaugmenting for delayed-type hypersensitivity. These authors therefore postulated that the enhancement of delayed-type hypersensitivity in vivo was a result of the selective elimination of precursors of suppressor T cells. Subsequent in vivo experiments led to the hypothesis that various immunoregulatory T cell subsets as well as effectors of both cellular and humoral immunity differed in their susceptibility to a given dose of CYP (1, 8, 9, 21, 22).

Although the immunoregulatory properties of CYP cannot be demonstrated in vitro, the recent availability of the synthetic compound 4-HC, which yields active metabolites in aqueous solution, has led to elucidation of these differing susceptibilities by murine regulatory and effector lymphocytes. In the experiments described here, 4-HC was used in conjunction with monoclonal antibodies to functional T cell subsets to further characterize regulatory T-B cell interactions in a human, PWM-driven polyclonal Ig secretion assay. The data presented clearly support the contention that 4-HC in vitro, like CYP administered in vivo, can be either immunosuppressive or immunoaugmenting for B cell differentiation and Ig secretion depending upon the concentration and the drug-treated cell population employed in a given assay. Several specific conclusions are also warranted from the results obtained in these studies: (a) T cell-helper function at high T:B cell ratios is resistant to low-dose 4-HC, although both [³H]Tdr incorporation and helper activity decay rapidly when the pretreatment

concentration of 4-HC exceeds 40 nmol/ml; (b) mitogen-induced T suppressor function, as measured either by augmentation in B effector function when T cells are pretreated or by Con A-induced suppressor activity is exquisitely sensitive to pretreatment concentrations of 4-HC that have no effect on helper or mature suppressor function; (c) treatment of B cells at concentrations of 20 nmol/ml blocks Ig secretion but this blockade can be overcome if treated B cells are cocultured in the absence of functional suppressor T cells; (d) differentiated T suppressor function, whether mediated by the OKT4⁻,OKT8⁺ subset or the mitogen-induced OKT4⁺,OKT8⁻ subset is resistant to concentrations of 4-HC in excess of those that abrogate helper activity or prevent differentiation or presuppressors; and (e) 4-HC appears to block mitogen-induced suppressor function by preventing the differentiation of mature suppressor-effector cells from OKT4⁺,OKT8⁻ precursors.

The cytotoxic effects of 4-HC on total T lymphocyte populations are undetectable until concentrations of ~30–40 nmol/ml are obtained, after which there is a sharp decline in [³H]TdR incorporation in response to the mitogens PHA, Con A, or PWM. Despite this apparent lack of T cell sensitivity at lower 4-HC concentrations, however, augmentation in secreted Ig levels was consistently observed even after pretreatment of T cells with as little as 10⁻³ nmol/ml. The augmentation observed after 4-HC pretreatment suggested the existence of a presuppressor subset within the total T population which differentiated in the presence of PWM and the maturation of which was blocked by pretreatment with 4-HC. This hypothesis was confirmed in the Con A-induced T-suppressor assay. Pretreatment of T cells before Con A activation with very low concentrations of 4-HC completely blocks the ability of activated T cells to suppress allogeneic B + T cell cocultures. That low-dose CYP exerts its antisuppressor effect at the level of the presuppressor was demonstrated by the inability of 4-HC to affect T suppressor function when used to treat T cells after a 48-h Con A activation, even at a concentration of 80 nmol/ml. Thus, low-dose 4-HC prevents the differentiation of suppressor-effector cells for B cell differentiation.

In experiments designed to determine the phenotype of the CYP-sensitive T subset, OKT8⁺- and OKT4⁺-enriched subsets were compared for their ability to suppress B + T cell cocultures after Con A activation as well as their ability to support Ig synthesis when recombined with autologous B cells. As has been previously described (13, 17, 23, 24) the OKT8⁺ subset fails to support Ig synthesis when cocultured with B cells alone. Pretreatment of OKT8⁺ cells with 4-HC or 2,000 rad x-irradiation before coculture with B cells also failed to augment the minimal Ig synthesis obtained with untreated OKT8⁺ cells, confirming the absence of any cells with inducer function within this subset (13, 17). Because the OKT4⁺ subset has been demonstrated to yield a T suppressor population when activated with PWM (19), each subset was also examined for the ability to generate Con A-induced suppressor function when pretreated with 4-HC. The results presented here confirm that the T suppressor precursor that is activated by Con A and which is sensitive to very low doses of CYP is of the OKT4⁺,OKT8⁻ phenotype. The OKT8⁺ cells are unable to collaborate in Ig secretion before Con A activation, and are active suppressors for allogeneic B + T cell cocultures after Con A activation. Further, OKT8⁺ cells have been demonstrated to actively suppress Ig production only in the presence of radiosensitive OKT4⁺ cells, and their suppressor activity is itself radiosensitive (17). In these experiments, however, the OKT8⁺ subset remained suppressive after Con A activation, and suppressor

activity was not abolished by pretreatment with 20 nmol/ml 4-HC. In experiments to be reported subsequently from this laboratory, OKT8-mediated suppressor function as well as cytotoxic activity has been found to be sensitive to 4-HC, although at much higher concentrations than differentiated mitogen-induced suppressor or helper activity, suggesting a hierarchy of human lymphocyte functional sensitivity to CYP analogous to that described in murine systems (4) with the differentiation of presuppressors being most sensitive, inducer function of intermediate sensitivity, and mature suppressor/cytotoxic functions least sensitive.

The data presented here also demonstrate an interesting and unpredicted effect of 4-HC pretreatment of the B cell population. Sensitivity of B cell function to CYP was among the earliest observations of the immunosuppressive properties of this agent (18, 20). Although B cells do appear to be sensitive to concentrations of 4-HC that block Con A-induced suppressor activity, immunoglobulin synthesis in coculture can be restored if T suppressor activity is itself blocked by x-irradiation or 4-HC pretreatment. There are at least two possible interpretations of this observation. B cell populations may themselves consist of subsets that are differentially susceptible to CYP. In this model, a partial reduction in the total number of responding B cell effectors would effectively increase the T/B cell ratio and thus favor increased suppression (17) unless T cell suppressor activity was blocked. Alternatively, all B cells within the treated population may be modified in their susceptibility to T suppression by CYP-induced injury, perhaps via alteration in B cell receptors as suggested by Shand and Howard (1). Definition of the actual mechanism involved must await more detailed analysis of the effects of 4-HC on B cell membrane integrity and intracellular metabolism.

The precise effects of 4-HC or CYP on T lymphocyte biology also remain unexplored. It remains unknown, for example, whether any of the observed effects of 4-HC on T subset function differ from those induced by low doses (<2,000 rad) of x-irradiation. It is possible, for example, that blockade of suppressor-effector induction at very low concentrations (≤ 10 nmol/ml) may be the result of extremely subtle changes induced in membrane receptors or protein synthesis because obvious effects on blastogenesis are not observed at these concentrations as they are when T cells are treated with 2,000-rad x-irradiation (16). The target T cell of CYP-induced suppressor blockade likewise remains undefined. Although these results conclusively demonstrate that low-dose 4-HC blocks the differentiation of suppressor precursors from within the OKT4⁺,OKT8⁻ subset, 4-HC may be acting directly on a mitogen-induced suppressor-precursor cell or on its inducer, both of which apparently share the same phenotype and are also radiosensitive as described by Thomas et al. (17, 19, 24). These data provide further evidence that the functional heterogeneity and regulation of human T cell populations may be analogous to the T cell network interactions that have been well described in murine systems (25). There is now evidence for a differentiated, mitogen-independent, OKT4⁻,OKT8⁺ radiosensitive suppressor/cytotoxic population that requires an OKT4⁺ inducer to mediate suppression (13, 17, 23), as well as for mitogen-induced OKT4⁺,OKT8⁻ suppressor cells, which retain the phenotype of the suppressor-precursor (17). In addition, the induction of suppressor function within the OKT4⁺,OKT8⁻ subset is itself both radiosensitive (19) and, as described in this report, sensitive to low doses of 4-HC. By analogy with murine T-T cell interactions, the OKT4⁺,OKT8⁻ target of both x-irradiation and low-dose 4-HC

may be the equivalent of the Lyt-123⁺, Qa1⁺ subset mediating nonimmune feedback suppression of Lyt-1 helper cells for antibody responses and which is sensitive to low-dose CYP in vivo (26). The future application of 4-HC as a probe of differential lymphocyte subset sensitivity in vitro, in combination with monoclonal antibodies, may be of great benefit in further dissecting T-B and T-T cellular interactions and immunoregulation in man.

Summary

The alkylating agent cyclophosphamide may suppress or enhance immune responses in vivo but is inactive in vitro unless metabolized by microsomal enzyme activation. 4-hydroperoxycyclophosphamide (4-HC) is a synthetic compound that is spontaneously converted in aqueous solution to the active metabolites. In this report, we examined the in vitro sensitivity of functional human T cell subsets to 4-HC in a polyclonal B cell differentiation assay and in the generation of mitogen-induced suppressor cells for effector B cell function. Con A-induced T suppression of B cell differentiation is completely abrogated by a 1-h pretreatment of T cells at very low concentrations of between 10^{-2} and 20 nmol/ml, whereas inducer T cell function is sensitive only to concentrations in >40 nmol/ml. The effects of 4-HC on suppressor T cells appear to occur at concentrations that do not result in DNA cross-linking or decreased blastogenesis. Con A-induced T suppressors are generated from within the OKT4⁺, OKT8⁻ subset and are sensitive to low-dose 4-HC only before activation, whereas differentiated suppressor cells are resistant to concentrations in >80 nmol/ml. Low-dose 4-HC pretreatment of the B cell population results in abrogation of immunoglobulin secretion when treated B cells are cocultured with unfractionated T cells, however, this effect is completely reversible if pretreated B cells are cocultured with T cells devoid of suppressor activity. These results demonstrate that human presuppressor cells for B-effector function differentiate in response to Con A from the OKT4⁺, OKT8⁻ subset and are exquisitely sensitive to low concentrations of CYP whereas mature suppressor and inducer functions are resistant to all but very high concentrations in vitro. The differential sensitivity of functional T and B cell subsets to 4-HC in vitro can be a very useful probe in dissecting immunoregulatory interactions in man.

We thank Dr. Gideon Goldstein of the Ortho Pharmaceutical Corp. for contributing the monoclonal antibodies and Mrs. Jeanne Kamysz for manuscript preparation.

Received for publication 8 September 1981.

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