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Evidence for the involvement of monocyte-derived toxic oxygen metabolites in the lymphocyte dysfunction of Hodgkin's disease

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SUMMARY

This study was performed to see if adherent cell-derived toxic oxygen metabolites contribute to the suppression of mononuclear cell blastogenic responses in Hodgkin's disease. Peripheral blood mononuclear cells from 10 patients with Hodgkin's disease were stimulated in culture with the mitogen PHA in the presence of the prostaglandin inhibitor indomethacin and the antioxidants catalase or vitamin E. Patient lymphocytes showed significant increases in PHA-induced proliferation at all PHA doses when cultured with indomethacin. Further augmentation of lymphocyte proliferation was achieved with the addition of catalase or vitamin E to indomethacin in the culture system. The increases in proliferation seen on culture with these agents were greatest in patients with more depressed initial PHA responses. When adherent cells were removed before culture, the agents no longer facilitated increases in proliferation. These data suggest that abnormal lymphocyte proliferative responses seen in Hodgkin's disease may result in part from the excessive production of toxic oxygen metabolites as well as prostaglandins by adherent cell populations.

INTRODUCTION

Some patients with Hodgkin's disease have a population of adherent mononuclear cells which can produce prostaglandin E_2 (PGE_2) in such quantities that *in vitro* lymphocyte proliferation to mitogens such as phytohaemagglutinin (PHA) is suppressed (Goodwin *et al.*, 1977a; Schechter, Wahl & Oppenheim, 1980). This suppression can be corrected in part *in vitro* either by removal of the adherent cells from the cell population or by the addition of the inhibitor of prostaglandin synthesis, indomethacin (Goodwin *et al.*, 1977a). Similarly, we have recently reported that the administration of oral indomethacin to Hodgkin's disease patients whose lymphocyte responses were depressed could in some cases correct this *in vitro* abnormality (deShazo, 1980). Our failure to correct these abnormalities completely or to influence delayed hypersensitivity skin test responses in these patients suggested that abnormalities other than or in addition to those produced by prostaglandin might exist.

Subsequent to that study, macrophage-derived oxygen products such as hydrogen peroxide have been implicated as mediators of macrophage-mediated suppression of lymphocyte proliferation (Metzger, Hoffeld & Oppenheim, 1980). This information suggested that antioxidant compounds known to protect cell membranes from toxic oxygen products when used in conjunction

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with indomethacin might effect greater improvement of cultured lymphocyte proliferative defects than that seen with indomethacin alone. This report presents observations which point out a probable role of toxic oxygen products in the lymphocyte dysfunction seen in Hodgkin's disease.

PATIENTS AND METHODS

Patients. Ten patients aged 15–35 years who were admitted for evaluation of primary or recurrent Hodgkin's disease volunteered to participate in this study. Clinical data concerning these patients are presented in Table 1. In addition, a group of 10 healthy, normal medical personnel aged 19–40 years volunteered to serve as normal controls.

Skin tests. Delayed hypersensitivity skin tests were performed by intradermal injection of 0·1 ml of each of the following antigens: Candida (1:100, 1:10, Hollister–Stier Laboratories, Spokane, Washington); streptokinase–streptodornase (4 U/1 U per ml and 40 U/10 U per ml, Lederle Laboratory, Pearl River, New York); mumps vaccine (full strength, Lilly Laboratories, Indianapolis, Indiana); intermediate strength PPD (5 TU, Parke–Davis Co., Detroit, Michigan); and trichophyton (1:30, Hollister–Stier). Tests were measured at 24 and 48 hr and read as positive if palpable induration of 10 mm or greater in diameter was present.

Lymphocyte blastogenic studies. Isolation procedures, incubations and measurement of lymphocyte proliferative responses to PHA were performed by standard microculture techniques as previously described (deShazo, 1980). Briefly, peripheral blood was collected in heparinized plastic syringes and the mononuclear cell fraction separated by density-gradient sedimentation over lymphocyte separation medium (LSM, Litton Bionetics Inc., Rockville, Maryland). The interface layer, containing greater than 95% mononuclear cells (MNC), was washed in Hanks' balanced salt solution before and after erythrocyte lysis with hypotonic saline. Cultures were prepared in enriched medium consisting of minimum essential medium (Microbiological Associates, Bethesda, Maryland) containing 2 mM glutamine, penicillin (50 units/ml), streptomycin (50 μg/ml) and 10% heat-inactivated fetal calf serum (Microbiological Associates). One hundred thousand MNC were cultured in each well of flat-bottomed microtitre plates in the presence of varying concentrations of PHA-P (DIFCO Laboratories, Detroit, Michigan). Other additions to the cultures, singly or in combination, included indomethacin, 1 μg/ml (gift of Dr Clement Stone, Merck, Sharp & Dohme Inc., West Point, Pennsylvania), catalase, 20,000 units/ml (bovine liver, twice crystallized, 32,000 Sigma units/ml), or vitamin E, 50 μg/ml (alpha-tocopherol, Sigma Chemical Co., St Louis, Missouri). The catalase was dialysed in order to eliminate any possible effects of thymol which is used as a preservative in this preparation. Indomethacin was dissolved in 95% ethyl alcohol at 10 mg/ml and diluted to a final concentration in which the ethyl alcohol was no greater than 0·1%, a concentration shown to have no effect on the blastogenic response of control cultures in Hodgkin's disease patients (Goodwin *et al.*, 1977a; deShazo, 1980). Vitamin E was similarly dissolved and diluted in enriched medium. Optimal non-toxic doses of additives were established in pilot studies and used thereafter.

Triplicate cultures were incubated at 37°C in 5% CO_2 for 72 hr. Cultures were pulsed with ^{3}H-thymidine (New England Nuclear Corp., Boston, Massachusetts, 1 μCi/well) at 48 hr, harvested onto glass-fibre filters 24 hr later with an automated harvester, and counted in a scintillation counter (Searle Model 81). Results were recorded as mean ± standard error of triplicate Δ c.p.m. where Δ c.p.m. equalled counts per minute in stimulated cultures minus background Δ c.p.m. obtained from mononuclear cells cultured in the absence of PHA (but which contained the same drug or drug combination, or medium). The standard error was generally less than 10% of the mean. Cell viability at 72 hr was greater than 85% as observed by trypan blue dye exclusion.

Removal of nylon wool-adherent cells. Separation columns were prepared by packing sterile nylon wool (0·3 g, Type 200, Fenwall Laboratories, Deerfield, Illinois) to a volume of 6 ml in 10-ml plastic syringes. After equilibration with enriched medium at 37°C for 1 hr, mononuclear cells ($1{\cdot}5 \times 10^8$) were added to the column and incubated at 37°C in 5% CO_2 for 45 min. The non-adherent cells were then eluted with 30 ml of enriched medium and washed (Folch, Yoshinaga & Waksman, 1973). The recovery was generally 30–40% of the initial mononuclear cell population

with >90% viability by trypan blue dye exclusion. Monocytes identified by alpha-naphthyl esterase staining (Yam, Li & Crosby, 1971) were less than 5% in the eluted, non-adherent MNC populations.

Data analysis. Results are reported as mean (±s.e.m.) per cent change in PHA response where:

$$\% \text{ change in PHA-induced blastogenic response} = \frac{\text{PHA response with drug(s)} - \text{PHA response without drug(s)}}{\text{PHA response without drug(s)}} \times 100$$

The calculated mean per cent change is the algebraic mean ± s.e.m. of the individual patient values. The individual patient values were calculated as the mean of triplicate stimulated cultures less unstimulated control cultures at each PHA dose. Numerical data between groups in this study were analysed using the paired Student *t*-test (Ostle, 1976).

RESULTS

Lymphocyte responses of the patient population

As listed in Table 1, five of the 10 Hodgkin's disease patients studied (patients 1–5) had PHA responses at 1, 2, 5, 10, 20 and 50 μg/ml of PHA which were less than 50% of the MNC PHA response of simultaneously cultured normal controls. This 'hyporeactive group' included two previously treated patients. Only three of the 10 patients were lymphopenic (<1,500 lymphocytes/mm^3) and total monocyte counts were normal except in two patients who had a monocytosis (>950 monocytes/mm^3).

Effects of monocyte depletion on proliferative responses

Lymphocyte proliferative responses to PHA were studied in three normal individuals and three Hodgkin's disease patients before and after passage of mononuclear cell populations through nylon-wool columns. As previously demonstrated (Goodwin *et al.*, 1977a), PHA-induced normal lymphocyte proliferation (across the range of six PHA doses) decreased (mean −61 ± 13%), but patient lymphocyte proliferation increased (mean +34 ± 10%) after depletion of adherent cells from cultures.

Table 1. Clinical characteristics of Hodgkin's disease patients in this study

Patient	Hodgkin's disease stage/type	Time since last treatment (years)	Positive delayed skin tests	Per cent of normal PHA response	Total lymphocyte count/mm^3*	Total monocyte count/mm^3*
1	4B/NS recurrent	1	0/5	4	584	1,460
2	2B/NS recurrent	1	0/5	6	945	805
3	4B/MC	None	0/5	6	1,944	396
4	2B/NS	None	1/5	28	1,548	690
5	3B/NS	None	1/5	31	2,846	333
6	2A/NS	None	2/5	50	1,488	558
7	3A/NS	None	1/5	53	3,367	1,813
8	3A/NS	None	1/5	57	2,033	428
9	3A/NS	None	2/5	73	2,016	756
10	4B/NS	None	3/5	108	2,745	510

* Wright's stain preparations.
MC = mixed cellularity; NS = nodular sclerosing.

Table 2. Per cent increase in PHA-induced proliferative response in normal volunteers and Hodgkin's disease patients

	Agents added to PHA in culture								
	Indomethacin (1 μg/ml)			Indomethacin (1 μg/ml)+ catalase (20,000 units/ml)			Indomethacin (1 μg/ml)+ vitamin E (50 μg/ml)		
PHA dose (μg/ml)	Normals ($n=10$)	All patients ($n=10$)	Hyporeactive patients ($n=5$)	Normals ($n=10$)	All patients ($n=10$)	Hyporeactive patients ($n=5$)	Normals ($n=10$)	All patients ($n=10$)	Hyporeactive patients ($n=5$)
1	8±3*	94±29	143±43	28±11	158±39	147±48	10±7	143±35	222±52
2	7±2	66±19	110±29	19±8	83±24	124±45	16±6	99±29	192±55
5	12±3	50±7	89±18	25±9	67±17	102±28	12±4	83±25	147±29
10	9±4	64±17	99±28	19±6	86±22	138±38	15±6	81±28	143±47
20	8±5	50±12	85±26	25±9	82±15	114±24	19±7	68±20	117±32
50	6±4	51±12	65±11	36±9	71±11	93±17	30±8	59±18	94±23

* Per cent increase at each PHA dose (mean±s.e.m.).

Effect of indomethacin, catalase and vitamin E on patient lymphocyte responses to PHA

As seen in Table 2, 1 μg/ml of indomethacin significantly ($P < 0.05$) increased the proliferative responses of lymphocytes of normals and patients across the range of PHA doses tested. Enhancement was greater in patients than in normals ($P < 0.05$) and greater in 'hyporeactive' patients than the group of 10 patients as a whole ($P < 0.05$).

The antioxidants catalase and vitamin E alone produced small increases in lymphocyte proliferation of both patients and normals. These increases were not significantly different between the two groups ($P > 0.05$). Patient responses averaged an increase of $8 \pm 2\%$ with 50 μg/ml of vitamin E alone and $6 \pm 6\%$ with 20,000 units/ml of catalase alone across the range of six PHA doses. Control responses increased an average of $7 \pm 2\%$ with vitamin E alone and $12 \pm 3\%$ with catalase alone.

Synergistic effects of indomethacin and antioxidants

Next, because of animal studies reporting synergistic effects of catalase and indomethacin on the reversal of the inhibition of suppressor macrophage function (Metzger *et al.*, 1980), combinations of antioxidants and indomethacin were added to lymphocyte cultures (Table 2). Greater augmentation of proliferative responses ($P < 0.05$) occurred with indomethacin (1 μg/ml) plus catalase (20,000 units/ml) or indomethacin plus vitamin E (50 μg/ml) than with indomethacin alone. This was true with cell cultures from both normal and Hodgkin's disease patients; however, increases in patient lymphocyte responses were greater ($P < 0.05$) in the hyporeactive group than in the patient group as a whole. Likewise, responses in the presence of indomethacin and vitamin E were significantly ($P < 0.05$) greater in the hyporeactive group than in the patient group as a whole. Representative data from patient 6 are shown in Fig. 1.

Effects of monocyte depletion on antioxidant-mediated proliferative enhancement

Studies in three patients with normal responses to optimal concentrations of PHA confirmed previous reports (Goodwin *et al.*, 1977a) that increases in patient lymphocyte proliferation by indomethacin no longer occur when adherent cells are removed from the MNC population prior to culture. Mean increase in lymphocyte proliferation with indomethacin in our three patients was $51 \pm 6\%$ at PHA 10. This increase dropped to $2{\cdot}7 \pm 1\%$ when adherent cells were removed prior to culture. Removal of adherent cells prior to culture also abrogated the additional augmentation of

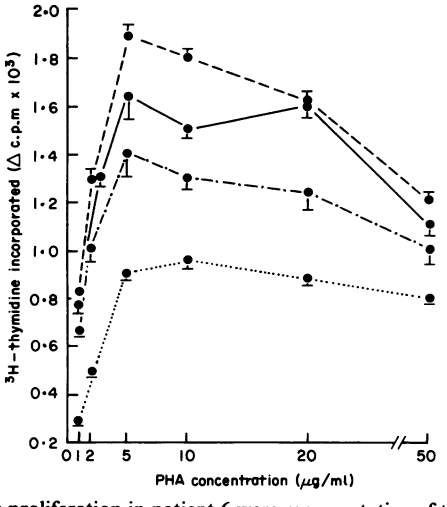

Fig. 1. Changes in lymphocyte proliferation in patient 6 were representative of those of the group as a whole. Data are presented as mean ± s.e. of triplicate Δ c.p.m. values. (●····●) Response to PHA alone, (●-----●) with indomethacin (1 μg/ml), (●—●) with indomethacin (1 μg/ml) and catalase (20,000 units/ml), and (●— — —●) with indomethacin (1 μg/ml) and vitamin E (50 μg/ml).

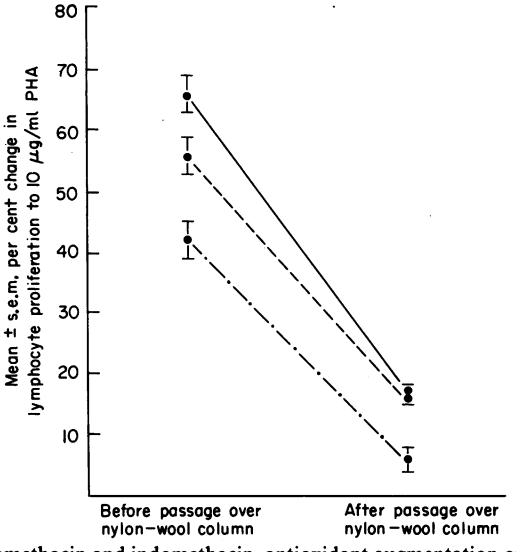

Fig. 2. Abrogation of indomethacin and indomethacin–antioxidant augmentation of lymphocyte proliferation to PHA by removal of adherent cells from culture prior to the addition of these agents. (●–·–·–●) indomethacin (1 μg/ml), (●---●) indomethacin (1 μg/ml) and vitamin E (50 μg/ml), and (●———●) indomethacin (1 μg/ml) and catalase (20,000 units/ml).

blastogenic responses by indomethacin–antioxidant combinations. Augmentation by indomethacin–vitamin E dropped from 76±5% to 6±3% after adherent cell removal. Augmentation by indomethacin–catalase similarly dropped from 84±6% to 12±3%. Data from a representative patient are shown in Fig. 2.

DISCUSSION

Evidence for enhanced production of prostaglandins (PG) by glass-adherent mononuclear cells has been noted in tumour-bearing animals (Pelus & Bockman, 1979) and man (Goodwin *et al.*, 1977a). Among the many regulatory effects exercised by prostaglandins on the immune system are inhibition of PHA-induced lymphocyte proliferative responses by E-series PG (Goodwin & Webb, 1980). These prostaglandins are produced in abnormally high concentrations by adherent mononuclear cells from patients with Hodgkin's disease (Goodwin, Bankhurst & Messner, 1977b). These high PG concentrations have provided a possible explanation for reported abnormalities of lymphocyte proliferative responses (Levy & Kaplan, 1974) in some Hodgkin's disease patients. Although we postulate that such PG production in Hodgkin's disease may result from macrophage 'activation' (Metzger *et al.*, 1980), macrophage-mediated suppression might also be produced by increases in the number of macrophages in the cultured population. Although differential counts of patient peripheral blood showed only two of our patients with absolute monocyte counts greater than 950/mm^3, there are data to suggest that mononuclear cell preparations prepared by density-gradient centrifugation from Hodgkin's disease patients may be unintentionally enriched with monocytes (Twomey *et al.*, 1980). Thus it is reasonable to assume that increases in both the macrophage number and activity may contribute to the suppressive effects noted here and elsewhere (Levy & Kaplan, 1974).

Our ability to correct depressed *in vitro* lymphocyte proliferation in Hodgkin's disease patients only partially by either *in vitro* or *in vivo* indomethacin therapy (deShazo, 1980) suggested that other, perhaps cytotoxic, macrophage-derived products (Nathan *et al.*, 1979) might also be involved in the abnormalities under consideration. These products include the highly reactive

oxygen metabolites hydrogen peroxide, superoxide, singlet oxygen, and hydroxyl radical which, like prostaglandin E_2, are produced in increased amounts by activated macrophages (Paswell, Dayer & Merler, 1979; Gemsa *et al.*, 1978; Babior, 1978; Johnson *et al.*, 1975; Weiss, King & LoBuglio, 1977). We therefore chose to study the effects of indomethacin, an inhibitor of prostaglandin synthesis (Vane, 1971) alone or in combination with either a free-radical scavenger, vitamin E (Baehner *et al.*, 1977; Oski, 1977), or with catalase, an enzyme promoting the degradation of H_2O_2 (Schonbaum & Chance, 1976).

Lymphocyte proliferative responses in the study group ranged from severely depressed to normal. Five of the 10 patients had responses less than 50% of those of normal controls across the PHA dose–response curve. Further studies to characterize the population showed that removal of adherent cells from the cultured mononuclear cell population enhanced proliferative responses in Hodgkin's disease patients while severely inhibiting those of normals (Schechter & Schonlen, 1978; Siblett, Bankhurst & Williams, 1978), thus confirming the inhibitory effects of adherent MNC from patients with Hodgkin's disease. Treatment of patient MNC with 1 μg/ml of indomethacin dramatically increased their proliferative responses while producing a lesser, although consistent, augmentation of normal MNC proliferation. Culture of PHA-stimulated MNC from either patients or normal individuals with either catalase (20,000 units/ml) or vitamin E (50 μg/ml) alone produced similar small increases in proliferation. However, incubation of patient MNC with either vitamin E or catalase in combination with indomethacin produced significantly augmented proliferation over that seen with indomethacin alone ($P < 0{\cdot}05$). Responses to both combinations were greater ($P < 0{\cdot}05$) in the five hyporeactive patients than in the group as a whole. The dependence of these effects on the macrophage was demonstrated by removal of the enhancing effect of these drugs when adherent cells were depleted from the MNC population. These findings parallel results in which activated murine macrophages are capable of suppressing lymphocyte proliferative responses *in vitro* (Metzger *et al.*, 1980). Catalase, while only minimally effective alone in blocking the suppressive effects of activated macrophages on lymphocyte proliferation, has a striking synergistic effect when combined with indomethacin (Metzger *et al.*, 1980). In those animal studies, this combination was capable of complete abrogation of the macrophage-mediated suppression. In our study the free-radical scavenger vitamin E was more effective in cultures from hyporesponsive patients than was catalase.

The apparent interplay between PG synthesis and oxygen radical generation by activated macrophages may be explained by independent observations reported from several laboratories. Hydrogen peroxide or lipid peroxides, which result from the action of oxygen radicals on cell membrane unsaturated lipids, have been reported to stimulate directly the cyclo-oxygenase enzyme (Hemler, Cook & Lands, 1979). Conversely, the addition of E-series prostaglandins to macrophages actively generating oxygen radicals inhibits oxygen radical production (Weidemann *et al.*, 1978). These data would suggest that the effect of indomethacin alone on activated macrophages would be the removal of the prostaglandin-mediated depression of oxygen radical production. This would lead to enhanced oxygen radical generation, the deleterious effects of which could be overcome by radical scavenging antioxidants, such as vitamin E. Similarly, the vitamin E alone could prevent the formation of the oxygen radical-induced hydrogen peroxide and membrane lipid peroxides, both of which can augment prostaglandin synthesis. These complex interactions may explain why indomethacin and vitamin E seem to be more effective in the removal of suppressive prostaglandins and oxygen radicals than either agent alone. In this regard, several relationships in Hodgkin's disease culture systems require further study. These include relationships between numbers of circulating monocytes and their state of activation, monocyte numbers in cultured populations, and the quantitation of specific monocyte toxic oxygen metabolite and prostaglandin levels.

The possibility that treatment of Hodgkin's disease patients with both indomethacin and free-radical scavengers such as vitamin E may augment depressed delayed hypersensitivity responses warrants further study. The involvement of macrophage-derived free radicals in other chronic inflammatory conditions with similar lymphocyte abnormalities is the subject of ongoing research in our laboratories.

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