

Studies of Cell Subpopulations Mediating Mitogen Hyporesponsiveness in Patients with Hodgkin's Disease

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ABSTRACT Hodgkin's disease (HD) is associated with a deficit in T-cell immunity characterized by skin test anergy and decreased lymphocyte responses to phytohemagglutinin (PHA). To investigate this mitogen hyporesponsiveness in HD, we separated peripheral blood mononuclear cells on Ficoll-Hypaque gradients and determined their response to various suboptimal concentrations of PHA. As was expected, patients with HD demonstrated marked mitogen hyporesponsiveness relative to normal controls; however, if the cell suspensions were first passed through glass wool columns to remove adherent cells, the PHA responsiveness of the hyporesponsive HD cells was markedly increased. In contrast, the responsiveness of normal controls was decreased so that the responses of nonadherent normal and HD cells were statistically indistinguishable. Evidently, a glass wool-adherent suppressor cell had been removed from patients with HD, while a glass wool-adherent cell which enhanced mitogenic responses had been removed from normal controls during column passage. Previous to column depletion, patients with HD had decreased proportions of E-rosettes and increased proportions of cells with surface α -fetoprotein; however, the proportion of these cells was not changed after column passage. Significant changes with column depletion of glass wool-adherent cells in HD were recorded in the proportions of monocytes (13.2 vs 5.8%) and lymphocytes with C-3 receptors (12.6 vs. 7.8%). The only significant change in normal controls was a decrease in the proportion of monocytes (10 vs. 1.7%). To determine if glass-adherent cells would have a suppressor effect, HD-adherent cells were added in progressively increasing numbers to mononuclear cell suspensions depleted of glass wool-adherent cells. PHA responsiveness returned

toward predepletion levels. In summary, patients with HD possess a glass wool-adherent suppressor cell which is responsible at least in part for in vitro mitogen hyporesponsiveness.

INTRODUCTION

Patients with Hodgkin's disease (HD)¹ are known to have a marked depression in T-cell responsiveness (1). The immunologic defect of early active HD is characterized by depression of delayed hypersensitivity and decreased homograft reactions (2, 3). Antibody formation is largely intact and peripheral lymphocyte counts are normal or slightly decreased, while a loss of phytohemagglutinin (PHA) responsiveness of lymphocytes in HD has been reported even before peripheral lymphocyte depletion has occurred (4). Thus a defect of T-lymphocyte reactivity is well established, but its role in the pathogenesis of the disease itself is uncertain.

Decreased responses by lymphocytes to mitogens, decreased delayed skin hypersensitivity, and decreased immunoglobulin levels have all been correlated with the severity and clinical stage of the disease (5). It seems possible that a suppressor cell subpopulation could be responsible in part for these observations in light of recent data demonstrating the contribution of suppressor T cells to immunologic suppression observed in fungal infections and some neoplastic or immunodeficiency diseases (6-9).

The present study was designed to examine the effect of selective depletion of certain cell populations on PHA responsiveness of peripheral blood lymphocytes in normal subjects and patients with HD. Previous evidence has been accumulated in both mouse (10-13) and man (6-9, 14, 15) for the existence of cells which suppress immunologic function. The cur-

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¹ Abbreviations used in this paper: AFP, α -fetoprotein; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; HBSS, Hanks' balanced salt solution; HD, Hodgkin's disease; PHA, phytohemagglutinin; ZY-C3, zymosan-C3.

rent study presents evidence that suppressor cells may indeed be involved in the mitogen hyporesponsiveness found in many patients with HD and provides preliminary characterization of these suppressor cell populations.

METHODS

Patients

10 patients with active HD and 8 normal controls were studied. All patients had active disease documented by physical examination, laparotomy, lymphangiography, spleen and liver scans, bone marrow aspiration, and characteristic tissue pathology. Patients with various histological HD subclasses and in different stages were included but were carefully selected so that they were receiving either no therapy or only local nodule irradiation. None were receiving or had recently received chemotherapy. Clinical and laboratory profiles are shown in Table I. Each patient received a battery of skin test antigens including purified protein derivative, mumps, trichophyton, streptodornase-streptokinase, and *Candida Albicans*. Concentrations of antigens and procedures for this skin testing have been previously described (16). Peripheral blood smears and total leukocyte counts were done to establish lymphocyte counts.

Cell cultures

Lymphocytes were cultured in RPMI 1640 supplemented with L-glutamine, penicillin-streptomycin, and 10% fetal calf serum (FCS) (Grand Island Biological Co., Grand Island, N. Y.). The PHA (Difco Laboratories, Detroit, Mich.) was titrated to determine a dose-response curve. A suboptimal range of PHA concentrations was subsequently used in the experiments which followed. Each microtiter well contained 1.5×10^5 cells. Samples were studied in duplicate and were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO₂ for 72 h. For evaluation of mitogen reactivity, 0.2 μ Ci of tritiated thymidine was added for the last 8 h of culture. Cultures were harvested after pulsing on a Mash II Harvester on glass wool filters and were counted in a liquid scintillation counter.

Removal of glass wool-adherent cells

Glass wool (Pyrex Wool, Corning Glass Works, Science Products Div., Corning, N. Y.) was packed to 8.0 ml in 10-ml plastic syringes and equilibrated with undiluted FCS. 5 ml of a cell suspension (20×10^6 cells) was incubated on the column at 37°C for 30 min and then eluted with 30 ml of warm FCS and washed (17). The yield of purified cells was generally 50%, with 99% viability as measured by trypan blue exclusion.

TABLE I
Clinical Profile of Patients with Hodgkin's Disease

Patient	Stage of disease	Skin Tests					Peripheral blood lymphocyte count	PHA responsiveness (percent of normal controls)§	Therapy (recent or current)
		Mumps	SS*	Trichophyton	PPD	Candida			
							cells/mm ³ †	%	
B. B.	IV	—	—	—	—	—	600	25	Local nodule irradiation (500 rad)
A. S.	II	—	—	—	—	—	980	75	Local nodule irradiation (500 rad)
C. A.	IV	—	+	—	—	—	600	5	Local nodule irradiation (500 rad)
D. W.	II	—	+	—	—	—	1,140	4	Local nodule irradiation (500 rad)
A. M.	IV	—	—	—	—	—	1,100	8	None [‡]
M. S.	III	—	—	—	—	—	1,300	8	None
D. J.	IV	—	—	—	—	—	240	122	None
J. W.	II	+	+	—	—	—	2,420	16	None
M. S.	III	+	+	—	—	—	2,010	8	Local nodule irradiation (500 rad)
S. S.	III	—	—	—	—	—	1,200	32	None

* Streptodornase-streptokinase.

† Cells/mm³ (normal range: $2,072 \pm 67$, mean \pm SEM).

§ Percentage of normal controls at 10 μ g/ml PHA.

[‡] These patients were untreated.

Preparation of adherent cell suspensions

Peripheral blood mononuclear cells were suspended in Hanks' balanced salt solution (HBSS), placed in plastic petri dishes, and incubated at room temperature for 45 min. Non-adherent cells were decanted, and the remaining adherent cells were washed three times with HBSS. Adherent cells were resuspended with gentle agitation by a rubber policeman and washed three times with phosphate-buffered saline. This procedure yielded a preparation of approximately 30–50% monocytes as identified by dibenzidine hydrochloride staining (18). The remaining nonmonocyte cells were lymphocytes showing minor changes in the distribution of cell surface markers in comparison with fractionated suspensions. In several experiments, indomethacin (Sigma Chemical Co., St. Louis, Mo.) at a final concentration of 10 μ g/ml was added to cultures.

Cell surface markers

Identification of monocytes. In all the succeeding assays, monocytes were identified by phagocytized latex beads. Latex beads (1.1 μ m, Dow Diagnostics, Indianapolis, Ind.) were diluted 1:100 in HBSS and added to an equal volume of mononuclear cells at 10×10^6 cells/ml which were then incubated at 37°C for 30 min. Monocytes were identified by cytoplasmic inclusion of latex beads and excluded from lymphocytes surface marker assays. The number of monocytes identified by latex ingestion correlated closely with cells identified by dibenzidine staining (18).

Spontaneous sheep erythrocyte rosette formation (E-rosettes). Fresh sheep erythrocytes (Colorado Serum Co., Denver, Colo.) were washed three times with HBSS with 5% FCS. 0.1 ml of mononuclear cells (10×10^6 cells/ml) prepared on Ficoll-Hypaque gradients were added to 0.3 ml of 1% sheep erythrocytes in 75 \times 12-mm glass tubes and incubated for 30 min at 37°C. The tubes were centrifuged at 200 g for 10 min and placed at 0°C overnight. The pellet was gently resuspended with a Pasteur pipette and rosettes were counted on a hemocytometer (19).

Lymphocytes forming bovine erythrocyte (IgG-coated) rosettes (EA-IgG). Bovine erythrocytes (Colorado Serum Co.) were washed four times with HBSS and then the packed cells were adjusted to 5%. An equal volume of rabbit anti-bovine erythrocytes (IgG) (Cappel Laboratories, Inc., Downingtown, Pa.) diluted 1:50 was added to the 5% erythrocyte suspension and the mixture was incubated at 37°C for 1.5 h and subsequently washed four times with HBSS. The IgG fraction of the anti-bovine erythrocyte antiserum was not contaminated by other immunoglobulins detectable by immunodiffusion techniques. The sensitizing dilution of IgG was that concentration which produced a maximum number of rosettes on a subagglutinating plateau titration curve of sensitizing antibody. The sensitized erythrocytes were then diluted to 2% and added in equal volume to a Ficoll-Hypaque-enriched lymphocyte suspension (4×10^6 cells/ml) in 12 \times 75-mm glass tubes which were then centrifuged at 200 g for 5 min. The tubes were incubated first at room temperature for 15 min and then at 0°C for 30 min. The erythrocyte pellet was gently resuspended with a Pasteur pipette and the percent rosettes read on a hemocytometer (200 cells counted) (20).

Lymphocytes forming bovine erythrocyte (IgM-coated) rosettes (EA-IgM). Peripheral blood lymphocytes were separated as described earlier and placed into culture at 0.5×10^6 cells/ml in medium 199 with 20% FCS supplemented with penicillin and streptomycin. The cells were cultured for 12 h at 37°C. Bovine erythrocytes were coated

with IgM rabbit anti-bovine erythrocyte antibody as follows: A 2% suspension of washed bovine erythrocytes was added to 0.4 ml of a 1:40 dilution of rabbit anti-bovine erythrocyte IgM preparation, incubated at room temperature for 30 min, washed three times with HBSS, and finally diluted at 0.4 ml (21). The IgM antibody was prepared by i.v. immunization of rabbits with bovine erythrocyte stroma. Bleedings were obtained at 10–14 days. The IgM was isolated by DEAE-column chromatography and subsequently subjected to immunoabsorption with insolubilized goat anti-rabbit IgG to remove all IgG. Acrylamide-gel analysis of these IgM preparations showed essentially no IgG contamination. An equal volume of cultured lymphocytes at 4×10^6 cells/ml and IgM-coated bovine cells were combined, centrifuged at 200 g for 5 min, and incubated at 0°C for 1 h. The pellet was gently resuspended and read on a hemocytometer (200 cells counted).

Zymosan-C3 receptors (Zy-C3). Zymosan (Sigma Chemical Co.) was boiled for 60 min in phosphate-buffered saline, diluted to 1 mg/ml, and incubated with fresh normal human serum at 0.5 ml of serum/mg zymosan for 30 min at 37°C. The zymosan was washed three times with HBSS, diluted to 0.5 mg of zymosan/ml, and mixed with equal volumes of lymphocytes at 4×10^6 cells/ml in 12 \times 75-mm glass tubes. The tubes were centrifuged at 600 g for 5 min, incubated for 30 min at 0°C, and subsequently read on a hemocytometer (22). Concentrations of reactants in this assay were chosen so that a maximum number of zymosan-C3 rosettes were produced.

Lymphocytes with surface α -fetoprotein (AFP). An equal volume of purified AFP (0.75 mg in 1.0 ml of saline, gift of Dr. S. Yachnin, Chicago, Ill.) was mixed with an equal volume of complete Freund's adjuvant and injected into four subcutaneous sites of a virgin, white, female New Zealand rabbit. 2 and 4 wk later, the rabbit received identical immunizations. 10 days after the last immunization, the rabbit was bled. The rabbit anti-AFP antiserum was absorbed two times (equal volumes of absorbent and antiserum) at 0°C for 1 h with glutaraldehyde-insolubilized normal human serum. The IgG fraction of the antiserum was isolated by DEAE ion-exchange chromatography and digested with pepsin (23). The F(ab')₂ fragment was isolated by Sephadex G-150 (Pharmacia Fine Chemicals, Inc.) gel chromatography and its purity verified by immunodiffusion. The F(ab')₂ fragment was then conjugated with fluorescein isothiocyanate (FITC) (24) and after conjugation showed an F/P ratio of 1.5–3.0. The assay for lymphocytes with surface AFP was established with a dilution of FITC F(ab')₂-anti-AFP which produced a maximum number of fluorescent cells on a plateau titration curve. 0.1 ml of FITC F(ab')₂-anti-AFP (0.8 mg/ml) was incubated with 0.1 ml of lymphocytes (10×10^6 /ml) in 12 \times 75-mm glass test tubes for 30 min on ice, washed twice with cold HBSS, and examined immediately by ultraviolet microscopy with a Zeiss fluorescence microscope with epillumination (Carl Zeiss, Inc., New York). At least 200 cells were counted.

Identification of lymphocyte surface immunoglobulin Lymphocyte surface Ig was identified by a direct fluorescent technique. 0.1 ml (10×10^6 cells/ml) lymphocytes were incubated at 0°C for 30 min with 0.1 ml (1 mg/ml in phosphate-buffered saline) of a fluoresceinated F(ab')₂ fragment of rabbit anti-human F(ab')₂. This was followed by two washes with phosphate-buffered saline, and at least 200 cells were counted under the fluorescent microscope. The preparation of these reagents and use of this technique have been described previously (24).

Labeling of bovine cells with FITC. 5 mg of FITC was dissolved in 1 ml of 0.12 M NaHCO₃ buffer at pH 9.23. 1 ml of this solution was mixed with 0.5 ml of packed bovine

erythrocytes; the mixture was incubated for 30 min at 20°C and subsequently washed four times with HBSS (25).

Double surface marker assays. The double marker studies used a combination of E-rosettes and EA-IgM or EA-IgG and were done under the same conditions as the single marker EA studies. 0.1 ml of lymphocytes, 0.1 ml of fluoresceinated bovine erythrocytes, and 0.1 ml of unlabeled sheep erythrocytes were incubated in the double marker assay. These double markers were then read under the fluorescent microscope with the numbers of single and double rosettes being recorded separately. The single marker assays were always done in parallel to make certain that the markers did not interfere with one another. The proportion of E-rosettes detected in this double marker assay did not significantly differ from the proportion of E-rosettes detected by conventional overnight E-rosette assay.

Microscopy

The preparations were examined with a Zeiss photomicroscope equipped with a vertical illuminator IIIRS and a mercury light source (Carl Zeiss, Inc.). The following filter combination was used for fluorochrome visualization: BG12 exciter filter, FL500 chromatic beam-splitter, and barrier filter 50. Experiments evaluating the percentage of labeled lymphocytes in cell suspensions were performed by counting fluorescent cells in the ultraviolet field and then switching to light field illumination to count the total cell numbers.

RESULTS

The clinical and laboratory characteristics of the 10 patients with HD are shown in Table I. Five patients were anergic to all skin test antigens while nine patients had decreased PHA responses.

PHA response of normal subjects and patients with HD. The dose responses to PHA of eight patients with HD and six normal subjects were compared (Fig. 1 A). It is apparent that the patients with HD were hyporesponsive in comparison with normal controls at PHA concentrations between 0 and 10 $\mu\text{g/ml}$ ($P < 0.01$ at all PHA concentrations). When lymphocytes were cultured for 24 h in the absence of mitogens before assaying their PHA response, there was still a highly significant depression of responsiveness in patients with HD vs. normal controls ($P < 0.05$ at all PHA concentrations).

The effect of glass wool column passage on PHA responsiveness. In an effort to remove a suppressor cell population from cells of patients with HD, lymphocyte suspensions were passed through glass wool columns. The response of six normals was decreased while the response of the eight patients with

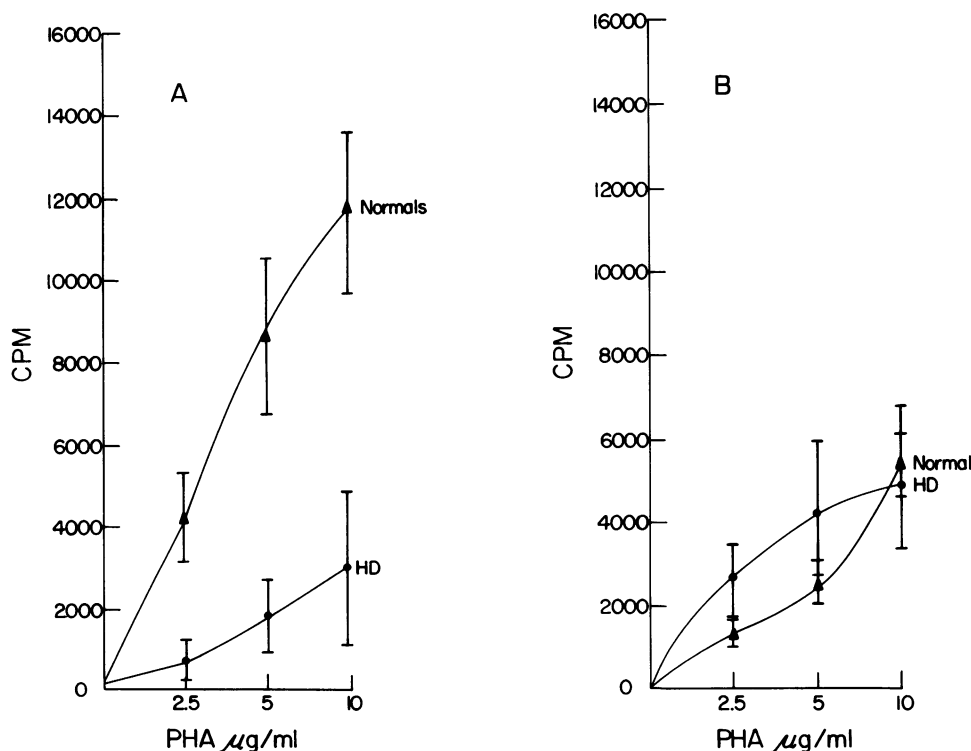


FIGURE 1 (A) PHA response of normal controls (six individuals) and patients with HD (8 individuals) expressed as mean cpm \pm 1 SD. (B) PHA response (mean cpm \pm 1 SD) of normal controls (six individuals) and patients with HD (eight individuals) after passage of cell suspensions through glass wool columns.

HD was enhanced (Fig. 1 B). Two sets of cultures were always done in parallel, one with glass wool-depleted lymphocytes (Fig. 1 B) and the other with nondepleted lymphocytes (Fig. 1 A). The glass wool-depleted cells from both groups demonstrated no statistical difference in their response ($P < 0.3$) to all mitogen concentrations. In other words, glass wool depletion decreased mitogen responsiveness in normal and increased responsiveness in patients with HD to the extent that glass wool-depleted lymphocytes from patients and normals were statistically indistinguishable.

Comparison of surface markers in Hodgkin's disease patients vs. normal controls. Seven patients with HD and five normal subjects were compared as to differences in proportions of lymphocytes with surface markers. As demonstrated in Table II, there were no significant differences between patients and normals in lymphocytes with Zy-C3 receptors and lymphocytes with surface immunoglobulin. However, there was a significant difference ($P < 0.01$) between patients and normals in the proportions of lymphocytes forming E-rosettes and possessing surface AFP. Patients with HD formed E-rosettes equal to $36.2 \pm 21.3\%$ (mean \pm SD) and surface AFP of $11.0 \pm 3.5\%$, while normals had $65.7 \pm 7.6\%$ and $4.6 \pm 1.2\%$, respectively. According to double-marker assays, approximately 24% of lymphocytes with surface AFP in patients with HD formed E-rosettes. Virtually all lymphocytes bearing receptors

for IgM also formed E-rosettes in both normal controls and patients with HD. Lymphocytes were then evaluated as to the changes in the proportion of surface markers after depletion of glass wool-adherent cells. Table II shows that with normal individuals the only significant change in proportions of surface markers was a marked decrease in monocytes (10.0 to 1.7%) ($P < 0.05$); however, Zy-C3 receptor-bearing cells also decreased from 12.6 to 7.8% ($P < 0.05$) in marked contrast to the lack of decrease in normals. E-rosette lymphocytes and cells bearing surface AFP remained essentially unchanged after glass wool depletion in both normal and patient populations. Thus, the only significant changes occurring after depletion of glass wool-adherent cells were a decrease in proportions of monocytes and cells bearing the Zy-C3 receptor in patients with HD. In normal subjects, the only change after column depletion was a decrease in the proportion of monocytes. The profile of surface markers among the adherent cells was also examined. These results are shown in Table III. When the profile of surface markers of adherent cells was compared with nonadherent cells, the only significant differences included an increase in cells with Zy-C3 receptors in the adherent cells and, as expected, an increase in monocytes among the same populations.

Studies on the addition of adherent cells to cultures depleted of adherent cells. Leukocyte preparations

TABLE II
Surface Markers of Normal Controls and Patients with Hodgkin's Disease
before and after Depletion of Glass Wool-Adherent Cells

	Normals		HD	
	Before	After	Before	After
E-rosette	65.7 \pm 7.6*	62.3 \pm 6.4	36.2 \pm 21.3	34.4 \pm 11.9
Surface AFP	4.6 \pm 1.2†	3.7 \pm 3.1	11.0 \pm 3.5	9.6 \pm 2.1
EA-IgG	12.3 \pm 3.5§	16.0 \pm 11.0	16.3 \pm 13.6	10.3 \pm 8.4
EA-IgM	14.0 \pm 1.7	13.6 \pm 2.6	11.8 \pm 9.3	10.2 \pm 6.8
Surface immunoglobulins	7.7 \pm 2.3	6.0 \pm 2.0	12.6 \pm 4.3	12.8 \pm 7.1
Zy-C3	9.0 \pm 1.0¶	9.7 \pm 2.5	12.6 \pm 5.0	7.8 \pm 2.4¶
Monocytes	10.0 \pm 1.0	1.7 \pm 0.6¶	13.2 \pm 8.7	5.8 \pm 5.5¶
Combined E-rosette and AFP				
a. E-rosette alone	61.6 \pm 7.4	ND	35.6 \pm 20.5	ND
b. AFP alone	2.0 \pm 1.7	ND	7.0 \pm 3.5	ND
c. cells bearing both markers	1.7 \pm 1.2	ND	2.2 \pm 1.3	ND
Combined E-rosette and EA-IgM				
a. E-rosette alone	51.0 \pm 8.7	ND	30.6 \pm 20.3	ND
b. EA-IgM alone	2.0 \pm 1.0	ND	0.6 \pm 0.8	ND
c. cells bearing both markers	16.3 \pm 7.1	ND	10.0 \pm 2.4	ND

ND, no difference.

* Results expressed in percentages \pm 1 SD.

† Mean \pm 1 SD of a larger number of normal controls (8) = 2.8 ± 1.0 .

§ Mean \pm 1 SD of a larger number of normal controls (8) = 15 ± 5.0 .

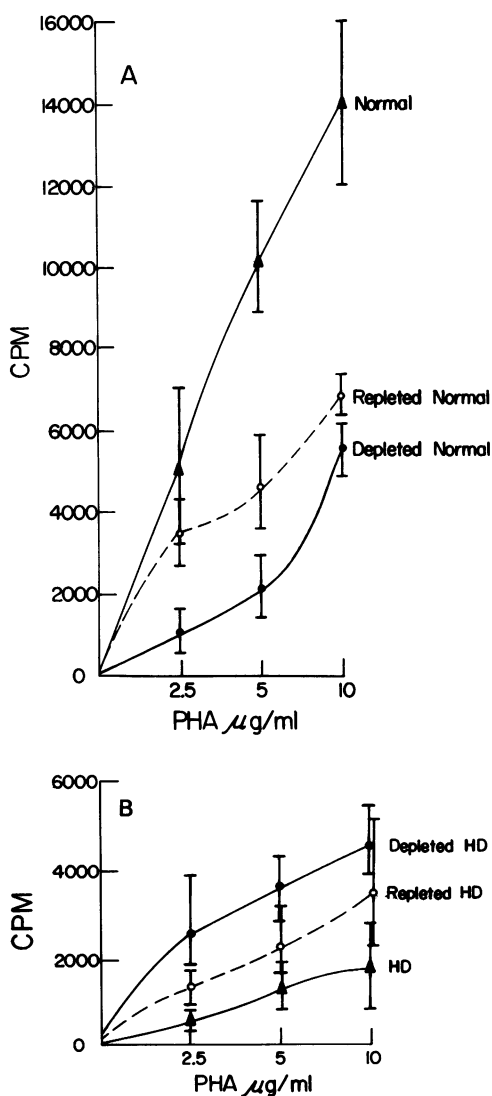
¶ Mean \pm 1 SD of a larger number of normal controls (8) = 13 ± 4.8 .

¶ These percentages are statistically different from nondepleted cell suspensions ($P < 0.05$).

TABLE III
Profile of Cell Markers on Nondepleted, Non-glass-adherent, and Glass-adherent Mononuclear Cell Populations

	E-rosette	EA-Ig	Surface immunoglobulins	Zy-C3	Monocytes
Undepleted					
Normal	69±7.0*	10±2.1	7±3	10±0.7	10±1.4
HD	27±7.9	18±10	11±1.1	11±2.3	14±6.0
Depleted					
Normal	66±1.4	7±1	10±2.8	8±2	2±0
HD	32±9.6	11±7.8	15±8.1	7±4	4±1
Adherent					
Normal	65±7.0	14±1.4	11±4.2	15±2.8	27±3.5
HD	21±7.8	19±7.6	12±3.6	13±3.6	28±2.8

* Results expressed in percentages±1 SD (two normals and three HD patients).



from three normal subjects and four patients with HD were studied. As can be seen in Fig. 2A, the PHA response of normal controls was decreased significantly after glass wool column passage. In contrast, the PHA response of patients with HD (Fig. 2B) was enhanced. There was no significant difference in the PHA response of depleted cells when normals were compared with patients with HD. When enriched autologous adherent cell suspensions were added to depleted cells, the PHA response of normals vs. patients with HD became significantly different again ($P < 0.5$) with the normals gaining increased responsiveness, while the cells from patients with HD showed less responsiveness (Figs. 2A and B). A representative experiment demonstrating the effect of the addition of increasing numbers of adherent cells is shown in Fig. 3. It can be seen that there is a progressive diminution of the PHA response when autologous adherent cells were added to nonadherent cells in the case of HD versus the progressive increase in PHA response when normal adherent cells were added to autologous nonadherent cells. In Fig. 3 is also shown the effect when indomethacin (10 µg/ml) was added to the cultures. Indomethacin caused a marked increase in the response of the HD patient with only minor change in the normal subject so that the two responses were indistinguishable. The suppressive effect of the addition of adherent cells to nonadherent cells and the enhancement effect with depletion in HD was only observed in patients who were markedly hyporesponsive.

FIGURE 2 (A) PHA response (mean cpm ±1 SD) of normal controls (three individuals) after depletion of glass wool-adherent cells and after repletion with monocyte-enriched suspensions. (B) PHA response (mean cpm ±1 SD) of patients with HD (four individuals) after depletion of glass wool-adherent cells and after repletion with monocyte-enriched suspensions.

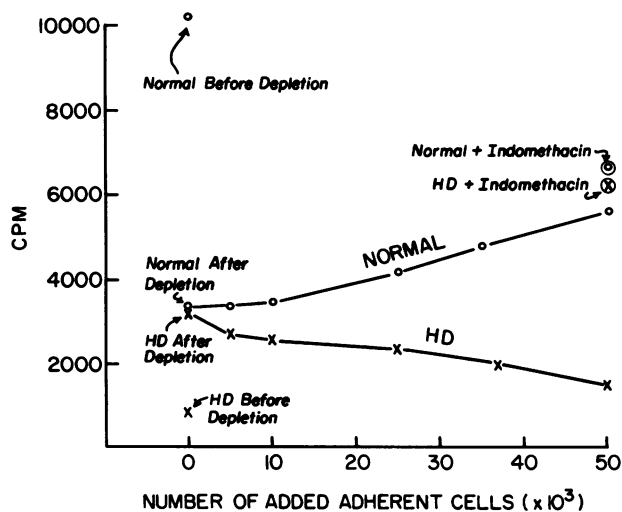


FIGURE 3 A representative experiment showing the effect on PHA responsiveness of the addition of increasing numbers of adherent cells to nonadherent cells. PHA concentration = 10 μ g/ml. Number of nonadherent cells = 5×10^4 cells. Indomethacin concentration = 10 μ g/ml.

DISCUSSION

Defective immunologic reactivity, which is associated with a number of disease states, has recently stimulated investigation into the role of leukocyte subpopulations as mediators of impaired immunologic function (6–9). Cellular interactions involved in the suppression of immunologic responsiveness are complex and may involve T cells, B cells, and macrophages as well as numerous humoral substances and lymphokines (6–13, 26–28). Waldmann et al. (7, 8), Stobo et al. (6), and others (9) have recently demonstrated the existence of a population of suppressor leukocytes which appear to be involved in immune dysfunction in a variety of clinical disorders. Many patients with HD have a profound disorder of cellular immunity characterized by a decrease in delayed hypersensitivity, a hyporesponsiveness to mitogens, and a markedly increased prevalence of viral and mycotic infections (1–3). Some defect in T cell function is generally thought to be responsible either because of T-cell depletion, intrinsic T-cell abnormalities, or the presence of a cell subpopulation suppressing normal T-cell responses (29). In the results presented here, lymphocytes from normal subjects and patients with HD demonstrated a marked difference in *in vitro* T-cell responsiveness. Similar results have been reported by other investigators (2, 4, 5). The depletion of glass wool-adherent mononuclear cells from normal subjects decreased PHA responsiveness; this was associated with the loss of monocytes and the consequent decrease in macrophage-dependent lymphoproliferative responses (30). In contrast, glass wool depletion of ad-

herent cells from patients with HD increased mitogen responsiveness, suggesting that a glass wool-adherent suppressor cell was removed. Cell marker studies after glass wool depletion indicated that normal cells decreased only in the proportion of monocytes while lymphocytes from patients with HD decreased in both monocytes and cells bearing C3 receptors. These results are consistent with those obtained by Twomey et al. (31) employing a mixed leukocyte reaction assay which suggested that a nonmonocyte mononuclear glass wool-adherent cell was responsible for suppression of the mixed leukocyte reaction. In the latter study, no extensive profile of cell surface markers on the mononuclear cells remaining after depletion of adherent cells was reported; however, the effect of adherent cells on the mixed leukocyte reaction assay as reported by Twomey and co-workers appeared to be consistent with suppression. In our experiments, a monocyte-enriched suspension was added to glass wool-depleted cells and this resulted in the suppression of the HD lymphocyte mitogen response. The glass-adherent cells removed in our study included both monocytes and lymphocytes. We have not distinguished in our studies whether the monocytes or lymphocytes could be mediating the suppression of PHA reactivity in Hodgkin's disease. The more selective depletion by iron ingestion by monocytes in Twomey's study would be consistent with the fact that the nonphagocytic cells rather than phagocytic monocytes are the suppressor leukocytes. Pertinent to this point are previous studies of Folch et al. which supported the existence of a glass-adherent T cell as being important in suppressor activity (17). Studies shown in Table II indicate no change in proportion of cells bearing AFP, surface Ig, receptors for EA-IgG, EA-IgM, or E-rosettes when depletion of suppressor cell activity by glass wool columns was accomplished. In contrast, a significant decrease in the proportion of monocytes and C3 receptor-bearing lymphocytes was recorded. These results are consistent with the existence of a suppressor cell in HD which is a C3 receptor-bearing lymphocyte, a monocyte, or another subpopulation of adherent lymphocytes. Precise characterization of this suppressor cell is now indicated.

Numerous studies in patients with HD have demonstrated a depletion in T cells as evidenced by a decrease in sheep E-rosette formation, especially in patients during the late stages of the disease (32, 33). More recently, this depletion has been shown to be only apparent since normal T cell numbers could be demonstrated using assays which employed an anti-T-cell antiserum (34). This latter study suggested that T cells identified with anti-T-cell antisera might constitute a different subpopulation from those which are capable of forming sheep cell rosettes. Indeed the problem of precise methods for identifying various T-

cell populations has as yet not been completely resolved (35). In support of the fact that T cells are not substantially decreased in HD is a recent report by Fuks et al. which indicated that decrease in T-cell rosettes was due to the interference of an inhibitor protein. HD patients in the present study showed low T cells by E-rosette formation. Apparently normal proportions of E-rosette-forming lymphocytes are not necessary for a normal PHA response, since E-rosette-forming proportions were essentially unchanged after glass wool depletion while PHA responsiveness was enhanced in patients with HD. Similarly, Ng et al. found that PHA responsiveness increased without any increase in proportions of E-rosette-forming cells when transfer factor was given to patients with HD (36).

Recently it has been shown that several diseases associated with immune hyporesponsiveness had increased levels of serum AFP or increased proportions of lymphocytes with surface AFP (37). Our study demonstrated an increase in AFP-bearing lymphocytes in patients with HD when compared with normals. In normal subjects and in patients with HD, approximately 25% of the AFP cells were T cells by E-rosette formation. Keller et al. have found similar results in various neoplasms, especially in malignant lymphomas (37). In our study, however, AFP-bearing cells were not selectively depleted by glass wool adherence, and this suggests that AFP-bearing lymphocytes in HD are not the suppressor cells. Lymphocytes bearing receptors for IgM were evaluated in patients with HD and were found to be slightly decreased from normal. In normal controls and patients with HD, essentially all IgM receptor-bearing cells identified in double marker studies were also E-rosette-forming cells. A recent report by Moretta et al. suggested that lymphocytes bearing a receptor for IgM may be a subpopulation of helper T cells (38).

Whatever cells are involved in the suppression of T cell function in patients with HD, this suppression appears to be reversible. Levamisole and transfer factor derived from leukocytes of patients with HD can apparently restore normal in vitro and in vivo lymphocyte responses (39). Ramot et al. have reported an increase in lymphocyte cyclic AMP in patients with HD relative to normal, which appears to decrease to normal levels with levamisole administration (39). Such increases in cyclic AMP could be due to a variety of reasons. It is possible that the secretion of an active substance by the suppressor cells causes an increased intracellular cyclic AMP. Prostaglandins can cause such an increase, and it is possible they might be involved (40-42). Preliminary in vitro evidence from a limited number of patients with HD in our laboratory has demonstrated a dramatic increase in PHA responsiveness of HD lymphocytes upon administration of indomethacin, a prostaglandin synthetase inhibitor. An

example of the enhancement of PHA responsiveness in patients with HD is seen in Fig. 3. Similar inhibition of suppressor cells by indomethacin has been demonstrated in mouse splenic lymphocyte cultures (12).

The studies reported above are consistent with the concept that the T-cell dysfunction present in patients with HD may be mediated by a subpopulation of suppressor leukocytes. It is possible that glass-adherent suppressor cells are mediating their effect by secretion of soluble factors such as prostaglandins. Studies directed toward the further delineation of these cells and their precise mechanism of immunosuppression may lead to decisive advances in the treatment of the malignancy as well as some of its infectious complications.

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REFERENCES

1. Aisenberg, A. C. 1966. Manifestation of immunological unresponsiveness in Hodgkin's Disease. *Cancer Res.* **26**: 1152-1160.
2. Aisenberg, A. C. 1966. Immunologic status of Hodgkin's Disease. *Cancer.* **9**: 385-394.
3. Chase, M. W. 1966. Delayed type hypersensitivity and the immunology of Hodgkin's Disease with a parallel examination of sarcoidosis. *Cancer Res.* **26**: 1097-1120.
4. Papac, R. J. 1970. Lymphocyte transformation in malignant lymphomas. *Cancer.* **26**: 279-286.
5. Hersh, E. M., and W. S. Irvin. 1964. Blastogenic responses of lymphocytes from patients with untreated and treated lymphomas. *Lymphology.* **2**: 150-160.
6. Stobo, J. D., S. Paul, R. E. Van Scoy, and P. E. Hermans. 1976. Suppressor thymus-derived lymphocytes in fungal infection. *J. Clin. Invest.* **57**: 319-328.
7. Waldmann, T. A., S. Broder, R. M. Blaese, M. Durm, M. Blackman, and W. Strober. 1974. Role of suppressor T-cells in pathogenesis of common variable hypogammaglobulinemia. *Lancet.* **II**: 609-613.
8. Waldmann, T. A., S. Broder, M. Durm, M. Blackman, R. Krakauer, and B. Meade. 1975. The role of suppressor T-cells in the pathogenesis of hypogammaglobulinemia with a thymoma. *Clin. Res.* **23**: 477A. (Abstr.)
9. Broder, S., R. Humphrey, M. Durm, M. Blackman, B. Meade, C. Goldman, W. Strober, and T. Waldmann. 1975. Impaired synthesis of polyclonal (non-paraprotein) immunoglobulins by circulating lymphocytes from patients with multiple myeloma. Role of suppressor cells. *N. Engl. J. Med.* **293**: 887-892.
10. Rich, R. R., and C. W. Pierce. 1973. Biological expression of lymphocyte activation. II. Generation of population of thymus-derived suppressor lymphocytes. *J. Exp. Med.* **137**: 649-659.

11. Gershon, R. K., and K. Kondo. 1970. Cell interactions in the induction of tolerance. The role of thymus lymphocytes. *Immunology*. 18: 723-737.
12. Webb, D. R., Jr., and A. T. Jamieson. 1976. Control of mitogen-induced transformation: characterization of splenic suppressor cell and its mode of action. *Cell. Immunol.* 24: 45-57.
13. Cantor, H., F. W. Shen, and E. A. Boyse. 1976. Separation of helper T-cells from suppressor T-cells expressing different Ly components. II. Activation by antigen: after immunization, antigen-specific suppressor and helper activities are mediated by distinct T-cell subclasses. *J. Exp. Med.* 143: 1391-1401.
14. Shou, L., S. A. Schwartz, and R. A. Good. 1976. Suppressor cell activity after concanavalin treatment of lymphocytes from normal donors. *J. Exp. Med.* 143: 1100-1110.
15. Bresnihan, H., H. E. Jasin, and M. Ziff. 1976. Impaired suppressor function of peripheral blood mononuclear cells in systemic lupus erythematosus. *Clin. Res.* 64: 55.
16. Van Epps, D. E., D. L. Palmer, and R. C. Williams, Jr. 1974. Characterization of serum inhibitors of neutrophil chemotaxis associated with anergy. *J. Immunol.* 113: 189-200.
17. Folch, H., M. Yoshinaga, and B. H. Waksman. 1973. Regulation of lymphocyte responses *in vitro*. III. Inhibition by adherent cells of the T-lymphocyte response to phytohemagglutinin. *J. Immunol.* 110: 835-839.
18. Yam, L. T., C. Y. Li, and W. H. Crosby. 1971. Cytochemical identification of monocytes and granulocytes. *Am. J. Clin. Pathol.* 55: 283-290.
19. Jondal, M., G. Holm, and H. Wigzell. 1972. Surface markers of human T and B lymphocytes. I. A large population forming non-immune rosettes with sheep red blood cells. *J. Exp. Med.* 136: 207-215.
20. Ferrarini, M., G. P. Tonda, A. Risso, and G. Viale. 1975. Lymphocyte membrane receptors in human lymphoid leukemias. *Eur. J. Immunol.* 5: 89-93.
21. Moretta, L., M. Ferrarini, M. L. Durante, and M. C. Mingari. 1975. Expression of a receptor for IgM by human T-cells *in vitro*. *Eur. J. Immunol.* 5: 565-569.
22. Huber, C. H., and H. Wigzell. 1975. A simple rosette assay for demonstration of complement receptor sites using complement-coated zymosan beads. *Eur. J. Immunol.* 5: 432-435.
23. Nisonoff, A., F. C. Wissler, and L. N. Lipman. 1960. Properties of the major component of a peptic digest of rabbit antibody. *Science (Wash. D.C.)*. 132: 1770-1771.
24. Husby, G., R. G. Strickland, J. L. Caldwell, and R. C. Williams, Jr. 1975. Localization of T and B cells and alpha-fetoprotein in hepatic biopsies from patients with liver disease. *J. Clin. Invest.* 56: 1198-1209.
25. Moller, G. 1974. Effect of B-cell mitogens on lymphocyte subpopulations possessing C3 and Fc receptors. *J. Exp. Med.* 139: 969-982.
26. Gershon, R. K. 1974. T-cell control of antibody production. In *Contemporary Topics in Immunology*. M. D. Cooper and N. L. Warner, editors. Vol. 3. Plenum Press, New York.
27. Ha, T. Y., and B. H. Waksman. 1973. Role of the thymus in tolerance. X. "Suppressor" activity of antigen-stimulated rat thymocytes transferred into normal recipients. *J. Immunol.* 110: 1290-1297.
28. Uhr, J. W., and G. Moller. 1968. Regulatory effect of antibody on the immune response. *Adv. Immunol.* 8: 81-127.
29. Order, S. E., and S. Hellman. 1972. Pathogenesis of Hodgkin's Disease. *Lancet*. I: 571-573.
30. Twomey, J. J., O. Sharkey, Jr., J. A. Brown, A. H. Laughter, and P. H. Jordan, Jr. 1970. Cellular requirements for the mitotic response in allogeneic mixed leukocyte cultures. *J. Immunol.* 104: 845-853.
31. Twomey, J. J., A. H. Laughter, S. Farrow, and C. C. Douglass. 1975. Hodgkin's Disease: an immunodepleting and immunosuppressive disorder. *J. Clin. Invest.* 56: 467-475.
32. Ramot, B., M. A. Biniaminov, A. Many, and E. Aghai. 1973. Thymus-derived lymphocytes (T-cell) depletion in Hodgkin's Disease. *Isr. J. Med. Sci.* 9: 657-659.
33. Levy, R., and H. S. Kaplan. 1974. Impaired lymphocyte function in untreated Hodgkin's Disease. *N. Engl. J. Med.* 290: 181-186.
34. Kaplan, H. S., A. M. Bobrove, Z. Fuks, and S. Stober. 1974. T and B cells in Hodgkin's Disease. *N. Engl. J. Med.* 290: 971.
35. Strickland, R. G., E. Diaz-Jouanen, and R. C. Williams, Jr. 1975. Application of lymphocyte markers as a probe in various human disease states. In *Lymphocytes and Their Interactions*. R. C. Williams, Jr., editor. Raven Press, New York. 4: 133-155.
36. Ng, P. P., C. J. Moran, C. G. Alexopoulos, and A. J. Bellingham. 1975. Transfer factor in Hodgkin's Disease. *Lancet*. II: 901-903.
37. Keller, R. H., R. J. Dattwyler, E. O. Harrison, and J. B. Tomasi, Jr. 1975. Alpha-fetoprotein in human lymphoproliferative diseases. *Blood*. 46: 1035A. (Abstr.)
38. Morretta, L., S. R. Webb, C. E. Grossi, P. M. Lydyard, and M. D. Cooper. 1976. Functional analysis of two subpopulations of human T lymphocytes and their distribution in immunodeficient patients. *Clin. Res.* 448A. (Abstr.)
39. Ramot, B., M. Biniaminov, C. Shoham, and E. Rosenthal. 1976. Effect of levamisole on E-rosette-forming cells *in vivo* and *in vitro* in Hodgkin's disease. *N. Engl. J. Med.* 294: 899-911.
40. Plescia, O. J., A. H. Smith, and K. V. Grinwich. 1975. Subversion of immune system by tumor cells and role of prostaglandins. *Proc. Natl. Acad. Sci. U.S.A.* 72: 1848-1851.
41. Strausser, H., and J. Humes. 1975. Prostaglandin synthesis inhibition: effect on bone changes and sarcoma tumor induction in balb/c mice. *Int. J. Cancer*. 15: 724-730.
42. Quagliata, F., J. W. Lawrence, and J. M. Phillips-Quagliata. 1973. Prostaglandin E₁ as a regulator of lymphocyte function. Selective action on B lymphocytes and synergy with procarbazine in depression of immune responses. *Cell. Immunol.* 6: 457-465.