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Research Article

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Quantitation of Immunocompetence in Hodgkin's Disease

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ABSTRACT In vitro cellular immunocompetence was
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INTRODUCTION

Lymphocyte activation by phytohemagglutinin (PHA)[1]
has been extensively studied in patients with Hodgkin's
disease in whom a deficit in cell mediated immunity is
known to exist (1-4). The conventional single dose
maximum PHA stimulation discloses a lymphocyte ab-
normality on relatively few patients (5-7). Correlation
with skin test reactivity, (5, 6, 8) stage (5, 9, 10),
peripheral blood lymphocyte count, and systemic symp-
tomatology (5, 9, 10) has shown considerable variation.
However, since many studies were conducted on patients
during the various phases of their disease and treatment,
the inconsistency of these observations may reflect the
heterogeneity and insensitivity of the methodology and
the timing of the studies as well as the status of these
patients' immune competence.

We have previously shown that lymphocyte trans-
formation to a wide range of PHA concentrations,
encompassing the entire spectrum of lymphocyte re-
sponse capabilities (11), is more discriminating than
the conventional method of a single dose PHA stimula-
tion. This approach, confirmed by others (12, 13), dis-
closes a lymphocyte abnormality in patients with Hodg-
kin's disease and disseminated solid tumors more readily
than the single dose PHA assay.

The present report confirms and extends our previous
observations and demonstrates the sensitivity of this
approach for detecting an underlying lymphocyte defect
in previously untreated patients with Hodgkin's disease.
It also examines the quantitative aspects of the various
lymphocyte response patterns obtained and their cor-
relation with prognostically valuable clinical parameters
and with the ultimate course of the disease.

METHODS

Patients. 35 consecutive, previously untreated patients
with Hodgkin's disease were studied. The group consisted
of 23 males and 12 females with a mean age of 40 yr
(range 4-78). The histologic subtypes were identified ac-
cording to the Rye modification of the Lukes-Butler clas-
sification. There were 5 patients with lymphocyte pre-
dominance, 15 with nodular sclerosing, 14 with mixed cellu-
larity, and 1 with lymphocyte depletion. All patients were
carefully staged, including lymphangiography, bone marrow
biopsy, and exploratory laparotomy with splenectomy, the
latter being performed in 17 patients with clinical stages
less than III B. Bone and liver scans and lung tomo-
grams were performed as indicated. The distribution of
the patients by stages was as follows: I A = 2, II A = 6,
III A = 3, IV A = 0, I B = 0, II B = 5, III B = 13, and
IV B = 6.

Skin testing. All patients were skin tested with the fol-
lowing intradermal recall antigens: purified protein deriva-
tive (PPD), mumps antigen, streptokinase-streptodornase,
and *Candida albicans*. Their ability to be sensitized by 2,4-
dinitrochlorobenzene was also studied. Skin tests were ap-
plied and interpreted before laparotomy and therapy.

Lymphocyte separation and cultures. In all cases blood
was drawn before staging and therapy. As previously re-
ported (14), 30-35 ml of heparinized blood (preservative
free, Connaught Laboratories, Toronto, Canada), was sedi-
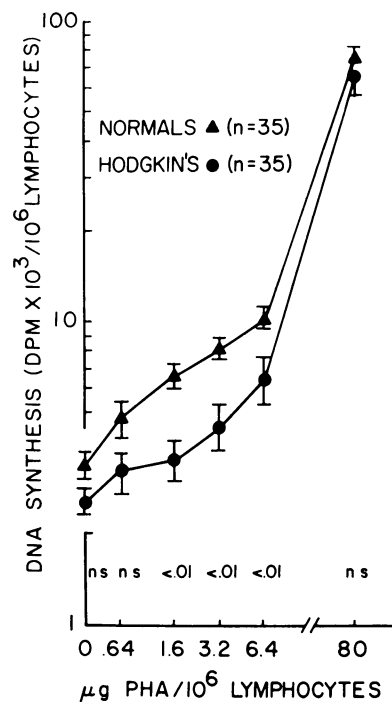mented with dextran (mol wt 250,000, Sigma Chemical Co.,

FIGURE 1 Lymphocyte responsiveness in Hodgkin's and normals. Lymphocyte responsiveness to PHA from normals ($\blacktriangle$, $n = 35$) and patients with Hodgkin's disease ($\bullet$, $n = 35$). The data is expressed as DNA synthesis/10^6 lymphocytes and presented as mean±SE for each PHA concentration. Significance of difference (P value) between normals and Hodgkins disease as indicated at each PHA concentration.

St. Louis, Mo.). The buffy coat passed through a glass bead column to yield a lymphocyte suspension which was comparable in normals and patients in terms of lymphocyte purity (75–95%) and nonlymphoid cell contamination. Each culture was made of 1×10^6 lymphocytes in a 2-ml volume of minimal essential medium (Gibco, Grand Island Biological Co., Grand Island, N. Y.), 20% fetal calf serum (Gibco), antibiotics (penicillin 100 U/ml, streptomycin 100 μg/ml, and mycostatin 100 U/ml), and the appropriate concentration of PHA. A single batch of PHA-M (no. 562543, Difco Laboratories, Detroit, Mich.) was used throughout the study in doses from 0.64 to 80.0 μg PHA-protein (total protein content measured by spectrophotometry, Beckman Instruments Inc., Fullerton, Calif.). This spectrum of PHA concentrations was used after demonstration that in our system less than 0.64 μg PHA failed to significantly activate normal lymphocytes, whereas 80 μg resulted in maximum lymphocyte responses beyond which deoxyribonucleic acid (DNA) synthesis decreased due to the cytotoxic effect of PHA. After 68 h incubation at 37°C, DNA was pulse labeled (4 h) with 0.5 μCi of tritiated thymidine (New England Nuclear, Boston, Mass., sp act 6.7 Ci/mmol), precipitated with 10% trichloroacetic acid (TCA) ($\times 2$), washed with 95% methanol ($\times 2$), dissolved in 0.5 ml of Nuclear Chicago Solubilizer, and counted in a toluene-based scintillation fluid. Counts per minute were measured at ambient temperature in an LS-150 Beckman spectrometer with a counting efficiency of

45±0.9%, calculated according to the external standardization method (15).

The mean and standard error of the three samples at each PHA concentration were calculated. Significant DNA synthesis ($P < 0.05$) was determined by statistical analysis (Student's t test) comparing DNA synthesis from each PHA concentration to the unstimulated controls. The threshold of lymphocyte responsiveness was identified by the minimum stimulatory concentration of PHA capable of eliciting significant DNA synthesis. Maximum lymphocyte transformation was defined by the DNA synthesis of the maximally stimulated cultures.

RESULTS

Delayed hypersensitivity reactions. 21 patients (60%) exhibited anergic skin reactions. This does not differ significantly from 55% found in a large recent study (16). Of the 14 patients who had one or more positive skin test, their response was as follows: 2,4-dinitrochlorobenzene 86%, Candida 79%, streptokinase-streptodornase 36%, mumps 36%, and PPD 21%.

Lymphocyte responsiveness in normal donors. As previously reported, 1.6 μg PHA-protein/10^6 lymphocytes, which induced significant DNA synthesis over and above that of unstimulated control cultures, represented the least stimulus capable of consistently initiating lymphocyte activation in all normal subjects. Each subsequent increment in PHA concentration invariably resulted in augmented DNA synthesis reaching a maximum in response to 80 μg of PHA-protein/10^6 lymphocytes. This PHA dose-response pattern was present in each individual tested. The pooled data can be seen in Fig. 1.

As previously reported (14), the magnitude of DNA synthesis showed considerable intra and interpersonal variation at each PHA concentration tested, however, the threshold of lymphocyte responsiveness and the pattern of PHA dose-response curve elicited remained predictably reproducible.

Lymphocyte responsiveness in the Hodgkin's group. It can be appreciated in Fig. 1 that the shape of the curve from the Hodgkin's group (pooled data) is at variance with that of normals. Although the base line, unstimulated DNA synthesis was not significantly subnormal; these cells were relatively insensitive to low concentrations of PHA, requiring twice more PHA (3.2 μg) than normal to initiate lymphocyte activation. Because of this elevated threshold to PHA stimulation beyond which PHA concentration-dependent rate of DNA synthesis appears to progress normally, the magnitude of DNA synthesis in Hodgkin's lymphocytes was statistically subnormal in response to 1.6, 3.2, and 6.4 μg PHA-protein. However, these cells were capable of a normal response when maximally stimulated, as shown by the magnitude of DNA synthesis elicited by 80 μg PHA-protein.

The Hodgkin's population was subdivided according to skin test reactivity, constitutional symptomatology,
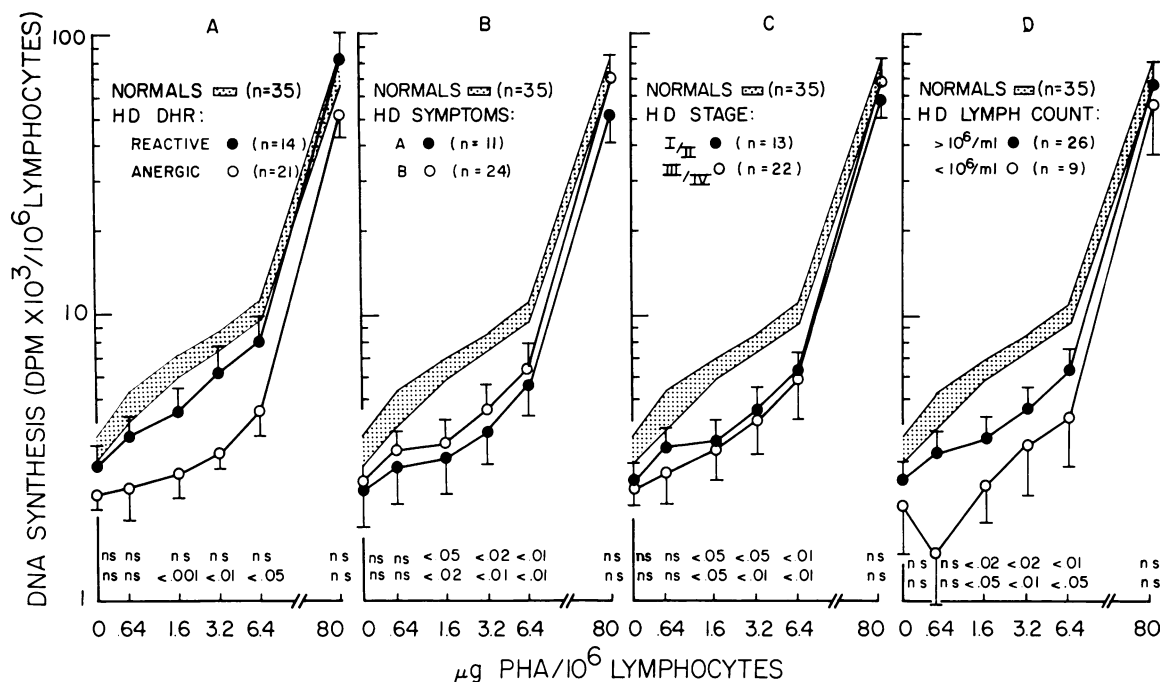
FIGURE 2 Lymphocyte responsiveness in Hodgkin's according to clinical status. Lymphocyte responsiveness to PHA from patients with Hodgkin's disease grouped according to skin tests (A), constitutional symptoms (B), stage of disease (C), and circulating lymphocyte counts (D), as indicated in each panel, are compared to normal responses and presented as mean ±SE. Significance of difference (P value) between normals and each patient subgroup as indicated at each PHA concentration.

peripheral blood lymphocyte count, and extent of disease. As shown in Fig. 2A, significant differences in lymphocyte responses to PHA were seen between reactive and anergic patients. While patients who reacted to one or more skin tests retained normal responses to PHA, anergic patients were clearly abnormal in the pattern and the magnitude of their responses, reflected by a high threshold for PHA activation with significant subnormal responses to low PHA concentrations, but no impairment in the spontaneous unstimulated DNA synthesis nor in response to maximum PHA stimulation. The presence or absence of constitutional symptomatology, however, had no effect on the pattern or magnitude of lymphocyte responses to PHA, as shown in Fig. 2B. Although patients with advanced disease (stages III and IV) or lymphopenia showed greater impairment of lymphocyte responsiveness than did patients with early stages and normal lymphocyte count, respectively, these differences were not statistically significant (Fig. 2C and D).

Individual patterns of response in Hodgkin's patients. When lymphocyte responsiveness to PHA stimulation was analyzed for each individual patient, three patterns of response emerged (Fig. 3). Pattern I included nine individuals (26%). These patients showed no detectable

abnormality. Indeed, unstimulated DNA synthesis, lymphocyte sensitivity to PHA, the magnitude of PHA-dependent DNA synthesis, and the pattern of response were indistinguishable from normal. Pattern II included 20 patients (57%) who showed lymphocyte insensitivity to low PHA concentrations. Twice as much PHA as normal or more was required to initiate activation of these cells which were capable, however, of a normal response to maximal stimulation (80 μg PHA). Finally, pattern III was identified in six patients (17%) in whom grossly abnormal DNA synthesis was present throughout, including unstimulated cultures. Only in this latter group of patients would an abnormality have been readily detected by using the conventional, single dose maximum lymphocyte transformation assay. In contrast, our approach of investigating the individual threshold for PHA activation discloses lymphocyte insensitivity to PHA in 74% of patients (patterns II and III), most of whom (pattern II) retain normal response capabilities to maximal stimulation.

When individual patterns of lymphocyte responses to PHA stimulation were correlated with the patient's clinical status (Table I), it was observed that almost all (91%) anergic patients exhibited abnormal lymphocyte responses to PHA. In contrast, only 50% of pa-
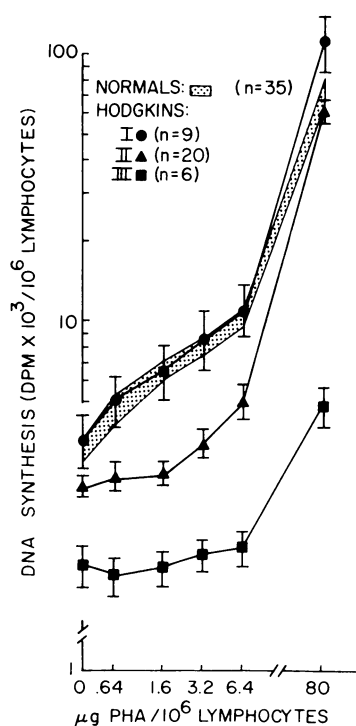
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FIGURE 3 Patterns of lymphocyte responsiveness in Hodgkin's. Hodgkin's disease, expressed as mean±SE for each pattern observed (see text), as compared to normal. Significance of difference (P value) between normal and pattern I is NS, pattern II is < 0.01 at 1.6, 3.2, and 6.4 μg PHA, and pattern III is < 0.001 at all PHA concentrations.

tients with reactive skin tests showed impaired lymphoblastogenesis and this consisted mostly of the subtle defect associated with pattern II. The presence or absence of consitutional symptoms and the presence of a normal circulating lymphocyte count seemed to exert no influence on the percentage nor the degree of detected lymphocyte abnormality. The effect of lymphopenia on lymphoblastogenesis could not be analyzed separately by virtue of the concomitant anergy exhibited by these

patients. Thus, the patterns of response from lymphopenic patients are reminiscent of those observed with anergy alone. An abnormality was detected as frequently in patients with stages I/II (77%) as in those with stages III/IV (73%). However, patients with early disease exhibited exclusively the subtle lymphocyte abnormality associated with pattern II. The favorable influence of positive skin tests, and to a lesser extent of stages I and II, and conversely the unfavorable influence of anergy upon lymphocyte responsiveness to PHA were observed to predominate over any other clinical parameter or combination thereof.

Lymphocyte responsiveness in previously treated Hodgkin's. The abnormality of lymphocyte responsiveness present in untreated patients was appreciably greater after radiation or combination chemotherapy. Analysis of individual responses (Table II) showed that 24% of treated patients had a normal dose-response curve (pattern I), 29% showed lymphocyte insensitivity to submaximal PHA stimulation (pattern II), and 47% had abnormal responses at all levels of PHA stimulation (pattern III). It is noteworthy that although the overall incidence of lymphocyte abnormality (76%) is similar to that of previously untreated Hodgkin's (74%), contrary to the latter, most of these patients exhibited the greatest degree of lymphocyte abnormality. Interestingly, of eight patients restudied during unmaintained complete remission over a period of 1–7 yr, half exhibited normal lymphocyte responses, the remainder showing mostly the subtle defect of pattern II. All four restudied patients who died in relapse exhibited the greatest lymphocyte abnormality of pattern III.

DISCUSSION

The marked variation of PHA-induced lymphocyte transformation observed among normals (14, 17) and in a given individual upon repeated testing (14) hampers precise definition of normal and abnormal lymphocyte responsiveness to PHA stimulation. The conventional single PHA concentration, aiming at maximal

TABLE I
*Patterns of Lymphocyte Responsiveness and Clinical Status**

PHA-response pattern	All HD ($n = 35$)	Skin tests		Constitutional symptoms		Lymphocyte count		Stage	
		Positive ($n = 14$)	Negative ($n = 21$)	"A" ($n = 11$)	"B" ($n = 24$)	Normal ($n = 26$)	Low ($n = 9$)	I/II ($n = 13$)	III/IV ($n = 22$)
I	26	50	9	28	25	30	11	23	27
II	57 ⎱74‡	43 ⎱50‡	67 ⎱91‡	55 ⎱73‡	58 ⎱75‡	58 ⎱70‡	56 ⎱89‡	77 ⎱77‡	46 ⎱73‡
III	17 ⎰	7 ⎰	24 ⎰	18 ⎰	17 ⎰	12 ⎰	33 ⎰	0 ⎰	27 ⎰

* Percentage distribution of lymphocyte responsiveness to PHA in patients with Hodgkin's disease according to patterns of response and grouped according to clinical status as indicated in each panel.
‡ Indicates total abnormality detected in each subgroup of patients.
HD, Hodgkin's disease.

PHA-response patterns	Pre-therapy (n = 35)	Post-therapy (n = 17)	Complete remission (n = 8)	Dead (n = 4)
I	26	24	50	0
II	57⎤74‡	29⎤76‡	38⎤50‡	0
III	17⎦	47⎦	12⎦	100

* Percentage distribution of lymphocyte responsiveness to
PHA in patients with Hodgkin's disease according to patterns
of response and grouped as indicated in each panel.
‡ Indicates total abnormality detected in each subgroup of
patients.

lymphocyte stimulation, discloses impaired PHA trans-
formation in only 20–40% of patients with Hodgkin's
disease (5–7). Furthermore, the generally poor cor-
relation between lymphoblastogenesis and delayed hyper-
sensitivity reactions in the cell donor before (7) and
after therapy (5, 6, 9, 10) suggests that the conven-
tional transformation assay does not reliably reflect the
status of immune competence (6, 18). Although still
controversial (6, 18), the discrepancies of these observa-
tions may reflect the quantitative rather than qualita-
tive nature of immune phenomena: powerful stimulation
(by maximum concentrations of mitogens, antigens or
HL-A [19]) may overcome an underlying subtle lym-
phocyte defect, disclosed only by de-escalating the in-
tensity of the stimulus, whether in vitro (11) or in
vivo (16). The present study confirms and extends our
previous observations of a subtle lymphocyte defect in
most patients with previously untreated Hodgkin's di-
sease, utilizing the PHA dilution technique. It differs
from other published reports (5–10, 12, 13, 20) in two
important aspects. First, the three distinct patterns of
PHA response observed in Hodgkin's disease identified
patients with severe (17%), mild (57%), and lack of
(26%) in vitro abnormality, thus permitting quantita-
tive estimation of the lymphocyte function. Secondly, we
have correlated the magnitude of the observed lympho-
cyte defect with prognostically valuable clinical param-
eters in each individual. A direct relationship was noted
with skin reactivity, absolute lymphocyte count, and
extent of the disease. The severity of in vitro defect
increased with progression of the disease. However,
the demonstration of defective lymphocyte function in
patients with stage I/II, normal skin tests, and lympho-
cyte count, and its independence from constitutional
symptoms, suggest this defect to be inherent to the
disease and not a reflection of its complications.

It is disturbing that the conventional PHA trans-
formation assay and skin test reactivity have no prog-
nostic value (6, 7, 21). Although the availability of
effective therapy may mask any possible contribution

by the immune system (7), such findings may reflect
the insensitivity of the qualitative tests routinely utilized
in assessing the integrity of the immune system. It is
of interest, however, that PPD negative patients after
Calmette-Guerin bacillus (BCG) vaccination exhibited
greatly shortened survival when compared to reactive
individuals (22). The return to normal PHA trans-
formation after prolonged unmaintained remissions ob-
served in this and other studies (6, 8, 10) may be due
to the reappearance of cells depleated during active di-
sease. The relationship of this phenomenon to improving
immunocompetence is supported by the reappearance of
reactive skin tests during unmaintained remission
(3, 23).

Although the nonpurified PHA utilized in this study
contains nonmitogenic protein contaminants, we have
obtained similar results using PHA-M from two dif-
ferent sources, PHA-P as well as a pure PHA fraction.
It is of interest that the last six patients of the present
series were concomitantly studied with a highly pure,
single mitogenic fraction of PHA with similar quantita-
tive results. A similar functional abnormality has also
been described in Hodgkin's lymphocytes stimulated
with pure PHA and evaluated by their RNA synthesis
capabilities (13). However, quantitation of the defect
was not attempted and no correlation was found with
symptomatology, skin reactivity, and absolute lympho-
cyte count. Another sensitive approach is the study of
the entire time-course of the PHA response (20) that
disclosed abnormal Hodgkin's lymphocyte responses in
all patients studied. However, the in vitro abnormality
observed over an 8-day incubation period may reflect
magnified in vitro cell death as much as refractoriness
to mitogenic stimulation. In that study no quantitation of
the lymphocyte impairment was attemped and no clinical
correlations were offered.

Several possible mechanisms could be implicated in
the impaired PHA response seen in Hodgkin's disease:
(a) Laboratory artifacts seem unlikely by virtue of the
predictability of responses in normals, the dose-de-
pendence of transformation observed in normals and
responders, and the reproducibility of replicate cultures
(14). (b) Serum factors reportedly diminish the PHA
response of otherwise PHA-responsive cells (8, 24, 25).
However, the failure of Hodgkin's serum to diminish
normal lymphocyte responsiveness to PHA (26) and
the persistence of the Hodgkin's lymphocyte defect after
removal of autologous plasma (8, 27) argue against
the role of serum factors in the functional lymphocyte
abnormality reported here. (c) Removal of "helper
cells" during the lymphocyte separation procedure (28,
29). This is unlikely by virtue of the similar cell re-
covery, purity, and degree of nonlymphoid cell con-
tamination of the final lymphocyte suspensions obtained
from both patients and controls. Moreover, highly pure

lymphocyte suspensions have elicited similar results (20). (*d*) An intrinsic functional abnormality of all PHA-responsive lymphocytes or depletion of these cells (11, 20). Dilution of T-lymphocytes by chronic lymphatic leukemia cells seems to account for the abnormal PHA transformation seen in this disease (30). Similarly, the impaired lymphoblastogenesis observed in Hodgkin's may represent normal response by cells depleted of a certain T-cell subpopulation, as recently described (31). However, some of our patients with impaired PHA transformation exhibited normal T- and B-cell population ratios.

The various patterns of Hodgkin's lymphocytes response to PHA shown in the present study suggest the presence of two abnormalities: the first represents quantifiable lymphocyte insensitivity to PHA stimulation. Although the nature of this lymphocyte defect has not been elucidated, recent work in our laboratory (32) has demonstrated quantitatively and qualitatively normal binding of pure ^{125}I-PHA to Hodgkin's lymphocytes. These cells, however, exhibited increased thresholds for PHA activation and were hyporesponsive to normally bound PHA, thus suggesting a functional metabolic cell defect located beyond membrane receptor sites; the second, reflected in the markedly different shape of pattern III (with significantly subnormal spontaneous DNA synthesis), may relate to an endogenous qualitative defect superimposed to the qualitative lymphocyte insensitivity to PHA. This combined defect may reflect in vitro cell death (5, 6, 8) or it may relate to in vivo stimulation leading to PHA refractory T-lymphocytes (11, 20), loss of these cells recirculating capacity (33), or their depletion from peripheral blood (11, 20, 31). Although the direct relationship shown in this report between spontaneous and PHA-induced DNA synthesis in Hodgkin's disease favors the former concept, the presence of "large lymphoid cells" engaged in DNA synthesis found in Hodgkin's blood (34) and the recent discovery of Hodgkin's disease tumor-associated antigens (35) provide a strong basis for the latter. It is conceivable that these two phenomena are related, and patients with pattern III may be at the end of a spectrum of patients with gradually increasing functional lymphocyte abnormality.

Our findings of a functional lymphocyte defect in most patients with localized disease, which is intensified as the disease progresses, with the most profound impairment found in anergic, lymphopenic patients with advanced disease suggest the following hypothesis: an intrinsic functional lymphocyte abnormality inherent to Hodgkin's, possibly reflecting in vivo stimulation of these cells by Hodgkin's tumor-specific antigens, would develop early in the course of the disease. With disease progression, this initially subtle (and therefore elusive) impairment becomes more pronounced leading to progressive immunoincompetence with loss of immunologic memory (16, 21), shortened cell survival reflected by in vitro cell death (5, 6, 8), and in vivo cell depletion (11, 20, 31), with ensuing lymphopenia found mostly in far advanced disease (36).

This study was not intended to establish the prognostic usefulness of the impaired PHA response observed in Hodgkin's disease. However, if cellular immune mechanisms constitute the main determinant of host resistance in this disease (22), quantitative evaluation of immunocompetence, as illustrated in this report, may eventually be of paramount importance to the clinician in following the course of the disease and the adequacy of treatment. Such a goal will require new techniques of greater sensitivity and specificity.

ACKNOWLEDGMENTS

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