Heterogeneous Mechanisms of Impaired Lymphocyte Responses in Non-Hodgkin’s Lymphoma

By Ronald L. Whisler, Stanley P. Balcerzak, and J. Lee Murray

Peripheral blood mononuclear cells (PBMC) from 18 untreated patients with non-Hodgkin’s lymphoma (NHL) were studied to characterize the cellular mechanisms contributing to impaired in vitro lymphocyte responses after stimulation by the mitogen concanavalin A (Con-A). In vitro reactivity was quantitated by the 3H-thymidine incorporation in response to an optimal dose of Con-A. All patients demonstrated impaired in vitro reactivities compared to normal controls. These in vitro impairments were partially reversible since patient’s cells precultured in media alone for 3 days demonstrated enhanced Con-A responses. In greater than half of the patients, the hyporeactive PBMC suppressed the enhanced reactivities of autologous precultured PBMC when assayed in cocultures. Suppressor activity was detected mainly in those untreated patients presenting with either constitutional symptoms or diffuse histology and in general was not marked compared to the severity of impairments. Adherent monocytes were shown to participate in the suppression of autologous lymphocyte reactivity but only appeared partially responsible for the in vitro impairments. In those patients lacking detectable suppressive activity, preculturing also enhanced Con-A reactivities and was compatible with the presence of a reversible, inhibitory mechanism differing from active suppression. Many patients’ hyporeactive PBMC, however, failed to demonstrate normal responses after preculturing. This failure could not be directly attributed to aberrant regulatory populations, but rather appeared to possibly represent an additional intrinsic impairment of potentially reactive populations.

A BERRANT STATES of in vivo and in vitro immune reactivity are frequently associated with human malignant lymphomas. Considering the complexities of normal immune reactivity with interactions between responding populations and subsets of regulatory cells, heterogeneous mechanisms likely contribute to the states of immune impairment. For example, in Hodgkin’s disease, suppressor cells are present in patients with diminished in vitro lymphocyte responses. However, other factors, including the lack of “helper” cells, aberrant reactive cells, circulating immune complexes, and other soluble inhibitory materials, may be equally important.

Among patients with NHL, marked heterogeneity of in vivo immunocompetence is well documented. Thus, patients with diffuse histology and constitutional symptoms display more severely impaired skin test reactivity compared to those with nodular histology and lacking symptoms. The in vitro mechanisms of impaired lymphocyte responses that may be associated with the specific manifestations of NHL are unknown. In the present study, the cellular mechanisms are directly analyzed by examining both hyporeactive and reactive autologous cells in cocultures stimulated by the mitogen Con-A. In all patients with NHL, the preculturing of hyporeactive cells for 3 days in media alone resulted in enhanced proliferative responses.

In the patients with diffuse histology or constitutional symptoms, this reversible impairment was closely associated with suppressor cell activity, which appeared to be mediated by highly pure populations of adherent monocytes. Among those patients lacking detectable suppressor cells, precultured cells also demonstrated enhanced responsiveness and directly suggested reversible, inhibitory mechanisms other than suppressor cells. Finally, most patients’ cells failed to acquire normal responsiveness during preculture, therefore indicating either a more severe and/or additional type of defect among the potentially reactive populations.

MATERIALS AND METHODS

Patients

Eighteen untreated patients with NHL were studied between April 1977 and November 1978 after obtaining their informed consent. All patients were carefully evaluated for extent of disease as previously detailed and were staged according to the Ann Arbor criteria. Lymph node biopsy specimens were classified histologically according to Rappaport’s criteria and no patient had leukemia. The clinical features of patients studied are shown in Table I. All immunologic studies were performed prior to therapy. Control subjects were normal men and women in good health and who ranged from 27 to 60 yr of age.

Lymphocyte Separation and Cell Cultures

Peripheral blood mononuclear cells (PBMC) from patients and normal controls were obtained by centrifugation over Ficoll-Hypaque gradients. The PBMC were washed in RPMI 1640 media (Grand Island Biological Co., Grand Island, N.Y.) and cultured in the presence of 10% fetal calf serum. PBMC were cultured in a total volume of 1 ml at a concentration of 2 x 10⁶ cells/ml. PBMC were cultured in media alone, media plus 10% normal human serum, or in media plus autologous PBMC. Autologous PBMC were obtained at the time of culture and were pretreated with mitomycin (0.1 μg/ml) for 30 minutes in RPMI 1640 media. Cultures were returned to the incubator and were fed weekly with fresh media. Cell cultures were harvested after 5 days of culture. Cell cultures were tested reactions for optimal responses using concanavalin A (Con-A).

From the Department of Medicine, Division of Hematology and Oncology, and Division of Immunology, the Ohio State University, Columbus, Ohio.

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Address reprint requests to Ronald L. Whisler, M.D., Director, Division of Immunology, The Ohio State University, 456 Clinic Drive, Columbus, Ohio 43210.

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come, Beckenhaum, England) in a microculture system exactly as geneic stimulator cells from normal controls in flat-bottom micro-stained smears. Unidirectional mixed leukocyte cultures (MLC) cultures did not exceed 1.5%. Blast transformation was assessed in samples in response to the optimal concentration of Con-A. Spurious alterations in culture reactivities secondary to differing optimal proliferative responses. This was necessary to prevent occurred at 96 hr; therefore, this culture time was used in comparing compared. Early experiments documented that maximal responses among FPBMC, RPBMC, and FPBMC plus RPBMC were experiments. The optimal responses to Con-A after 96 hr incubation of Con-A. To determine if hyporeactive FPBMC might be capable of influencing the reactivity of autologous RPBMC, cell mixing of Con-A. The adherent monocyte preparations consisted of greater than 95% cells and 1% phagocytic cells were present in the MD preparations. Ethylenedinitrilotetra acetic acid tetrasodium salt (EDTA) (Matheson, Coleman, and Bell, Norwood, Ohio) followed by harvesting with a rubber policeman. The FAM were immediately rinsed twice in warm RPMI 1640 prior to culturing with cells depleted of monocytes. To prepare RAM, the FAM were incubated at 1.7 x 10^6/ml in tissue culture tubes (no. 2057, Falcon Plastics) with RPMI 1640 plus 6% FBS. After 3 days, RAM were removed, and rinsed twice with RPMI 1640 prior to addition to cultures. MD populations were obtained by incubation of the nonadherent PBMC with carbonyl iron (GAF Corporation, New York) followed by passage through a magnetic field. Less than 2% esterase-positive cells and 1% phagocytic cells were present in the MD preparations. The adherent monocyte preparations consisted of greater than 95% positive for nonspecific esterase activity identified by the method of Li et al. with α-naphthyl butyrate (Sigma Chemical Co.) buffered to pH 7.5 as substrate. More than 90% of the adherent cells phagocytized latex particles (1.1 μm, Dow Diagnostics Division, Indianapolis, Ind.) according to the method of Sibbitt et al. In addition, as judged by morphology, after Giemsa staining more than 95% of the adherent cells were monocytes.

Table 1. Clinical Features of Patients with NHL

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<th>Diffuse</th>
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<td>Total number</td>
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<td>Advanced</td>
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<td>Symptoms†</td>
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<td>B (Present)</td>
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<td>3</td>
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<td>Mean age in years (range)</td>
<td>64 (48-77)</td>
<td>57 (37-65)</td>
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</table>

*Seven of 11 patients with diffuse histology were histiocytic. The remaining patients had lymphocytic histology.
†B classification includes those patients with night sweats, unexplained fevers greater than 38°C, or unexplained weight loss of more than 10% of the body weight in the 6 mo prior to admission.

With 9 μg, 18 μg, and 36 μg/ml conconavalin-A (Con-A) (Wellcome, Beckenham, England) in a microculture system exactly as previously described.13 The concentrations of Con-A were determined by a dose-response curve and represent a range resulting in optimal proliferative responses. This was necessary to prevent spurious alterations in culture reactivities secondary to differing cellular requirements for Con-A. After an 18-hr pulse with 1 μCi 3H-thymidine/microwell, the PBMC were processed at 96 hr of culture with a microplate harvester. The incorporated radioactivity per microculture was determined in a liquid scintillation counter. Data are expressed as the mean counts per minute for triplicate samples in response to the optimal concentration of Con-A. Variation of 3H-thymidine incorporation among the triplicate cultures did not exceed 15%. Blast transformation was assessed in certain experiments by microscopic examination of Wright Giemsa stained smears. Unidirectional mixed leukocyte cultures (MLC) were performed by incubating 10^5/ml responder cells from patients with NHL or control subjects in combination with 10^5/ml fresh mitomycin-C (Sigma Chemical Co., St. Louis, Mo.) treated allogeneic stimulator cells from normal controls in flat-bottom microtiter plates (No. 3040, Falcon Plastics, Oxnard, Calif.). Medium was RPMI 1640 supplemented with 15% fetal bovine serum (FBS) (lot no. 93481, Microbiological Associates, Walkersville, Md.). After culturing for 120 hr at 37°C, 1 μCi 3H-thymidine was added per microtiter well and harvested 18 hr later.

Preincubation and PBMC Coculturing Method

Fresh PBMC (FPBMC) drawn on day 1 were immediately cultured with the 3 doses of Con-A. The remaining cells were placed in 25 sq cm tissue culture flasks (no. 3013, Corning) at a final density of 1.7 x 10^6/ml in RPMI 1640 supplemented with 6% FBS. After 3 days of culture, the cells were removed from the tissue culture flasks, washed twice with RPMI 1640, and counted. These recultured PBMC (RPBMC) were then incubated with the 3 doses of Con-A. To determine if hyporeactive FPBMC might be capable of influencing the reactivity of autologous RPBMC, cell mixing experiments were performed. FPBMC were drawn on day 3 and cocultured in various ratios with autologous RPBMC, therefore keeping the final cell density constant at 1.7 x 10^6/ml in all experiments. The optimal responses to Con-A after 96 hr incubation among FPBMC, RPBMC, and FPBMC plus RPBMC were compared. Early experiments documented that maximal responses occurred at 96 hr; therefore, this culture time was used in comparing the response of FPBMC and RPBMC. For the MLC, the ability of fresh and precultured cells to respond after stimulation by normal allogeneic, mitomycin-C-treated cells was compared.

Control for Cell Mixing Studies

Normal PBMC were either irradiated with 2000 rad from a cobalt source or treated with 75 μg/ml of mitomycin-C (Sigma Chemical Co.) to impair the blastogenic responses to Con-A. These hyporeactive cells were then cocultured with autologous reactive cells exactly as the cocultures from normals and patients. The degree of suppression by patients' hyporeactive cells was calculated by comparing the reactivity of patients' cocultures to the diminished responses that resulted from identical ratios of irradiated or mitomycin-C-treated autologous cells. Thus, if the coculture reactivities were half that could be attributed to dilution by unreactive cells, then the degree of suppression equaled 50%. Similarly, if the coculture reactivities were identical to the dilutional effect, then only hyporeactivity equaling 0% suppression was present.

Preparation of Fresh Adherent Monocytes (FAM), Monocyte Depleted (MD) Populations, and Recultured Adherent Monocytes (RAM)

FAM were prepared by incubating PBMC at a density of 2 x 10^5/ml in RPMI 1640 with 20% human serum in plastic Petri dishes (no. 2502, Falcon Plastics). After 6 hr, the nonadherent PBMC were removed following rinsing of the adherent cell monolayer 6 times with RPMI 1640 plus 5% bovine serum albumin (BSA). FAM were detached by incubation at 4°C for 10 min with phosphate-buffered saline (PBS) containing 1% BSA plus 0.5% disodium ethylenedinitrilotetra acetic acid tetrasodium salt (EDTA) (Matheson, Coleman, and Bell, Norwood, Ohio) followed by harvesting with a rubber policeman. The FAM were immediately rinsed twice in warm RPMI 1640 prior to culturing with cells depleted of monocytes. To prepare RAM, the FAM were incubated at 1.7 x 10^5/ml in tissue culture tubes (no. 2057, Falcon Plastics) with RPMI 1640 plus 6% FBS. After 3 days, RAM were removed, and rinsed twice with RPMI 1640 prior to addition to cultures. MD populations were obtained by incubation of the nonadherent PBMC with carbonyl iron (GAF Corporation, New York) followed by passage through a magnetic field. Less than 2% esterase-positive cells and 1% phagocytic cells were present in the MD preparations. The adherent monocyte preparations consisted of greater than 95% positive for nonspecific esterase activity identified by the method of Li et al. with α-naphthyl butyrate (Sigma Chemical Co.) buffered to pH 7.5 as substrate. More than 90% of the adherent cells phagocytized latex particles (1.1 μm, Dow Diagnostics Division, Indianapolis, Ind.) according to the method of Sibbitt et al. In addition, as judged by morphology, after Giemsa staining more than 95% of the adherent cells were monocytes.

RESULTS

Reversible Impairment of PBMC Reactivity in NHL

As shown in Fig. 1, thymidine incorporation in response to Con-A was not significantly different among FPBMC (88,427 ± 7,389, mean ± SD), RPBMC (82,556 ± 10,161), or cocultures containing a 1:1 ratio of FPBMC plus RPBMC (97,892 ± 12,744) from normal controls (p > 0.05). Identical experiments performed with FPBMC, RPBMC, and FPBMC plus RPBMC from patients with NHL revealed quite different results. First, the reactivity of
IMMUNE DEFECTS IN NON-HODGKIN'S LYMPHOMA

patients' FPBMC (16,501 ± 4,947) was substantially less than FPBMC from normal controls (p < 0.001). Second, in contrast to the equivalent reactivity of FPBMC and autologous RPBMC from normals, the patients' RPBMC had a threefold mean increase in thymidine incorporation compared to their autologous FPBMC. Third, the thymidine incorporation of FPBMC plus RPBMC or patients was intermediate (37,331 ± 9,568) between either FPBMC (16,501 ± 4,947) or RPBMC (56,828 ± 8,149) alone. In 8 of the patients studied, serial coculture experiments revealed similar reactivity patterns. Because the degree of thymidine incorporation correlated well with morphological blast transformation, artifacts resulting from differences in cold thymidine pools were excluded (data not shown). The greater reactivity of patients' RPBMC compared to FPBMC was also observed in response to allogeneic stimulation during the unidirectional MLC, with a 3.2–6.0 increase observed in 6 patients examined (p<0.001). No such increase was noted in identical experiments performed with normal controls.

The FPBMC and RPBMC cell surface markers, characterized exactly as previously described, revealed that the preculturing of cells from either normal controls or patients in media alone for 72 hr did not significantly enrich or deplete T-cell or B-cell subpopulations, being 74% ± 12% and 11% ± 5%, respectively. No difference in the relative percent of monocytes was detected between FPBMC (22% ± 9%) and RPBMC (24% ± 7%) in patients or normal controls. The viability for each type of preparation was greater than 93% as determined by trypan blue exclusion. The mean recovery of patient cells after culture, however, was less than normal controls (75% compared to 90%).

**Effect of Hyporeactive PBMC on Autologous Reactive Cells**

The diminished Con-A reactivity of patients' precultured cells in the presence of autologous hyporeactive cells could represent either active suppression or the dilution of reactive by hyporeactive populations. To determine the dilution effect of hyporeactive autologous cell populations, the following experiments were performed. Hyporeactive populations were produced by treating PBMC from 7 normal subjects with mitomycin-C or 2000 rad irradiation (see Materials and Methods). The responses of the treated hyporeactive cells to Con-A were then compared to untreated autologous cells and to cocultures containing a 1:1 ratio of treated plus untreated cells. The final cell density of all cultures was 1.7 x 10⁶/ml, an identical density to the mixing experiments performed with patients' cells. Both mitomycin-C treatment and irradiation markedly inhibited lymphocyte reactivity, which averaged only 10% of untreated autologous PBMC (Fig. 2). The mixing of untreated reactive cells with treated hyporeactive cells resulted in responses that averaged 76% of the reactive populations alone. For each subject, these responses were never less than 71% of the reactive cells alone. Thus, those cocultures with responses between 71% and 100% of RPBMC represent the dilution of reaction populations by autologous hyporeactive cells. Conversely, those cocultures demonstrating responses less than 71% of RPBMC can be attributed to active suppression mediated by the hyporeactive populations.

**Qualitative and Quantitative Differences in FPBMC Suppressor Activity**

The ability of hyporeactive cells to suppress autologous reactive populations in cocultures was calculated...
for each of the 18 patients and expressed as percent suppression. Note that marked heterogeneity existed in the magnitude of suppression mediated by the hyporeactive cells (Fig. 3). In 11 of 18 patients, active suppression was detected among the hyporeactive cells ranging between 4% and 88%. In 9 of these 11 patients, the intensity of suppression was relatively modest, not exceeding 50%. The coculture responses of cells from two patients were identical to those observed secondary to the dilutional effects of unreactive populations, therefore indicating impaired proliferation, but the absence of active suppression. Five patients displayed coculture reactivities resembling the normal controls.

**Severely Impaired FPBMC and Augmented RPBMC Reactivity Occurring in the Absence of Suppressor Activity**

For the seven patients with no detectable suppressor activity among hyporeactive cells, preculturing augmented the reactivities between 1.6 and 6.0-fold (Table 2). In four patients (nos. 4, 5, 6, and 7), this absence of suppression coincided with minimally impaired reactivities (50,445 ± 20,389) compared to the responses of normals (88,427 ± 7,389). However, cells from three patients (nos. 1, 2, and 3) displayed marked hyporeactivity (8424 ± 4061) yet failed to actively suppress reactive autologous precultured cells.
the degree of suppression present within autologous hyporeactive FPBMC. Figure 4 shows that increasing suppressor activity in FPBMC correlated with the degree of reactivity recovered by the RPBMC as compared to autologous FPBMC ($p < 0.001$). No such correlation was observed between the intensity of suppressor activity and the severity of impaired responses by fresh cells.

The relationship between the intensity of suppressor activity, histology, and constitutional symptoms is shown in Fig. 5. Compared to controls, patients with diffuse histology had significant FPBMC suppressor activity ($p < 0.001$), but no significant suppressor activity was detected in those with nodular histology ($p > 0.05$). Similarly, patients lacking constitutional symptoms demonstrated no significant suppressor activity while considerable suppression was mediated by hyporeactive cells from those with symptoms ($p < 0.001$). The correlation of histology and symptoms with suppressor activity was imperfect. Thus, greater suppression among the patients with diffuse histology did not necessarily occur in association with constitutional symptoms. Since only two patients studied had localized disease, no comparison to disease stage was possible.

**Monocyte Mediation of Reversible Suppression**

PBMC were exhaustively depleted on monocytes resulting in MD populations with only 2% or less phagocytic, esterase-positive contaminants. Highly pure monocyte preparations were prepared as described in Materials and Methods. In normal controls, the reactivities of MD populations were reduced 3.8-fold ($24,051 \pm 3,052$) compared to unseparated PBMC ($95,875 \pm 21,647$). Addition of FAM to

![Fig. 4. Relationship between the magnitude of Con-A reactivity recovered during reculturing and the degree of suppressor activity. The ratio of RPBMC to FPBMC reactivity was compared to the ability of FPBMC to suppress in coculture for each of 18 patients.](image)

![Fig. 5. Comparison of the ability of hyporeactive cells to suppress Con-A reactivity in different clinical subsets of patients with NHL. The degree of FPBMC suppressor activity within patient groups differing in histology or constitutional symptoms was compared to normal controls. The number of subjects studied is indicated in parentheses.](image)
reactive lymphocytes, since the percent monocytes assessed by phagocytosis and nonspecific esterase stain were 26% ± 11% and 21% ± 8%, respectively, for the patient and control PBMC.

In contrast to the 3.2-fold augmentation observed after adding monocytes to monocyte-depleted cells among normal controls, freshly prepared monocytes from patients with NHL suppressed autologous depleted populations 2.5-fold, resulting in a response not different from the hyporeactive FPBMC. After preculturing, however, this suppressive effect diminished since the 3-day cultured monocytes failed to suppress the populations depleted of monocytes. In all cocultures, the final cell density was 1.7 × 10⁶/ml, therefore excluding artifacts secondary to differing cell densities or media exhaustion.

**DISCUSSION**

In the present study, a direct autologous coculture system analyzed the heterogeneous mechanisms contributing to impaired in vitro lymphocyte responses in NHL.

Most patients displayed impaired in vitro responses following stimulation by Con-A with improved reactivities after preculturing the cells 3 days in media alone. In over half of the patients, the hyporeactive populations demonstrated suppressive activity when assayed in cocultures containing the reactive, autologous precultured cells. The magnitude of suppression was not marked when compared to the severity of in vitro impairment, with only two patients demonstrating intense suppression. In addition, highly purified monocyte preparations were observed to participate in the mediation of this suppressive effect. Although the presence of greater suppressor activity correlated with the increased recovery of reactivities by precultured cells, enhanced reactivity also occurred in the absence of suppressive influences. Thus, a reversible, inhibitory mechanism distinct from active suppression contributed to impaired responsiveness. Besides the suppressive and inhibitory mechanisms, an intrinsic abnormality possibly existed among the responding cell populations. This was suggested by the failure of the majority of patients’ cells to acquire normal responsiveness after preculture.

Several points regarding the relationship between the presence of suppressive activity and inhibited responses should be emphasized. First, although the recovery of reactivity by patients’ precultured cells correlated with the magnitude of suppression detected among hyporeactive populations, some patients demonstrated markedly impaired responses in the absence of suppression (Table 2). Therefore, the degree of suppression did not coincide with the severity of impaired responsiveness, implicating other inhibitory types of mechanisms. Soluble inhibitory materials have been described in both lymphoid and solid malignancies,¹⁶ and we recently observed that approximately 40% of NHL sera contain such materials.¹⁷ Though preculturing may augment patients’ cell responses by the membrane shedding of these soluble inhibitory materials, we have not yet been able to detect substantial augmentation following exposure of patients’ cells to pronase, trypsin, or neuraminidase. Similarly, the addition of indomethacin to hyporeactive cultures has yielded varying results, indicating that prostaglandins might be responsible for the impairments in certain subsets of patients with NHL but not to the extent noted in HD.¹⁸ It is possible, however, that the present system may underestimate prostaglandin-mediated suppression since others have described the relative resistance of precultured cells to the suppressive effects of prostaglandins.¹⁹,²⁰

Second, it must be emphasized that the suppression mediated by monocytes only partially accounts for the in vitro impairments since exhaustive depletion of monocytes fails to result in normal responsiveness (Fig. 6). This observation, along with the finding that the majority of patients’ cells fail to demonstrate
normal reactivities following preculture, suggests either a more irreversible type of suppressive-inhibitory component or an intrinsic defect among the potentially reactive populations. Although a relatively longer-lived population of suppressor cells has not entirely been excluded, the failure of preculturing to restore normal responses in those patients without suppressor activity argues against this type of possibility. Thus, we presently favor the existence of an intrinsic defect, but current work is more directly examining this possibility.

Interestingly, more intense suppression was observed in those patients with diffuse histology and constitutional symptoms that are characteristics previously reported to be associated with severely impaired in vivo immunocompetence. Longitudinal studies may be helpful in discriminating whether this relatively more intense suppressive activity is a secondary type of phenomenon, representing more severe disease manifestations, or alternatively, is indicative of a more primary immune abnormality and poorer prognosis.

Regardless, these data directly demonstrate that the impaired lymphocyte responses in patients with NHL are a result of heterogeneous mechanisms. Future studies should determine if the further clarification of these mechanisms may be pertinent to the in vivo situation and approaches to treatment.

REFERENCES

Heterogeneous mechanisms of impaired lymphocyte responses in non-Hodgkin's lymphoma

RL Whisler, SP Balcerzak and JL Murray