Increased Sensitivity to Normal Adherent Suppressor Cells in Untreated Advanced Hodgkin's Disease

By Richard I. Fisher, Christian Vanhaelen, and Frieda Bostick

Mononuclear leukocytes isolated from the peripheral blood of 10 untreated patients with advanced stages of Hodgkin's disease and 26 normal volunteers were stimulated with allogeneic cells from normal donors in a one-way mixed lymphocyte culture. When the patients' cells were stimulated by an increased number of normal cells, the baseline mixed lymphocyte culture was suppressed 77.1% ± 4.1%. In contrast, when the normal responder cells were cultured with increased numbers of normal stimulator cells, mean suppression was only 46.6% ± 4.4% (p < 0.001). The removal of phagocytic cells from the high concentration of normal stimulators totally abolished the suppression observed with the patients' cells, resulting in a mean increase in stimulation of 84% above that found in the baseline culture. The suppression could also be reversed by depletion of adherent cells from stimulator leukocytes. When adherent and nonadherent stimulator cells were recombined, significant suppression of proliferation was again observed. The increased suppression was not caused by the presence of autologous plasma in patients' cultures, since similar results were obtained utilizing AB serum. The addition of indomethacin to the cultures only partially reversed the suppression observed with the patient's cells. These studies demonstrate that mononuclear leukocytes from the peripheral blood of untreated patients with advanced stages of Hodgkin's disease have increased sensitivity to a normal adherent suppressor cell system.

MATERIALS AND METHODS

Subjects

Ten consecutive patients with advanced stages of Hodgkin's disease, admitted to the Medicine Branch of the NCI, were studied. The clinical characteristics of these patients are shown in Table I. No patient had received prior radiation therapy or chemotherapy. All patients were staged according to the scheme proposed at the Ann Arbor Conference. The control population consisted of 26 healthy, normal volunteers (15 males, 11 females) of similar age distribution.

Mononuclear Cell Preparation

Peripheral blood mononuclear cells were separated from freshly drawn heparinized blood by centrifugation over Ficoll-Paque (Pharmacia Chemicals, Piscataway, N.J.) utilizing a modification of the method of Boyum. The cells were then washed three times in RPMI 1640 (Grand Island Biological Co., Grand Island, N.Y.) supplemented with 2 mM glutamine, 100 μg/ml penicillin, and 50 mg/ml streptomycin (NIH Media Unit), hereafter referred to as medium.

Mixed Lymphocyte Cultures

One-way mixed lymphocyte cultures were performed utilizing 2 x 10^6 responding cells in 100 μl medium plus 20% autologous plasma in flat-bottom plastic microtiter plates (Falcon Plastics, Oxnard, Calif.). In later studies, 20% pooled AB serum (North American Biologicals, Miami, Fla.) was used instead of autologous plasma. Stimulator cells were first radiated with 2500 rad by a 137Cs source at 1650 rad/min; then 2 x 10^6 or 8 x 10^3 stimulating cells in 100 μl medium were added to each well. The plates were incubated at 37°C in a humidified 5% carbon-dioxide-air atmosphere for 5 days. Each well was then pulsed with 1.0 μCi of tritiated thymidine, with a specific activity of 6.7 Ci/mmol (New England Nuclear Co., Boston, Mass.). After an additional 24 hr of incubation, the cells were harvested on a Multiple Automated Sample Harvested II (Microbiological Associates, Washington, D.C.). The glass fiber filter paper (Whatman Inc., Clifton, N.J.) containing the samples was washed with distilled water and ethanol and transferred to glass counting vials. After adding 15 ml of Aquasol (New England Nuclear...
SENSITIVITY TO SUPPRESSION IN HODGKIN'S

Determination of Suppression

The suppression induced by higher numbers of stimulating cells was calculated by the following formula:

\[
\text{% Suppression} = \frac{\text{MLC}(2) - \text{MLC}(8)}{\text{MLC}(2)} \times 100
\]

where MLC(2) was the mixed lymphocyte response to \(2 \times 10^3\) stimulators and MLC(8) the response to \(8 \times 10^3\) stimulators. Differences in the MLC and in suppression between patients with Hodgkin's disease and normal controls were evaluated statistically by the Wilcoxon-Mann-Whitney rank sum test.

Removal of Phagocytic Cells

Phagocytic cells were removed from the mononuclear cells by modification of the method of Poplack et al. Twenty million mononuclear cells were suspended in 1 ml of media plus 10% heat-inactivated fetal calf serum (Grand Island Biological Co.) and added to 3 ml of fetal calf serum in 15-ml plastic tubes (Falcon Plastics). The tube was then filled with 11 ml of an iron-filing-containing solution (Lymphocyte Separation reagent, Technicon Instrument Co., Tarrytown, N.Y.) and placed on a rotating platform for 45 min at 37°C. The iron particles were pulled to the bottom of the tube with a magnet. The cells in the supernatant were collected and centrifuged on a Ficoll-Paque gradient. Finally, the nonphagocytic cells were recovered from the interface and washed twice in medium.

Separation of Adherent Cells

Adherent cells were separated from the mononuclear cells by modification of the method of Sakane and Green. Mononuclear cells were suspended in medium plus 5% AB serum (North American Biologicals, Miami, Fla.) at \(2 \times 10^7\) cells/ml and plated in 100 \(\times 15\) mm Petri dishes (Falcon Plastics). The dishes were incubated for 60 min at 37°C in a 5% carbon-dioxide-air atmosphere. The dishes were then agitated gently and the nonadherent cell population collected. These cells were then washed twice in medium without protein. Residual nonadherent cells were removed from the dishes following vigorous pipetting with 5 ml medium. Calcium- and magnesium-free Hank's balanced salt solution (Grand Island Biological) was added to the dishes, which were then placed on ice for 30 min. The adherent cells were then collected by scraping with a rubber policeman and by vigorous pipetting. The adherent cells were then washed twice in medium. The nonadherent cells contained greater than 97% lymphocytes as judged by morphology and nonspecific esterase stain. The adherent population contained greater than 70% monocytes, as judged by the same criteria. Cell viability as judged by trypan blue dye exclusion exceeded 95%.

Cryopreservation of Mononuclear Cells

Mononuclear cells at a concentration of \(1-2 \times 10^6\) cells/ml in medium plus 40% heat-inactivated fetal calf serum were plated on ice. Slowly, an equal volume of cold medium plus 20% dimethyl sulfoxide (Crown Zellerbach Corp., Camas, Wash.) was added with gentle agitation. One milliliter of the suspension was aliquoted into each cold Nunc vial (Intermed Co., Copenhagen, Denmark) and the vials placed into a Cryo-Med Programmable Freezing System (Cyro-Med, Mt. Clemens, Mich.). The cells were cooled at 1°C/min until a temperature of –80°C had been reached. They were then transferred to a liquid nitrogen freezer (Union Carbide Corp., Indianapolis, Ind.) for storage at –180°C.

In order to recover the cells, the vials were removed from the freezer and immediately placed in a 37°C water bath with agitation. Before the last ice crystal melted, the vials were placed on ice for 2 min. They were then transferred to a 15-ml plastic tube at room temperature. One drop of medium plus 20% fetal calf serum was added. Every 2 min, the volume of the diluting medium being added was doubled until the cells were suspended in 15 ml. The mononuclear cells were then centrifuged and washed twice in medium without protein. Greater than 70% of cells were recovered. Cell viability as judged by trypan blue dye exclusion exceeded 95%.
Effect of Indomethacin

Indomethacin was kindly provided by Dr. Clement A. Stone, Merck Sharp & Dohme Research Laboratories, West Point, Pa. Indomethacin was prepared and added to the mixed lymphocyte cultures containing $8 \times 10^5$ stimulator cells at a final concentration of 1 $\mu$g/ml as previously described.

RESULTS

Mixed Lymphocyte Cultures

Ten untreated patients with advanced stages of Hodgkin’s disease were each studied in a one-way mixed lymphocyte culture utilizing $2 \times 10^5$ responder cells from the patient and $2 \times 10^5$ radiated stimulator cells from a normal donor. Results of the MLC for each patient are shown in Table 1. In addition, mixed lymphocyte cultures were performed among pairs of 26 normal volunteers under identical conditions. The mean stimulation of the MLC for the patients with Hodgkin’s disease, $67,263 \pm 13,168$ dpm, did not differ statistically from the mean of the normal controls, $55,000 \pm 5,000$ dpm ($p > 0.30$).

Suppression of the MLC by Increased Numbers of Normal Stimulator Cells

When $2 \times 10^5$ responder cells from the patients with Hodgkin’s disease were stimulated with $8 \times 10^5$ radiated stimulator cells from the normal donors, the MLC was suppressed in each patient (Table 1). The percent suppression for each patient, as well as the percent suppression for the MLC of each of the 26 normal volunteers, stimulated with $8 \times 10^5$ allogeneic normal stimulator cells is shown in Fig. 1. The mean suppression for the patients with Hodgkin’s disease, $77.1\% \pm 4.1\%$, is significantly greater than that observed in the normal population, $46.6 \pm 4.4\%$ ($p < 0.001$).

Effect of Phagocytic Cells on Suppression

Phagocytic cells were removed from the normal stimulator cells by utilizing an iron depletion technique. When the cells from patients with Hodgkin’s disease were stimulated with $8 \times 10^5$ normal, phagocyte-depleted mononuclear cells, the suppression of the mixed lymphocyte culture was totally abolished. In fact, the mean observed stimulation increased 84% above that observed when the patient’s cells were stimulated by $2 \times 10^5$ normal mononuclear cells.

Effect of Adherent Cells on Suppression

Normal mononuclear stimulator cells were allowed to adhere to plastic and the nonadherent and adherent cells isolated. Results of a characteristic experiment utilizing one patient with Hodgkin’s disease and one normal as sources for responder cells and another normal individual as a source of stimulator cells are shown in Table 2. The patient’s MLC was suppressed 82% when stimulated with $8 \times 10^5$ mononuclear cells. When $8 \times 10^5$ nonadherent cells were used as stimulators, the suppression was totally reversed and the MLC actually increased (mean suppression, $-112\%$). When a normal served as the source of responder cells, suppression with $8 \times 10^5$ mononuclear cells was 38% and with $8 \times 10^5$ nonadherent cells, $-11\%$. When 20–30% adherent cells were added to nonadherent cells, the original suppression was again observed for both the patient and normal responder.

Utilization of Cryopreserved Mononuclear Cells as Responder Cells

Since treatment for the patients with Hodgkin’s disease was initiated shortly after referral to the NCI, repeated pretreatment blood samples were often not...
available. Therefore, mononuclear cells were cryopreserved and tested later to determine whether cryopreserved cells could be utilized as responder cells in the MLC, thereby allowing repetition and verification of the initial results. Four patients were studied before and after cryopreservation. A single normal donor served as the source of stimulator cells for any given patient. Mean suppression of the assays performed with fresh mononuclear cells was 66% ± 7%, while that of the assays performed on frozen cells was 68% ± 6%.

Effects of Autologous Plasma on Suppression

All initial cultures were performed in plasma from the responder. Since suppressor substances have been described in the serum of patients with Hodgkin’s disease,15,16 simultaneous cultures were performed utilizing responder cells from patients in either autologous plasma or pooled AB serum. Five patients were studied. The mean change in suppression was only 2.8% ± 4.8%.

Effect of Indomethacin on Suppression

When 1 μg/ml indomethacin was added to the cultures of 6 normal responders, the change in suppression was minimal (mean change in suppression, −0.7% ± 2.3%). The addition of 1 μg/ml indomethacin to the cultures of 6 patients with Hodgkin’s disease resulted in a decrease in suppression (mean change in suppression −9.0% ± 3.7%). Identical results were obtained with higher concentrations of indomethacin, i.e., 10 and 20 μg/ml.

DISCUSSION

Laughter and Twomey have described an adherent cell suppressor system that was present in normal individuals and limited the proliferation of mononuclear leukocytes.17 The system was demonstrated by increasing the number of stimulator cells in a one-way mixed leukocyte reaction. The suppressive effect was eliminated by removing adherent cells from the stimulator population. Additional characteristics of these suppressor cells were their lack of genetic restriction, their resistance to radiation or mitomycin treatment, and their inactivation by carageenan.

We have modified this adherent cell suppressor system in order to study the effect of increasing numbers of normal mononuclear stimulator cells on the proliferative response of cells from untreated patients with advanced stages of Hodgkin’s disease. Our cultures contained 2 x 10⁹ responder cells instead of 10⁸ and were harvested after 6 days instead of 7. Both systems utilize 2 x 10⁸ stimulator cells in the baseline MLC. Our conditions were chosen so that stimulation of normal responder cells by 8 x 10⁹ normal allogeneic stimulator cells resulted in 47% less proliferation than observed in the baseline MLC. With this magnitude of suppression one could hopefully detect either increased or decreased sensitivity to suppression when cells from patients with Hodgkin’s disease were used as responders.

When mononuclear cells from the patients with Hodgkin’s disease were stimulated with standard numbers of stimulator cells (2 x 10⁹) in a baseline MLC, the proliferative response was normal—an observation also made by other investigators.12,22 However, when the patients’ cells were stimulated with 8 x 10⁹ normal mononuclear cells, the proliferative response was suppressed by 77% compared to the baseline MLC. This suppression was significantly greater than the suppression observed when normal responder cells were stimulated with an increased concentration of normal stimulator cells. It is important to recognize that the same number of adherent cells from the patients with Hodgkin’s disease were present in both the baseline MLC and the MLC containing a higher concentration of normal stimulator cells. Since the baseline MLC of the patients was not suppressed relative to that observed in other normals, the increased suppression seen when the patients’ cells were stimulated by larger numbers of normal stimulator cells cannot be attributed to increased suppressive activity of the patients’ adherent cells. Thus, the mononuclear cells from untreated patients with advanced stages of Hodgkin’s disease have increased sensitivity to a normal adherent suppressor cell.

The suppression was completely abolished by removing either phagocytic or adherent cells from the stimulator cells. The recombination of adherent and nonadherent cells reconstituted the suppressive capability. Although initial cultures were all performed with autologous plasma, subsequent experiments revealed that substitution of pooled AB serum yielded identical results. Thus, in contrast to the suppressive effects that may be caused by serum from Hodgkin’s patients in certain assays,13,16 the suppression in this system is not mediated via a host serum component.

Goodwin et al. have demonstrated that phytohemagglutinin-induced proliferation of mononuclear cells from patients with Hodgkin’s disease is subnormal and can be reversed by the addition of indomethacin. This suppression of proliferation has been shown to be caused by increased monocyte production of prostaglandins and not increased sensitivity to prostaglandins.14 In our adherent cell suppressor system, the addition of indomethacin to the cultures had no effect on the suppression when normal stimulators and
normal responders were studied. This finding is in agreement with that of Laughter and Twomey. However, when indomethacin was added to cultures using cells from Hodgkin’s patients as responders, the suppression was decreased by 9%. Since the suppression observed with the Hodgkin’s responders was 30% greater than that found with normal responder cells, the data demonstrate that only part of the increased suppression may be caused by prostaglandins in the culture. Another mechanism of monocyte suppression of proliferation must explain the remaining suppression.

The data presented in this paper are consistent with that of Hillinger and Herzig but may provide an alternative explanation for their observations. They added increasing numbers of mitomycin-treated responder cells to a mixed lymphocyte culture. Cells from normal donors were used as stimulator cells in all cases. When increased numbers of cells from patients were added to cultures using patients as responders, a mean suppression of 78% was noted. When increasing numbers of normal cells were added to cultures using normal cells as responding cells, mean suppression was only 21%. Furthermore, when increased numbers of cells from patients with Hodgkin’s disease were added to cultures using normal cells as responders, low levels of suppression were again observed. The authors postulated that these findings could be explained by increased numbers of genetically restricted suppressor cells in patients with Hodgkin’s disease. However, their results are also consistent with our observation that patients with Hodgkin’s disease have increased sensitivity to a normal suppressor cell that could be found in patients and normals alike. In the case, the magnitude of suppression would be determined by the sensitivity of the responder and not the source of suppressor cells. Our system does differ from that of Hillinger and Herzig in one important regard. Although the suppressor cell was characterized as a monocyte in six of their ten patients with Hodgkin’s disease, four patients did appear to have suppression mediated via T lymphocytes. Engleman et al. have also reported the presence of a T suppressor cell in Hodgkin’s disease. In this study and in the studies of Twomey, no evidence was obtained to suggest that T lymphocytes caused the observed suppression.

The results of this study suggest that in order to improve our understanding of the pathogenesis of immunosuppression associated with certain disease states, we must study not only the number and activity of immunoregulatory cells, but also the responsiveness of the targets of the immunoregulatory cells. The mechanisms responsible for this heightened sensitivity to suppression in Hodgkin’s disease are currently unknown. Successful modulation of the immune response in these patients may only be possible when the interactions of regulatory cells and their targets are completely defined.

REFERENCES

20. Poppelbeck DG, Bonnard GA, Holiman BJ, Blaese RM: Mono-


Increased sensitivity to normal adherent suppressor cells in untreated advanced Hodgkin’s disease

RI Fisher, C Vanhaelen and F Bostick