Impact of Specific Immunotherapy in Acute Myelocytic Leukemia

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Introduction

During the last ten years immunotherapy has become an important tool in the treatment of human leukemias. Mathé et al. [1,2] demonstrated the therapeutic value of irradiated allogeneic myeloblasts in combination with BCG in treating childhood lymphoblastic leukemia. Similar studies were conducted by Powles et al. [3] and Gutterman et al. [4] in patients with acute myelocytic leukemia involving chemotherapy with or without irradiated allogeneic myeloblasts plus BCG. These studies consistently show that immunized patients sustain a somewhat longer remission duration than those without immunization. Also, after the first relapse immunized patients are reported to have higher frequency and greater "ease" of reinduction. BCG has been used in conjunction with cultured leukemia cells in the immunization of patients with chronic myelocytic leukemia by Sokol et al. [5]. Under optimal conditions prolongation of median survival of CML patients was attained in patients who were treated with busulfan and immunotherapy as compared to controls who received busulfan alone. In an attempt to find a more standardized immunological adjuvant, Weiss et al. [6] conducted extensive studies with MER, the methanol extraction residue of BCG. They were able to demonstrate therapeutic advantage of MER, especially in murine leukemias.

Neuraminidase of Vibrio Cholerae origin has been used successfully in increasing the expression of tumor specific antigenicity of autochthonous and syngeneic tumors. This phenomenon is exclusively due to the enzymatic cleavage of surface membrane bound N-acetylneuraminic acid from the tumor cells [7]. The effectiveness of neuraminidase (N'ase) modified spontaneous and transplantable tumors as immunogen in both immunoprophylactic and chemoimmunotherapeutic experiments in syngeneic mice was positively established [8–11]. We have demonstrated that neuraminidase-treated E_2G leukemic cells which, like the AKR leukemia, are Gross virus induced, but are completely different at the H_2 genetic locus from the AKR mice, were as effective as the syngeneic leukemic cells in prolonging the survival of leukemic AKR mice [12]. This suggests the existence of a cross-reacting common viral membrane antigen, and would suggest that if similar etiology existed for human acute leukemia, it would not be essential to use autologous leukemia cells for immunization. These data provided the basis for using neuraminidase-treated allogeneic myeloblasts in human immunotherapeutic investigations. It will be demonstrated here that combined with

an effective remission inducing and sustaining chemotherapy in patients with acute myelocytic leukemia, neuraminidase modified allogeneic myeloblasts have an important therapeutic value when administered in a systematic program of chemoimmunotherapy.

Remission and Sustaining Chemotherapy

The chemotherapy protocol on this study is based in maximal chemotherapeutic reduction of leukemic burden. This is achieved by induction therapy with a regimen of cytosine arabinoside continuously administered intravenously for 7 days at 100 mg per square meter of body surface area per day, and daunorubicin at a dose of 45 mg per square meter of body surface area per day by direct injection on days 1, 2 and 3. This regimen has induced approximately 70 per cent of patients into remission. All patients were between the ages of 15 and 70. All received cyclical maintenance chemotherapy every 4 weeks. This consisted of 5 day courses of AraC in addition to 6-thioguanine, cyclophosphamide, CCNU, or daunorubicin sequentially with each course repeated at 4 months cycles.

Collection of Allogeneic Myeloblasts for Immunotherapy

Patients became eligible for collection of myeloblasts after satisfying the following criteria: Negative HA-A as determined by radioimmunoassay, no previous chemotherapy, total WBC higher than 25000/μl, and higher than 70% myeloblasts in the peripheral blood. The myeloblasts were obtained by leukophoresis. In the last five years we collected myeloblasts from 93 patients between the ages of 14 and 72 years and have not encountered any important side-effects during the two to four hour procedure. After leukophoresis, the myeloblasts were separated from contaminating red blood cells by sedimentation at 37°C. After sedimentation leukemic cells were mixed with special freezing media (free of calcium and magnesium) containing 15% autologous or AB plasma and 10% DMSO. The final cell concentration was $0{,}3-1{,}0\times 10^8$ cells/ml. Myeloblasts were frozen by programmed freezing at a temperature drop of 1,5°C per minute until – 38°C was reached, and then rapidly to 80°C. The frozen cells were immediately stored in the vapor phase of liquid nitrogen.

Treatment of Allogeneic Myeloblasts with Neuraminidase

Myeloblasts were thawed and were washed twice with mixed salt and glucose media at 4°C and further purified on a 22 per cent human albumin gradient, layered over 45% surcrose for the separation of viable from nonviable blast cells. After purification, blast cells were washed and incubated with N'ase at a concentration of 50 units of enzyme per 5×10^7 cells/ml in

sodium acetate buffer, for 45 minutes at 37°C. The cells were then washed and resuspended in physiological saline and used as immunogen within 30 minutes.

Immunization with Allogeneic Myeloblasts and MER

Immunization with neuraminidase treated allogeneic myeloblasts was performed by intradermal injections. In order to get maximum exposure to the immunogen, sites were widely spread in the supraclavicular, infraclavicular, arm, forearm, parasternal, thoracic, suprainguinal and femoral regions draining into several node bearing areas.

Dose dependent cellular titration was performed with each immunization with 0,5, 1,5, $2,5 \times 10^8$ and 0 cells. The total immunization load was about 10^{10} cells at 48 body sites. The injections of neuraminidase treated myeloblasts produced no local lesions other than the delayed type cutaneous hypersensitivity reaction (Table 1) and none of the patients developed chill, fever, or adenopathy. No hypersensitivity reaction was apparent at the site of injection of physiological saline, heat denatured neuraminidase, or supernatant of cell incubation media. In patients randomized to receive MER too, we used ten intradermal sites of 100 µg/.1 ml each totaling 1,0 mg of MER.

Table 1. Delayed hypersensitivity response to X-irradiated or neuraminidase treated myeloblasts

Immunization Cycles	Induration[a]						
	Number of n'ase treated myeloblasts injected per site $\times 10^8$				Number of X-irradiated myeloblasts injected per site $\times 10^8$		
	0.5	1.0	2.0	3.0	1.0	2.0	3.0
1	3.5 ± 1.5	6.2 ± 2	14 ± 4	18 ± 3	3.0 ± 1	5.4 ± 1.5	7.8 ± 2
6	7.1 ± 2	12.9 ± 3	19.8 ± 6.1	24 ± 6	4.2 ± 2.1	7.5 ± 3	8.4 ± 1.2
12	8.3 ± 2.4	14.1 ± 2.9	20.3 ± 5	25.1 ± 7	4.6 ± 1.6	7.2 ± 2.4	8.1 ± 2.5

[a] Mean induration in mm obtained from at least 40 injected sites. measured 48 hours after the intradermal injection of myeloblasts.
Standard error of mean

Impact of Specific Immunotherapy in Patients with Acute Myelocytic Leukemia

Based on experimental observations, a successful chemoimmunotherapy trial was conducted in patients with acute myelocytic leukemia. The interim analysis presented below is calculated by standard life table methods and is subdivided into several subsets. The data represents 91 patients with AML who were allocated in three groups following successful remission induction

using cytosine arabinoside and daunorubicin. Patients designated to receive immunotherapy were injected (i.d.) in approximately 48 sites every 28 days with 10^{10} N'ase treated allogeneic myeloblasts. For 27 of the 91 acute myelocytic leukemic patient, the remission duration on the chemotherapy alone was 243 days; for those receiving N'ase modified allogeneic myeloblasts as immunogen the mean remission was 686 days (Fig. 1). The difference in remission duration between the two treatment groups is highly significant: $p = .001$ using Breslow's, Logrank and Cox regression analysis. Combination of specific plus adjuvant immunotherapy did not act synergenistically in the treatment of AML patients. Fig. 2 shows the behavior of patients immunized with N'ase treated myeloblasts plus the full prescribed dose of MER with a mean remission duration of 336 days. This was compared to another group of patients, in whom, based on the demonstration of the presence of suppressor cells and supporting clinical evidence, the MER dose was attenuated or omitted. This modality provided considerable improvement in the remission duration (of 630 days) but still not has reached the level attained with N'ase treated myelobasts alone (see Fig. 1).

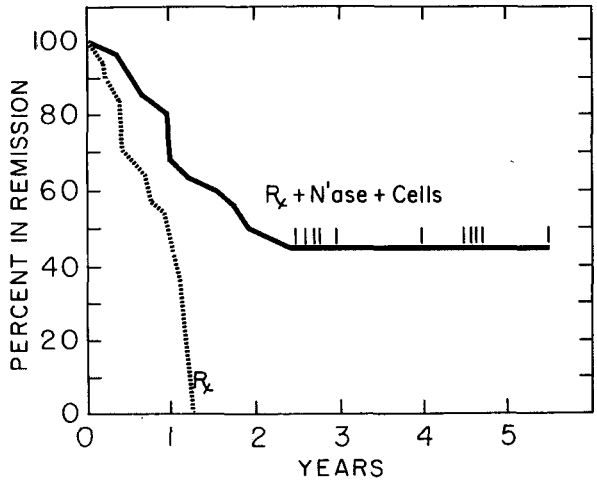

Fig. 1. Duration of complete remission in acute myelocytic leukemia in patients immunized with neuraminidase treated allogeneic myeloblasts.

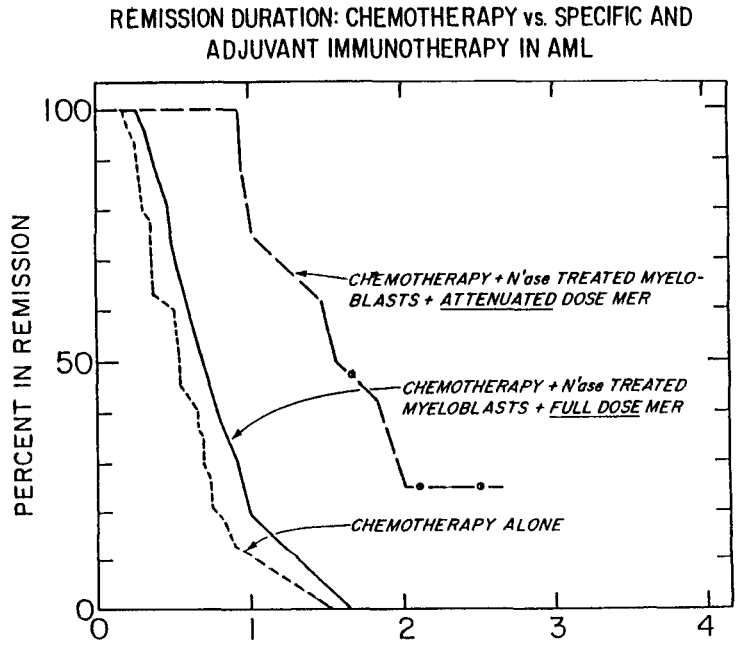

Fig. 2. Effect of chemotherapy and chemotherapy plus neuraminidase treated allogeneic myeloblasts plus MER on remission duration in patients with AML

However the difference between the control vs. cell + MER is significant at $p = .03$. It appears that inclusion of MER in this immunotherapy protocol adds no value to chemoimmunotherapy when added to N'ase treated allogeneic myeloblasts as immunogen in AML patients.

Response to Recall Antigens

The in vivo immunological status of the immunotherapy patients at various stages of their treatment was measured by DCH response to five recall antigens; PPD, mumps, candida, varidase and dermatophytin. Interpretation of the skin tests was based on the induration as measured in millimeters in two directions at 48 hours and considered positive if the diameter of induration exceeded 5 mm. Fig. 3 shows that there were significant improvements in the response to recall antigens in patients immunized with N'ase treated myeloblasts. However, patients who received N'ase treated myeloblasts plus MER, after an initial improvement, the DCH response to recall antigens gradually declined and was ultimately eradicated. The decline of in vivo response to antigens often preceeded subsequent relapse of those individuals who received full dose of MER in addition to N'ase treated myeloblasts.

Impact of Immunotherapy on the Immunological Status of AML Patients

On each immunization day, remission lymphocytes were isolated from freshly drawn heparanized blood by the Ficoll-Hypaque gradient method for the following in vitro assays: Surface markers by E and EAC rosettes,

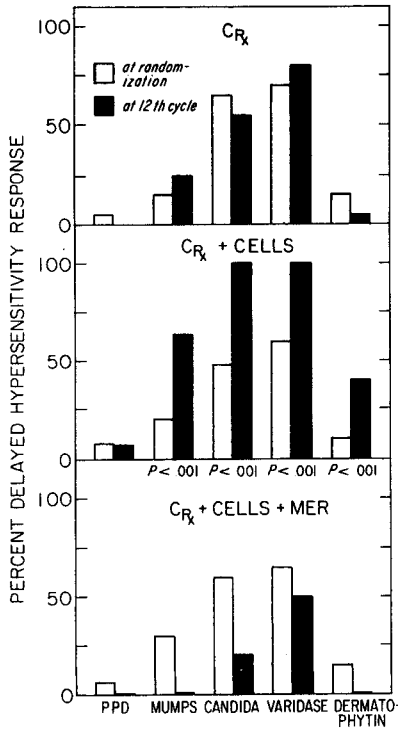

Fig. 3. Change of delayed hypersensitivity response to recall antigens during the course of chemoimmunotherapy

phytohemagglutinin (PHA) and pokeweed mitogen (PWM) induced lymphoblastogenesis and tumor leukocyte culture (MLTC) with immunizing allogeneic myeloblasts.

Although the quantification of E and EAC rosette forming lymphocytes from AML patients in the protocols were routinely performed, we are only showing in Table 2 two test periods: a) The initial E and EAC values at the time of randomization, and b) the impact of immunotherapy on the T and B lymphocyte surface markers. The median value for normal donors of E-rosetting PBL is 74,4%, with 1,986 as the number of absolute T-lymphocytes. For EAC rosetting the normal PBL values are 22,1% with 521 as the number of absolute lymphocytes. Patients at the time of randomization, still under recovery from induction and consolidating chemotherapy, have shown significantly lower percentage (49,2 and 51,7) and absolute number (412 and 487) of T-cells as well as lower percentage (16,2 and 16,5) and absolute number (169 and 195) of EAC rosetting lymphocytes.

Patients in both chemotherapy regimen showed a significant increase of T- and B-lymphocytes as compared to values at the time of randomization both in percentage and in absolute number.

Maximum lymphocyte blastogenesis was attained at 0,15 μg per well for PHA and 30 μg per well for PWM, for normal donors, as well as for the remission lymphocytes from patients in either of the therapeutic regimen. Lymphocytes obtained from patients receiving chemotherapy alone showed consistently lower degree of stimulation to both mitogens all through the observation periods. Lymphocytes obtained from AML patients who have been immunized with N'ase treated myeloblasts showed, despite the fact that they have been receiving chemotherapy, nearly normal lymphocyte function (Table 2). Countraiwise, patients immunized with N'ase treated myeloblasts plus MER have shown in the first six months of immunotherapy a continuous improvement in their response to mitogens but not to tumor cells. This was followed by a gradual decline in lymphocyte function. The fact that patients treated with N'ase treated myeloblasts plus MER have similar E-rosetting PBL as patients treated with cells alone, but have significantly altered in vivo and in vitro lymphocyte function (Fig. 4), raised the possibility of the presence of an inhibitory mononuclear cell population in the blood of such immunized patients. This hypothesis was tested and the data are summarized in Fig. 5. Isolated enriched T-cell fractions from normal donors or from patients immunized with N'ase modified myeloblasts gave similar uptake of H^3TdR as the unseparated PBL. However, isolated enriched T-cell fraction from AML patients who received N'ase treated myeloblasts plus MER, and have shown declining in vivo and in vitro immunological responses, gave 3–7 times greater H^3TdR incorporation in response to PHA than their unseparated PBL. The response of the enriched T-cells was strongly inhibited by addition of autologous but not normal donors' adherent mononuclear cells. These findings suggest that depression of cell mediated immunity is seen in most of the tested AML patients who received N'ase treated myeloblasts plus full dose of MER, but not among the patients immunized with N'ase modified myeloblasts alone,

Table 2. Lymphocyte function and surface membrane markers of remission lymphocytes in AML patients in the immunotherapy study

	PHA		PWM		MLTC[a]		MLTC[b]		E-Rosettes		EAC-Rosettes	
	max. stimul. c.p.m. × 10^3	SI	max. stimul. c.p.m. × 10^3	SI	max. stimul. c.p.m. × 10^3	SI	max. stimul. c.p.m. × 10^3	SI	%	absolute number	%	absolute number
Normal subjects N 79	102.2 ±8.7	257	95.1 ±6.4	194					74.4	1,986 ±251	22.1	521 ±52
Randomization Chemotherapy N 25 After 8th course	41.2 ±5.1	93	37.8 ±5.3	67	15.1 ±3.4	29	19.2 ±4.9	36	49.1	412 ±17	17.5	146 ±6
	51.7 ±6.1	117	49.3 ±5.9	89	19.6 ±4.2	37	22.3 ±6.1	39	56.3	725 ±17	21.3	274 ±14
Randomization Immunotherapy with cells N 34 After 12th course	37.1 ±4.4	90	35.8 ±6.4	73	18.3 ±6.1	32	21.5 ±6.9	35	49.2	429 ±21	18.5	161 ±8.2
	79.5 8.2	212	75.3 ±7.6	142	28.7 ±8.5	59	20.5 ±3.2	29	70.1	1,122 ±120	20.2	323 ±27
Randomization Immunotherapy cells + mer N 19 After 12th course	38.3 ±5.4	85	37.1 ±4.9	79	17.6 ±4.1	28	18.7 ±3.5	31	51.7	487 ±45	22.3	210 ±21
	51.2 ±6.0	113	46.6 ±6.3	68	20.9 ±2.7	29	21.3 ±5	37	70.1	1,099 ±118	21.5	337 ±37

Allogeneic myeloblasts [a] used for immunotherapy or [b] not used for immunotherapy. the mixed tumor-leukocyte culture was performed: Myeloblasts incubated with mitomycin-C at 30 µg/ml of 2×10^6 cell suspension for 30 minutes at 37°C. After washing 2×10^5 myeloblasts were distributed in each of the replicate wells of the falcon microplates containing 10^5 responding lymphocytes per well in RPMI 1640 media supplemented with 20% heat inactivated autologous plasma. After 90 hours of incubation 1 µci ^{3}H-Tdr was added per well. Cultures were harvested 18 hours later with the addition of excess cold thymidine.

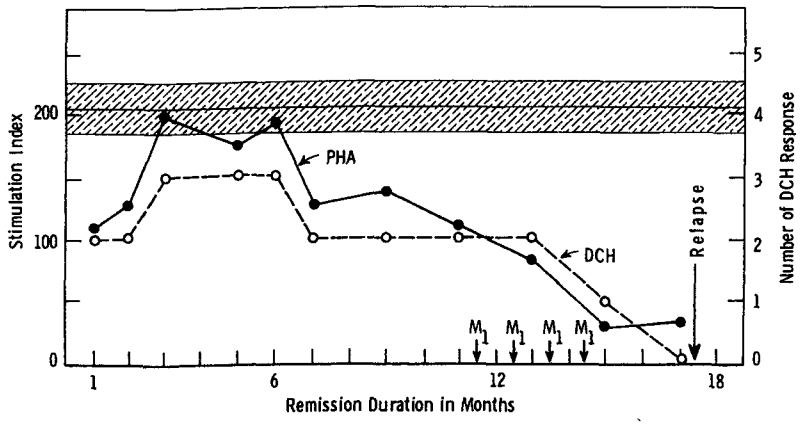

Fig. 5. Appearances of suppressor cell activity in AML patients treated with N'ase treated myeloblasts plus MER

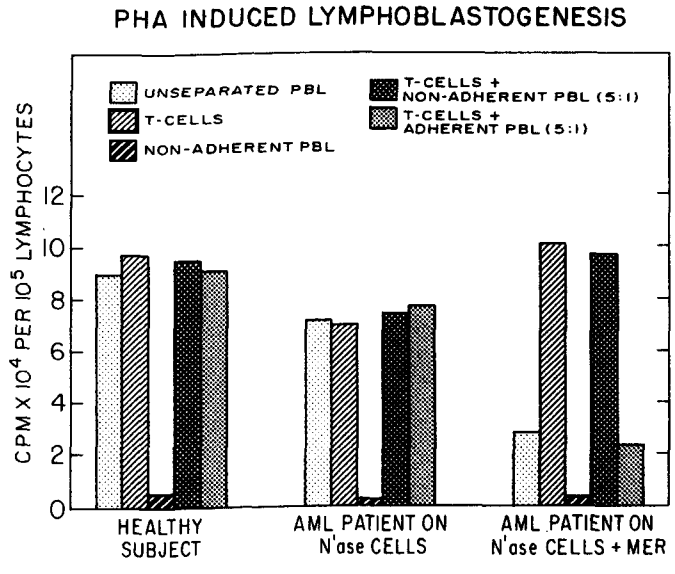

Fig. 4. Impact of N'ase modified myeloblasts plus MER on various immunological parameters in AML patients

maybe due to the suppression of certain T-cell functions by circulating monocytes affected by MER. The time of appearance of the apparent suppressor cell activity was different from patient to patient and omission of MER from the treatment in most cases prompted recovery of the patients' in vivo and in vitro immunological parameters and a gradual decrease of suppressor cell population.

Summary

Our studies clearly show that significantly longer remission duration was attained in groups of AML patients immunized with neuraminidase treated allogeneic myeloblasts as compared to patients who received chemotherapy alone or neuraminidase treated myeloblasts plus MER. It is clear that MER, albeit apparently active alone in certain other clinical studies impairs the immunotherapeutic value of neuraminidase treated allogeneic myelo-

blasts in AML patients. The in vivo and in vitro immunological test results reflect the host's immunological status in each arm of the protocol and correlate well with the duration of remission achieved with specific vs. combination of specific plus adjuvant immunotherapy.

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