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Blastogenic Response of Lymphocytes derived from Patients with Hematopoietic Malignancies to Antigens of a Type C Retrovirus isolated from a Burkitt's Lymphoma Cell Line*

ZIVIA SCHWARZBARD,† TAMAR GOTLIEB-STEMATSKY,†‡§ AMI VONSOVER,‡ MEIR DJALDETTI,‖ AHARON HALLAK¶ and ABRAHAM YANIV†

†*Department of Human Microbiology, Sackler School of Medicine, Tel-Aviv University, Tel Aviv, Israel,* ‡*The Central Virology Laboratory, Ch. Sheba Medical Center, Tel-Hashomer, 52621, Israel,* ‖*Department of Medicine "B" and Haematologic Clinic, Hasharon Hospital, Petach-Tikav, Israel and* ¶*Internal Medicine, Ichilov Hospital, Tel-Aviv, Israel*

Abstract—*Cellular immune response to antigens associated with a type C retrovirus derived from Burkitt's lymphoma (BL) lymphoblastoid cells was studied in patients with hematopoietic malignancies, noncancer patients and healthy controls. Repsonse was determined by lymphocyte blastogenesis assay measuring* [^{3}H]*-thymidine incorporation, thereby enabling the calculation of stimulation indices (SI). Positive response (SI* >2.0*) was demonstrated in patients with multiple myeloma (MM), chronic lymphocytic leukemia (CLL), chronic myelocytic leukemia (CML) and BL. No response was demonstrated in the non-cancer and healthy controls. Specific blastogenic response was obtained towards antigen extracted from three different cell lines infected with the tested type C retrovirus. No response was evident towards purified whole or purified disrupted virions or antigen extracted from murine myeloma MOPC-315 cells secreting a murine type C retrovirus or an antigen extracted from Mason–Pfizer monkey virus-infected NC-37 cells. The correlation between human hematopoietic malignancies and the* in vitro *lymphocyte blastogenic response to the BL-derived type C retrovirus was statistically highly significant.*

INTRODUCTION

RETROVIRUSES are involved in the etiology of sarcomas, hematopoietic malignancies or carcinomas in a large number of animal species, including primates [1]. The establishment of continuously growing cell lines derived from patients with leukemias and lymphomas has led to the isolation of a number of type C retroviruses [2–5]. The structural proteins of retroviruses possess antigenic determinants capable of inducing host immune response of the humoral and cellular types. Specific antigens of mammalian retroviruses have been detected in human leukemic cells. Evidence for the presence of Wooley monkey and gibbon ape virus p30 antigens was obtained by radioimmunoassays in human leukemia cells [6, 7]. An antigen immunologically related to gp52 of the mouse mammary tumor virus has been identified in paraffin sections of human breast cancers by means of the indirect immunoperoxidase technique [8]. ^{51}Cr-release cytotoxic assays performed with white blood cells of acute leukemia patients and simian sarcoma virus, endogenous virus of baboon or Rauscher murine leukemia virus demonstrated that a high proportion of tested subjects possess antigens related to these viruses [9].

It has been suggested that possibly both an RNA tumor virus and Epstein–Barr virus (EBV) participate in the etiology of Burkitt's lymphoma [10]. A type C virus was in fact isolated in our laboratory from the P3HR-1 Burkitt's lymphoma (BL)-derived lymphoblastoid cell line [3, 11]. Although antigenic relationship of this virus to

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§To whom requests for reprints should be addressed.

the type D retrovirus of the squirrel monkey was demonstrated, a significant level of viral information was also detected in the spleen DNA of a patient with multiple myeloma (unpublished results).

The presented lymphocyte blastogenesis assays demonstrate that patients with hematopoietic malignancies display a significant cell-mediated immune response towards an antigen related to the type C virus isolated from a BL-derived cell line.

MATERIALS AND METHODS

Cells and culture conditions

The P3HR-1 lymphoblastoid cell line, originally established from a patient with Burkitt's lymphoma, was found to be a producer of EBV [12]. The DG-75 cell line established from a patient with lymphoblastic lymphosarcoma was found to lack receptors for EBV [13]. The NC-37 is a human lymphoblastoid cell line which is chronically infected with Mason–Pfizer monkey virus (MPMV) [14]. The human lymphoblastoid cell lines grown in suspensions in RPMI-1640 medium supplemented with 15% fetal calf serum (FCS) (Gibco Laboratories, Grand Island, NY) were maintained at 37°C in a humidified incubator supplied with 5% CO_2. The murine myeloma MOPC-315 cells were propagated in Eagle's modified medium containing 10% calf serum [15]. A human amnion-derived cell line (HuAm) was cultured in Eagle's minimal essential medium (MEM) supplemented with 15% FCS. This cell line was kindly supplied by Professor N. Goldblum, Head, Channok Institute of Virology, Hadassah Medical School, Jerusalem. Cell lines were tested and found to be mycoplasma-free.

Infection of cell cultures with P3HR-1 type C virus

Lymphoblastoid and trypsinized HuAm cells were washed in growth medium, counted and resuspended in fresh growth medium to the appropriate concentration. Cells (5×10^6) were infected with 1.0 ml of cell-free virus suspension. After 2 hr adsorption at 37°C, the cells were resuspended in fresh growth medium to 3×10^5 cells/ml and incubated at 37°C for another 3 days. Five-milliliter aliquots of culture fluids obtained from type C virus-infected and non-infected cells were examined for the presence of particle-associated DNA polymerase activity in an exogenous templated reaction employing the synthetic oligo$(dT)_{12-18}$ · poly(rA) template [16].

Purification of virus

Culture medium of P3HR-1 cells secreting type C virus was harvested. Medium was clarified and the viral particles were purified by sucrose density centrifugation as previously described [17]. In addition, purified banded viral particles were disrupted by treatment with 0.2% Nonidet P-40 (NP-40) for 10 min on ice [16].

Preparation of antigens

Antigen preparations for lymphocyte stimulation experiments were obtained from P3HR-1, DG-75 and HuAm cells infected with and secreting type C retrovirus as well as from the respective control cells. Viral antigen preparations were also obtained from MPMV-infected NC-37 cells and from the murine myeloma MOPC-315 cells secreting a murine type C retrovirus. For the preparation of each viral antigen, 5×10^7 cells were centrifuged at 250 *g* for 15 min and culture medium was removed. The cell pellet was resuspended in 0.1 ml glycine buffer, pH 9.0 (Fluka), and centrifuged at 250 *g* for 15 min. This procedure was repeated three times, followed by dilution to 10% (v/v) in 0.1 M glycine buffer. The cell suspension was sonicated on ice for five bursts of 60 sec each in an MSE 150-W ultrasonic disintegrator at an amplitude of 8 μm, then incubated for 18 hr at 4°C and centrifuged at 250 *g* for 15 min. Supernatants containing viral antigen were supplemented with 200 units/ml penicillin and 200 μg/ml streptomycin and stored in 0.5-ml portions at −70°C. Protein content of each antigen preparation was determined by the method of Lowry *et al.* [18]. A single batch of each antigen was employed in all experiments throughout the study.

Preparation of peripheral blood lymphocytes

Heparinized blood containing 50 IU/ml of thromboliquin (Organon) was centrifuged at 750 *g* for 15 min at 15°C.

The plasma was removed and the buffy-coat leukocytes were collected and resuspended in 5 ml RPMI-1640 medium supplemented with 200 units/ml penicillin and 200 μg/ml streptomycin. The cell suspension was layered over 3 ml of lymphoprep (Nyegaard & Co., Oslo, Norway) in a 12-ml sterile conical plastic tube (Nunc, Denmark) and centrifuged at 400 *g* for 35 min at 15°C. The interface lymphocyte band was collected, resuspended in RPMI-1640 medium and washed three times. The resulting cell suspension demonstrated a viability of 99% as counted by the trypan blue exclusion technique.

Lymphocyte blastogenesis technique

A modification of the technique described by Böyum was employed [19]. Lymphocytes at a concentration of 10^6 viable cells/ml were suspended in RPMI-1640 medium supplemented

with 15% FCS, 0.4 mM glutamine (Fluka), 200 units/ml penicillin and 200 μg/ml streptomycin. Microcultures containing a total of 2×10^5 lymphocytes were established in plastic plates with flat-bottomed wells (Nunc, Denmark). Cultures were sensitized by antigens extracted from type C virus-infected cell lines and by antigens extracted from the respective control cells. The non-specific mitogen phytohemagglutinin (PHA; Wellcome Reagents, Beckenham, U.K.) served as an indicator of the immune competence of the tested lymphocytes. Antigens were added to cultures at a protein concentration of 25 μg/ml and PHA at a concentration of 1 mg/ml. Lymphocyte blastogenic response was additionally assessed towards purified whole and disrupted virions of the tested type C retrovirus, viral antigens extracted from MPMV-infected NC-37 and from murine myeloma MOPC-315 cells. Cultures were performed in quadruplicate for each antigen and the plates were incubated for 7 days at 37°C in a humidified incubator supplied with 5% CO_2. Six hours before termination of the experiment, cultures were charged, each well receiving 1 μCi [^{3}H]-thymidine (25 Ci/mmol; Amersham International, Amersham, U.K.), and returned to the incubator. Lymphocyte cultures were harvested onto Whatman GF/C glass microfiber paper (Reeve Angle, Cifton, NJ) with a Tubingless Cell Harvester (Linca Lamon Instrumentation Co.). Filters were washed, dried, immersed in 3 ml scintillation fluid (0.4% 2,5-bis-2(5-tert-butyl-benzoaxozolyl)thiophene in toluene) and counted in a Packard Tri-Carb PRIAS liquid scintillation counter. Lymphocyte blastogenic response was reflected by the increase in DNA synthesis as measured by incorporation of [^{3}H]-thymidine into newly synthesized DNA. Mean cpm values of quadruplicate cultures were calculated.

Stimulation index determination

Stimulation index (SI) determinations for each antigen were based on the ratio of mean cpm in cultures treated by antigens extracted from virus-infected cell lines to mean cpm in cultures treated by antigens extracted from the respective non-infected cells. SI >2.0 was regarded as expressing positive lymphocyte blastogenic response.

Statistical analysis

The Statistical Consulting Unit of the Tel-Aviv University kindly processed our data. The statistical methods applied were Welch's modification of the t test (double-tailed) and the correlated 2×2 table [20].

RESULTS

Lymphocyte blastogenic response was assayed in 59 patients with hematopoietic malignancies including multiple myeloma (MM), chronic lymphocytic leukemia (CLL), chronic myelocytic leukemia (CML) and Burkitt's lymphoma (BL). Twenty of the 59 patients were undergoing chemotherapeutic treatment. The control group comprised 20 healthy volunteers (clinical staff, secretaries, etc.) as well as 20 non-cancer patients suffering from various diseases such as polycythemia, thrombocytopenia, appendicitis, hernia and heart disease. Table 1 provides information on the studied groups. As can be seen, the mean age of the control groups was similar to that of the test subjects, except for the very young Burkitt's lymphoma patients. Each experiment also included lymphocytes from one non-cancer patient and one healthy volunteer. Lymphocyte cultures derived from test subjects and from the controls were sensitized by antigens extracted from type C retrovirus-infected P3HR-1, DG-75 and HuAm cell lines and by antigens extracted from the respective non-infected cell lines, which were previously tested for particle-associated DNA polymerase activity as indicated in Materials and Methods. Experiments establishing dose response of lymphocyte cultures towards the type C retrovirus antigen at three protein concentrations were carried out. From Fig. 1 it can be seen that optimal dose response was obtained at 25 μg/ml protein concentration. Figure 2 presents stimulation indices (SI) for the lymphocytes derived from each evaluated individual against the type C viral antigen extracted from the three virus-infected cell lines employed. It is evident that a cut-off value of SI = 2.0 excludes 100% of the non-cancer patients and healthy volunteers. Lymphocytes derived from patients with MM, CLL, CML and BL showed SI ranging from 2.0 to 8.0 or more. Thus positive response (SI >2.0) was demonstrated in 12 out of 15 (80%) patients with

Table 1. Description of study groups

Group		No. of patients	Age (mean ± S.E.)
MM	non-treated	17	66.47 ± 2.85
	treated	4	61.25 ± 4.77
CLL	non-treated	16	66.69 ± 3.26
	treated	11	62.18 ± 4.95
CML	non-treated	3	57.67 ± 5.49
	treated	3	62.33 ± 7.42
Total (excluding BL)	non-treated	36	65.83 ± 2.03
	treated	18	62.00 ± 3.29
BL	non-treated	3	8.33 ± 3.18
	treated	2	5.00 ± 1.00
Non-cancer patients		20	58.40 ± 2.71
Healthy volunteers		20	31.35 ± 2.67

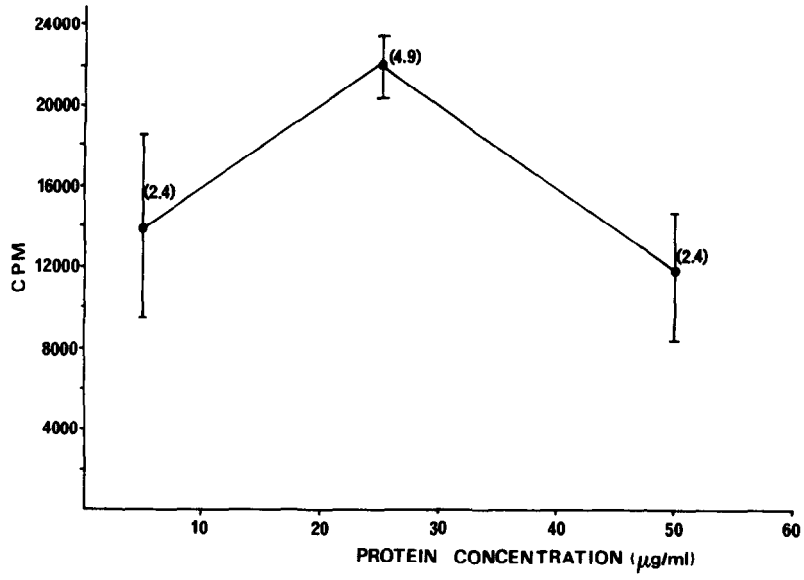


Fig. 1. Dose response to antigen extracted from type C virus-infected P3HR-1 cells. Numbers in parentheses represent stimulation values and vertical lines at each point represent the standard error of four replicate experiments.

MM, 21 out of 26 (80%) with CLL, 5 out of 6 (84%) with CML and in all 5 (100%) patients with BL. Statistical analysis by the correlated 2×2 table demonstrated no significant differences in the response when tested with the type C antigen extracted from the three cell lines.

To establish the significance of the lymphocyte blastogenic response of cancer patients in each group vs the controls, statistical analysis by the *t* test was performed. This was done with groups of patients composed of a statistically adequate number, such as the non-treated MM and non-treated and treated CLL patients (Table 2). It is clear that the difference in response towards the three cell line-derived viral antigens between lymphocytes obtained from the controls and from groups of patients is either significant ($P < 0.05$) or highly significant ($P < 0.0001$).

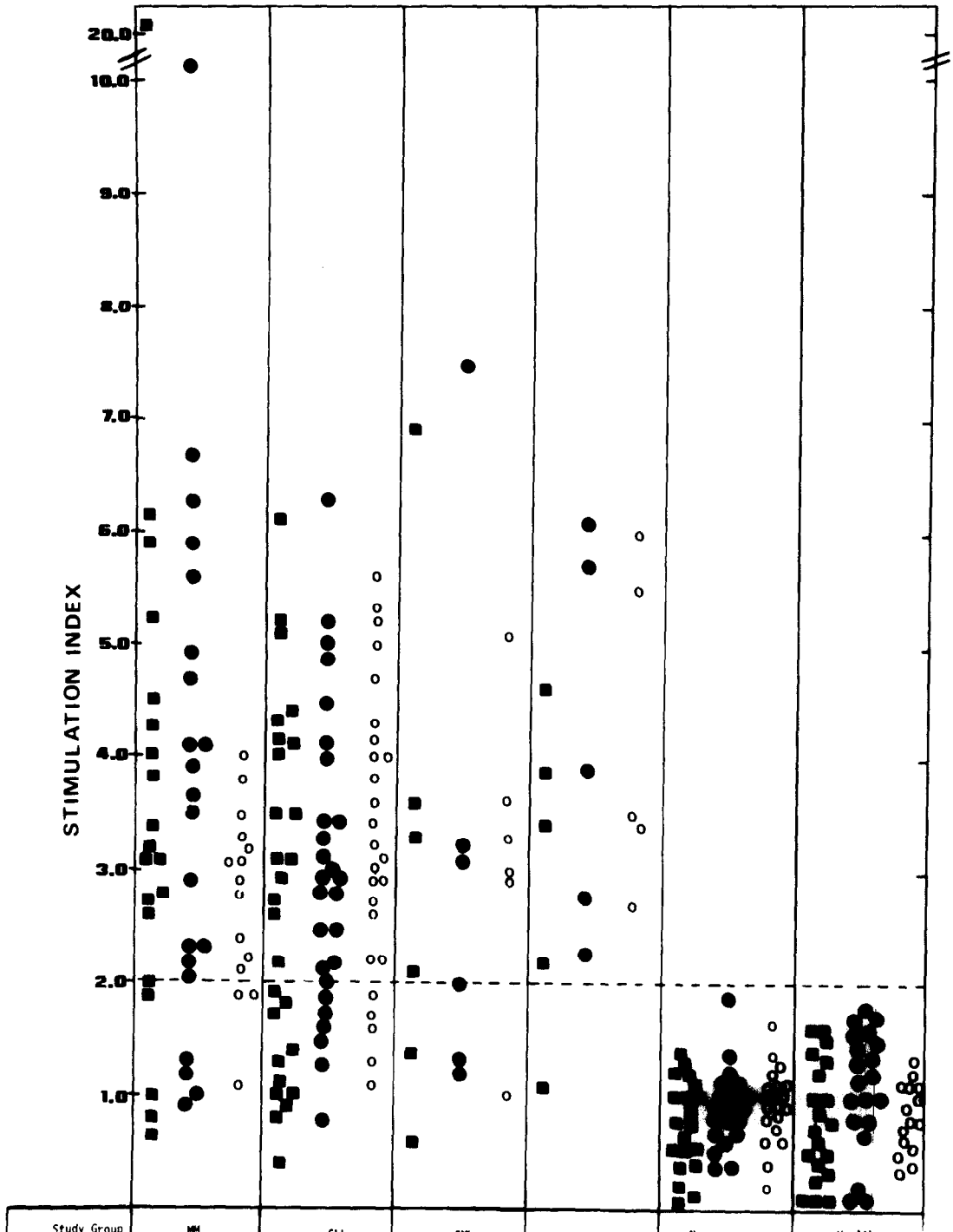
Table 3 presents the mean stimulation indices provided by the whole group of non-treated and treated MM, CLL and CML patients (the BL group which differed significantly in mean age was excluded), as well as by the control groups. The *t* test analysis of the hematopoietic malignancies as compared to the controls clearly indicated that the blastogenic response of the non-treated patients was highly significant ($P < 0.0001$) with all three cell line-derived viral antigens, while that of the treated patients was either highly significant ($P < 0.0001$) or significant ($P < 0.05$).

For reasons of simplicity, results were presented by SI values. Table 4 demonstrates mean cpm and variance of representative experiments in which lymphocyte cultures were stimulated by antigens extracted from non-infected and type C-infected DG-75 cells, as well as non-stimulated and PHA-stimulated lymphocytes.

The group of patients with MM consisted of 17 non-treated and 4 treated patients, the group of CLL consisted of 16 non-treated and 11 treated patients, and the group of treated and non-treated CML patients consisted of 3 patients each. The non-cancer patients and healthy volunteer groups consisted of 20 individuals each. The data show that mean cpm values obtained in lymphocytes stimulated by antigen extracted from type C-infected cells are significantly higher than those obtained in lymphocytes stimulated by antigen extracted from non-infected cells. Calculated SIs for PHA-stimulated cultures were based on the ratio to cpm in non-challenged cultures. It can be seen that lymphocytes, whether of treated or non-treated patients, were immune-competent.

In order to understand better the nature of the stimulating factor, lymphocyte blastogenesis was assayed towards purified whole and disrupted type C virions and compared to the response obtained towards crude type C virus-antigen. Lymphocytes were obtained from 4 patients with MM, 9 patients suffering from CLL, 3 patients with CML and 1 patient with BL. Positive responses ranging from $SI = 2.2$ to $SI = 6.9$ were obtained with crude viral antigen preparation, while no response ($SI < 2.0$) was observed when the same lymphocyte cultures were sensitized by purified whole or disrupted purified virions (Fig. 3).

To test the specificity of the blastogenic response towards our type C viral antigen, lymphocytes derived from patients with various



Study Group	MM			CLL			CML			BL			Non-cancer patients			Healthy volunteers		
Cell line	P3HR-1	DG-75	HuAm	P3HR-1	DG-75	HuAm	P3HR-1	DG-75	HuAm	P3HR-1	DG-75	HuAm	P3HR-1	DG-75	HuAm	P3HR-1	DG-75	HuAm
SI ≥ 2.0 #positive/#tested	15/20	16/21	12/15	16/27	20/27	21/26	4/6	3/6	5/6	4/5	5/5	5/5	0/20	0/20	0/20	0/20	0/20	0/15
percentage	75	76	80	59	74	80	67	50	84	80	100	100	0	0	0	0	0	0

Fig. 2. Lymphocyte blastogenic response, expressed by the stimulation index (SI), of patients with hematopoietic malignancies and of control groups towards antigens extracted from type C virus-infected P3HR-1 (■), DG-75 (●) and HuAm (○) cell lines.

malignancies were also sensitized by antigens extracted from type C virus-secreting murine myeloma MOPC-315 cells and MPMV-infected NC-37 cells. Figure 4 clearly demonstates that in contrast to the high stimulation indices manifested towards antigens extracted from the type C virus-infected DG-75 cell line, none responded towards antigens extracted from murine myeloma MOPC-315 or MPMV-infected NC-37 cells. Statistical analysis by the correlated 2×2 table showed that the difference in response of lymphocytes towards the type C virus-associated antigen and towards the murine virus or MPMV antigens was highly significant ($P < 0.0001$), thus reflecting the specificity of the lymphocyte blastogenic response.

DISCUSSION

Lymphocyte blastogenesis by specific antigens constitutes a useful device for measuring cellular immune response. The present study demonstrates that lymphocytes derived from patients with hematopoietic malignancies undergo blastogenesis when sensitized by virus-associated antigens of a type C virus isolated from P3HR-1 cells and extracted from three virus-infected cell lines: P3HR-1, DG-75 and HuAm, while no such response was obtained when the same lymphocytes were sensitized by antigens extracted from the respective non-infected cells. No blastogenic response towards the type C virus-associated antigens was demonstrated by lymphocytes derived from healthy subjects and non-cancer patients. Similar lymphocyte blastogenesis assays [21, 22] showed that among healthy subjects including pregnant women, few demonstrated cellular immune response towards mouse mammary tumor virus or simian oncornavirus antigen, respectively.

The lymphocyte-stimulating factor derived from the type C virus-infected cells was not a general non-specific mitogen like PHA [23], nor was it associated with FCS, because lymphocytes cultured in RPMI-1640 growth medium containing 15% FCS were not stimulated when no viral antigen was added. To rule out the possibility that EBV antigen might be the stimulating factor, virus was grown in DG-75 lymphoblastoid cell line and in HuAm monolayer, which do not harbor or permit adsorption or growth of EBV. The fact that no differences in mean SI were obtained by stimulation with the type C antigen extracted from any one of the three cell lines excludes the possible involvement in the induction of blastogenesis of EBV or cellular components associated with P3HR-1 cells. Stimulation was obtained only when lymphocytes were sensitized by an antigen preparation extracted from type C virus-infected cells. No stimulation could be demonstrated with purified whole or disrupted virions. Hence it seems that the active stimulating factor may be comprised of both viral and cellular components. It was also suggested that insertion of a partial retrovirus genome possibly containing the LTR region may play the part of a promoter and thus induce the expression of a specific cellular gene.

It was further ascertained that the lymphocyte blastogenic response was specific for our type C virus-related antigen, because antigens extracted from murine myeloma MOPC-315 cells secreting type C particles and from MPMV-infected NC-37 cells did not induce blastogenic response when administered to lymphocyte cultures under the same conditions.

Lymphocytes of patients subjected to chemotherapy appeared to be less responsive to stimulation in that highly significant differences in response were found between the non-treated and control groups, whereas merely significant differences were detected between the treated patients and the controls. The differences in response between treated and non-treated patients may reflect the cytostatic and cytotoxic effects of the drugs usually administered in chemotherapy, although no such effect could be observed on the stimulation indices when the lymphocyte blastogenic response of these treated patients was assayed towards PHA. Being a non-specific mitogen, PHA stimulates a large number of

Table 2. Statistical analysis of lymphocyte blastogenic response of healthy volunteers and non-cancer patients as compared to treated and non-treated patients with MM and CLL

Healthy volunteers and non-cancer patients vs patients with:	No. tested (n)	Significance values (t test) Type C (P3HR-1)*	Type C (DG-75)*	Type C (HuAm)*
Non-treated MM	17	$P < 0.05$	$P < 0.0001$	$P < 0.0001$ ($n = 13$)
Non-treated CLL	16	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$
Treated CLL	11	$P < 0.05$	$P < 0.05$	$P < 0.0002$ ($n = 10$)

*Antigen extracted from type C virus-infected P3HR-1, DG-75 or HuAm cell lines.

Table 3. Mean SI and significance values (P) of non-treated and treated patients with hematopoietic malignancies (MM, CLL, CML) as compared to non-cancer patients and healthy volunteers

Study group		No. in group (*n*)	Stimulation index (mean ± S.E.) Type C (P3HR-1)*	*P*	Type C (DG-75)*	*P*	Type C (HuAm)*	*P*
Hematopoietic malignancies	non-treated	36	3.95 ± 0.59	<0.0001	3.86 ± 0.33	<0.0001	3.40 ± 0.19 (*n* = 32)	<0.0001
	treated	18	1.88 ± 0.30	<0.05	2.28 ± 0.31	<0.05	2.45 ± 0.26 (*n* = 15)	<0.0001
Non-cancer patients		20	1.01 ± 0.05		1.02 ± 0.05		1.02 ± 0.05	
Healthy volunteers		20	0.88 ± 0.12		1.13 ± 0.12		0.82 ± 0.08 (*n* = 15)	

*Antigen extracted from type C virus-infected P3HR-1, DG-75, or HuAm cell lines.

Table 4. Mean counts per minute (cpm) and variance values of stimulated lymphocytes of non-treated (NT) and treated (T) patients and control groups

Stimulating antigen extracted from:	Study group	cpm (mean ± S.E.) MM	CLL	CML	Non-cancer patients	Healthy volunteers
DG-75 cells	NT	2762 ± 194	2169 ± 148	3041 ± 105	1808 ± 118	2408 ± 102
	T	1357 ± 102	2078 ± 133	2022 ± 434		
Type C-infected DG-75 cells	NT	12188 ± 1161	8859 ± 612	8079 ± 565	1856 ± 93	1947 ± 136
	T	3173 ± 311	6058 ± 570	7115 ± 686		
None	NT	1223 ± 51	1587 ± 63	1687 ± 142	1472 ± 77	1296 ± 86
	T	1164 ± 67	1602 ± 107	1321 ± 137		
PHA	NT	35175 ± 739 *(29.0 ± 0.8)	29249 ± 874 (29.0 ± 0.7)	29615 ± 2039 (28.0 ± 0.8)	35147 ± 1201 (27.0 ± 1.2)	33354 ± 398 (29.0 ± 1.3
	T	34402 ± 917 (34.0 ± 0.9)	28625 ± 1383 (28.0 ± 0.9)	31852 ± 3489 (27.0 ± 0.8)		

*Numbers in parentheses represent SI values ± S.E.

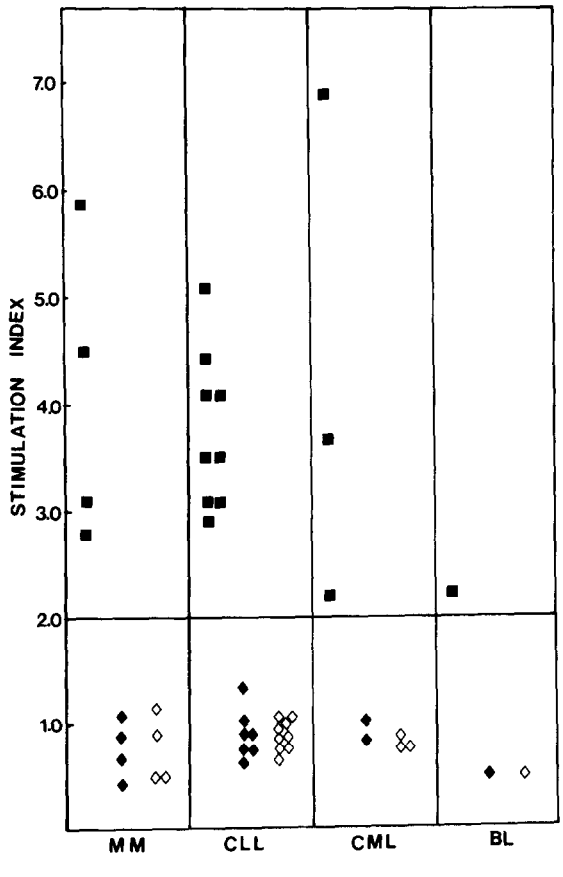


Fig. 3. Lymphocyte blastogenic response of patients with hematopoietic malignancies towards purified whole (◆) and disrupted (◇) virions and towards crude antigen (■) extracted from type C virus-infected P3HR-1 cells.

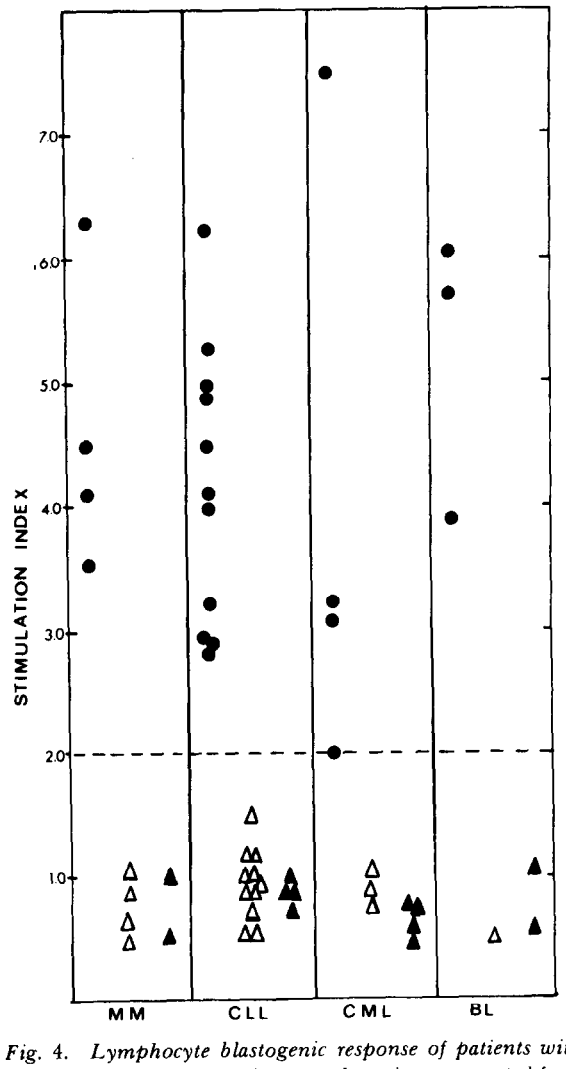


Fig. 4. Lymphocyte blastogenic response of patients with hematopoietic malignancies towards antigens extracted from the type C virus-infected DG-75 (●) cell line and towards antigens extracted from MOPC-315 type C virus-secreting (△) and MPMV-infected NC-37 (▲) cell lines.

lymphocytes, in contrast to specific antigens, which are probably capable of stimulating only cells specifically sensitized to the antigen in question. This may also explain the relatively high stimulation indices obtained towards PHA as compared to the lower ones obtained with lymphocyte cultures treated by the specific type C antigen. The findings of the present studies point to the possibility that the antigen recognized by the lymphocytes derived from patients with multiple myeloma, chronic lymphocytic leukemia, chronic myelocytic leukemia or Burkitt's lymphoma may be virus-coded, but it is probably not a structural protein.

While the tested type C virus is related to the squirrel monkey retrovirus, our results gain added significance in light of recent isolations of type C viruses from human T leukemias [4, 5] which suggest that type C viruses may figure in the etiology of certain human leukemias and lymphomas.

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