JOURNAL OF IMMUNOLOGICAL RESEARCH

Vol. 2, No. 4, December 1990

Tolerance induction in adult mice intrathymically injected with Moloney murine leukemia virus and treated with cyclophosphamide

Paola Zanovello, Elisabetta Vallerani, Vincenzo Bronte, Antonio Rosato, Luigi Chieco-Bianchi, Dino Collavo

Summary. We investigated viral antigen expression and T cell response in M-MuLV i.t. injected adult mice that had been treated with a CY to temporarily reduce the recipient T cells reactive toward virus-induced antigens. Thymic and splenic T and B lymphocytes in these mice expressed virus-induced antigens, as shown by virus-specific CTL lysis and staining by anti-M-MuLV fluorescent serum; moreover, the percentage of M-MuLV-infected cells increased with time after virus inoculation. The evaluation of virus-specific cytotoxic T lymphocyte precursor frequency indicated a progressive immune response decline that correlated with the establishment of the lifelong viremia and lymphomas development in a high percentage of M-MuLV i.t. injected mice. These findings suggest that the lack of immunocompetent T cells induced by CY administration associated with a persistent virus infection, produced a tolerant status to M-MuLV antigens that was followed by leukemia development.

Key words: Cyclophosphamide, intrathymus-injection, tolerance.

Abbreviations: CTLp, cytotoxic T lymphocyte precursor; CY, cyclophosphamide; i.t., intrathymus; MLTC, mixed leukocyte tumor cell culture; M-MuLV, Moloney-murine leukemia virus.

Introduction

Extensive studies of specific immunologic tolerance to a variety of antigens using mainly murine model systems, suggest that three major mechanisms are operational in the induction and maintenance of an antigen-specific unresponsiveness status: regulation by suppressor T cells (clonal suppression), functional inactivation of responding cells (clonal anergy), responding cell elimination (clonal deletion) [1,2]. Tolerance to self-antigens has been shown to develop during ontogeny, as a result of the deletion of self-reactive cells in the thymus [3]; the observation that T cell receptor specificity after gene rearrangements remains constant, indicates that this mechanism is effective in maintaining self-tolerance even within peripheral lymphoid organs.

These mechanisms can also work to achieve tolerance to self-replicating infectious agents. In reference to murine oncogenic retroviruses a clonal deletion of virus-specific CTL within the thymus appears to be mainly responsible for the immune unresponsiveness observed following perinatal activation of endogenous sequences, or neonatal infection by exogenous viruses [4]. In this last case, infection with Gross [5], Graffi [6], or Moloney (M) [7] murine leukemia viruses (MuLVs) induces early expression of MuLV antigens on T lymphocytes, persistent viremia, deficient antibody production and absent transplantation resistance towards syngeneic MuLV-induced leukemic tissue. M-MuLV-neonatally infected mice also fail to generate virus-specific CTL [8] and show a considerable reduction in CTL precursors frequency on limiting dilution analysis [9]. On the other hand, MuLV infection in adult mice of conventional strains results in immune reactivity rather then tolerance toward virus antigens [10]. Although the host age at the first antigen exposure is crucial in determining whether tolerance or immunity occurs, we recently observed that the site of virus inoculation also affects virus expression [11]. In fact, intrathymic (i.t.) injection of M-MuLV in adult mice is followed by persistent infection of thymic and peripheral T cells, despite a well detectable specific CTL response [12]. Since these animals did not become viremic and did not develop leukemia, it was suggested that the cellular immune response is sufficient for preventing neoplastic transformation.

We present evidence that a temporary reduction of the immune response in M-MuLV i.t. injected adult mice, achieved by CY administration, induced virus infection spread to both T and B peripheral lymphocytes, as well as long-lasting unresponsiveness toward virus-induced antigens and ultimately, lymphoma development.

Cattedra di Immunologia, Istituto di Oncologia, Centro Interuniversitario per la Ricerca sul Cancro, Università, Padova

Received July 20th, 1990

Materials and methods

Animals

Inbred C57BL/6 (B6) mice were purchased from the Charles River Laboratories (Calco Como, Italy) and maintained in our colony for several generations by sister × brother matings.

Virus and tumor cells

Virus preparation procedures are reported in detail elsewhere [13]. M-MuLV, which had a titer of 5×10^8 PFU/ml evaluated on SC-1/XC cells, was injected i.t. into adult mice at a dose of 0.05 ml. To assay M-MuLV production 2% w/v tail extracts were tested on SC-1 cells with the use of the UV/XC procedure [14]. MBL-2, a M-MuLV-induced lymphoma of B6 mice, was maintained by weekly passage of the ascitic form in syngeneic recipients.

Protocol of cyclophosphamide treatment

The sterile CY-sodium chloride preparation (Endoxan, Asta-Werke, Brackwide, Germany) was diluted in PBS at a concentration of 20 mg/ml, and administered i.p. at a dose of 50 mg/kg in a single weekly injection. This treatment was initiated 7 days after the M-MuLV injection, and continued for 4 weeks; total CY dose administered was 200 mg/kg.

Cell cultures

Mixed leukocyte tumor cell culture systems were carried out as previously described [15]. Briefly, 25×10^6 responder spleen cells were co-cultured with 5×10^6 irradiated MBL-2 lymphoma cells in a total volume of 15 ml complete medium in a 30-ml flask (Falcon Plastic Co., Los Angeles, CA). Complete medium consisted of Dulbecco's MEM (Gibco, Grand Island, NY) supplemented with L-glutamine, Hepes, 2-ME, antibiotics and 10% heat-inactivated FCS (Flow Lab. Inc., U.K.).

Limiting dilution micro-MLTC were performed according to Brunner et al. [16] with minor modifications [9]. These cultures, consisting of limiting numbers of responder cells, 3×10^4 irradiated MBL-2 and 5×10^5 irradiated syngeneic spleen cells, were set up in 96 round-bottom microtiter plates (Costar, Cambridge, MA) in a final volume of 0.2 ml of conditioned medium containing an appropriate dilution of EL-4 supernatant as the source of IL-2 (20 U/ml). After 7 days the cultures were tested for cytotoxic activity.

T lymphocytes were selectively stimulated by adding 5 ug/ml of Con A (Pharmacia, Uppsala, Sweden) to thymus or spleen cells, that had previously been passed through a nylon wool column [17] to achieve T cell fraction enrichment. To obtain selective B lymphocyte stimulation, spleen cells were pretreated with anti-Thy 1.2 mAb and C and then stimulated with 25 ug/ml of LPS (lipopolysaccharide B E. Coli 0.55: B6, Difco, Detroit, MI).

Cytolytic assay

MLTC cytolytic activity was determined by incubating serial dilutions of effector cells with 2×10^3 ^{51}Cr-labelled (Na_2 $^{51}CrO_4$, NEN, Dreieich, FRG) target cells in round-bottom microtiter plates (Sterilin, Teddington, Middlesex, UK) for 4h, as previously described [15]. The plates were centrifuged, and 0.1 ml supernatant removed for counting; the percentage of specific ^{51}Cr release was calculated according to the expression, 100 × (Experimental release-spontaneous release/Maximum release-spontaneous release). To assay micro-MLTC cytolytic activity, 100 ul of supernatant from each microculture were replaced with 2×10^3 ^{51}Cr-labelled target cells in 0.1 ml of medium. The microplates were incubated at 37° C for 4 h, centrifuged, and 0.1 ml of supernatant was then removed for counting. Spontaneous release was determined in control microculture groups set up without responder cells.

Calculation of CTL-p frequencies

Twenty-four microcultures were scored as positive or negative, with positive cultures defined as those in which ^{51}Cr release values exceeded mean spontaneous release by more than 3 SD. The percentage of negative cultures was plotted against the number of responder cells plated, and the CTL-p frequency was determined by linear regression analysis, as described by Taswell et al. [18].

Cell surface markers

mAb specific for the Thy 1.2 antigens diluted 1:5000 with PBS, were incubated with the cells for 30 min on ice, and then, after washing, with FITC-conjugated antimouse Ig F(ab)$_2$ (Nordic Imm. Lab., The Netherlands). M-MuLV-induced antigens were identified by direct immunofluorescence, with a 1:40 dilution of FITC-labelled anti-tween-ether disrupted M-MuLV goat serum (produced by Becton and Dickinson and Co. Research Center under contract from the Divsion of Cancer Cause and Prevention, NCI).

Results

Viral antigen expression on different cell populations from M-MuLV i.t. injected mice treated with CY (i.t. + CY treated mice).

Following i.t. injection of M-MuLV in adult mice, thymus cells and peripheral T lymphocytes expressed virus-induced cell surface antigens which were shown by anti-M-MuLV fluorescent serum and recognized by specific CTLs [12]. Although the percentage of infected peripheral T lymphocytes progressively increased throughout the life of these animals, B lymphocytes failed to express viral antigens. To explore whether a transient impairment of the immune response associated with a viral in-

fection would favour virus spreading and subsequent cell infection in the peripheral lymphoid organs, adult mice injected i.t. with M-MuLV received a total CY dose of 200mg/kg, according to the above protocol. Previous studies demonstrated that this CY dosage in adult normal mice temporarily decreases allo- or virus-specific CTLp frequencies in the spleen, without affecting those in the thymus [18,19]. Total recovery of immunocompetence is observed on day 12 after treatment [19]. In M-MuLV injected mice, in particular, CY treatment after virus inoculation, may constitute an efficient method to transiently reduce recipient T cells reactive against virus antigens, as demonstrated in several tolerance-inducing methods [20].

The percentage of infected cells was evaluated in the thymus and spleen of i.t.+CY treated mice, sacrificed at various intervals following M-MuLV injection. We observed that the percentage of cells stained by anti-M-MuLV fluorescent serum increased progressively from 40% to 78% in the thymus, and from 22% to 60% in the spleen at 30 and 120 days, respectively (figure 1). Thus, while thymus values were similar to those found in the i.t. injected adult animals, spleen values were consistently higher, and very close to those obtained in M-MuLV neonatally injected mice which later became permanent virus carriers [8].

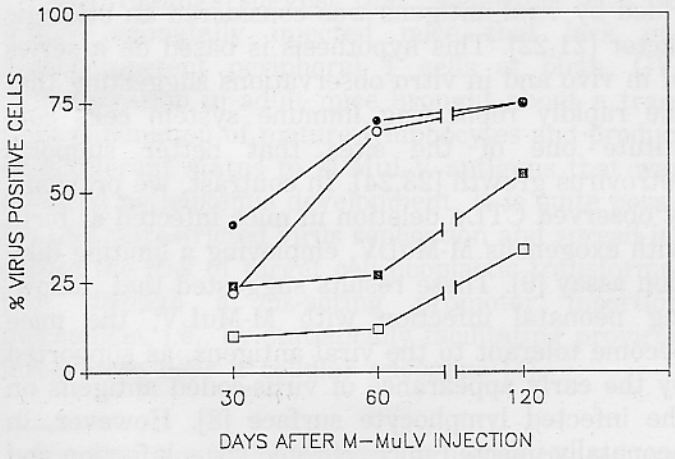

Figure 1. - Percent of virus-positive cells from i.t. and i.t.+CY treated mice at different days after M-MuLV inoculation. Thymus (circles) and spleen (squares) cells from i.t. (open symbols) and i.t.+CY (filled symbols) treated mice were incubated with anti-M-MuLV fluorescent serum. Similar findings were obtained in three experiments. The results of one experiment are shown.

M-MuLV-induced antigen expression was also evaluated on peripheral T and B lymphocytes obtained from i.t.+CY treated mice. Animals were sacrificed 30, 60, and 120 days after virus injection, and ^{51}Cr-labelled Con-A- and LPS-induced spleen blasts were used as targets in a short-term cytotoxicity assay. Virus-specific CTLs, obtained in secondary MLTC, exerted a strong cytotoxic effect on Con-A-induced T blasts at all times tested.

On the other hand, LPS-induced B blasts were also lysed, even if viral antigen expression was detectable in these cells only 60 days after M-MuLV inoculation (figure 2).

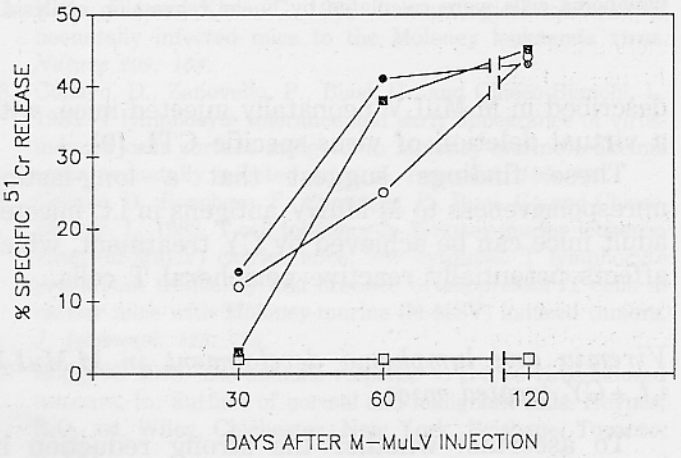

Figure 2. - Lytic activity mediated by virus-specific CTL against peripheral T and B lymphocytes obtained from M-MuLV i.t. and i.t.+CY treated mice. Con-A (circles) and LPS (squares) induced blast cells from i.t. (open symbols) and i.t.+CY (filled symbols) treated mice were used as targets of virus-specific CTL obtained in secondary MLTC at an effector: target cell ratio of 45: 1.

Virus-specific CTL-p frequencies in M-MuLV i.t.+CY treated mice

A persistent virus-specific T cell response prevents lymphoma development in adult mice injected i.t. with M-MuLV, despite progressive virus infection of their thymus and peripheral T cells [12].

Virus-specific CTL generation was determined in spleen cells from i.t.+CY treated mice at 30, 60 and 120 days after virus injection by means of a limiting dilution analysis of CTLp.

Evaluation of CTL-p frequency, determined from the slope of the linear regression curves, demonstrated that CTLp frequencies in i.t.+CY treated mice were lower than those previously reported in M-MuLV i.t. injected adult animals, at all time intervals after virus inoculation (table I). The reduction was more striking with time; at 120 days after M-MuLV injection, the CTLp frequency in i.t.+CY treated mice was very similar to that

Table I

Virus-specific CTLp frequency in i.t. and i.t. + CY treated mice evaluated at different days after M-MuLV inoculation.

Days after M-MuLV injection	i.t. + CY		i.t.	
	reciprocal of mean frequency (range) *			
30	39.250	(29.000-49.500)	10.000	(5.000-15.000)
60	42.000	(33.000-51.000)	12.400	(11.600-13.200)
120	150.000	(130.000-180.000)	32.500	(31.000-34.000)

* Minimal estimates of the frequencies of CTLp specific for MBL-2 lymphoma cells were calculated by linear regression analysis.

described in M-MuLV neonatally injected mice, with a virtual deletion of virus-specific CTL [9].

These findings suggest that a long-lasting unresponsiveness to M-MuLV antigens in i.t. injected adult mice can be achieved by CY treatment, which affects potentially reactive peripheral T cells.

Viremia and lymphoma development in M-MuLV i.t.+CY treated mice

To ascertain whether the strong reduction in virus-specific CTLp frequencies is associated with the spread of M-MuLV, the presence of infectious virus was evaluated by the UV-XC plaque method. High virus titers were observed in tail tissue extracts of i.t.+CY treated mice tested 60 days after virus inoculation while i.t. injected mice were virus free (data not shown). The establishment of life-long viremia in these animals correlated with a high incidence of lymphoma development; in fact 14 out of 15 i.t.+CY treated mice developed lymphomas (93%), mostly of thymic origin, within 7 months after virus inoculation (figure 3). No lymphomas were observed in adult mice treated with CY only. The T cell origin of several lymphomas was determined using anti-Thy 1.2 mAb.

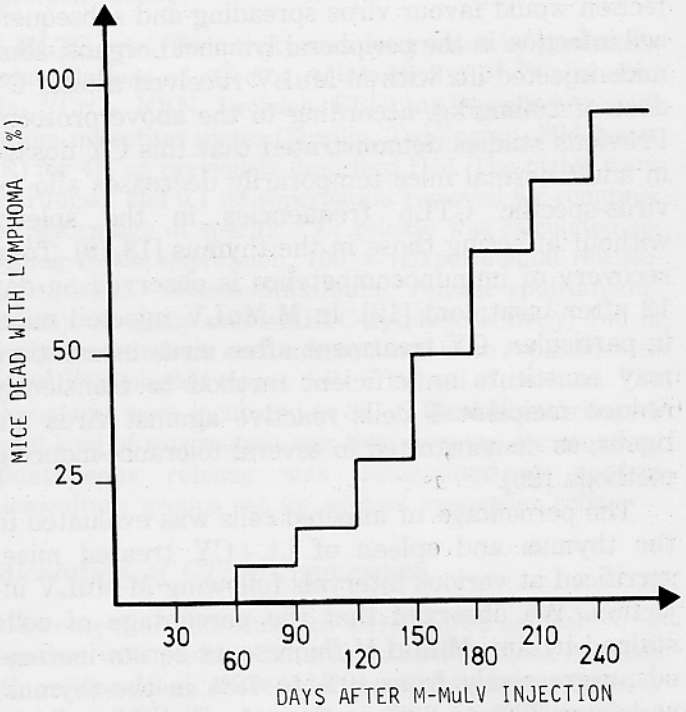

Figure 3. - Cumulative lymphoma incidence in M-MuLV i.t.+CY treated mice.

Discussion

Unresponsiveness to specific antigens can be induced in adult animals, but the mechanisms involved are not well understood. Our data indicate that unresponsiveness, which led to lymphoma development in M-MuLV i.t.+CY treated mice, might be due to a virtual deletion of virus-specific CTLp. This finding partially clarifies the role of immune reactivity in lymphoma development in mice with persistent M-MuLV infection. In fact, much controversy surrounds the role played by virus-specific CTL-mediated immunity in the resistance against M-MuLV induced lymphomagenesis. In addressing the problem of how slow acting, oncogene-devoid MuLVs induce lymphomas, chronic immunostimulation provided by viral antigens was considered an etiologic factor [21,22]. This hypothesis is based on a series of in vivo and in vitro observations suggesting that the rapidly replicating immune system cells constitute one of the sites that better supports retrovirus growth [23,24]. In contrast, we previously observed CTLp deletion in mice infected at birth with exogenous M-MuLV, employing a limiting dilution assay [9]. These results suggested that, following neonatal infection with M-MuLV, the mice become tolerant to the viral antigens, as supported by the early appearance of virus-coded antigens on the infected lymphocyte surface [8]. However, in neonatally injected mice, chronic virus infection and lack of specific CTL reactivity are invariably associated; consequently, whether or not lymphoma may be induced by the persistence of virus-infected cells in the presence of an efficient immunological response remains an open question. The features of the M-MuLV i.t. injected adult mouse model enable us to address this important issue. In fact, these animals have a persistent cell-mediated reactivity, but they do not develop lymphomas despite continuous M-MuLV infection of their thymus and

peripheral T cells [12]. This suggests that the residual CTL response does not represent a substrate for subsequent cell transformation, but instead restricts M-MuLV spread by either preventing multiple cycles of target cell reinfection, or destroying virus infected cells. Short-term treatment with CY at a dosage that markedly reduces virus-specific CTLp [19] favoured virus spreading and lymphoma development in these mice in much the same way the phenomenon is seen in neonatally injected mice. Virus-induced cell surface antigen expression was correlated with a major virus spread, as shown by 60% of the spleen cells stained by anti-M-MuLV fluorescent serum, or lysed by virus-immune CTL at 120 days after virus-inoculation. More interestingly, the same effector cells were able to lyse LPS-induced blasts obtained from i.t.+CY treated hosts, thus demonstrating that B lymphocytes were also infected by the virus. It is noteworthy that B cell infection was never observed in i.t. injected adult mice. The increase in the number of infected peripheral cells paralleled the decline in cytotoxic activity observed in i.t.+CY treated animals. In fact, their CTLp frequencies at various intervals after virus-inoculation disclosed that cytotoxic activity was always less than in i.t. injected mice; at 120 days post-inoculation, the reduction was striking. The impaired T lymphocyte reactivity observed in these mice appears to correlate with the long-lasting viremia observed, and the onset of T-cell lymphoma.

Our findings suggest that, analogous with M-MuLV neonatally injected mice that lack immunocompetent peripheral T cells at birth, CY-administration in adult mice brought about a transient elimination of mature lymphocytes and produced a tolerant status to M-MuLV antigens that was followed by leukemia development. It is quite possible that unrestricted virus replication and spread increase the risk of target cell neoplastic transformation through a cis-acting promoter insertion mechanism, i.e., by statistically facilitating provirus integration near a cellular oncogene [4].

We tank Mr. Silvio Mezzalira for competent technical assistance, Mrs. Patrizia Segato for expert help in the preparation of the manuscript and Mrs. Wilma Tognon for secretarial assistance.

This work was supported by grant from Ministero Pubblica Istruzione, Consiglio Nazionale delle Ricerche (Progetto Biotecnologie e Biostrumentazione n. 89.00236), and Associazione Italiana per la Ricerca sul Cancro.

References

1. Weigle, W.O. 1973. Immunological unresponsiveness. *Adv. Immunol. 16: 61.*
2. Nossal, G.J.V. 1983. Cellular mechanisms of immunological tolerance. *Ann. Rev. Immunol. 1: 33.*
3. Kappler, J.W., Staerz, U., White, J., and Marrack, P.C. 1988. Self-tolerance eliminates T cell specific for MLS-modified products of the major histocompatibility complex. *Nature 332: 35.*
4. Chieco-Bianchi, L., Collavo, D., and Biasi, G. 1988. Immunologic unresponsiveness to murine leukemia virus antigens: mechanisms and role in tumor development. *Adv. Cancer Res. 51: 277.*
5. Old, L.J., Boyse, E.A., and Stockert, E. 1965. The G (Gross) leukemia antigen. *Cancer Res. 25: 813.*
6. Chieco-Bianchi, L., Senso, F., Aoki, T., and Barrera, O.L. 1974. Immunologic tolerance to antigens associated with murine leukemia viruses: T-cell unresponsiveness? *J. Natl. Cancer Inst. 52: 1345.*
7. Klein, E., and Klein, G. 1966. Immunological tolerance of neonatally infected mice to the Moloney leukaemia virus. *Nature 209: 163.*
8. Collavo, D., Zanovello, P., Biasi, G., and Chieco-Bianchi, L. 1981. T lymphocyte tolerance and early appearance of virus-induced cells surface antigens in Moloney-murine leukemia virus neonatally injected mice. *J. Immunol. 126: 187.*
9. Collavo, D., Ronchese, F., Zanovello, P., Biasi, G., and Chieco-Bianchi, L. 1982. T cell tolerance in Moloney-murine leukemia virus (M-MuLV) carrier mice: low cytotoxic T lymphocyte precursors frequency and absence of suppressor T cells in carrier mice with Moloney-murine (M-MSV) induced tumors. *J. Immunol. 128: 774.*
10. Hogg, N. 1978. The immune response to C-type virus-induced tumours. In: Surface of normal and malignant cells. Heynes, R.O., ed. Wiley, Chichester, New York, Brisbane, Toronto, p. 287.
11. Zanovello, P., D'Andrea, E., Ronchese, F., De Rossi, A., Collavo, D., and Chieco-Bianchi, L. 1984. Role of infectious virus expression and immune response in retrovirus-induced oncogenesis. *Tumori 70: 1.*
12. Zanovello, P., Collavo, D., Ronchese, F., De Rossi, A., Biasi, G., and Chieco-Bianchi, L. 1984. Virus-specific T cell response prevents lymphoma development in mice infected by intrathymic inoculation of Moloney leukaemia virus (M-MuLV). *Immunol. 51: 9.*
13. Collavo, D., Zanovello, P., Leuchars, E., Davies, A.J.S., Chieco-Bianchi, L., and Biasi, G. 1980. Moloney murine sarcoma virus oncogenesis in T-lymphocyte-deprived mice: biologic and immunologic studies. *J. Natl. Cancer Inst. 64: 97.*
14. Rowe, W.P., Pugh, W.E., and Hartley, J.W. 1970. Plaque assay techniques for murine leukemia viruses. *Virology 42: 1136.*
15. Collavo, D., Parenti, A., Biasi, G., Chieco-Bianchi, L., and Colombatti, A. 1978. Secondary in vitro generation of Cytolytic T-Lymphocytes (CTL's) in the Murine sarcoma virus system. Virus-specific CTL induction across the H-2 Barrier. *J. Natl. Cancer Inst. 61: 885.*
16. Brunner, K.T., MacDonald, H.R., and Cerottini, J.C. 1980. Antigenic specificity of the cytolytic T lymphocyte (CTL) response to murine sarcoma virus-induced tumors II. Analysis of the clonal progeny of CTL precursors stimulated in vitro with syngeneic tumor cells. *J. Immunol. 124: 1627.*
17. Julius, M.H., Simpson, E., and Herzenberg, H.A. 1973. A rapid method for the isolation of functional thymus-derived murine lymphocytes. *Eur. J. Immunol. 3: 645.*
18. Taswell, C., MacDonald, H.R., and Cerottini, J.R. 1979. Limiting dilution analysis of alloantigen-reactive T lymphocytes. II. Effect of cortisone and cyclophosphamide on cytolytic precursors frequencies in the thymus. *Thymus 1: 119.*

19. Collavo, D., Ronchese, F., Zanovello, P., Cerundolo, V., and Biasi, G. 1984. Reduction in precursors of cytotoxic T lymphocytes and of cells with natural killer-like activity in spleens of cyclophosphamide-treated mice. *Int. J. Immunopharmac. 6: 529.*
20. Mayumi, H., and Good, R.A. 1989. Long-lasting skin allograft tolerance in adult mice induced across fully allogeneic (multimajor H-2 plus multiminor histocompatibility) antigen barriers by a tolerance-inducing method using cyclophosphamide. *J. Exp. Med. 169: 213.*
21. Lee, J.C., Horak, I., and Ihle, J.N. 1981. Mechanisms in T cell leukemagenesis. II. T cell responses of preleukemic BALB/c mice to Moloney leukemia antigens. *J. Immunol. 126: 715.*
22. Lee, J.C., and Ihle, J.N. 1981. Chronic immune stimulation is required for Moloney leukaemia virus-induced lymphomas. *Nature 289: 407.*
23. Bendinelli, M., Matteucci, D., and Friedman, H. 1985. Retrovirus-induced acquired immunodeficiencies. *Adv. Cancer Res. 45: 125.*
24. Meruelo, D., and Bach, R. 1983. Genetics of resistance to virus-induced leukemias. *Adv. Cancer Res. 40: 107.*

Reprints from:
Paola Zanovello
Istituto di Oncologia
Via Gattamelata 64
35128 Padova