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Virus-specific T cell response prevents lymphoma development in mice infected by intrathymic inoculation of Moloney leukaemia virus (M-MuLV)

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Summary. Previous work on mice neonatally injected with Moloney-murine leukaemia virus (M-MuLV) had shown that T cell lymphoma development correlates with virus infection of lymphoreticular cells (T and B lymphocytes and macrophages) as well as with a lack of generation of virus-specific cytotoxic T lymphocytes (CTL) due to clonal deletion of CTL precursors. In the present report, viral antigen expression and T cell response in mice injected as adults with M-MuLV intrathymus (i.t.) was investigated. Only thymic and splenic T lymphocytes from these mice express virus-induced antigens since they were lysed by virus-specific CTL, and stained by anti-M-MuLV fluorescent serum. In addition, the percentage of M-MuLV-infected T cells increased with increasing post-inoculation times. However, these mice could mount a strong cellular immune response against M-MuLV-infected cells, as detected by massive mixed leucocyte tumour cell culture and by evaluation of virus-specific CTL precursor frequency. Finally, i.t. injected mice were not viraemic and did not develop lymphomas during an observation period of 12–15 months. These data, in contrast with the recent hypothesis that T cell lymphoma development depends on a chronic stimulation of virus-specific T lymphocytes, indicate that the cellular immune response is sufficient for prevention of neoplastic transformation, despite a persistent viral infection of the thymus and peripheral T lymphocytes.

Abbreviations: M-MuLV, Moloney-murine leukaemia virus; CTL, cytotoxic T lymphocyte; i.t., intra-thymus; i.p., intra-peritoneum; MLTC, mixed leucocyte–tumour cell culture; IL-2, interleukin 2; C, complement.

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INTRODUCTION

Type C retroviruses are considered related aetiologically to T cell leukaemia and lymphoma development in mice, although the mechanisms leading to T cell transformation are not fully understood. This particularly holds for slowly transforming murine leukaemia viruses (MuLV), i.e. viruses which are devoid of transforming genes and induce lymphomas, mostly of T cell origin, with a prolonged latent period (Teich *et al.*, 1982).

It is clear, however, that lymphoma development represents the final result of a complex series of events involving both virus and host gene controlled phenomena (Teich *et al.*, 1982). Among these, the immunological response against MuLV-induced antigens appears critical since, in the majority of systems investigated, an efficient host immune reactivity counteracts lymphoma development (Hogg, 1978). This view has been recently challenged by a new model of leukaemogenesis which suggests that chronic stimulation of T cells by viral antigens may lead ultimately to

neoplastic transformation (McGrath *et al.*, 1980; Lee, Horak & Ihle, 1981; Lee & Ihle, 1981).

Mice injected as newborns with Moloney (M)-MuLV represent a useful model for these studies, since these animals become virus carriers (carrier mice) for life and develop T cell lymphomas with high incidence within 4–6 months of age (Chieco-Bianchi & Collavo, 1976). We observed that thymus cells, mature T and B lymphocytes, and macrophages from M-MuLV carrier mice express virus-induced cell surface antigens as early as 10 days after infection (Collavo *et al.*, 1981). The appearance of these antigens on the majority of lymphoreticular cells at an early stage of immune system development induces in carrier mice a T cell tolerance characterized by the failure to generate virus-specific cytotoxic T lymphocytes (CTL) due to virtual deletion of CTL precursors (Chieco-Bianchi *et al.*, 1974; Chieco-Bianchi *et al.*, 1980; Collavo *et al.*, 1981; Collavo *et al.*, 1982). Therefore, these findings support the hypothesis that lymphoma development depends on lack of reactivity to virus-infected cells. However, since chronic virus infection and T cell tolerance in M-MuLV carrier mice are related phenomena, whether or not lymphoma may be induced by persistence of virus-infected cells in the presence of an efficient immunological response remains an open question.

In this study, in adult mice injected intra-thymus (i.t.) with M-MuLV, a persistent viral infection of thymus and peripheral T lymphocytes was observed despite a strong cellular immune response and absence of lymphoma development.

MATERIALS AND METHODS

Mice

Inbred C57BL/6J(B6) mice were originally purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained in our colony for several generations by sister × brother matings.

Viruses

The procedures for virus preparation are reported in detail elsewhere (Collavo *et al.*, 1980). The M-MuLV, whose titer evaluated on SC-1/XC cells was 5×10^8 plaque-forming units (PFU) per ml, was injected subcutaneously into newborn mice at a dose of 0·05 ml. Adult mice (8 weeks old) received 0·05 ml of M-MuLV i.t. or 0·2 ml i.p.

Tumour cell lines

MBL-2, a M-MuLV-induced lymphoma of B6 mice, was maintained by weekly passage of the ascitic form in syngeneic recipients.

Cell cultures

CTL were generated *in vitro* in a secondary mixed leucocyte tumour cell culture (MLTC) system as previously described (Collavo *et al.*, 1981). Briefly, 20×10^6 responder spleen cells and 4×10^6 mitomycin C (m)-treated MBL-2 lymphoma cells were cultured in a 30-ml flask (Falcon Plastics Co., Los Angeles, CA) in a total volume of 15 ml complete medium. Complete medium consisted of Dulbecco's modified minimal essential medium (MEM; Gibco, Glasgow, U.K.) supplemented with L-glutamine, HEPES, 2-mercaptoethanol, antibiotics, and 10% heat-inactivated foetal calf serum (FCS; Gibco). The cultures were incubated for 6 days at 37° in a water-saturated atmosphere containing 5% CO_2 in air.

Limiting dilution micro MLTC were set up according to Brunner, MacDonald & Cerottini (1980) with minor modifications (Collavo *et al.*, 1982). Micro MLTC were prepared in culture medium supplemented with 10% FCS and 25% secondary mixed leucocyte culture supernatant as a source of IL-2 (Brunner *et al.*, 1980). Each culture contained limiting numbers of responder cells, 3×10^4 m-treated MBL-2 lymphoma cells, and 10^6 m-treated syngeneic spleen cells, in a final volume of 0·2 ml in round-bottom microwells (Greiner, Nürtingen, West Germany). After 7 days of culture, cell growth was assessed microscopically, and aliquots were removed to measure cytolytic activity.

Selective stimulation of T lymphocytes was achieved by addition of 5 μg/ml of concanavalin A (Con A; Pharmacia, Uppsala, Sweden) to spleen cells, obtained following passage through nylon wool column (Julius, Simpson & Herzenberg, 1973). To obtain selective stimulation of B lymphocytes, spleen cells, pretreated with anti-Thy 1.2 monoclonal antibodies and complement (C), were stimulated with 25 μg/ml of LPS (lipopolysaccharide B *E. Coli* 0·55:B6, Difco, Detroit, MI). Anti-Thy 1.2 antibodies plus C generally lysed 94% of blast cells from Con A-stimulated cultures; in LPS-stimulated cultures, 2% of blast cells were Thy 1.2 positive, and anti Ig fluorescent serum stained 92% of cells.

^{51}Cr-release assay

The cytotoxic activity of MLTC was evaluated in a short-term incubation assay (Collavo *et al.*, 1981),

using 2×10^4 target cells, labelled with 0·1 ml of $Na_2[^{51}Cr]O_4$ (Sorin, Saluggia, Italia), and different effector cell doses. Cytotoxicity was expressed as the percentage of specific lysis, calculated as follows: 100 × (Experimental release − spontaneous release/ Maximum release − spontaneous release).

To assay cytolytic activity of micro MLTC, 100 μl of supernatant from each microculture were removed, and 2×10^3 ^{51}Cr-labelled target cells in 100 μl of medium were added. After a 4-hr incubation, 100 μl of supernatant were removed for counting. Spontaneous release was determined in control microcultures prepared in the same manner as the experimental groups but without responder cells.

Calculation of CTL-precursor frequencies

Twenty-four micro MLTC were scored as positive and negative, with positive cultures defined as those in which ^{51}Cr-release values exceeded the mean spontaneous release by more than three standard deviations (SD). The percentage of negative cultures was plotted against the number of responder cells plated, and the CTL precursor (p) frequency was determined by linear regression analysis, as described by Taswell, MacDonald & Cerottini (1979).

Cell typing methods

To detect Thy 1.2 positive cells, cells were incubated, at 37° for 45 min, with anti-Thy 1.2 monoclonal antibodies (NEN, Dreieich, Germany), diluted 1:500, in the presence of agar-adsorbed rabbit serum, diluted 1:9, as a source of C. The cytotoxic effect was determined by the eosin Y exclusion method. Direct immunofluorescence, with fluorescein isothiocyanate (FITC)-labelled anti-tween-ether disrupted M-MuLV goat serum (produced by Becton and Dickinson and Co. Research Center under contract from the Division of Cancer Cause and Prevention, NCI) diluted 1:40, was used to identify M-MuLV-induced antigens. Direct immunofluorescence, with rabbit anti-mouse FITC-labelled serum (Nordic Imm. Lab., The Netherlands) diluted 1:30, was used to detect surface immunoglobulins.

RESULTS

Presence of virus-induced antigens on different cell populations from adult mice injected i.t. with M-MuLV

We had previously observed that, long before leukaemia onset, thymocytes, peripheral T and B lymphocytes and macrophages from M-MuLV-carrier mice express virus-induced cell-surface antigens which are detected by virus-immune CTL (Collavo *et al.*, 1981). However, these antigens are not present on cells from mice injected i.p. with the virus in adult life. Since in different MuLV systems, e.g. Rad-MuLV and Abelson-MuLV (Teich *et al.*, 1982; Cook, 1982), it has been shown that i.t. injection in adult mice enhances virus oncogenicity, we considered it worthwhile to evaluate

Table 1. Lytic activity by virus-immune CTL against different cell populations obtained from M-MuLV i.t. injected mice at various days after inoculation*

	% Specific ^{51}Cr-release					
	30 days†			120 days		
Target cells	45:1‡	15:1	5:1	45:1	15:1	5:1
Con A-stimulated thymus	42	30	15	65	45	19
Con A-stimulated spleen	35	28	14	60	29	11
LPS-stimulated spleen	1	1	1	1	1	1
Macrophages§	2	2	1	2	1	1

* CTL obtained in secondary MLTC consisting of spleen cells from i.p. M-MuLV injected B6 mice restimulated *in vitro* with MBL-2 leukaemic cells.

† Time from M-MuLV injection.

‡ Effector:target cell ratio.

§ Adherent peritoneal resident cells.

whether viral antigens are expressed on cells from M-MuLV i.t. injected adult mice. Therefore, mice were killed 30 or 120 days after virus injection, and ^{51}Cr-labelled Con A- and LPS-induced blast cells and peritoneal cavity macrophages were used as targets in a short-term incubation cytotoxicity assay. As reported in Table 1, virus-immune CTL, obtained in secondary MLTC, exerted a strong cytotoxic effect against Con A-stimulated thymus cells and spleen cells; no activity was however detected against LPS-induced blast cells or macrophages. As expected, the same effector cells failed to lyse blast cells obtained from adult i.p. M-MuLV-injected donors, or from uninjected control mice (data not shown). Since Con A and LPS induce a selective blast transformation of T and B lymphocytes respectively, these results indicate that, following i.t. injection, only thymus cells and peripheral T cells express M-MuLV-induced antigens and therefore are clearly infected by virus.

In order to evaluate the percentage of infected T cells, unstimulated thymus and spleen cells and Con A-stimulated spleen cells, from mice killed at various times after M-MuLV i.t. injection, were stained with anti-M-MuLV fluorescent serum. As shown in Fig. 1, the fluorescent positive cells increased progressively at various intervals after inoculation. In contrast, thymus and spleen cells from i.p. injected mice were negative (data not shown).

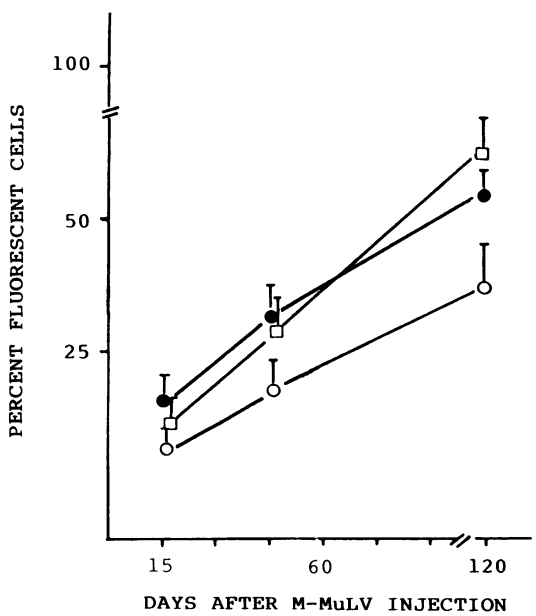

Figure 1. Percent of virus-positive cells from M-MuLV i.t. injected adult mice at different days after inoculation. Unstimulated thymus (□), unstimulated spleen (○) and Con A-stimulated spleen cells (●) were incubated with anti M-MuLV fluorescent serum diluted 1:40. Each point represents the mean ± standard deviation of data obtained in three mice.

These results indicate that viral antigen expression in i.t. injected mice is an early phenomenon and that the percentage of infected T cells increases with time.

The presence of infectious virus, in the different groups of mice employed in the above experiments, was also evaluated by the SC-1/XC plaque method. Tail tissue extracts from all mice injected i.t. or i.p. as adults were negative for virus presence up to 120 days after M-MuLV injection; in contrast, high titres of virus (ranging from 1×10^4 to 1×10^5 PFU/ml) were detected in carrier mice early after M-MuLV injection and throughout their life (data not shown).

Virus-specific CTL generation by spleen cells from mice injected i.t. with M-MuLV

We had observed that the failure of carrier mice to generate virus-specific CTL was due to strong reduction in CTL p (Collavo *et al.*, 1981, 1982). To establish whether adult mice injected i.t. with M-MuLV also show an impaired cellular immune response, spleen cells from these mice killed at various intervals following virus injection, were assayed for virus-specific CTL generation. The cytotoxic response was evaluated up to 120 days after injection as most of M-MuLV carrier mice develop lymphomas by this time; older mice were not used since the ability to generate CTL declines with advancing age (Makinodan & Kay, 1980). As shown in Table 2, strong cytotoxic activity is generated at 30 and 60 days after virus i.t. injection, even though it is less than that observed in i.p. injected control mice. At 120 days, however, only a weak lytic effect was detected in the former group of mice.

To evaluate whether the progressive fall in cytotoxicity, could be ascribed to a low frequency of CTL p, a limiting dilution micro MLTC system (Brunner *et al.*, 1980; Collavo *et al.*, 1982) was employed. As shown in Fig. 2, virus-specific CTL p in the spleens of i.t. injected mice varied from 1/7100 to 1/11600 cells at 60 days and from 1/31000 to 1/34200 cells at 120 days after inoculation. These values were lower than those observed at similar times in i.p. injected mice, where CTL p frequency varied from 1/1600 to 1/2100 cells, respectively (data not shown).

Therefore, unlike M-MuLV carrier mice, i.t. in-

Table 2. Time course of virus-specific CTL generation in spleens of i.t. or i.p. M-MuLV-injected adult mice*

Exp	Days after M-MuLV injection	% Specific ^{51}Cr-release					
		i.t. injected			i.p. injected†		
		45:1‡	15:1	5:1	45:1	15:1	5:1
1	30	55	37	19	70	55	40
	60	62	52	37	78	65	44
	120	13	9	7	52	40	24
2	60	58	41	30			
	120	17	8	3		ND§	
3	60	59	48	31			
	120	34	25	13		ND	

* Spleen cells obtained from B6 mice and restimulated *in vitro* with MBL-2 leukaemic cells (secondary MLTC).

† Mice injected i.t. or i.p. with M-MuLV when 6–8 weeks old.

‡ Effector: target cell ratio. MBL-2 leukaemic cells were used as targets.

§ND = not done.

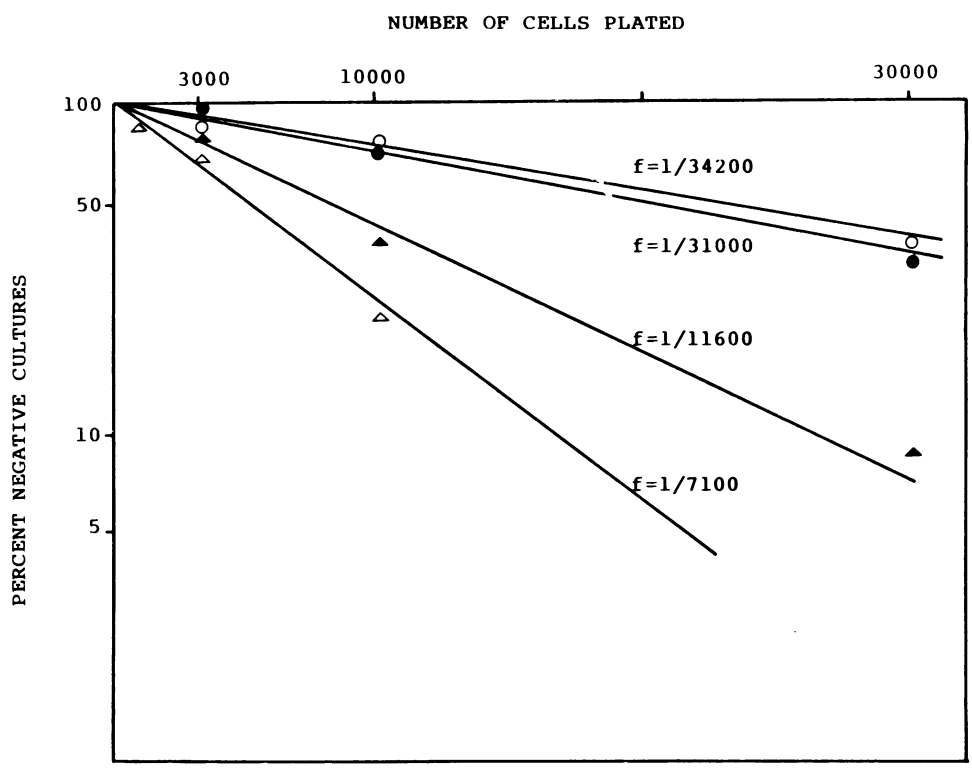

Figure 2. Minimal estimate of the frequencies (f) of CTL precursors specific for MBL-2 leukaemia cells in i.t. injected mice evaluated on day 60 (△▲) and 120 (○●) after M-MuLV inoculation and calculated by linear regression analysis of the data.

jected mice are able to generate virus-specific CTL, although CTL p frequency is lower than that of i.p. injected mice and decreases as time increases after inoculation.

Leukaemia development in i.t. injected mice

To ascertain if i.t. injected mice develop M-MuLV-induced lymphomas, 24 mice were kept under observation and killed 12–15 months after M-MuLV inoculation. Nineteen mice survived the observation period and, at the macroscopic examination, were disease-free. Five mice died 2–5 months after i.t. injection and at autopsy showed spleen enlargement. Histologically, the spleen and lymph nodes of these mice were not infiltrated by neoplastic cells, but presented depletion of lymphoid tissue and replacement by histiocytes, a picture compatible with that of a chronic graft versus host reaction (Simonsen, 1962). On the other hand, 23 out of 28 carrier mice developed lymphomas, mostly of thymic origin, within 6 months of birth.

No lymphomas were observed among 25 mice injected i.p. with M-MuLV as adults.

DISCUSSION

The expression of virus-induced antigens on different cell populations from M-MuLV-injected mice depends not only on the time but also on the site of virus inoculation. In fact, previous results indicated that viral antigens are present on the majority (more than 65%) of thymus, peripheral T and B lymphocytes and macrophages from neonatally injected mice, but not on cells from i.p. injected adult mice (Collavo *et al.*, 1981, and unpublished results). The present report shows that infection of thymus and peripheral T lymphocytes in adult mice is also possible when the virus is i.t. injected. In fact, following i.t. injection, most thymus cells were lysed by virus-immune CTL and stained by anti-M-MuLV fluorescent serum. The percentage of infected peripheral T lymphocytes was low soon after inoculation, but increased as time increased after inoculation. On the other hand, virus-immune CTL did not lyse LPS-stimulated B lymphocytes or macrophages. It should be mentioned that a low percentage (9–18%) of LPS-induced blast cells obtained not only from i.t. injected mice but also from i.p. injected or normal control mice, reacted with anti-M-MuLV serum. This reactivity, was never observed with Con A blast cells and might be related to the observation that LPS is more active than Con A in eliciting endogenous type C virus induction in spleen cells stimulated *in vitro* (Greenberger *et al.*, 1975).

Previous studies (Collavo *et al.*, 1981; 1982) had indicated that M-MuLV lymphocyte infection in carrier mice is associated with lack of cytotoxic activity of massive MLTC and with a dramatic reduction in virus-specific CTL p (from 1/250000 to 1/320000 spleen cells). In contrast, the peculiarity of i.t. injected mice lies in the fact that virus-specific CTL were generated despite persistent viral infection of thymus and peripheral T cells. However, in these mice the cytotoxic activity in MLTC and CTL p frequency were lower than in i.p. injected immune mice, and decreased with time after inoculation.

The decline in cytotoxic activity correlates with the increasing number of M-MuLV-infected T lymphocytes in the spleen of i.t. injected mice. It seems unlikely that the presence of these cells might inhibit competitively the lytic effect against labelled target cells since MuLV-infected cell addition to micro MLTC did not affect CTL p frequency calculation (Collavo *et al.*, 1982). In our opinion this fall of cytotoxicity has a different explanation. We have observed that the few M-MuLV specific CTL clones detected in neonatally injected mice do not express virus-induced antigens (Collavo *et al.*, in preparation). Therefore, this finding suggests that virus-infected CTL cannot be directed against antigens that they present on their own surface. In addition, it may be that continuous lysis of infected cells by uninfected CTL could ultimately lead to exhaustion of virus-specific clones. Such chronic autoaggressive phenomena would explain the spleen enlargement observed in five of the 24 mice kept under observation for several months.

The present data furnish clear-cut evidence that M-MuLV lymphocyte infection *per se* is not sufficient for lymphoma induction if CTL are generated. In fact, i.t. injected mice, which have a persistent T cell-mediated immune reactivity, do not develop lymphomas at a late age even though their thymus and peripheral T cells are progressively infected by M-MuLV. Instead, M-MuLV-carrier mice, where a virtual deletion of virus-specific CTLp was observed, showed a high incidence of T cell lymphomas within 4–6 months of birth (Collavo *et al.*, 1982). However, it is also likely that other T cell subpopulations might collaborate in lymphoma prevention. In this regard, good correlation between M-MuLV-specific helper T cell reaction, antibody production and resistance to viraemia and lymphoma development and resistance

to viraemia and lymphoma development has been demonstrated (Boyer *et al.*, 1982). Accordingly, using a radiolabelled protein A assay (D'Andrea, De Rossi & Chieco-Bianchi, 1982), we observed that sera from i.t. injected mice had the same high titres of anti-M-MuLV antibodies as sera from i.p. injected immune mice. This finding might explain the absence of infectious virus in the tail tissue extracts of i.t. injected mice. In contrast, no free antibodies were detected in sera of highly viraemic M-MuLV-carrier mice (unpublished results). Taken together, these observations suggest that the immune response restricts the spreading of M-MuLV and the reinfection of target T cells, thus reducing the risk of neoplastic transformation according to the promoter insertion hypothesis (Teich *et al.*, 1982).

It has been suggested that chronic stimulation of virus-specific T lymphocytes by M-MuLV antigens is essential for lymphoma induction. As evidence of this hypothesis, the presence of a helper T cell subpopulation highly responsive to M-MuLV gp 70 blastogenesis has been described in preleukaemic mice (Lee *et al.*, 1981), and lack of T cell responses against the virus in viraemic leukaemia resistant CBA/N mice has been reported (Lee & Ihle, 1981). Although in the present study the activity of a different T cell population has been evaluated, our data on the whole do not support this hypothesis, but instead suggest that continuous stimulation of virus-specific T lymphocytes by M-MuLV-infected cells might effectively prevent leukaemogenesis.

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