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Frequency analysis of tumor-reactive cytotoxic T lymphocytes in peripheral blood of a melanoma patient vaccinated with autologous tumor cells

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Abstract. A limiting-dilution assay was developed and used to determine the frequency of autologous tumor-reactive cytotoxic T lymphocytes (CTL) in peripheral blood of a melanoma patient MZ2, who has been free of detectable disease since several years. In this patient, the frequencies of tumor-reactive CTL spontaneously varied only by a factor of 1.5. After vaccinations with autologous mutagenized and lethally irradiated tumor cells a two- to tenfold increase in frequencies of tumor-reactive CTL was found within the first 2 weeks. Thereafter, CTL frequencies returned to values measured prior to vaccinations. We conclude, that the limiting-dilution assay applied in this study can detect changes in the T cell response to autologous tumor cells. The frequency of tumor-reactive CTL determined with this approach can serve as an immunological parameter for monitoring the T cell response to autologous tumor cells in individual cancer patients receiving tumor cell vaccinations.

Key words: Human melanoma – Limiting-dilution assay – Frequency of tumor-reactive CTL – Tumor cell vaccination

Introduction

During the past decade several groups have isolated autologous tumor-reactive cytotoxic T lymphocyte (CTL) clones from peripheral blood lymphocytes (PBL) of cancer patients in vitro (for review see [15]). These CTL were found to recognize multiple antigens in association with HLA class I molecules on autologous tumor cells [7, 11, 13, 22, 24].

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In the human melanoma model derived from patient MZ2, tumor-reactive CTL clones were repeatedly isolated via autologous mixed lymphocyte/tumor cell cultures (MLTC). They lysed autologous tumor cells (MZ2-MEL) but not autologous Epstein-Barr-virus(EBV)-transformed B lymphocytes or the natural killer (NK) target K562 [8]. These CTL clones recognized six different antigens on MZ2-MEL [22]. Recently, the gene MAGE-1, encoding the CTL-defined melanoma antigen MZ2-E, was identified [23]. The expression of this antigen is not restricted to MZ2-MEL but is also found on allogeneic tumor cells of various histological origins. The expression of MAGE-1 is confined to tumor tissues and is not detectable in non-malignant cells with the exception of the testis [3]. These findings suggest that MZ2-E can serve as a potential tumor-rejection antigen in vivo in a T-cell-mediated antitumor response occurring spontaneously or by induction through immunotherapy.

Numerous attempts have been initiated to stimulate antitumor immune responses in cancer patients (for review see [14]). In single cases partial or complete tumor regressions have been reported following vaccinations with whole melanoma cells or melanoma cell lysates. However, to date reliable parameters to determine and measure vaccine-induced, tumor-directed immune responses are not available. The frequency of tumor-reactive CTL in PBL of cancer patients may represent such a parameter.

Limiting-dilution analysis (LDA) has been used to determine the frequency of CTL with a defined specificity in lymphocyte populations [12, 16, 18, 20, 21]. In the work described here, PBL, separated from the blood of melanoma patient MZ2, were seeded at limiting dilutions and were repeatedly stimulated with autologous tumor cells. Culture conditions were largely identical to well-established MLTC conditions [8] applied to propagate and enrich tumor-reactive effector cells from patients' PBL. Split-well analysis was used to determine the lytic activity and specificity of each LDA responder subpopulation [19]. Frequencies of tumor-reactive CTL were calculated according to Taswell et al. [20].

With the test system described in this study, we obtained reproducible frequencies of autologous tumor-reactive CTL. An increase in CTL frequencies after vaccinations with mutagenized and lethally irradiated autologous tumor cells was readily detectable.

Materials and methods

Patient and cell lines. The cell line MZ2-MEL was established in 1982 from an adrenal metastasis of patient MZ2 with metastatic amelanotic melanoma of unknown primary origin. After ineffective cytostatic chemotherapy MZ2 received surgical resections of multiple lymph nodes and visceral metastases in 1982 and in 1983. Thereafter, the patient stayed free of detectable disease. From 1983 on, MZ2 was repeatedly vaccinated with mutagenized and lethally irradiated autologous melanoma cell clones [22]. The vaccination program was approved by an ethics committee and informed consent was obtained from the patient.

The tumor cell clone MZ2-MEL-43 was derived from the parental melanoma cell line MZ2-MEL by cloning and subsequent mutagenization [1] and has been described elsewhere [8]. All tumor antigens defined by autologous CTL on MZ2-MEL were also expressed on MZ2-MEL-43 [22]. Tumor cells were maintained in Dulbecco's modified Eagle's medium (Gibco Laboratories, Grand Island, N.Y.) containing 10 mM HEPES buffer, L-arginine (116 mg/ml), L-asparagine (36 mg/ml), L-glutamine (216 mg/ml), penicillin (10 IU/ml), streptomycin (100 µg/ml), and 10% fetal calf serum (FCS). This medium is subsequently further referred to as medium A. MZ2-EBV-B is an Epstein-Barr-virus-transformed lymphoblastoid B (EBV-B) cell line derived from patient MZ2. EBV-B cells were cultured in RPMI-1640 medium (medium B), supplemented as described for medium A. Cell cultures were kept in a water-saturated atmosphere with 5% CO_2 at 37° C.

Mixed lymphocyte/tumor cell culture (MLTC). PBL were separated from heparinized blood of patient MZ2 by centrifugation on a Ficoll-Paque gradient (Pharmacia, Uppsala). MLTC experiments were performed as described previously [8]. Samples containing 10^6 PBL were cocultured with 10^5 autologous melanoma cells (MZ2-MEL-43, irradiated with 100 Gy from a ^{137}Cs source) on 24-well tissue-culture plates (Greiner, Nürtingen, Germany) in 2 ml RPMI-1640 medium supplemented as described above, but with 10% human serum (referred to as medium C). Natural human interleukin-2 (IL-2, Biotest GmbH, Dreieich, Germany) was added from day 3 onwards at a final concentration of 10 ng/ml. Regularly, 3×10^5 responder lymphocytes were restimulated with 5×10^4 irradiated tumor cells (MZ2-MEL-43) at weekly intervals in 2 ml medium C containing interleukin-2 (IL-2).

Limiting-dilution assay (LDA). PBL from patient MZ2 were seeded at limiting dilutions in each microculture of round-bottom microtiter plates (Nunc, Roskilde, Denmark) with 3×10^3 irradiated autologous melanoma cells (MZ2-MEL-43) in 200 µl medium C. Unless otherwise indicated, PBL were seeded at 10000, 5000, 2500, 1250 and 625 cells/well. At least 96 microcultures were prepared for each dilution of PBL. On day 3, IL-2 was added at a final concentration of 10 ng/ml. Responder lymphocytes were restimulated on days 7 and 14 with 100 µl fresh medium C containing 3×10^3 irradiated autologous tumor cells (MZ2-MEL-43) and IL-2. Where indicated, 5×10^4 irradiated autologous EBV-B cells (MZ2-EBV-B) were added to each microculture on days 7 and 14. On day 18, a split-well analysis was carried out (see below).

Assay for cytolytic activity. ^{51}Cr-release assays were performed as described [4]. Target cells were incubated at 10^7 cells/ml in fetal calf serum for 1 h at 37° C with $Na^{51}CrO_4$ at 200 µCi/ml (Institut des Radioelements, Fleurus, Belgium). Labeled cells were washed three times and resuspended in medium A at 1×10^4 cells/ml. MLTC responder lymphocytes were serially diluted in duplicates in 50 µl medium A in conical 96-well microtiter plates. LDA responder lymphocytes of each subpopulation were divided into four aliquots of 50 µl and were then transferred to conical 96-well microtiter plates for split-well analysis. A 50-µl sample of medium A alone or with an excess of unlabeled K562 was added to each microwell containing MLTC or LDA responder lymphocytes. After the addition of 1×10^3 labeled target cells in 100 µl medium A to each well, the plates were incubated for 4 h at 37° C in a 5% CO_2 atmosphere. Assays were harvested by centrifugation at 200 *g* for 5 min and 100 µl supernatant was collected for counting in a gamma counter (LKB 1272, LKB, München, Germany). The percentage of specific ^{51}Cr release was calculated according to the formula:

specific ^{51}Cr release (%) = (experimental release – spontaneous release)×100/(maximum release – spontaneous release).

Maximum ^{51}Cr release was obtained by adding 100 µl NP-40 (Sigma Chemical, St. Louis, USA) to labeled targets. Spontaneous ^{51}Cr release ranged between 10% and 20% of the total label incorporated into cells. Targets of the split-well analysis were: ^{51}Cr-labeled MZ2-MEL-43 cells mixed with a 40-fold excess of unlabeled K562 cells [MZ2-MEL-43*+K562(1:40)]; ^{51}Cr-labeled MZ2-EBV-B cells mixed with a 40-fold excess of unlabeled K562 cells [MZ2-EBV-B*+K562(1:40)]; ^{51}Cr-labeled K562 cells mixed with a 40-fold excess of unlabeled K562 cells [K562*+K562(1:40)]; ^{51}Cr-labeled K562 cells alone [K562*].

Determination of CTL frequency. In split-well analysis LDA responder lymphocytes in each microwell were tested for lytic activity against different targets.

For a single microculture,

A. The presence of CTL with lytic activity restricted to autologous tumor cells was assumed if specific lysis of [MZ2-MEL-43*+K562(1:40)] exceeded 20% and was at least twofold higher than lysis of [K562*+K562(1:40)] and [MZ2-EBV-B*+K562(1:40)]. At the same time specific lysis of [K562*+K562(1:40)] had to be lower than 15% to ensure efficient NK blockade
B. The presence of CTL lysing autologous EBV-B cells was assumed if specific lysis of [MZ2-EBV-B*+K562(1:40)] exceeded 20% and was at least twofold higher than lysis of [K562*+K562(1:40)]. Again at the same time specific lysis of [K562*+K562(1:40)] had to be lower than 15%
C. The presence of NK activity was assumed if specific lysis of K562* exceeded 10%.

The frequencies of CTL with lytic activity restricted to autologous tumor cells were determined by Poisson distribution analysis and verified according to the method of χ^2 minimization [20]. Only CTL frequencies with a *P* value higher than 0.1 were accepted and are presented here.

Results

Determination of culture parameters for LDA

The conditions chosen for limiting-dilution cultures were similar to those described for the generation of tumor-reactive CTL in MLTC [8]. In an initial experiment, PBL separated from the blood of patient MZ2 were stimulated with autologous tumor cells at weekly intervals (Fig. 1). As variables we investigated the influence of the IL-2 concentration, the timing of split-well analysis and the effect of autologous EBV-B cells as feeders on the generation of lytic activity against different targets. Lysis data of split-well analyses on responder lymphocytes are shown in Fig. 1. On the basis of these data and by using the definitions for lytic specificity of single microcultures, as given in Materials and methods, the fractions of microcultures

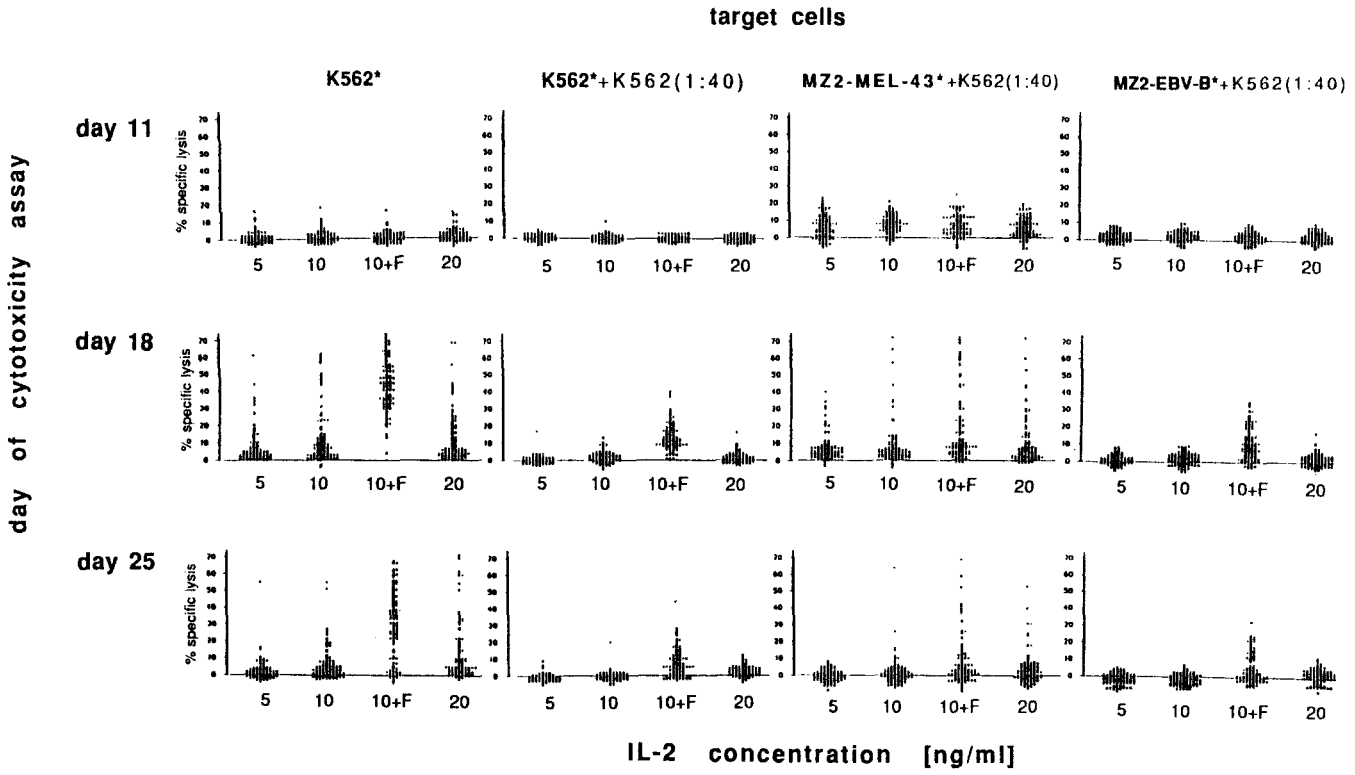

Fig. 1. Effect of culture parameters on the lytic specificity of limiting-dilution assay (LDA) responder lymphocytes. Peripheral blood lymphocytes (PBL) from melanoma patient MZ2 were seeded in 96-well microtiter plates (10000/well) and were stimulated with autologous tumor cells (MZ2-MEL-43) at weekly intervals. From day 3 on, interleukin-2 (IL-2) was added to the microcultures at concentrations indicated. Autologous feeders were added where shown (+*F*). On days 11, 18 and 25 split-well analyses were performed to test the responder cells of each microculture for their lytic activity against MZ2-MEL-43*, K562* and MZ2-EBV-B*, all in the presence of a 40-fold excess of unlabeled K562, and against K562* without K562 competitors. Each point represents the specific lysis of a single microculture

containing CTL with lytic activity restricted to autologous tumor cells, natural killer (NK) activity and CTL lysing autologous EBV-B cells were determined (Fig. 2).

On day 11 no target-specific lysis of LDA responder cells was seen (Figs 1, 2). The percentages of microcultures lysing autologous tumor cells, K562 cells and autologous EBV-B lymphocytes reached a peak on day 18 and dropped thereafter. On days 18 and 25 the percentages of microcultures with lytic activity against autologous tumor cells (MZ2-MEL-43) increased with rising IL-2 concentrations. In addition, a positive correlation between the appearance of NK activity and the concentration of IL-2 was observed. After addition of MZ2-EBV-B cells as autologous feeders, lytic activity against autologous tumor cells as well as K562 and MZ2-EBV-B cells appeared in a higher percentage of wells. However, NK activity was only partially blocked in the presence of a 40-fold excess of unlabeled K562 competitors while complete blockade was possible in the absence of feeders.

On the basis of these results, LDA culture conditions for subsequent experiments were chosen in order to support preferentially the proliferation of tumor-reactive CTL: (a) IL-2 at 10 ng/ml from day 3 on; (b) absence of feeder cells; (c) split-well analysis on day 18 after two restimulations.

Increase of CTL frequency in MLTC responder lymphocytes

It was previously demonstrated that tumor-reactive CTL were regularly generated from PBL of patient MZ2 after repeated stimulations with autologous tumor cells in MLTC [8]. We wanted to find out whether a LDA using the culture conditions described in the previous section would allow us to detect the enrichment of tumor-reactive CTL in the course of autologous MLTC.

PBL were freshly separated from the blood of patient MZ2 at a time when the patient had not been vaccinated for more than 8 months. With these PBL a MLTC was initiated. LDA were performed using unstimulated blood lymphocytes on day 0 (LDA-A1) and MLTC responder lymphocytes on day 7 (LDA-A2), day 14 (LDA-A3) and day 21 (LDA-A4).

In parallel, responder lymphocytes of MLTC were tested at weekly intervals for their lytic activity. From day 10 on, cytolytic activity against MZ2-MEL-43 was observed and was present until day 38 (Fig. 3).

As shown in Fig. 4, a 72-fold increase of CTL frequency was seen during the first 2 weeks of autologous MLTC. Thereafter, the frequency decreased. NK activity in LDA and MLTC was completely blocked by addition of a 40-fold excess of unlabeled K562 to the tests. Autologous MZ2-EBV-B cells were not lysed (data not shown).

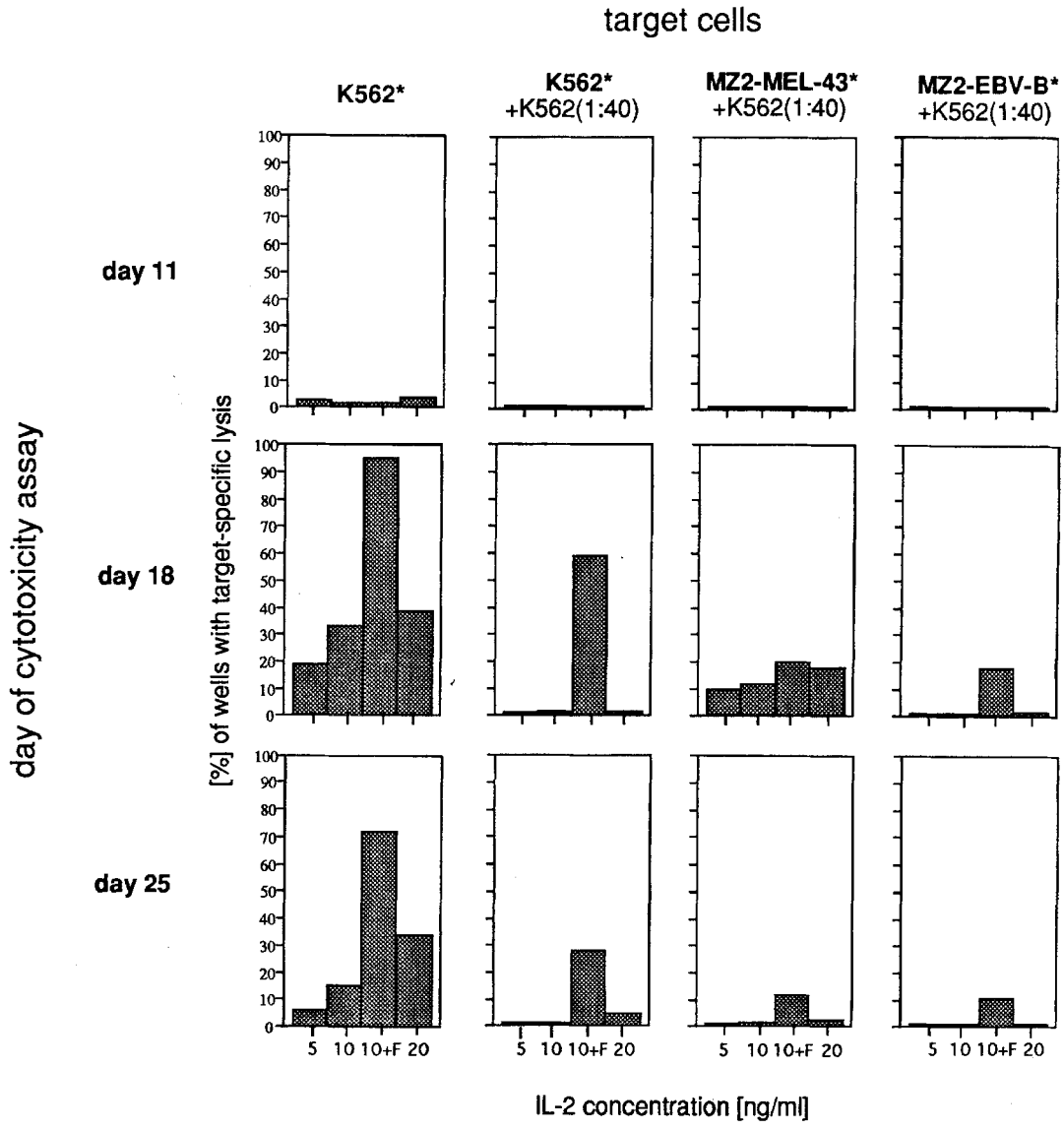

Fig. 2. Effect of culture parameters on the lytic specificity of LDA responder lymphocytes: evaluation of lysis data shown in Fig. 1. The percentages of microwells containing natural killer activity [*K562**, *K562*+K562(1:40)*], cytotoxic T lymphocytes (CTL) with lytic activity restricted to autologous tumor cells [*MZ2-MEL-43*+K562(1:40)*] and CTL lysing autologous EBV-B cells [*MZ2-EBV-B*+K562(1:40)*] were determined by using the definitions given in Materials and methods

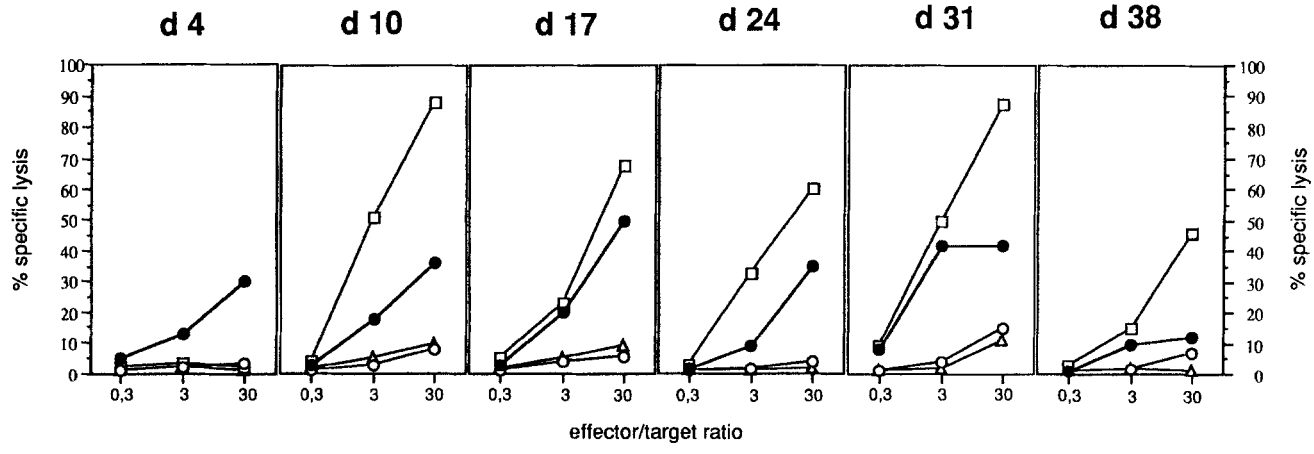

Fig. 3. Cytolytic activity of mixed lymphocyte/tumor cell culture (MLTC) responder lymphocytes. Fresh PBL separated from blood of patient MZ2 were weekly stimulated with autologous tumor cell clone MZ2-MEL-43 in a MLTC. Lytic activity and specificity of responder lymphocytes were determined at weekly intervals (days 4–38). ^{51}Cr-release assays were performed on the following targets: MZ2-MEL-43*+K562(1:40) (□); K562* (●); K562*+K562(1:40) (○); MZ2-EBV-B*+K562(1:40) (△)

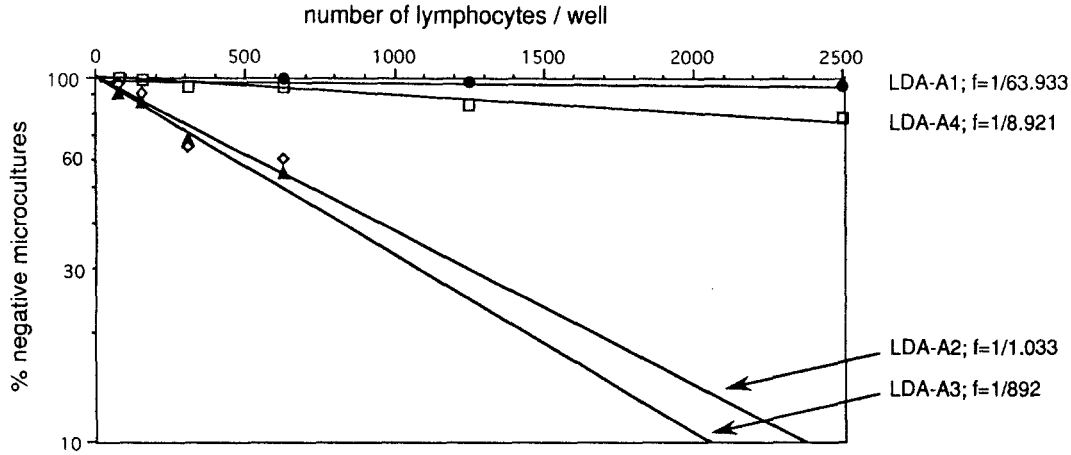

Fig. 4. Increase in frequency of tumor-reactive CTL among MLTC responder lymphocytes. LDA were performed with responder lymphocytes of the autologous MLTC described in Fig. 3 on day 0 (LDA-A1; ●), day 7 (LDA-A2; ◇), day 14 (LDA-A3, ▲) and day 21 (LDA-A4; □). LDA responders were stimulated with autologous tumor cells (MZ2-MEL-43) at weekly intervals in the presence of IL-2, as described in Materials and methods. On day 18 of each LDA, split-well analyses were carried out with the targets given in Fig. 3. Using the definition shown in Materials and methods, the frequencies (*f*) of autologous tumor-reactive CTL among MLTC responders were determined according to Taswell [20]

Spontaneous variation of CTL frequency in blood lymphocytes of patient MZ2

To determine the frequencies of autologous tumor-reactive CTL in the blood of patient MZ2, LDA (A1, B, C) were performed with fresh PBL at 4- to 8-week intervals. The first LDA (A1) was started at a time when MZ2 had not been vaccinated for more than 8 months. Frequencies of tumor-reactive CTL ranged from 1/51.690 (LDA-A1) to 1/78.288 (LDA-C) (Fig. 5). In all microcultures strong NK activity was seen, which was completely blocked by addition of a 40-fold excess of unlabeled K562 (data not shown). Lytic activity against autologous EBV-B cells was not observed.

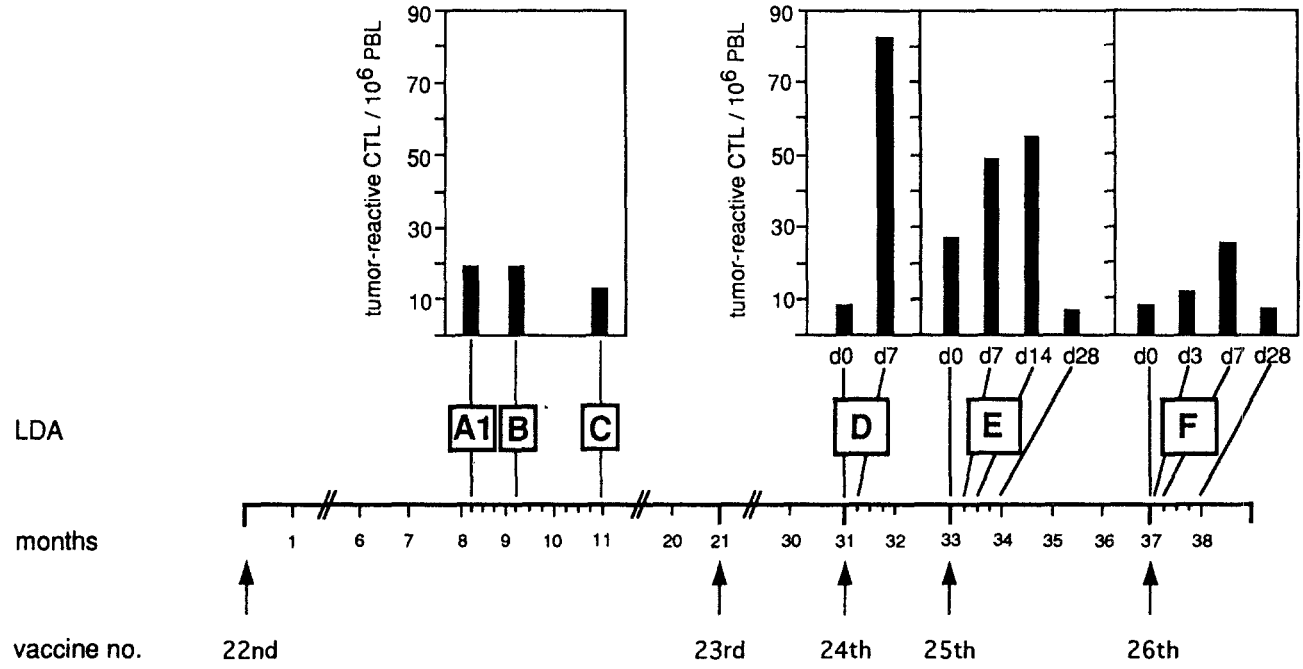

Fig. 5. Frequency analysis of tumor-reactive CTL in peripheral blood of MZ2: spontaneous variation of CTL frequency and its time course after vaccinations with autologous melanoma cells. After being free of clinically detectable tumor manifestations for more than 5 years, patient MZ2 received the 22nd to 26th melanoma vaccinations at the times indicated by *arrows*. LDA (□) were set up with fresh PBL separated from the patient's blood. LDA-A1, LDA-B and LDA-C were performed in a vaccination-free interval. Serial LDA were initiated each around the 24th (LDA-D), the 25th (LDA-E) and 26th (LDA-F) vaccination on the days of vaccination (*d0*) and at different times thereafter (*d3–d28*). On day 18 of each LDA split-well analyes were performed and CTL frequencies were determined as described in Fig. 4. Data shown in the diagrams represent the number of autologous tumor-reactive CTL in 10^6 PBL

Increase of CTL frequencies after vaccinations with autologous melanoma cells

To find out whether vaccinations with autologous tumor cells led to an increase in the frequency of tumor-reactive CTL, serial LDA were performed around the 24th (LDA-D), the 25th (LDA-E) and 26th (LDA-F) vaccination. PBL were separated from the blood of patient MZ2 and were directly applied in LDA.

As shown in Fig. 5, by comparing CTL frequencies in fresh MZ2-PBL separated before and 7 days after the 24th melanoma vaccine, a tenfold relative increase was detected [LDA-D(day 0):1/120.328, LDA-D(day 7):1/12.271]. An up to threefold relative increase in CTL frequencies was found during the first 2 weeks after the 25th and the 26th melanoma vaccines [LDA-E(day 0):1/34.700, LDA-E(day 14):1/17.197; LDA-F(day 0):1/143.990, LDA-F(day 7):1/44.718). CTL frequencies returned to values measured prior to vaccinations within the following 2–3 weeks [LDA-E(day 28): 1/172.038; LDA-F(day 28):1/110.209].

Discussion

Our aim was to develop a LDA that would allow us to detect changes in frequencies of autologous tumor-reactive CTL in peripheral blood of tumor patients. LDA is a widely used approach for estimating CTL frequencies in lymphocyte populations. However, there is no consensus regarding the culture parameters of LDA. Some authors find it crucial not to restimulate limiting-dilution cultures with antigen, since selection of activated lymphocytes may occur and only memory effector cells will then be detected [19]. Instead they prefer to provide growth conditions allowing each T cell clone to proliferate. However, assuming that the frequency of tumor-reactive CTL in peripheral blood of cancer patients is very low, a LDA supporting the proliferation of each single T cell appears impracticable.

It was demonstrated earlier that repeated stimulations of MLTC responder lymphocytes with autologous tumor cells are necessary to enable clonal maturation and proliferation of tumor-reactive CTL [8]. These weekly stimulations with autologous tumor cells were indispensable to generate sufficient amounts of LDA responder lymphocytes for a specificity analysis of lytic activities against a set of different targets in split-well analysis. As we could not distinguish whether lytic responder cells descended from primed or unprimed T cells we used the general term "CTL frequency".

By adding IL-2 at 10 ng/ml and by omitting the use of feeder cells, the outgrowth of NK cells was reduced. These findings are supported by observations of other groups [16, 17]. Residual NK activity was blocked by adding a 40-fold excess of unlabeled K562 cells as competitors to labeled targets. We previously verified that, under these conditions, unlabeled K562 cells do not reduce lysis by tumor-specific CTL (data not shown).

Recently, a similar LDA was published for determining the frequency of autologous tumor-reactive CTL in peripheral blood of tumor patients [6]. In that assay, testing lytic activity of LDA responders on day 18 after two restimulations with autologous tumor cells and omitting the use of feeder cells was also found to be preferable. Optimum growth conditions for responder lymphocytes included IL-4 for growth support. In addition, criteria for the specificity of responder lymphocytes in single microcultures were different. Therefore, comparisons of absolute CTL frequencies obtained in both test systems, even on the same patient, are not possible.

We first applied the LDA to determine CTL frequencies in MLTC responder populations. The maximum frequency (1/892) was found in a 14-day-old MLTC (Fig. 4). This frequency can be regarded as surprisingly low considering the lytic activity of bulk MLTC responders around day 14 (Fig. 3). However, it has to be taken into account that 14-day-old MLTC lymphocytes were carried on for an additional 18 days under limiting-dilution conditions until split-well analysis. During that period no feeder lymphocytes were supplied. This explains why only a proportion of tumor-reactive CTL enriched in MLTC was detectable. Still, a 72-fold relative increase of CTL frequency was seen. On the basis of these results we regarded the LDA described here as suitable to detect changes of CTL frequencies in ex vivo PBL that are less marked than those induced in the course of MLTC but are still within the range of biological relevance (see below).

The frequencies of autologous tumor-reactive CTL in fresh PBL of patient MZ2 varied only by a factor of 1.5 during a phase without evidence of disease, when melanoma vaccinations were not performed. Previously, the frequencies of allo-reactive CTL were reported to vary by a factor of 1.5 within a healthy individual over a period of 1 month [25].

Frequencies of tumor-reactive CTL were measured in the patient's PBL during the first weeks after each of three consecutive vaccinations with irradiated autologous tumor cells. A maximum increase was regularly observed after 1–2 weeks. On day 28, after the 25th and 26th vaccines, frequencies returned to values measured prior to vaccinations. Maximum CTL frequencies declined from the 24th vaccine to the 26th vaccine. This finding might indicate that, after repeated vaccinations at short intervals, inducibility of CTL responses, as detectable in peripheral blood, declines. The differences between CTL frequencies measured before and after vaccinations exceeded the spontaneous variation of CTL frequencies described above.

Increases in frequency of tumor-reactive CTL following melanoma vaccinations, as observed in patient MZ2 may be biologically relevant. By using limiting-dilution methods it was found that, in patients with a well-functioning kidney graft showing no signs of graft rejection, the frequency of donor-reactive CTL in PBL decreased more than tenfold within 3–8 months after kidney transplantation in some cases. In the same study no such decrease was seen in patients showing clinical symptoms of rejection [9]. In addition, in investigations of patients infected with cytomegalovirus or human immunodeficiency virus a positive correlation between the frequency of anti-viral CTL and the extent of virus replication was observed. CTL frequencies ranged from approximately 1/4000 to 1/20000 showing an up to fivefold increase at a time of strong virus replication [2, 10].

Our study in patient MZ2 indicates that LDA is useful in monitoring patients vaccinated with autologous tumor cells. However, further investigations with other patients will be needed to show if such vaccinations regularly induce a measurable increase in autologous CTL frequencies. Increases in CTL frequencies might be seen only within a certain period of time following vaccination. LDA methods based on restimulations of lymphocytes with autologous tumor cells are only suitable for determining intraindividual frequency variations. Frequencies obtained in different individuals are not comparable as they might depend on the ability of individual tumor cell lines to stimulate autologous lymphocytes.

The use of a LDA combined with split-well analysis should also allow the determination of the frequencies of CTL reactive against distinct antigens on tumor cells [13, 22]. This approach would then be useful in identifying those antigens on tumor cells that are most immunogenic in vivo. The characterization of these antigens is at present being pursued in several human tumor models [5].

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