

Improved IL-2 detection for determination of helper T lymphocyte precursor frequency in limiting dilution assay

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Abstract

In the context of allogeneic bone marrow transplantation, an accurate estimate of the risk of developing graft-versus-host disease (GVHD) is of major interest. The pre-transplant frequency of donor's helper T-lymphocyte precursors (HTLp) directed against host's antigens may be helpful in predicting this risk. This technique relies on an indirect measurement of interleukin-2 (IL-2) secreted by the HTLp, as assessed by the proliferation of an IL-2 dependent cell line. Many authors use the murine CTLL-2 cell line in this assay, but these cells do not respond to the presence of minute amounts of IL-2 in the culture medium, and thus do not discriminate between the absence or the presence of very low levels of IL-2. We therefore decided to compare CTLL-2 with another IL-2 dependent cell line, the murine A9.12 cell line. A comparison was made using serial dilutions of recombinant human IL-2, limiting dilutions of baby hamster kidney (BHK) cells transfected with human IL-2 gene and in the context of clinical tests performed for the detection of pre-transplant HTLp. Both the sensitivity and reliability of the tests were better using A9.12. We conclude that the A9.12 cell line might be a more suitable tool for pre-transplant HTLp determinations before allogeneic bone marrow transplantation or whenever low IL-2 levels are to be measured. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: IL-2 detection; HTLp; GVHD; A9.12 cell line; CTLL-2 cell line

1. Introduction

Allogeneic bone marrow transplantation (BMT) has become a widely used treatment for both malig-

nant and genetic blood disorders. Despite much progress, BMT remains a risky treatment, mainly due to graft rejection, opportunistic infections and the potentially severe syndrome named graft-versus-host disease (GVHD) (Ferrara, 1994; Theobald, 1995; den Haan et al., 1995).

Risk factors for GVHD such as disparities between donor and recipient for HLA antigens (Bradley and Jeannet, 1993), the use of an unrelated donor (Bearman et al., 1994), sex mismatch, donor and/or recipient in an older age group (Kalhs, 1993) and previous infections with herpes viruses (Boström et al., 1992; Appleton and Sviland, 1993, Appleton et

Abbreviations: BHK: baby hamster kidney; BMT: bone marrow transplantation; C.I.: confidence interval; CTLp: cytotoxic T lymphocyte precursor; DMSO: dimethylsulfoxide; GVHD: graft-versus-host disease; HLA: human leukocyte antigen; HTLp: helper T lymphocyte precursor; IL-2: interleukin 2; IL-4: interleukin 4; LDA: limiting dilution assay; MLR: mixed lymphocyte reaction; SD: standard deviation

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al., 1995) are well established. Nevertheless, any evaluation based on these general risk factors is insufficient to predict GVHD in a given donor–recipient pair.

To predict individual risk of GVHD after BMT, functional *in vitro* tests have been designed. The most widely used has been the mixed lymphocyte reaction (MLR). Nevertheless, there is no correlation between a high response index measured by MLR and post-transplant complications (GVHD or rejection) (de Gast et al., 1992; Hansen et al., 1995). Limiting dilution assays (LDA) estimating the frequency of cytotoxic or helper T lymphocyte precursors (CTLp or HTLp) have therefore been developed. These tests have been shown to give a better prediction for GVHD or rejection (Goulmy et al., 1985; Kaminski et al., 1989; Theobald et al., 1992; Batchelor et al., 1993; Nierle et al., 1993; Roosnek et al., 1993; Schwarzer et al., 1993; Theobald and Bunjes, 1993; Bunjes et al., 1995).

To estimate HTLp frequency, IL-2 secretion is assessed by the proliferation of an IL-2 dependent cell line. The CTLL-2 murine cell line is often used for this purpose but we have noted that these cells lack sensitivity. Therefore we have tested a modification of the usual protocol to improve sensitivity and reliability in the detection of IL-2 secreted by donor anti-host HTLp: the use of the A9.12 murine cell line (a kind gift from Dr. C.C.Y. Shih) instead of the usual CTLL-2 cell line. We compared these two cell lines for IL-2 detection in rhIL-2 serial dilutions, BHK-pBEH limiting dilution supernatants and HTLp supernatants.

2. Materials and methods

2.1. Harvesting and cryopreservation of donor and patient cells

Heparinized blood from healthy volunteer donors or from patients and their HLA-identical sibling donor was separated on Ficoll-Hypaque (density = 1.077, International Medical, Belgium), washed 3 times and cryopreserved in liquid nitrogen at 5×10^6 cells/ml in Dulbecco modified Eagle's medium (DMEM; Gibco) containing 20% heat-inactivated

normal AB human pooled serum, glutamine (1.5 mM), penicillin (50 U/ml), streptomycin (50 $\mu\text{g}/\text{ml}$), gentamycin (500 $\mu\text{g}/\text{ml}$) and 10% dimethylsulfoxide.

2.2. CTLL-2 culture

The CTLL-2 cell line was maintained in DMEM supplemented with 10% heat-inactivated fetal calf serum, glucose (3.5 g/l), 2-mercaptoethanol (2×10^{-5} M), arginine (0.55 mM), asparagine (0.24 mM), glutamine (1.5 mM), hepes buffer (10 mM), penicillin (50 U/ml), streptomycin (50 $\mu\text{g}/\text{ml}$), and gentamycin (500 $\mu\text{g}/\text{ml}$). Recombinant human IL-2 (Proleukin[®], Eurocetus) was added to reach a final concentration of 100 IU/ml. Cells were fed every two or three days and were not allowed to exceed the maximal concentration of 4×10^5 cells/ml.

2.3. A9.12 culture

This murine helper T cell line was obtained from an SJL anti-AKR limiting dilution microcytotoxicity assay. The cells proliferated in response to alloantigen, IL-2 or Con A. The response to antigen or mitogen was abrogated by CsA and this abrogation could be reversed by adding IL-2. This cell line was maintained in culture for more than 1 year (Shih et al., 1983).

The A9.12 cell line was cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, glutamine (1.5 mM), hepes buffer (10 mM), sodium pyruvate (2 mM), 2-mercaptoethanol (5×10^{-5} M), penicillin (50 U/ml), streptomycin (50 $\mu\text{g}/\text{ml}$), and gentamycin (500 $\mu\text{g}/\text{ml}$). IL-2 was added to reach a final concentration of 100 IU/ml.

Cells were fed every 2 or 3 days and were not allowed to exceed the maximal concentration of 4×10^5 cells/ml. Population doubling occurred over 16 to 18 h. In the early subculturing, 2 ml aliquots were frozen in complete culture medium containing 10% DMSO and were kept in liquid nitrogen until used. About $3\text{--}4 \times 10^6$ cells were frozen in each aliquot. Every 6 or 7 weeks, a new batch was started by thawing a previously frozen aliquot to ensure that all cells were IL-2 dependent.

2.4. BHK culture

This cell line was a kind gift from Dr. H. Hauser. It was transfected with the human IL-2 gene and was found to secrete 4.5×10^3 units/ 10^6 cells/24 h (Conradt et al., 1989). This cell line was maintained in 25 cm² culture flasks (Falcon) in the same medium as the A9.12 cell line, except for IL-2 addition. The population doubling time was 14 to 16 h. Every 2 or 3 days, cells were trypsinized to separate them from the culture flask, washed 3 times and subcultured. Culture supernatant was filtered through 0.45 μ m filter (Millipore) and kept at -70°C until used.

These three cell lines were kept in an humidified atmosphere with 5% CO₂ at 37°C.

2.5. Limiting dilution assay with BHK-IL-2 secreting cells

BHK cells were harvested from the culture flasks, washed three times and diluted to obtain 1.25×10^4 cells/well/100 μ l. This solution was then serially two-fold diluted in flat-bottomed plates (Falcon) with the last dilution containing 0.1 cell/well. Sixteen replicates were set up for each dilution. The limiting dilution plates were incubated for 5 h at 37°C and were then frozen until analysed for IL-2 content.

2.6. Limiting dilution assay for HTLp frequency determination

All cells used in the HTLp assay were thawed on the test day and washed 3 times. Responding cells from the bone marrow donor were diluted to obtain a final concentration of 8×10^4 cells/well/100 μ l. These cells were plated in round-bottom plates and serially two-fold diluted with the last dilution at 2.5×10^3 cells/well. Stimulating cells from the patient were irradiated (20 Gy, rate: 29.6 Gy/min, source of γ radiation: ¹³⁷Cs) and 10^5 cells were added in 100 μ l to responding cells. Twenty-four replicates of each dilution were set up, and also 24 replicates with stimulator cells alone for determination of positivity threshold, and 16 replicates with responder cells alone. The stimulating capacity of patient cells was evaluated with 16 replicates con-

taining responding cells from two unrelated, HLA mismatched healthy volunteer controls. The responding capacity of donor cells was evaluated with the same controls irradiated to serve as stimulator cells. The cultures were performed in DMEM medium supplemented with 10% heat-inactivated normal AB human pooled serum, glutamine (1.5 mM), penicillin (50 U/ml), streptomycin (50 μ g/ml), and gentamycin (500 μ g/ml). Cells were kept in an humidified atmosphere (5% CO₂, 37°C) for 72 h. After 72 h, 100 μ l of the supernatant were removed in new, flat-bottom plates and frozen at -70°C for further analysis. In some experiments, instead of removing supernatants, the plates were irradiated (25 Gy) to prevent lymphocyte proliferation before the indicator cell line was added for the determination of IL-2 in the microcultures.

On the day of analysis, plates containing the supernatants were slowly thawed (4°C for 6 h, 37°C for 30 min). During this time, IL-2 dependent cells were washed three times in 50 ml culture medium, counted, diluted to obtain a final concentration of 4×10^3 cells/well/50 μ l and added to the supernatants. A9.12 cells were diluted in an enriched medium (normal culture medium supplemented with 7 mM sodium pyruvate and 3 mM 2-mercaptoethanol). In some experiments, A9.12 cells were starved during 5 ± 1 h before being added to the supernatants. They were incubated in a humidified atmosphere (5% CO₂, 37°C) for 24 or 42 h, following experiments, with the last 18 h in the presence of 2 μ Ci/well of ³H-thymidine. Thymidine incorporation was measured by harvesting cells to filters and counting β radiation with a β scintillation counter (Beckman LS 6000 SE or Topcount, Packard Instruments).

2.7. IL-2 or IL-4 detection within serial dilutions

Two-fold serial dilutions of rh IL-2 or rh IL-4 (Genzyme, Belgium) were prepared in triplicate in 100 μ l of HTLp culture medium. The dilutions started with an IL-2 concentration of 500 IU/ml to reach 0.03 IU/ml or with an IL-4 concentration from 5000 IU/ml to 0.02 IU/ml. Plates containing dilutions to be tested were frozen at -70°C until used. On the test day, these plates were slowly

thawed (4°C for 6 h, 37°C for 30 min) and treated in the same way as the LDA supernatants.

2.8. Statistical analysis

Evaluations of HTLp frequency were obtained using the χ_2 minimalisation for the zero term of the

Poisson distribution between the number of responder cells and the fraction of negative wells (Taswell, 1981, 1984). The result was calculated with GLIM software (NAG, Oxford, UK). p values > 0.1 were considered as fitting the Poisson model.

In HTLp tests, the threshold of positivity for IL-2 secretion was calculated from the 24 replicates set up

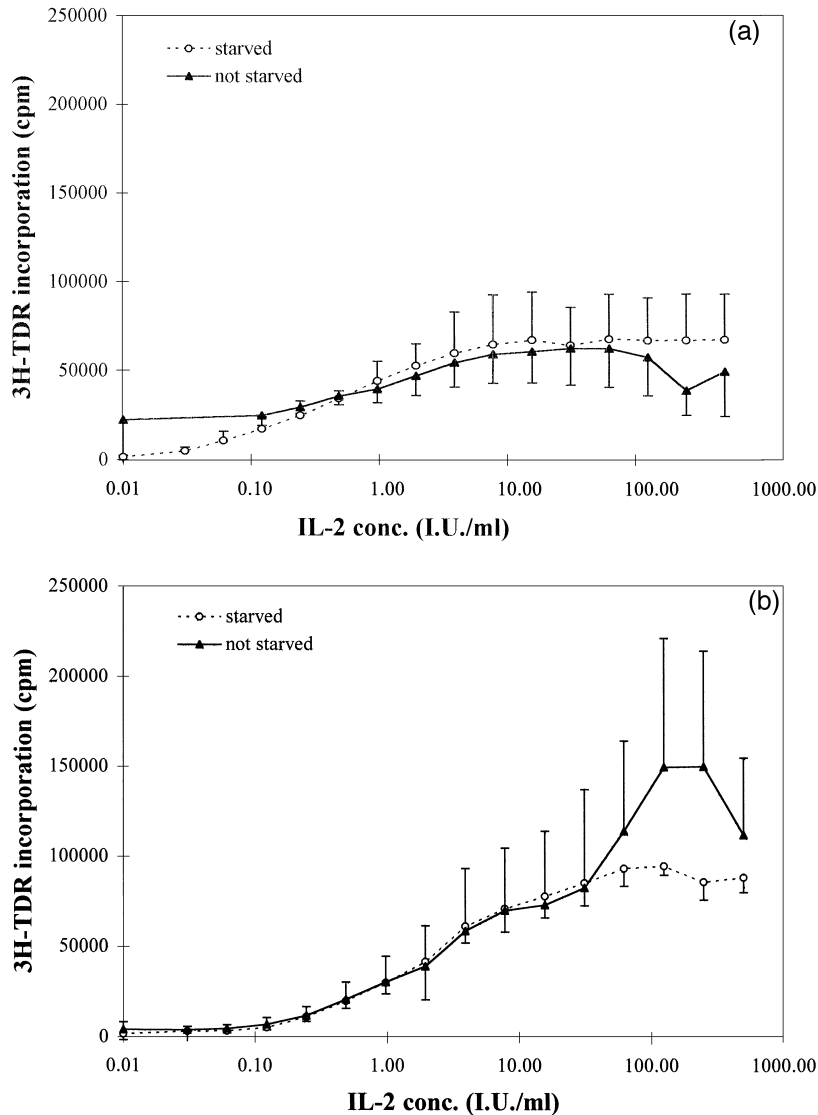


Fig. 1. (a) A total of 4×10^3 A9.12 cells were incubated for 24 h after being starved (open circles) or not (full triangles) and proliferation was measured by ³H-thymine incorporation. Each point represents the mean of three separate experiments and the bar indicates one standard deviation. (b) A total of 4×10^3 A9.12 cells were incubated for 42 h after being starved (open circles) or not (full triangles), and proliferation was measured by ³H-thymidine incorporation. Each point represents the mean of three separate experiments and the bar indicates one standard deviation.

without responder cells as follows: percentile 75 + (percentile 75 – percentile 25).

Comparisons of the mean sensitivity of A9.12 and CTLL-2 cells were made using the Mann–Whitney rank test.

3. Results

Although the CTLL-2 line is well known and described (Gillis et al., 1978), we needed to determine optimal conditions for the use of the A9.12 cell

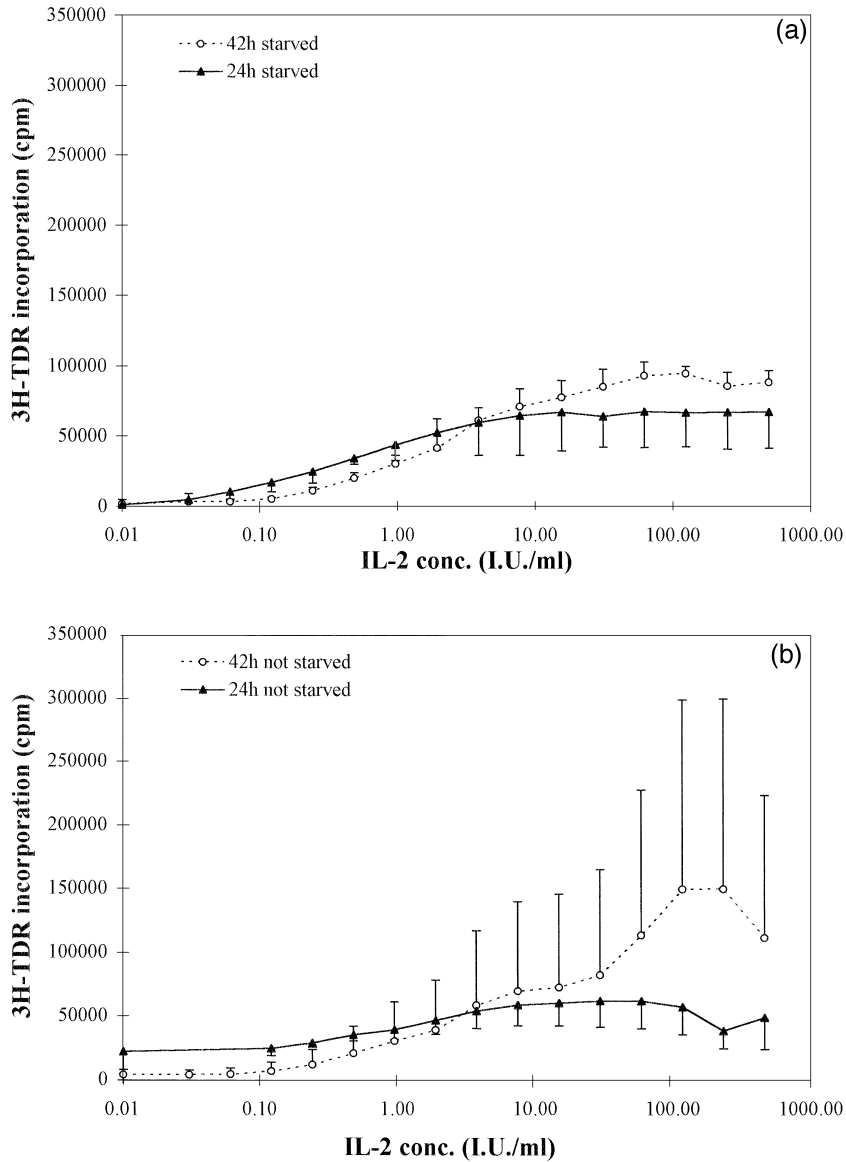


Fig. 2. (a) A total of 4×10^3 A9.12 cells were incubated in IL-2 dilutions for 24 (triangles) or 42 h (circles), after they had been starved, and proliferation was measured by ³H-thymidine incorporation. Each point represents the mean of three separate experiments and the bar indicates one standard deviation. (b) A total of 4×10^3 A9.12 cells were incubated in IL-2 dilutions for 24 (triangles) or 42 h (lozenges), without starving, and proliferation was measured by ³H-thymidine incorporation. Each point represents the mean of three separate experiments and the bar indicates one standard deviation.

line to detect IL-2. We evaluated the cell number that would give a good sensitivity, the starvation period and the total incubation time to detect IL-2 with the highest discriminatory power, and also the best correlation with IL-2 in the culture wells. We finally ensured that A9.12 cells did not proliferate in response to human IL-4.

3.1. Starvation of sensitive cells

We compared the use of the A9.12 cell line with or without starvation before adding the cells to IL-2 dilutions. A total of 4×10^3 A9.12 cells were incubated for 24 (Fig. 1a) or 42 h (Fig. 1b), after being starved for 5 ± 1 h or not. If the total incubation time was 24 h, there was an elevated background, and the discrimination power in the first part of the curve was lower. If cells were incubated for 42 h (Fig. 1b), there was little difference between starved or fed cells. The dosage range was larger for cells that were not starved, but with these culture conditions, there was more variation between wells and between different experiments. We therefore decided to work with starved cells.

3.2. Incubation time

We compared two different incubation times: 24 h (which is often used with the CTLL-2 cell line) or 42 h. Fig. 2a and b show results obtained in three distinct experiments. Proliferation of 4×10^3 cells/well was measured after 24 or 42 h of incubation in IL-2 dilutions, with (Fig. 2a) or without (Fig. 2b) starving. With 24 h incubation, cells that were not starved did not exhibit enough sensitivity (elevated background, weak slope of the curve). The small IL-2 concentrations were detected, but there was a plateau effect at lower concentrations compared to cells incubated for 42 h. There were also greater intra- and interassays variations when cells were incubated for 24 h only.

3.3. Number of cells required

We plated $1-16 \times 10^3$ cells in serial IL-2 dilutions after they had been starved for 5 ± 1 h, and allowed them to grow for 42 h. Fig. 3 shows proliferation in response to IL-2. Obviously, 1×10^3 or 2×10^3 cells were associated with less sensitivity than 4×10^3 cells. A total of 4×10^3 cells re-

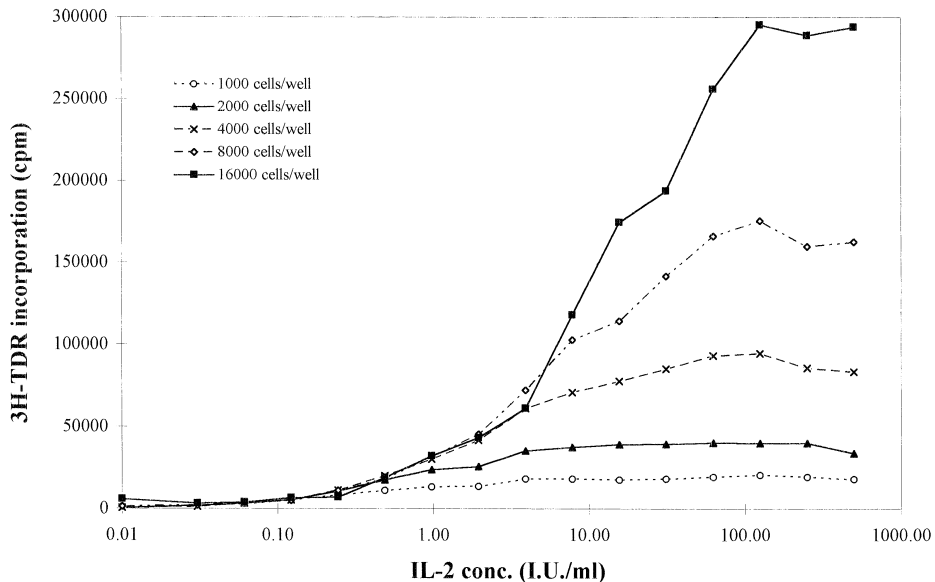


Fig. 3. About $1-16 \times 10^3$ A9.12 cells were incubated in IL-2 serial dilutions for 42 h after being starved, and proliferation was measured by ^3H -thymidine incorporation. Each point represents the mean of three separate experiments.

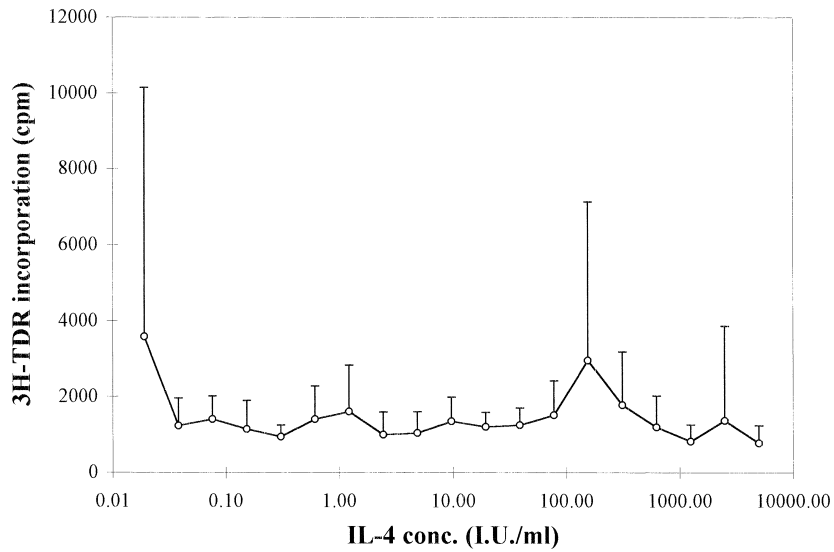


Fig. 4. A total of 4×10^3 A9.12 cells were incubated for 42 h in the presence of rhIL-4 serial dilutions, and proliferation was measured by ^3H -thymidine incorporation. Each point represents the mean of three separate experiments and the bar indicates one standard deviation.

sponded to a wider range of IL-2 concentrations (no maximum effect until approximately 50 IU/ml). When a larger number of cells was used, the maximal proliferation was proportional to the cell number, but occurred within the same range of IL-2

concentration (about 50 IU/ml). On the other hand, the first part of the curve (from 0.2 to 7 IU/ml) was almost the same with 4×10^3 , 8×10^3 or 16×10^3 cells per well. Thus, for the measurement of small amounts of IL-2, we did not increase the cell number

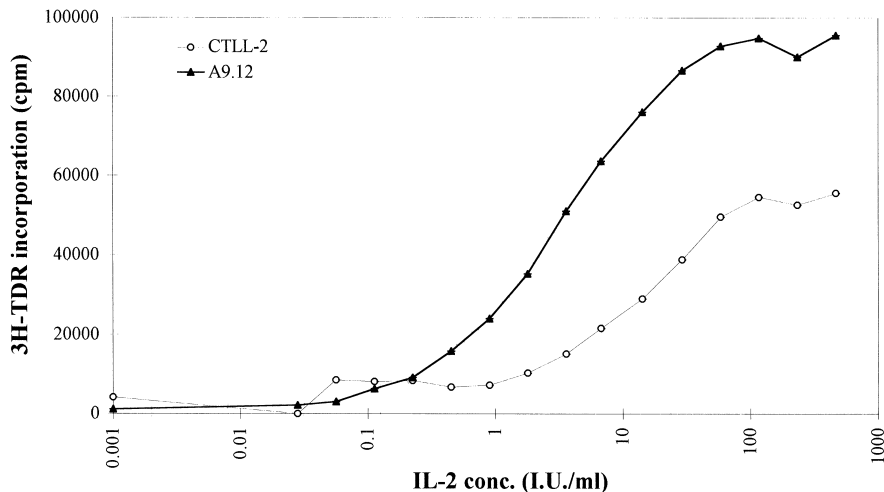


Fig. 5. A total of 4×10^3 cells were incubated in IL-2 dilutions (for 24 h in the case of CTLL-2 (open circles), and 42 h in the case of A9.12 (full triangles)), and proliferation was measured by ^3H -thymidine incorporation. Each point represents the mean of eight separate experiments.

beyond 4×10^3 per well. If IL-2 concentrations in the range 10–500 IU/ml were to be measured, it would be preferable to use 16×10^3 cells or more per well.

3.4. Sensitivity to rhIL-4

Some authors have reported a sensitivity and growth dependence of CTLL-2 to human IL-4 (Menu et al., 1990; Theobald and Bunjes, 1993). To ensure that A9.12 cells did not grow in response to this cytokine, we performed serial dilutions of IL-4 as we did for IL-2 and tested 4×10^3 A9.12 cells growth within 42 h. Fig. 4 shows that there was no relationship between rhIL-4 concentration and A9.12 proliferation, in comparison with that obtained with IL-2.

3.5. Sensitivity of IL-2 dependent cell lines

Fig. 5 compares the two IL-2 sensitive cell lines. A total of 4×10^3 cells were plated in IL-2 dilutions and incubated for 24 h for CTLL-2 or for 42 h for A9.12 (this cell line having been starved for 5 ± 1 h). The sensitivity of eight cell batches derived from different frozen aliquots was measured. The total observation period lasted for at least 6 months. Over

this period, A9.12 cells proved more sensitive than CTLL-2, especially at low concentrations, with lower background and higher maximal proliferation. The dose-proliferation plot showed a longer linear portion with the A9.12 cell line (from 0.06 to 100 IU/ml vs. 1 to 100 IU/ml for CTLL-2), permitting dosage over a wider concentration range. IL-2 detection (given by the first dilution that stimulated higher proliferation than the background) was always better with A9.12 cells (0.05 IU/ml vs. 1.22 IU/ml, $p < 0.001$, Mann–Whitney rank test) (Table 1).

In Fig. 6, the distribution of proliferating A9.12 or CTLL-2 cultures in response to low doses of rhIL-2 (0 or 0.5 IU/ml) is shown. The data were pooled from sixteen separate experiments. CTLL-2 cells responded in a largely overlapping fashion to 0 or 0.5 IU/ml of IL-2 (Fig. 6a). Conversely, the A9.12 cell line proliferated at completely distinct levels in response to 0 or 0.5 IU/ml (Fig. 6b). These data demonstrated again the greater sensitivity of the A9.12 cell line to small amounts of IL-2.

3.6. Reproducibility of IL-2 detection

Table 1 represents batch-to-batch variability in IL-2 detection for the two cell lines. Maximal and minimal proliferation were compared for the two cell

Table 1
Batch-to-batch reproducibility of IL-2 detection

	³ H-TDR incorporation in medium with 100 IU/ml rhIL-2 (cpm)		³ H-TDR incorporation in medium with 0 IU/ml rhIL-2 (cpm)		IL-2 detection (IU/ml)	
	CTLL-2	A9.12	CTLL-2	A9.12	CTLL-2	A9.12
Batch 1	33 821	99 438	1349	747	0.48	0.06
Batch 2	58 995	92 293	1639	863	0.48	0.06
Batch 3	48 305	106 681	2203	423	0.73	0.03
Batch 4	62 952	92 736	1299	345	1.80	0.03
Batch 5	90 025	71 005	8356	733	0.45	0.12
Batch 6	62 490	82 817	3234	530	1.80	0.03
Batch 7	35 878	127 317	3282	381	3.60	0.06
Batch 8	44 388	74 113	11 430	100	0.45	0.03
Mean	54 607	93 300	4099	515	1.22	0.05
SD	18 254	18 324	3748	254	1.12	0.03
SD/mean	33%	20%	91%	49%	92%	59%

Eight batches were analysed for minimal and maximal proliferation in response to known IL-2 amounts (0 or 100 I.U./ml) as described for Fig. 5. Triplicate cultures were analysed from each batch. Mean and standard deviation (SD) were calculated from the eight batches. IL-2 detection was indicated by the first dilution that stimulated higher proliferation than the background obtained in wells with culture medium only (mean + 3 SD).

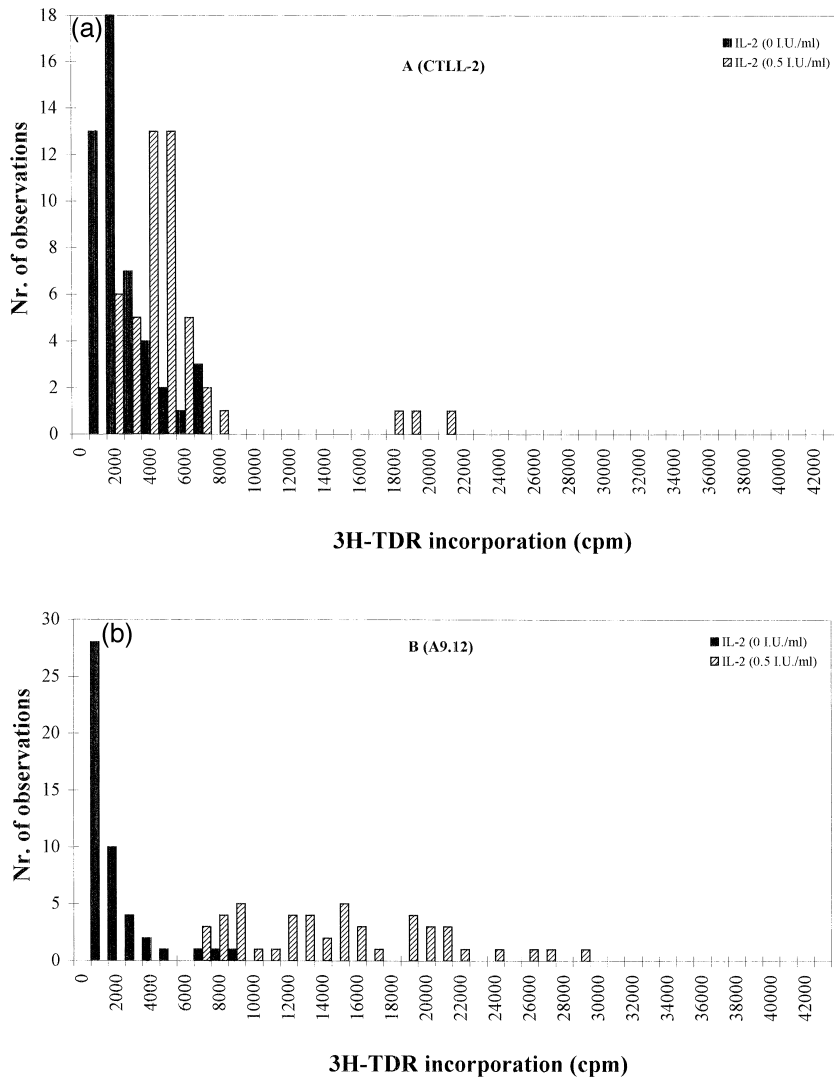


Fig. 6. Proliferation of individual cultures of CTLL-2 (panel A) or A9.12 (panel B) in response to 0 or 0.5 IU/ml rhIL-2. Proliferation was measured by ^3H -thymidine incorporation after 24 or 42 h incubation. Data are pooled from 16 separate experiments.

lines. More important batch-to-batch differences were observed with CTLL-2 cells vs. A9.12 cells, with an elevated ratio of standard deviation to mean (33% vs. 20% for maximal proliferation, 91% vs. 49% for background, respectively). IL-2 detection was less reproducible with CTLL-2 cells (ratio of standard deviation to mean: 92% vs. 59% for A9.12).

All these experiments led us to choose the following experimental conditions to measure IL-2 produc-

tion in HTLp supernatants: starving cells for 5 ± 1 h, total incubation time of 42 h, and the use of 4×10^3 cells per well.

Next, we ensured that this cell line could detect IL-2 secretion from a single cell. For this purpose, a BHK cell line transfected with the human IL-2 gene was plated in limiting dilution experiments. We then compared the A9.12 cell line with the CTLL-2 cell line for IL-2 detection in these assays.

Table 2

Comparison of the ability to detect one IL-2 secreting cell in limiting dilution experiments set up with a transfected BHK cell line

	BHK cell frequency ⁻¹ (95% confidence interval)	
	CTLL-2	A9.12
Expt 1	2.68 (1.94–3.7)	0.18 (0.13–0.26)
Expt 2	10.6 (7.1–15.7)	1.83 (1.32–2.53)
Expt 3	–	1.54 (1.2–2.3)

BHK cells were incubated in limiting dilution numbers (from 1.25×10^4 to 0.1 cells per well) for 5 h. A total of 4×10^3 A9.12 or CTLL-2 cells were added to LDA plates, incubated for 24 or 42 h, and proliferation was measured by ³H-thymidine incorporation. BHK frequency was estimated with the zero term of Poisson law.

3.7. Capacity to measure IL-2 in IL-2-producing-BHK limiting dilution supernatants

To assess whether the two cell lines were able to detect IL-2 production from one IL-2 secreting cell, we set up limiting dilution experiments with a transfected BHK cell line that was producing glycosylated human IL-2 at high levels (Conradt et al., 1989). The same experiments were done three times to ensure reproducibility. The A9.12 cell line was clearly able to detect a single IL-2 producing cell, but this was not the case with CTLL-2 (Table 2).

3.8. Capacity to measure IL-2 in HTLp supernatants

3.8.1. Positivity threshold determination

Our final aim was to evaluate pretransplant HTLp frequency with the greatest sensitivity and reliability. This frequency is calculated using the ratio of negative cultures to the total number of cultures for a

given number of responder cells. Thus, the determination of the positivity threshold is of crucial importance. In the negative control wells, the majority of cultures could not support A9.12 cell growth, but some ‘outlier’ wells displayed ³H-thymidine incorporation as high as 8×10^3 cpm (data not shown). When we calculated the positivity threshold by adding three standard deviations to the mean of the negative control wells, we obtained very high values. These threshold values were also very different from one experiment to another. Accordingly we chose to calculate the positivity threshold with the outlier labelling method (Iglewicz and Hoaglin, 1993). This is based on the median negative value observed and accounts for the false positive values, but is less influenced by them.

3.8.2. Comparison between supernatant removal and second irradiation

As IL-2 detection is based on cell line proliferation, it is important to avoid any proliferation from alloreactive lymphocytes in the HTLp assay. For this purpose, some authors perform a second irradiation of LDA plates (Schwarer et al., 1993; Gribben et al., 1996; Potolicchio et al., 1996). Other investigators harvest supernatant and add this directly to the indicator cell line (Theobald et al., 1992) or freeze it (Schanz et al., 1994; Bouma et al., 1996; Lewalle et al., 1996; Young et al., 1996; van der Meer et al., 1997). This method permits an easier management of LDA experiments.

Second irradiation or supernatant freezing was compared for the A9.12 and CTLL-2 cell lines. HTLp frequency was determined in one HLA fully mismatched healthy donor pair. Four identical sets of limiting dilution plates were set up to permit compar-

Table 3

HTLp assay with one HLA incompatible healthy control pair was performed in four sets. After 72 h incubation of alloreactive lymphocytes, either a second irradiation or supernatant removal and freezing was performed. Then 4×10^3 IL-2 sensitive cells were added to the LDA plates, incubated for 24 (CTLL-2) or 42 h (A9.12), and proliferation was measured by ³H-thymidine incorporation. HTLp frequency was estimated with the zero term of Poisson law

	1/HTLp frequency 10^{-3} (95% confidence interval)	
	CTLL-2	A9.12
(a) Irradiated plates	27.4 (20.2–37.2)	6.4 (4.7–8.7)
(b) Frozen supernatants	25.2 (19–33.5)	4.5 (3.3–6.1)

ison under identical conditions. Table 3 shows the results obtained: for each cell line, the HTLp frequency is equivalent, with 95% confidence intervals largely overlapping either when supernatants were removed and frozen or when plates were irradiated again. Here also, the A9.12 cell line proved to be more sensitive, detecting four-fold higher HTLp frequencies. As supernatant freezing was far easier to manage, allowing many plates to be analysed on the same day, this method was chosen for all HTLp experiments.

3.8.3. Ability to measure IL-2 in supernatants from HTLp assays

The CTLL-2 or A9.12 cell lines were used to estimate HTLp frequency from 107 different donor-recipient pairs (healthy controls or patients with blood disease). Table 4 (part 1) shows that CTLL-2 gave numerous problems: absence of proliferation in 48% of the experiments and poor concordance with the zero term of Poisson law in 40% of the experiments. Conversely, the A9.12 cell line never failed to respond to IL-2 present in culture supernatants. Moreover the concordance with Poisson law was obtained in 81% of the experiments.

Twenty-six experiments were set up to determine donor anti-host pretransplant HTLp frequency for bone marrow recipients. The results obtained were compared with the occurrence of acute GVHD (Table 4, part 2). When CTLL-2 cells were used as the indicator cell line, there was an important lack of specificity in predicting GVHD (20% vs. 71% for the A9.12 cell line). Poor predictive values were obtained (43% for positive predictive values, 50% for negative predictive values, compared to 80 and 71% for A9.12 cells, respectively).

Sensitivity for HTLp detection was compared for the two cell lines in 84 HTLp experiments (59 with A9.12 and 25 with CTLL-2) (Table 4, part 3). The threshold for HTLp detection (that is proliferation in negative controls) was calculated for these experiments. Median background proliferation was higher with CTLL-2 cells (3346 cpm vs. 2652 cpm with A9.12 cells). Median proliferation in microcultures from the dilution containing one HTLp per well was calculated. Higher responses were obtained with A9.12 cells (5858 cpm vs. 4841 for CTLL-2). Thus a better discrimination between positive and negative microcultures was obtained (ratio of proliferation in positive wells relative to negative control wells: 1.44 for the CTLL-2 cell line, 2.2 for the A9.12 cell line).

Table 4

Helper T lymphocyte precursor frequency was estimated in healthy volunteers and in prospective BMT patients with the A9.12 or the CTLL-2 lines

		Cell line used for IL-2 detection	
		CTLL-2	A9.12
Part 1	Nr. LDA experiments without cell proliferation in positive controls	23/48 (48%)	0/59 (0%)
	Nr. LDA experiments not matching the zero term of Poisson law	10/25 (40%)	11/59 (19%)
	Nr. LDA experiments matching the zero term of Poisson law	15/25 (60%)	48/59 (81%)
Part 2	Low HTLp frequency—GVHD 0–I (true negative)	1/9 (11%)	5/17 (32%)
	Low HTLp frequency—GVHD II–IV (false negative)	1/9 (11%)	2/17 (11%)
	High HTLp frequency—GVHD 0–I (false positive)	4/9 (44%)	2/17 (11%)
	High HTLp frequency - GVHD II–IV (true positive)	3/9 (33%)	8/17 (47%)
	Sensitivity	3/4 (75%)	8/10 (80%)
	Specificity	1/5 (20%)	5/7 (71%)
	Positive predictive value	3/7 (43%)	8/10 (80%)
	Negative predictive value	1/2 (50%)	5/7 (71%)
	Part 3	Threshold of HTLp detection (median cpm)	3346
Proliferation in one HTLp containing culture well (median cpm)		4841	5858

Part 1: Validity of HTLp experiments (data from 107 experiments).

Part 2: Use of pretransplant HTLp results in BMT patients for GVHD prediction (data from 26 experiments).

Part 3: Capacity to proliferate in response to IL-2 produced by a single HTLp (data from 84 experiments).

4. Discussion

The CTLL-2 cell line is widely used to detect IL-2 in culture supernatants. As also noted by Weston and Farrell (1996), we observed that the sensitivity of this cell line is not stable over time, especially following subculturing (data not shown). Moreover, in many HTLp supernatants, this cell line was unable to detect the small amounts of IL-2 produced. As this test was performed only once (due to the large number of patient's cells required), it was essential to detect IL-2 in the supernatants by the most accurate method. The A9.12 cell line met these requirements of sensitivity and reproducibility and therefore its use should be recommended whenever the IL-2 levels are very low. Other investigators (Lewalle et al., 1996; Keever-Taylor et al., 1997) have used it with success in HTLp detection assays.

The greater sensitivity of A9.12 might explain the proliferation observed in some negative control wells in the HTLp test. Some stimuli other than allogeneic cell recognition might be associated with these cultures. For instance, there might be recognition of allogeneic serum proteins present in the culture medium. The irradiation stress might also provoke release of a small amount of IL-2. This was shown *in vivo* (Xun et al., 1994) as well as *in vitro* (Galdiero et al., 1994). Hornick et al. (1997) also noted a source of 'unwanted' IL-2 both in the stimulating and in the responding population. They also noted a 'back stimulation' of stimulating cells activated by alloantigens from responder cells, leading to poor concordance with single-hit kinetics. To avoid these IL-2 sources, they depleted the responding population of HLA class II positive cells and the T cells from the stimulating population. These depletions allowed HTLp to achieve better concordance with the single-hit model. On the other hand, this led to a decrease of sensitivity and an increase of the number of stimulating cells required for the successive rounds of depletion. Moreover, this model deviates from the *in vivo* conditions encountered in bone marrow or solid organ transplantation.

Rather than performing depletions, we changed the determination of the positivity threshold. The new method of calculation takes into account the median proliferation rather than the mean because the mean showed larger variations due to a few

elevated values. Thus, the outlier labelling method was chosen. It gave a better inter experimental stability in positivity thresholds. Using this cell line, the number of cells, the starving duration and the incubation time were critical.

An accurate measurement of IL-2 might also prove useful in the evaluation of patients suffering from other immunological diseases such as AIDS, in which IL-2 plays an important role (Westendorp et al., 1994; Brinchmann et al., 1994; Collette et al., 1996; Yoo et al., 1996). In kidney and cardiac allografts, local and systemic IL-2 production has been correlated with rejection episodes and indicates the need to continue immunosuppression (Muluk et al., 1991; Schulick et al., 1993; DeBruyne et al., 1995). There is also an increasing interest in IL-2 dosage in autoimmune diseases such as multiple sclerosis, thyroiditis, myasthenia gravis, type I diabetes, thrombocytopenic purpura (Burns and Littlefield, 1993; De Maria et al., 1994; Lohr et al., 1994; Yi et al., 1994; Rep et al., 1996; Semple et al., 1996). The follow-up of IL-2 and other cytokines in all these diseases might permit a better understanding of the pathological processes and lead to better disease management.

In conclusion, we have established that the A9.12 line is preferable for detecting low IL-2 production in the HTLp assay compared to the conventionally used CTLL-2 cell line.

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