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ELISPOT cloning of tumor antigens recognized by cytotoxic T-lymphocytes from a cDNA expression library

Akiko Uenaka[1], Hidenori Hata[1], Sanda Win[1], Toshiro Ono[1], Hisashi Wada[2], and Eiichi Nakayama[1]

[1]Department of Immunology, Okayama University Graduate School of Medicine and Dentistry, 2-5-1 Shikata-cho, Okayama 700-8558, Japan
[2]Department of Surgery, Osaka University Graduate School of Medicine, 2-2 Yamada-oka, Suita 565-0871, Japan

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Abstract

The methodology of cloning genes coding for antigens recognized by T-cells from cDNA expression libraries was improved technically by using enzyme-linked immunospot (ELISPOT) assays instead of enzyme-linked immunosorbent assays (ELISA) or bioassays to detect cytokines produced by T-cells in response to antigens. Combining large and small scale ELISPOT assays for expression cloning has the following advantages compared to conventional cDNA expression cloning: i) the number of recombinant plasmids which can be screened is greater than 10,000 per well in a 24-well plate in a large scale ELISPOT assay compared to fewer than 100 per well in a 96-well plate in an IFN-gamma ELISA or a TNF-alpha bioassay; ii) the total number of recombinant plasmids which can be screened in a routine assay is 2×10^5 in only one 24-well plate in a large scale ELISPOT assay compared to 1×10^5 in ten 96-well plates in an IFN-gamma ELISA or a TNF-alpha bioassay. Thus the screening efficiency of large scale ELISPOT cloning is approximately 200 times that of conventional expression cloning approaches. The efficiency of the method was confirmed by detecting the model gene *RLakt* from a cDNA library of a murine leukemia RL male 1.

Introduction

The identification of tumor antigens that elicit an immune response in the autologous host has long been an objective in tumor immunology (1). Following the introduction of methodology for cloning genes coding for antigens recognized by T cells (2, 3), a range of human tumor antigens has been identified (4, 5, 6, 7, 8, 9). There are several methods currently employed for identifying tumor antigens recognized by cytotoxic T-lymphocytes (CTL). These are i) T-cell screening of transient transfectants of cDNA expression libraries (2, 3, 6), ii) direct acid elution and amino acid sequence analysis of MHC-associated peptides combined with (10, 11) or without (12, 13) mass spectrometry, iii) peptide motif analysis of the targeted protein in the cell (14, 15), and iv) T-cell screening of a synthetic peptide library based on the motif residues (16, 17). Of these methods, expression cloning of cDNA libraries and peptide elution have been the preferred methods to identify antigens recognized by T cells for which no molecular information is available. However, there are some intrinsic difficulties in applying these methods. In expression cloning of cDNA libraries, the number of recombinant plasmids in a pool should be less than 100, possibly even less than 50, when CTL sensitivity is low. Therefore to screen 1×10^5 recombinant plasmids, a minimum of 1,000 recombinant plasmid pools must be screened. The assay carried out detects cytokines such as IFN-gamma and TNF-alpha released in the culture supernatant. The purification of plasmids from a large number of bacterial pools, their transfection, and the subsequent T-cell assays for cytokines are not inconsiderable tasks. Similarly, peptide elution is technically demanding and it is practically impossible to have a fraction containing a single peptide even after repeated high performance liquid

chromatography using various phases (18). As a consequence, it has proven extremely difficult to resolve the reactive peptide sequence from contaminating peptide signals.

By using large and small scale ELISPOT assays instead of ELISA or bioassays to detect cytokines produced by T-cells in response to antigens, we have improved the method of cloning genes coding for antigens recognized by T-cells from cDNA expression libraries.

Results

Cytotoxicity and IFN-gamma ELISPOT assays using CTL clone B-24

CTL clone B-24 was established from spleen cells of an RL male 1-bearing BALB/c mouse by repetitive stimulation with RL male 1 cells. Specific recognition by CTL clone B-24 of RL male 1 and pRL1a peptide-pulsed P1.HTR targets was demonstrated in cytotoxicity and IFN-gamma ELISPOT assays (Fig. 1A and B). The control targets BALB/c radiation-induced leukemia RL female 8, BALB/c RadLV-induced leukemia RVC, BALB/c mineral oil-induced myeloma MOPC-70A, BALB/c methylcholanthrene-induced fibrosarcomas Meth A and CMS8 and unpulsed P1.HTR were not recognized in these assays. As shown in Figures 1C and D, B-24 cytotoxicity and IFN-gamma ELISPOT activity were blocked by anti-CD8 mAb and anti-H-2L^d mAb, but not anti-CD4 mAb, anti-H-2K^d mAb or anti-H-2D^d mAb, confirming B-24 as a CD8 CTL clone specific for the pRL1a peptide epitope bound to H-2L^d (13).

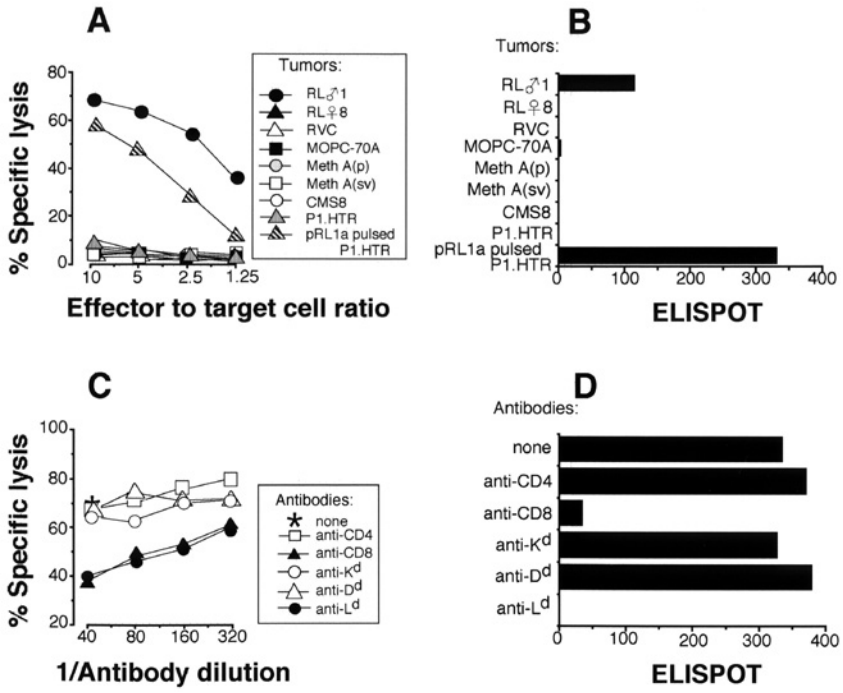

Figure 1. Specificity and antibody blocking of CTL clone B-24. Direct cytotoxicity (A) and IFN-gamma ELISPOT (B) of B-24 CTL against various target cells. Blocking of cytotoxicity (C) and IFN-gamma ELISPOT (D) of B-24 CTL by various antibodies. The number of B-24 cells used in the IFN-gamma ELISPOT assays (B and D) was 5000. The number of target cells in A and B was 1x10^4. pRL1a pulsed P1.HTR cells (1x10^4) were used as targets in C and D. Effector to target cell ratio in C was 10. Antibody dilution in D was 1/100. Refer to the text for details.

ELISPOTs produced by 5,000 B-24 cells in response to CMS8 stimulator cells pulsed with pRL1a and two other L^d binding peptides, as well as to different numbers of CMS8 cells pulsed with pRL1a were examined in 96-well

culture plates. As shown in Figure 2A, 629 spots were observed for 10,000 CMS8 stimulator cells pulsed with pRL1a peptide, whereas no spots were observed for CMS8 cells pulsed with peptides p2Ca or T2H. As shown in Figure 2B, the number of spots decreased with decreasing number of stimulator cells.

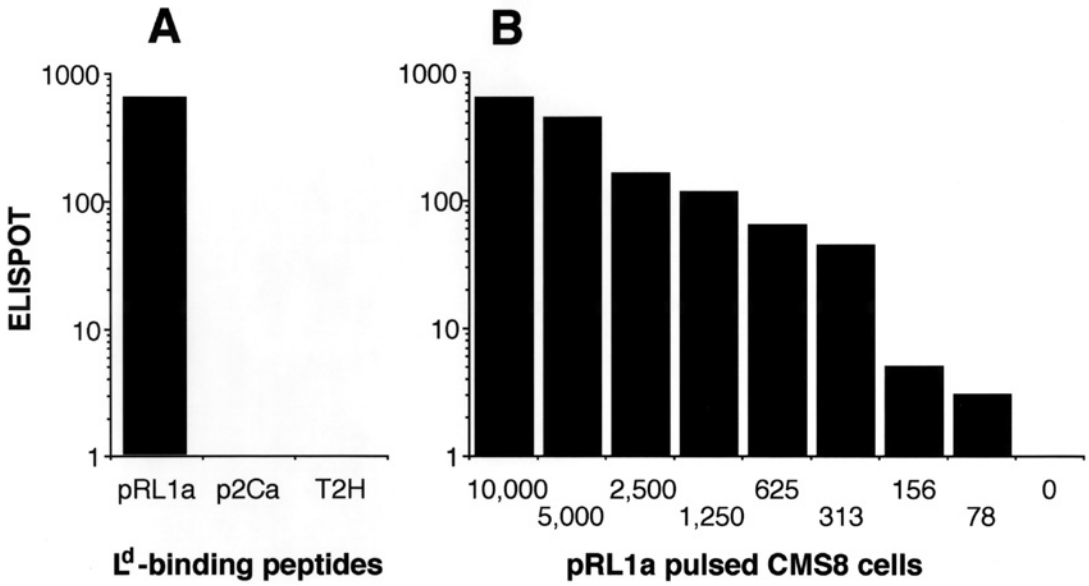

Figure 2. ELISPOT assays with B-24 CTL against CMS8 cells pulsed with synthetic peptides. CMS8 cells were pulsed with three L^d-binding peptides, pRL1a, p2Ca and T2H (A). ELISPOT assay of B-24 CTL against different numbers of CMS8 cells pulsed with pRL1a (B).

Small and large scale ELISPOT assays with CTL clone B-24 in response to CMS8 cells transfected with recombinant *RLakt*

We investigated the sensitivity of small and large scale ELISPOT assays by detecting cells transfected with the *RLakt* gene coding for the pRL1a antigen peptide recognized by B-24 CTL. In conventional small scale ELISPOT assays, a total of 100 ng of purified recombinant *RLakt* plasmid mixed with control enhanced green fluorescent protein (*EGFP*) plasmid at various weight ratios were transfected into 1 x 10^4 CMS8 cells in 96-well culture plates. After culture for 24 h, the transfectants were collected and transferred onto anti-IFN-gamma mAb-coated nitrocellulose membrane-based 96-well culture plates and 5,000 B-24 cells were added to each well. After overnight culture, spots were detected. As shown in Figure 3A, B-24 elicited 268 spots against CMS8 cells transfected with 100 ng *RLakt* and no *EGFP* plasmid. The number of spots detected was gradually decreased by lowering the amount of *RLakt*. For *RLakt*:*EGFP* ratios of 1:64 and 1:128, 25 and 8 spots were observed respectively. No spots were observed with control *EGFP* alone. Large scale ELISPOT assays were then performed to increase the sensitivity and enable the detection of lower ratios (<1:100) of *RLakt* plasmid to control *EGFP*. Transfections were carried out in 24-well plates with 3 µg DNA and 1 x 10^5 CMS8 cells. After culture for 24 h, the transfectants were collected and transferred onto anti-IFN-gamma mAb-coated, 14 mm diameter nitrocellulose membranes in 24-well culture plates and 50,000 B-24 cells were added. As shown in Figure 3B, as many as 82, 27, and 14 spots were observed for *RLakt* to *EGFP* ratios of 1:640, 1:2,500, and 1:10,000 respectively. In this particular experiment, 4 spots were observed with the control *EGFP* alone and a range of 0 - 5 spots were obtained in repeat experiments.

IFN-gamma ELISA assays using culture supernatants (100 µl) from transfected CMS8 and B-24 co-cultures in 96-well (small scale) and 24-well (large scale) plate assays were performed without nitrocellulose membranes in 96-well plates. A dose response curve similar to that obtained for the ELISPOT assays was observed for supernatants from a 96-well plate (Fig. 3C), but no IFN-gamma was detected even at a 1:40 *RLakt*:*EGFP* ratio for supernatants from a 24-well plate (Fig. 3D).

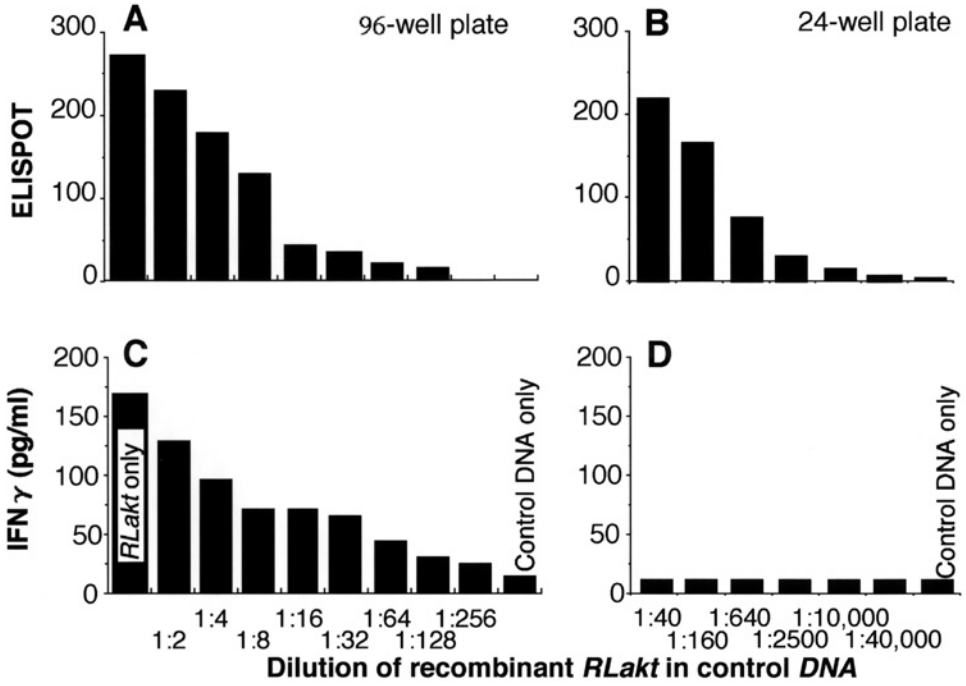

Figure 3. Sensitivity of small and large scale ELISPOT assays in detecting cells transfected with *RLakt* gene. ELISPOT and IFN-gamma ELISA assays with B-24 CTL against CMS8 cells transfected with *RLakt* plasmid mixed with control *EGFP* plasmid at various ratios. ELISPOTs produced by B-24 CTL in 96- (A) and 24- (B) well plates. IFN-gamma in the supernatant (100 µl) from each well of 96- (C) and of 24- (D) well plates were determined by ELISA. Refer to the text for details.

Identification of the *RLakt* gene by B-24 ELISPOT screening of an RL male 1 cDNA library

To evaluate the sensitivity and efficiency of the combined use of small and large scale ELISPOT assays for cDNA expression cloning, we applied this method to the detection of the antigenic *RLakt* gene from an RL male 1 cDNA library. As shown in Figure 4, five rounds of ELISPOT screening with different sized pools of recombinant plasmids were performed. The first two rounds of screening were large scale assays in 24-well culture plates, whereas the last three rounds of screening were small scale assays in 96-well plates. In the first round of screening, a total of twenty bacterial pools were prepared in which 10,000 bacterial colonies per pool were used. Plasmids purified from each bacterial pool (3 µg) were transfected into 1 x 10^5 CMS8 cells. After incubation for 24 h, the cells were collected, transferred onto anti-IFN-gamma mAb-coated nitrocellulose membranes in newly prepared 24-well culture plates as described previously and 50,000 B-24 cells added to each well. After overnight culture, more than 10 spots were observed for three plasmid pools. For the second round of screening, pool number 14 (with 20 spots) was chosen and diluted, yielding 40 pools each containing 1,000 bacterial colonies. Thirteen pools with more than 10 spots were obtained in this round of screening. For the third round of screening, pool number 11 which showed discernible large spots was chosen and diluted to obtain 40 pools containing 100 bacterial colonies per pool. Between 100 to 200 ng of plasmids were transfected into 1 x 10^4 CMS8 cells in 96-well plates. After incubation for 24 h, the cells were collected and transferred onto an anti-IFN-gamma mAb-coated nitrocellulose membrane-based 96-well plate. Next, 5,000 B-24 cells were added to each well and the spots detected following an overnight incubation. For the fourth round of screening, pool number 11 with 8 spots was chosen and diluted to obtain 40 pools containing 10 bacterial colonies per pool. Three pools with more than 20 spots were obtained. For the final round of screening, pool number 33 was chosen and 40 wells with a single bacterial colony were prepared. Two single-plasmid clones, number 17 and number 30, were detected.

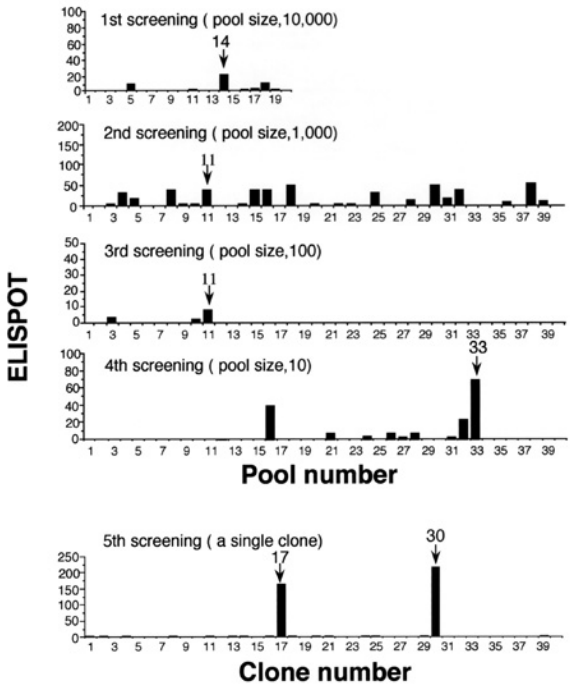

Figure 4. ELISPOT cloning of *RLakt* by B-24 CTL from an RL male 1 cDNA expression library. The first two rounds of screening were large scale assays in 24-well culture plates (10,000 and 1,000 bacterial colonies per pool) and the last three rounds were small scale assays in 96-well culture plates (100 and 10 bacterial colonies per pool and single clone). Refer to the text for details.

Determination of the presence of the *RLakt* insert by PCR

As shown in Figure 5A, *Sal*I and *Not*I restriction digests of the plasmid purified from clones number 17 and 30 reveal an insert size of 2.6 kb. This is consistent with the full length *RLakt* mRNA determined by Northern blot analysis (19). As shown in Figure 5B, PCR using *RLakt*-specific sense primer 5'-CAGCTTGGGGGTCTTTCAACAT-3' (*RLakt* 125) and anti-sense primer 5'-AGACACAATCTCCGCACCATAGA-3' (*RLakt* 1020) produced the predicted 896 bp band for clones 17 and 30. The insert from plasmid clone number 30 was sequenced and confirmed as being *RLakt*.

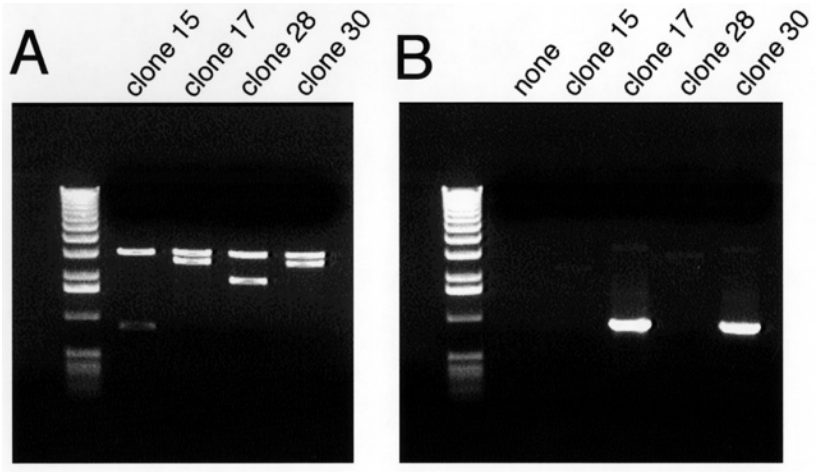

Figure 5. Determination of *RLakt* insert of plasmid clones 17 and 30. *Sal*I and *Not*I digestion of clone 17 and 30 plasmids produced two bands corresponding to the 2.6 kb *RLakt* insert and 3.0 kb pMET7 vector (A). Negative clones 15 and 28 were also included in the analysis. PCR using *RLakt*-specific primers (refer to text) produced the predicted 896 bp band for clones 17 and 30 (B).

Discussion

In this study we have improved the sensitivity and efficiency of T-cell antigen cloning from cDNA expression libraries by incorporating ELISPOT assays into the methodology. The ELISPOT assay detects the locally trapped cytokine produced by cells around a stimulator cell (20). Combining large and small scale ELISPOT assays for expression cloning has the following advantages compared to conventional cDNA expression cloning approaches using ELISA or bioassays: i) the number of recombinant plasmids which can be screened in a single well is more than 10,000 in a 24-well plate in a large scale ELISPOT assay compared to less than 100 in a 96-well plate in an IFN-gamma ELISA or TNF-alpha bioassay; ii) the total number of recombinant plasmids which can be screened in a routine assay is 2×10^5 in just one 24-well plate in a large scale ELISPOT assay compared to 1×10^5 in ten 96-well plates in a conventional expression cloning IFN-gamma ELISA or a TNF-alpha bioassay. Thus the screening efficiency of large scale ELISPOT cloning is over 200 times that of conventional expression cloning methods.

For cDNA expression cloning, the quality of the cDNA library is critical (18, 21). For cDNA libraries prepared using either oligo(dT) or random primers, the number of clones in the library should be as large as possible and the insert size should be sufficiently long. Even when cDNA libraries of sufficient quality are used, sorting positive clones from a large number of pools, each containing large number of clones, is subject to a certain ambiguity because of weak signals (2, 3, 6). By using a large scale ELISPOT assay for cloning, we could screen large numbers of clones representing a whole library in a single 24-well plate assay. This avoids misinterpretation of results with especially weak signals caused by background variations in individual assays and enabled us to evaluate library quality properly and identify positive signals without ambiguity. Furthermore, in the conventional method, cytokine assays performed on different occasions during prolonged screening of a single library may vary in quality and sensitivity. Sensitive T-cells are requisite for the detection of cDNA clones coding for antigenic epitopes in the screening. However, maintaining stable and responsive T-cells is challenging (22). In ELISPOT assays, we observed small-sized spots for CTLs producing low quantities of IFN-gamma, which proved to be advantageous particularly when T-cells with lower sensitivity were used. Variations in transfection efficiency in assays used to screen cDNA libraries are unpredictable and cause ambiguity in the screening results. The increased sensitivity achieved by combining large and small scale ELISPOT assays overcomes this and allows the unambiguous detection of cDNA clones.

We describe above the results of a study in which we detected a large number of positive clones in 40 recombinant plasmid pools, each containing 1,000 colonies derived from a positive pool (number 14) of 10,000 colonies. It is quite likely that this pool contained several *RLakt* clones. The ELISPOT cloning method described is currently being used to identify new antigens recognized by tumor specific CTL in various murine and human tumors.

Abbreviations

EGFP, enhanced green fluorescent protein

Acknowledgements

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Materials and methods

Tumors and cell lines

RL male 1 and RL female 8 are radiation-induced leukemias in BALB/c mice (23). RVC is a leukemia induced by injection of radiation-leukemia virus (RadLV) into a neonatal BALB/c mouse (24, 25). Meth A and CMS8 are methylcholanthrene-induced sarcomas in BALB/c mice (26, 27). Meth A (p) is the parental Meth A line. Meth A (sv) is a variant line which became sensitive to lysis by CTL and was provided by Dr. H. Shiku (Mie University School of Medicine, Mie, Japan). MOPC-70A is a mineral oil-induced myeloma in a BALB/c mouse (28). P1.HTR (29) is a subline of P815 (30) which is a methylcholanthrene-induced mastocytoma in a DBA/2 mouse.

Antibodies

Anti-L3T4 (CD4) mAb, a rat antibody of the IgG2b immunoglobulin class produced by hybridoma GK1.5 (ATCC TIB-207) (31), was provided by Dr. F. Fitch (University of Chicago, Chicago, IL) and anti-Lyt-2.2 (CD8) mAb, a mouse antibody of the IgG2a class produced by hybridoma 19/178, was provided by Dr. G. Hämmerling (Memorial Sloan-Kettering Cancer Center, New York). Anti-H-2K^d and anti-H-2D^d are mouse antibodies produced by hybridomas SF1-1.1.10 (ATCC HB159) and 34-5-8S (ATCC HB102) respectively. Anti-H-2L^d mAb is a mouse IgG2a antibody produced by hybridoma 30-5-7S (ATCC HB-31) (32). Anti-IFN-gamma mAb is a rat antibody of the IgG1 class produced by hybridoma R4-6A2 (ATCC HB-170) (33). Polyclonal rabbit

anti-IFN-gamma serum was produced by immunization with recombinant murine IFN-gamma. Alkaline phosphatase conjugated goat anti-rabbit IgG was purchased from Southern Biotechnology (Burmingham, AL).

Synthetic peptides

L^d binding peptides pRL1a (13), IPGLPLSL, p2Ca (34), LSPFPFDL, and T2H (35), ISTQNHRALDLVA, were synthesized by standard F-moc (9-fluorenylmethoxycarbonyl) solid phase chemistry (36) using a peptide synthesizer (model 430A; Perkin-Elmer Applied Biosystems, Foster City, CA).

CTL clone B-24

CTL clone B-24 was established from spleen cells of an RL male 1-bearing BALB/c mouse and maintained by weekly stimulation with mitomycin C-treated RL male 1 stimulator cells and mitomycin C-treated BALB/c splenic feeder cells in the presence of recombinant human IL-2 (37).

Cytotoxicity assays

Tumor cells were labeled by incubating 2 x 10^6 cells with 2 MBq of $Na_2{}^{51}CrO_4$ (New England Nuclear, Boston, MA) in 0.3 ml medium for 90 min at 37°C in 5% CO_2. The cells were washed and used as targets. In direct assays, 10^4 labeled target cells (100 µl) were incubated with effector cells (100 µl). In antibody blocking assays, serially diluted mAb (100 µl) was added to the mixture of effector cells and 10^4 labeled target cells (100 µl). After incubation for 3.5 h at 37°C in 5% CO_2, supernatants were removed and their radioactivity measured. The percentage of specific lysis was calculated according to the following equation: (a-b)/(c-b) x 100, where a is the radioactivity of the supernatant of the target cells mixed with effector cells, b is that of the supernatant of the target cells incubated alone, and c is that of the supernatant after lysis of the target cells with 1% NP-40.

ELISPOT assays

The original ELISPOT assay for detecting cytokine-producing cells (20) was modified as follows. Briefly, nitrocellulose disk membranes (14 mm diameter) and 96-well nitrocellulose membrane-based plates (Millipore S4510, Millipore Corp., Bedford, MA) were coated with anti-IFN-gamma mAb (R4-6A2) in 0.05 M bicarbonate buffer (pH 9.6) at 4°C overnight. After washing, membranes were blocked with RPMI 1640 containing 10% FCS and 50 µM 2-mercaptoethanol for 60 min at 37°C. CTLs were incubated with stimulator cells on the coated membrane in 96- or 24-well plates for 18 h at 37°C in 5% CO_2. The membranes were then thoroughly washed with distilled water and incubated with polyclonal rabbit anti-IFN-gamma antibody for 90 min at 37°C. The IFN-gamma spots were developed by alkaline phosphatase-conjugated anti-rabbit IgG antibody, using a substrate kit (Bio-Rad, Hercules, CA), and counted under a dissecting microscope.

IFN-gamma ELISAs

CTL and stimulator cells were cocultured for 18 h, following which the culture supernatant was removed and assayed for IFN-gamma by the sandwich ELISA. Anti-IFN-gamma mAb (R4-6A2) was used as the capture antibody and polyclonal rabbit anti-IFN-gamma antibody as the detection antibody. Horseradish peroxidase-conjugated anti-rabbit IgG antibody (MBL, Nagoya, Japan) and o-phenylenediamine dihydrochloride were used as the indicator system.

Construction of pMET7-RLakt and pMET7-EGFP

The *RLakt* (19) 116-1784 fragment, amplified by RT-PCR using 5'-TTGACTGCCCAGCTTGGGGGT-3' as the forward primer and 5'-CATCCGAGAAACACATCAGGT-3' as the reverse primer, was ligated into the *Sal*I-*Not*I digested pMET7 vector (provided by Dr. A. Shibuya, Tsukuba University, Tsukuba, Japan). The pEGFP-N1 vector (Clontech, Palo Alto, CA) was digested with *Sal*I and *Not*I and the *EGFP* fragment corresponding to positions 640 to 1402 ligated into the pMET7 vector.

cDNA library construction

mRNA was isolated from RL male 1 cells using QuickPrep mRNA Purification Kit (Amersham Pharmacia Biotech, Piscataway, NJ) and first strand cDNA was synthesized using oligo(dT) primer. The cDNA was inserted

into the *Sal*I and *Not*I sites of expression vector pMET7 as described in the SuperScript™ Plasmid System (GIBCO BRL, Rockville, MD) manufacturer's instructions. The cDNA library was divided into pools of approximately 10,000 bacterial colonies.

Transfection and screening

Recombinant plasmids were purified from bacteria using the Wizard Plus Series 9600™ DNA purification system (Promega, Co., Madison, WI) and were transfected with plasmid pdl3027 containing the polyoma T antigen (38) using lipofectamine (GIBCO BRL, Rockville, MD) into 1 x 10^5 and 1 x 10^4 CMS8 cells in 24- and 96-well plates, respectively. Following culture for 24 h in 5% CO_2, the transfected cells were treated with 0.25% trypsin-0.02% EDTA-PBS and plated onto anti-IFN-gamma mAb-coated nitrocellulose membranes in culture plates and B-24 CTLs added. Spots were detected after overnight culture.

DNA sequencing and homology search

DNA sequence analysis was performed using the BigDye terminator cycle sequencing ready reaction kit (Perkin-Elmer Applied Biosystems, Foster City, CA) and an ABI PRISM DNA sequencer (Perkin-Elmer Applied Biosystems, Foster City, CA). The computer search for sequence homology was performed using the BLAST program on the GenBank database.

Contact

Address correspondence to:

Akiko Uenaka
Department of Immunology
Okayama University Graduate School of Medicine and Dentistry
2-5-1 Shikata-cho
Okayama 700-8558
Japan
Tel.: + 81 86 235-7192
Fax: + 81 86 235-7193
E-mail: uenaka@md.okayama-u.ac.jp