ELSEVIER

Journal of Immunological Methods 187 (1995) 95–101

**JOURNAL OF
IMMUNOLOGICAL
METHODS**

An assay for the identification of antigens recognized by cytotoxic T cells, based on transient transfection of COS cells

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Received 13 January 1995; revised 1 May 1995; accepted 22 June 1995

Abstract

The studies reported here describe methodology permitting the direct identification of antigens recognized by cytotoxic T lymphocytes. We demonstrated that bovine alloreactive CTL can detect a bovine MHC molecule transiently expressed in a COS cell population in a standard microcytotoxicity assay. We then showed that alloreactive CTL can detect cells expressing the bovine class I MHC molecule in a population of cells transfected with the plasmid containing the corresponding gene plus 100-fold as many plasmids containing an irrelevant gene. In addition, the transiently transfected COS cells can specifically restimulate CTL as detected by a standard microcytotoxicity assay using the target cell line. Overall, the results suggest that COS cells could be employed for the direct screening of an antigen or antigen gene library by immune CTL.

Keywords: Cytotoxic T lymphocyte; Cytotoxicity assay; Antigen identification

1. Introduction

The transient expression of cDNA libraries in COS cells has permitted the rapid isolation of genes encoding mammalian cell surface antigens (Seed and Aruffo, 1987; Aruffo and Seed, 1987).

These procedures have relied on the use of monoclonal antibodies to isolate COS cells expressing the desired antigen. The significant advantage of the COS cell system is that plasmids replicate episomally to high copy number after transfection into the cells (Mellon et al., 1981). This results in high levels of expression of the introduced genes, and allows rapid and easy recovery of the gene of interest from identified cells.

Recently, the COS cell system has been employed to identify antigens recognised by tumour-specific cytotoxic T lymphocytes (CTL). These studies have involved transfection of a cDNA library into COS cells and identification of the gene of interest by specific restimulation of

Abbreviations: CTL, cytotoxic T lymphocyte; DMEM, Dulbecco's modified Eagle's medium; mAb, monoclonal antibody; MHC, major histocompatibility complex; %CTX, percentage cytotoxicity; PBMC, peripheral blood mononuclear cells; Tp(M), *Theileria parva* (Muguga).

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the CTL. CTL restimulation was measured by the release of tumour necrosis factor (Brichard et al., 1993; Coulie et al., 1994).

Our research interests lie in the identification of those antigens of the protozoan parasite, *Theileria parva*, which are recognised by CTL from immune cattle. We wished to take advantage of the COS cell system to establish a procedure for the screening of a library of parasite genes directly with CTL. We have examined the ability of transiently transfected COS cells to be detected in a standard microcytotoxicity assay, and to restimulate CTL to lyse specifically the target cells. The results indicate that both systems could be employed to identify antigens recognised by bovine CTL.

2. Materials and methods

2.1. Cell lines

COS-I cells were obtained from the American Type Culture Collection, Rockville, MD, USA. The cells were maintained by passage in DMEM containing 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine and gentamicin at 50 µg/ml (complete DMEM). The cloned cell lines L4 and L10 are genomically transfected mouse L fibroblasts which express the KN104 and the w10 bovine class I MHC molecules, respectively (Toye et al., 1990). The *Theileria parva* (Muguga) (TpM)-infected lymphoblastoid cell lines D409 N2 TpM, E98 TpM, G383 TpM, G282 TpM and G277 TpM, were established and maintained as previously described (Goddeeris and Morrison, 1988). The cell lines were typed for bovine class I MHC antigens in a microlymphocytotoxicity assay (Teale et al., 1983).

2.2. Monoclonal antibodies

mAb IL-A88 is specific for a monomorphic determinant on bovine class I MHC molecules (Toye et al., 1990). mAb IL A4 is specific for the class I MHC molecule, KN104 (Kemp et al., 1988).

2.3. Eukaryotic expression plasmid containing the bovine class I MHC gene, KN104

We wished to obtain a full length copy of the cDNA encoding the KN104 molecule. A cDNA library was prepared from RNA derived from the TpM-infected bovine lymphoblastoid cell line, D409/N2. This cell line has the bovine class I MHC phenotype: W7,-/W10, KN104 (Goddeeris et al., 1990). The library was inserted into the COS cell expression vector, pCDM8 (Seed, 1987). In brief, the cDNA was ligated to *Bst* XI linkers and inserted into *Bst* XI-digested pCDM8. The library was transformed into *E. coli* MC1061/p3 by electroporation under standard conditions, and transformants were selected by resistance to ampicillin and tetracycline. The library was estimated to contain 150 000 colonies. The library was probed with a pUC19 plasmid containing part of the KN104 gene (Bensaid et al., 1991) which allowed isolation of a pCDM8 recombinant plasmid containing homologous DNA.

Sequence analysis of this plasmid revealed that it contained the entire KN104 gene minus a small part of the 5' open reading frame. This was repaired by inserting an appropriate oligonucleotide into the plasmid. Transfection of the reconstructed plasmid, called pCD-KN104, into COS cells resulted in cells reactive with mAb IL-A88 and IL-A4.

2.4. Transient transfection of COS cells

Transient transfection of COS cells was accomplished by the DEAE-Dextran method. The cells were grown to about 80% confluence in 75 cm² tissue culture flasks. After being resuspended by incubation in 0.05% trypsin, 0.02% EDTA ('Trypsin-EDTA'; Flow Laboratories, Irvine, Scotland), the cells were harvested and washed in complete DMEM. For transfection, 3×10^6 cells were suspended in 3 ml RPMI 1640 medium containing 50 mM Tris-HCl, pH7.5, 250 µg/ml DEAE-Dextran (average molecular weight 500 000; Sigma Chemical Company, St. Louis, MO) and 3 µg/ml pCD-KN104. The cells were incubated for 2 h at 37°C, with occasional mixing

by inversion. After incubation, the cells were washed in RPMI 1640 plus 50 mM Tris-HCl, pH7.5, and cultured in complete DMEM until required (usually 48–72 h later).

2.5. Stable transfection of COS cells

Stable transfection of COS cells was achieved using calcium phosphate-mediated gene transfection (Wigler et al., 1979). To facilitate incorporation of plasmid sequences into the COS cell genome, we deleted the SV40 *ori* from the pCD-KN104 vector by digestion of the plasmid with the restriction endonucleases *AccI* and *NheI* (Watson et al., 1983). The larger fragment resulting from this digestion contained the complete KN104 gene, and from pCDM8, the CMV-T7 promoter, SV40 splice and polyadenylation sequences, 3.3 kbp of polyoma origin sequence, the M13 *ori* and the sup F gene. The fragment was purified and co-transfected into COS cells with linearized pSV2neo (Southern and Berg, 1982).

After transfection, the COS cells were incubated in complete DMEM containing G₄₁₈ sulphate ('Geneticin', Gibco Life Technologies, Paisley, Scotland) at 1.0 mg/ml. The selection was removed after 6 days, and COS cells expressing the KN104 antigen were selected by fluorescence-activated cell sorting with mAb IL-A88. After two rounds of sorting, when about 70% of the sorted population expressed the KN104 molecule, the cells were cloned by limiting dilution. A cloned cell line, IL-X30, was selected for further analysis.

2.6. Cytotoxicity assays

The cytotoxic activity of alloreactive CTL on transfected cells or TpM-infected lymphoblasts was measured in a standard 4 h ⁵¹Cr microcytotoxicity assay (Goddeeris and Morrison, 1988). The assays employed 5×10^4 or 1×10^4 labelled target cells/well for lymphoblasts or COS/L cells respectively. Background release of label was always less than 20% of total incorporated label. Cytotoxic activity was expressed as percentage cytotoxicity (%CTX) (Goddeeris and Morrison, 1988).

2.7. Derivation of alloreactive, anti-KN104 CTL

The alloreactive, anti-KN104 CTL cell line used in these experiments was derived by inoculating an animal which does not express the KN104 molecule, with PBMC from animal E98 which is homozygous for the KN104 gene (Toye et al., 1990). A total of 2×10^8 PBMC of E98 were injected in equal numbers by the subcutaneous, intramuscular and intravenous routes. 14 days after inoculation, PBMC from the inoculated animal were employed in an allogeneic mixed lymphocyte reaction as modified from a previously described method (Teale et al., 1987). In essence, 2×10^4 PBMC were mixed with 5×10^3 irradiated TpM-infected E98 lymphoblasts (E98TpM) in each of 36 wells of round-bottomed microculture plates in the presence of T cell growth factor as conditioned medium (Teale et al., 1985). After 9 days, the lymphocyte population in each well was assayed for cytotoxic activity in a 4 h indium-111 release assay (Goddeeris and Morrison, 1988). One-third of the cells in each well were assayed on E98 TpM cells and one-third on non-KN104-expressing control cells. Analysis of the specific release of label indicated that four wells contained cells which specifically lysed the E98 lymphoblasts to greater than 10% CTX. The cells from a single well, which we assume to be clonal, were restimulated by incubation with E98 TpM until sufficient numbers were available for cytotoxicity assays. The specificity of this cell line for the KN104 molecule was established using a panel of bovine lymphoblasts and transfected L cells (Table 1).

Table 1
Specificity of the anti-KN104, alloreactive CTL

Cell line	Class I MHC type	%CTX ^a
E98TpM	w10,KN104/w10,KN104	91
G383TpM	-,KN104/w1,-	87
G282TpM	-,KN104/-,-	92
G277TpM	w1,-/w1,-	< 1
L4	KN104	87
L10	w10	< 1

^a At an E:T ratio of 2:1.

3. Results

3.1. COS cells permanently expressing bovine class I MHC molecules

In order to use COS cells in a CTL screening assay, we wished to produce COS cells stably transfected with a functional bovine class I MHC gene. Accordingly, COS cells were transfected with a plasmid containing the bovine class I MHC gene, KN104, by calcium phosphate-mediated transfection. The SV40 *ori*, which may inhibit stable integration of plasmid sequences (Watson et al., 1983), was removed from the KN104-containing plasmid before transfection. A cloned cell line, IL-X30, was derived from the transfected COS cell population enriched for KN104-expressing cells by two rounds of fluorescence-activated cell sorting with mAb IL-A88. Immunofluorescence analysis showed that IL-X30

Fig. 1. Immunofluorescence analysis of cells transfected with the KN104 gene. The permanently transfected, cloned COS cell line, IL-X30, and untransfected COS cells were analyzed with mAb IL-A4 (IgM) and an irrelevant IgM mAb (Con). The horizontal and vertical axes indicate fluorescence intensity and cell number, respectively.

Fig. 2. Cytotoxic activity of the alloreactive CTL clone against the permanently transfected IL-X30 clone (●), COS cells transiently transfected with pCD-KN104 (■) and untransfected COS cells (○), at various *E:T* ratios. For comparison, cytotoxic activity against a permanently transfected, cloned L cell line, L4, is also indicated (▲).

cells reacted specifically with mAb IL-A88 and with mAb IL-A4 (Fig. 1). The IL-X30 cell line has been maintained in continuous culture for at least 219 days after transfection with no obvious loss of expression of the KN104 molecule. To show that the KN104 molecule was being expressed on the surface of COS cells in a manner recognisable by CTL, IL-X30 cells were analyzed in cytotoxicity assays using an alloreactive CTL clone specific for the KN104 gene. As shown in Fig. 2, the alloreactive CTL clearly recognized the IL-X30 cells, but not the untransfected COS cells. These results confirm that the KN104 gene was expressed on the surface of COS cells in a form recognisable by bovine CTL.

It should be emphasized that, in the generation of a permanently transfected COS cell line, selection for the marker gene was maintained for only 6 days after transfection. It was observed that if selection was maintained beyond this point, no resistant colonies appeared. This may be due to the transient nature of the expression of the pSV2neo gene in COS cells. Nevertheless, 6 days of selection in medium containing G_{418} was suffi-

cient to kill all the cells in an untransfected (control) population, thus avoiding the problem of 'false positive' transfectants.

3.2. Cytotoxic activity on transiently transfected COS cells

We wished to determine whether bovine CTL could recognize the product of a transiently transfected gene in a microcytotoxicity assay. COS cells were transiently transfected with the plasmid containing the KN104 gene, and were used as targets in a standard microcytotoxicity assay after 72 h. Specific killing of the transiently transfected cells was clearly detectable (Fig. 2).

To determine whether this approach would be feasible for sibling analysis of a cDNA library, we transfected populations of COS cells with decreasing amounts of the KN104-containing plasmid, in the presence of a constant amount of a similar plasmid but containing an irrelevant gene. As indicated in Table 2, CTL clearly recognized cells in a population transfected with the KN104-containing plasmid 'diluted' 200-fold, and even 1000-fold, with a plasmid containing an irrelevant gene. This level of sensitivity should permit practicable screening of a cDNA library by sibling analysis.

3.3. A stimulation assay for identifying CTL antigens

An alternative approach to identify CTL antigens from COS cells transfected with a cDNA

Table 2
Detection of target cells in COS cell populations transfected with decreasing amounts of pCDM8-KN104

Plasmid transfected ^a		%CTX ^b
pCD-KN104	pCDM8-CD8	
10 µg	10 µg	33
50 ng	10 µg	15
10 ng	10 µg	7
0 ng	10 µg	1.1

^a Refers to the amount of each plasmid present when the COS cells were transfected.

^b At an E:T ratio of 2:1.

Fig. 3. Scatter plot of cytotoxic activity of alloreactive CTL stimulated with various numbers of transiently transfected COS cells. The dotted line represents the mean, plus three standard deviations of the mean, of the release from assays involving CTL stimulated with filler cells only. Eight wells were assayed for each combination of COS cells and filler cells.

library is to use the transfected COS cells as stimulator cells. Specific restimulation, which will occur only in wells containing COS cells expressing the relevant gene, is measured by a cytotoxicity assay on the target cell. To assess the sensitivity of this approach, various numbers of COS cells transiently transfected with KN104 were mixed with 10^5 bovine PBL, representing cells not transfected with the appropriate gene. Alloreactive CTL, which had been stimulated 14 days previously, were added to the wells. After 7 days, the CTL activity was measured in each well. As can be seen from Fig. 3, as few as 50 cells from the transfected population can specifically restimulate CTL, even in the presence of 10^5 'negative' cells.

4. Discussion

These studies were performed in order to establish the feasibility of screening a gene library transiently transfected into mammalian cells with CTL and to identify the corresponding CTL anti-

gen. COS cells were selected as the recipient cells because of the ease of transfection and the high levels of expression of transfected genes. In order to establish such a procedure, we demonstrated that COS cells could be stably transfected with a foreign class I MHC gene, and that transfected COS cells were efficiently lysed with xenogeneic CTL. We further demonstrated that alloreactive CTL can detect cells expressing the corresponding antigen in a transiently transfected COS cell population, and that the frequency of transfection is such that even when the relevant gene is 'diluted' 100-fold with irrelevant genes, specific cytotoxic activity is observed. By employing these cells in a sibling analysis, where the total library is divided into pools of 100 to 200 colonies, it should be possible to screen a library of genes directly with CTL to identify relevant antigens. This is supported by the demonstration that a TNF-based assay has been employed to identify CTL antigens by screening a cDNA library as pools of 200 plasmids transfected into COS cells (Brichard et al., 1993), which is the level of sensitivity of the direct cytotoxicity assay which we have described.

An alternative system is described where the transiently transfected sibling populations may be used as stimulators of CTL. Specific restimulation of the CTL is measured in a standard microcytotoxicity assay using the relevant cell line as targets. This method has the advantage of requiring only one target cell line to be labelled for use in the microcytotoxicity assay. The previous method requires each transfected sibling population to be labelled.

Acknowledgements

The authors would like to thank Drs. Alan Teale and Steve Kemp for typing the cell lines used in these experiments.

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The application of *in vitro* isolation, cryopreservation and patch-clamp microelectrode recording methods to adult rat thymic nurse cells

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Received 28 February 1995; revised 4 June 1995; accepted 26 June 1995

Abstract

Characterisation of the ionic mechanisms possessed by thymic cells has begun to reveal a range of transmembrane ion channel properties which may have immunological significance. Since thymic epithelial cells appear to influence selection of the T-cell repertoire, understanding their membrane physiology may be of importance. A method is therefore outlined for the recording of patch-clamp electrophysiological measurements in acutely isolated fresh and cryopreserved adult rat thymic nurse cells. These cells represent a distinct and predominantly cortical population of epithelium. Cells were separated by enzymatic and mechanical dispersal of thymus, enriched by sedimentation and identified on the basis of their characteristic lympho-epithelial multi-cellular morphology. Phase-contrast cells retaining this morphology survived freezing and the voltage-gated conductances in such cells are described. The merits of this approach include the preparation and storage of native unfixed and unfrozen phenotypes of immune cells for *in vitro* studies. Their preservation permits repeated separation of experiments, comparative experimentation on identical samples, optimised cell preparation in terms of cost and labour, and reduced animal and tissue requirements.

Keywords: Thymic nurse cell; Acute isolation; Cryopreservation; Patch clamp; Animal conservation; Adult rat

1. Introduction

Transmembrane voltage is a fundamental effector, not only capable of influencing ionic and metabolic transport processes, but also of deter-

mining the functional conformations of membrane-associated proteins. All cells maintain transmembrane voltage gradients, and ionic mechanisms are utilised in the maintenance of these electrochemical potentials (Hille, 1992). A growing body of work attests to the presence of ion channels on immune cells (Gallin, 1991). Moreover, a number of different ion channel activities have been correlated with immunologi-

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