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Inhibition of HLA B8-restricted recognition by unrelated peptides: evidence for allosteric inhibition

A. Fernan, S. R. Burrows, D. J. Moss, A. Saul and A. Suhrbier
Queensland Institute of Medical Research, Bancroft Centre, Herston, Brisbane, Queensland 4006, Australia

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1. Summary

A panel of synthetic peptides representing human lymphocyte antigen (HLA) B8, other class I and class II restricted T cell epitopes and two B cell epitopes, were all able to compete with recognition of a HLA B8 restricted epitope by a cytotoxic T cell clone. Competition was obtained when the competitor peptides were added either before or after the target epitope. The target epitope also had a slow off rate, implicating allosteric inhibition. The presence of non-specific, allosteric binding sites may interfere with experiments attempting to define immunologically relevant MHC binding specificities.

2. Introduction

Three epitopes have been described which are restricted to the human class I major histocompatibility antigen HLA B8; FLRGRAYGL from Epstein-Barr virus ([1] and S. T. Rodda, S. R. Burrows, A. Suhrbier, H. M. Geysen and D. J. Moss, submitted for publication) and NPPIPVGEIYKRWII and IETVPVKLKPGMDGPKVKQWPLTEE from HIV [2, 3]. Using previously reported methods for competition of cytotoxic T lymphocyte (CTL) epitopes [4, 5] we were unable to demonstrate competition of CTL killing through the synthetic peptide epitope FLRGRAYGL by either of the latter peptides (data not shown). Reasoning that competition might be difficult to demonstrate when a T cell can be activated by the recruitment of only a few hundred major histocompatability complex (MHC) molecules bearing the target epitope [7], the target cells were fixed [8]. As fixed cells do not permit the use of the chromium release assay, CTL recognition of target cells was measured using the BLT (benzyloxycarbonyl-L-lysine thiobenzyl ester) serine esterase assay. This assay measures the serine esterase activity of granzyme A, a protease released by CTLs during target cell lysis and gives results very comparable to the chromium release assay (A. Suhrbier, A. Fernan, S. R. Burrows, A. Saul and D. Moss, submitted for publication). In addition, the peptide HIRGRAYSL was used instead of FLRGRAYGL as the target peptide. We have previously shown that HIRGRAYSL is a 30-fold more active analogue of FLRGRAYGL by titration (Rodda et al., submitted); a higher competitor peptide/target peptide concentration ratio could thus be achieved [5].

A panel of synthetic peptides representing HLA B8, A2, B44, DR4 and DR1 restricted T cell epitopes and two known B cell epitopes (Table 1) were used to compete with HIRGRAYSL induced BLT activity.

Key words: Cytotoxic T cell; HLA B8; Competition; BLT esterase

Correspondence to: A. Fernan, Queensland Institute of Medical Research, Bancroft Centre, Herston, Brisbane, Qld. 4006, Australia.

3. Materials and Methods

3.1. *Synthesis of peptides*

The panel of synthhetic peptides (Table 1) was synthesised as described previously [9]. Lyophilised

TABLE 1

Synthetic peptide epitopes used as competitors.

Peptide	Epitope type	Reference
NPPIPVGEIYKRWII	HLA B8	[20]
IETVPVKLKPGMDGPKVKQWPLTEE	HLA B8	[3]
QLSDTPLIPLTIFVG	Class I HLA A2	[a]
EENLLDFVRF	Class I HLA B44	[21]
PRYVRQNTLRLAT	Class II T cell HLA DR1	[22]
YSYFPSVI	Class II T cell HLA DR4	[b]
ALEGRQGRFGSSGQ	B Cell, IgA	[23]
DNNLVSGP	B Cell, IgG	[24]

[a]Schmidt, C., Burrows, S.R., Sculley, T.B., Moss, D.J. and Misko, I.S., submitted for publication. [b]Suhrbier, A., Fernan, A., Burrows, S.R., Saul, A. and Moss, D., submitted for publication.

peptides were dissolved in RPMI-1640 containing 10% FCS.

3.2. *Cells*

A HLA B8-restricted EBV strain specific human CTL clone LC13 was used as the effector cell, and an autologous lymphoblastoid cell line (LCL) LC/Ag876 as the target (LC refers to the donor, and Ag876 is the transforming EBV strain). LCL LC/Ag876 cells were fixed for 20 min in 0.5% paraformaldehyde and washed three times with RPMI-1640 prior to use. LCL LC/Ag876 is not recognised by LC13 unless incubated with appropriate synthetic peptide target antigen, FLRGRAYGL or HIRGRAYSL [1].

Cell lines were maintained as described previously [10].

3.3. *Competition experiments*

3.3.1. *Competitor peptides added before HIRGRAYSL*

Competitor peptides were added at varying concentrations (0 – 2.56 mM) to fixed LCL LC/Ag876 cells and incubated overnight at 37 °C in a total volume of 70 μl (the pH of these solutions was 6.7 – 7.2, data not shown). HIRGRAYSL was then added in 20 μl to give a final concentration of 1 μM and the cells were incubated for 1 h at 37 °C. This is the minimum concentration and time to give maximum BLT esterase activity (data not shown). The LCLs were then washed 3 times with RPMI-1640 through an underlay of FCS, resuspended in RPMI-1640 containing 10% FCS, and added at 1×10^4 cells per well in 100 μl to 96-well round-bottomed microwell plates. 2×10^4 CTL LC13 were added in a volume of 100 μl to each well. Parallel control wells were identical, but lacked HIRGRAYSL. Cultures were centrifuged for 5 min at 500 rev./min prior to incubating for 18 h (overnight) at 37 °C. 100 μl of culture supernatant was then harvested and assayed for BLT esterase activity (see below).

3.3.2. *Competitor peptides added after HIRGRAYSL*

In a separate experiment, cultures were set up as described above, except that peptides were added in the reverse order. Fixed LCLs were first incubated with HIRGRAYSL for 1 h, washed 3 times as described above, then competitor peptides added overnight. The cells were then washed 3 times before incubation with CTL LC13. Control wells were treated identically except that HIRGRAYSL was omitted. BLT activity of the supernatants then was assessed as described below.

Two positive control (+C) samples without any competitor peptides were included; the first, +C1, consisted of fixed LCL LC/Ag876, which were incubated with 1 μM HIRGRAYSL for 1 h, washed and incubated with medium (instead of competitor peptides) overnight. The fixed cells were then washed and incubated with CTL LC13. +C2 consisted of fixed LCL LC/Ag876 incubated with medium instead of HIRGRAYSL, which were then

washed, incubated with medium overnight and then incubated with 1 μM HIRGRAYSL for 1 h. The cells were then washed 3 times and added to CTL LC13 and the supernatants assayed for BLT activity.

3.4. *BLT serine esterase assay*

This assay was performed essentially as described previously [10, 13]. Briefly, to 100 μl of supernatant was added an equal volume of 0.1 M Tris pH 8.1 containing approximately 0.4 mM *N*-α-benzyloxycarbonyl-L-lysine thiobenzyl ester (BLT) and 0.4 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB; Ellmans reagent) (Sigma, U.S.A.). The colour change was monitored over 30 min at 410 nm (automated EIA plate reader, EL310, Bio-Tek Instruments Inc., U.S.A.) with absorbance readings taken every 3 min and an absorbance change per min (ΔA/min) calculated.

Results are expressed as "mean ΔA/min of duplicate experimental wells – mean ΔA/min of control wells".

4. Results

4.1. *Competitor peptides added before HIRGRAYSL*

Human CTL clone LC13 recognised fixed LCL LC/Ag876, which had previously been incubated with 1 μM of the peptide epitope HIRGRAYSL, resulting in detectable BLT activity (Fig. 1; arrow). If the fixed LCL LC/Ag876 were first incubated with any of the peptides in Table 1, prior to the addition of HIRGRAYSL, HIRGRAYSL induced BLT activity of CTL clone LC13 was progressively reduced as the competitor concentration was increased (Fig. 1).

1 μM HIRGRAYSL added to a sample of fixed non-HLA B8 LCL, followed by washing, did not result in detectable BLT esterase activity (data not shown). The use of 20 μM FLRGRAYGL instead of 1 μM HIRGRAYSL in the above experiment resulted in similar inhibition at about 10-fold higher competitor concentrations (data not shown).

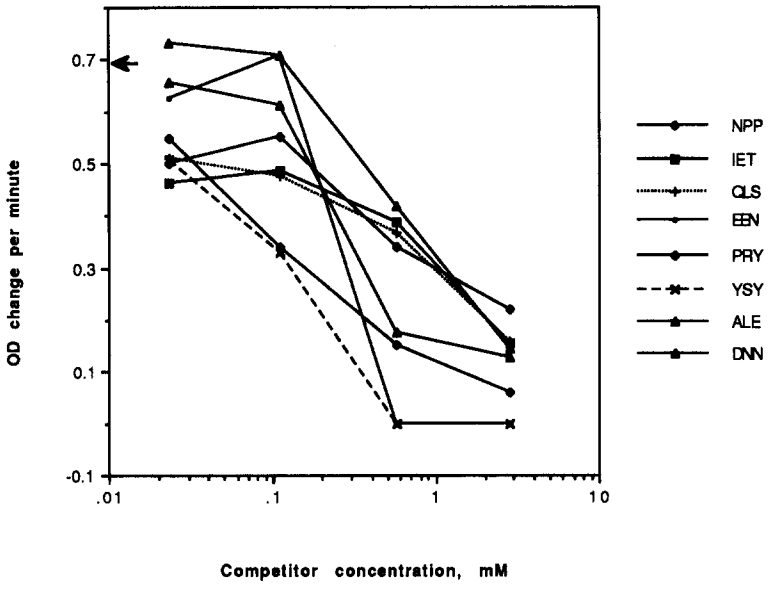

Fig. 1. BLT serine esterase activity of LC13 when added to LCL LC/Ag876 after incubation with competitor peptides followed by 1 μM HIRGRAYSL. Figure legend refers to the first three amino acids of each peptide shown in Table 1. Points are in duplicate, errors approximately ± 20%. Arrow – activity in the absence of competitor peptides.

4.2. *Competitor peptides added after HIRGRAYSL*

When target cells were incubated with HIRGRAYSL for 1 h first, followed by the addition of competitors overnight, the levels of competition were comparable to those observed in Fig. 1 (Fig. 2). Target cells incubated with 1 μM HIRGRAYSL, washed and given medium instead of competitors and then incubated overnight (+C1), resulted in a $\Delta A = 1.735 \pm$ S.D. 0.13. The BLT activity of cells given an identical HIRGRAYSL treatment just prior to the addition of CTLs (+C2) was $\Delta A = 1.873 \pm$ S.D. 0.09 (arrowhead and arrow, respectively, Fig. 2).

4.3. *Toxicity controls*

Competitor peptides and/or fixed LCL LC/Ag876 did not give significant BLT activity. LC13 + fixed LC/Ag876 or LC13 + fixed LC/Ag876 + HCC (LCL LC/Ag876, which had been incubated with the highest concentration (2.56 mM) of each of the eight competitor peptides overnight and had then been washed: see section 3.3.1.) or LC13 alone all gave similar BLT levels (a, b and c, Fig. 3).

Fixed LCL LC/Ag876 + HCC were not toxic to the CTLs. When 1×10^4 living LCL LC/BL74 (a cell line killed by LC13; [1]) were added to the above control wells, in which LC13 + LC/Ag876 or LC13 + LC/Ag876 + HCC or LC13 had been incubating for 18 h, similar levels of BLT activity were obtained (d, e and f, Fig. 3). Data for a – f (Fig. 3) and Fig. 1 were obtained during the same experiment.

A mixture of all the chemicals used in the synthesis of the peptides was used as a "competitor" (see section 3.3.1.) to ensure that any possible impurities in the peptides did not in themselves interfere with HIRGRAYSL induced BLT activity. A solution containing 1 mM of each of the following chemicals was prepared in medium and the mixture referred to as PSC (peptide synthesis chemicals); dichloromethane (Dow Chemicals, Australia), diisopropyl ethylamine, trifluoroacetic acid, *N,N'*-di-isopropyl carbodimide, (Sigma, U.S.A.), isopropanol, dimethyl formamide (Selby Anax, Australia), anisole (BDH, U.K.), diethyl ether, acetic acid (Ajax Chemicals, Australia), BOC-arginine, BOC-leucine, BOC-glycine (Biochem. Inc., U.S.A.). When fixed LC/Ag876 were incubated overnight with the PSC, washed (referred to as LC/Ag876 + PSC) and then added to LC13, no significant BLT activity was obtained (g, Fig. 3). LC/Ag876 was as capable as LC/Ag876 + PSC of inducing BLT activity after incubation with HIR-

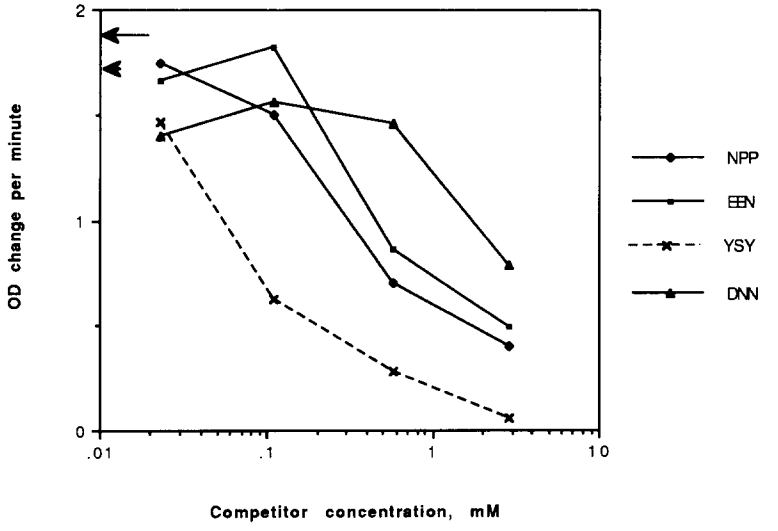

Fig. 2. BLT serine esterase activity of LC13 when added to LCL LC/Ag876 which had first been incubated with 1 μM HIRGRAYSL, then competitor peptides. Arrow head – +C1 (1 μM HIRGRAYSL added at the beginning of experiment); arrow – +C2 (1 μM HIRGRAYSL added at the end of experiment).

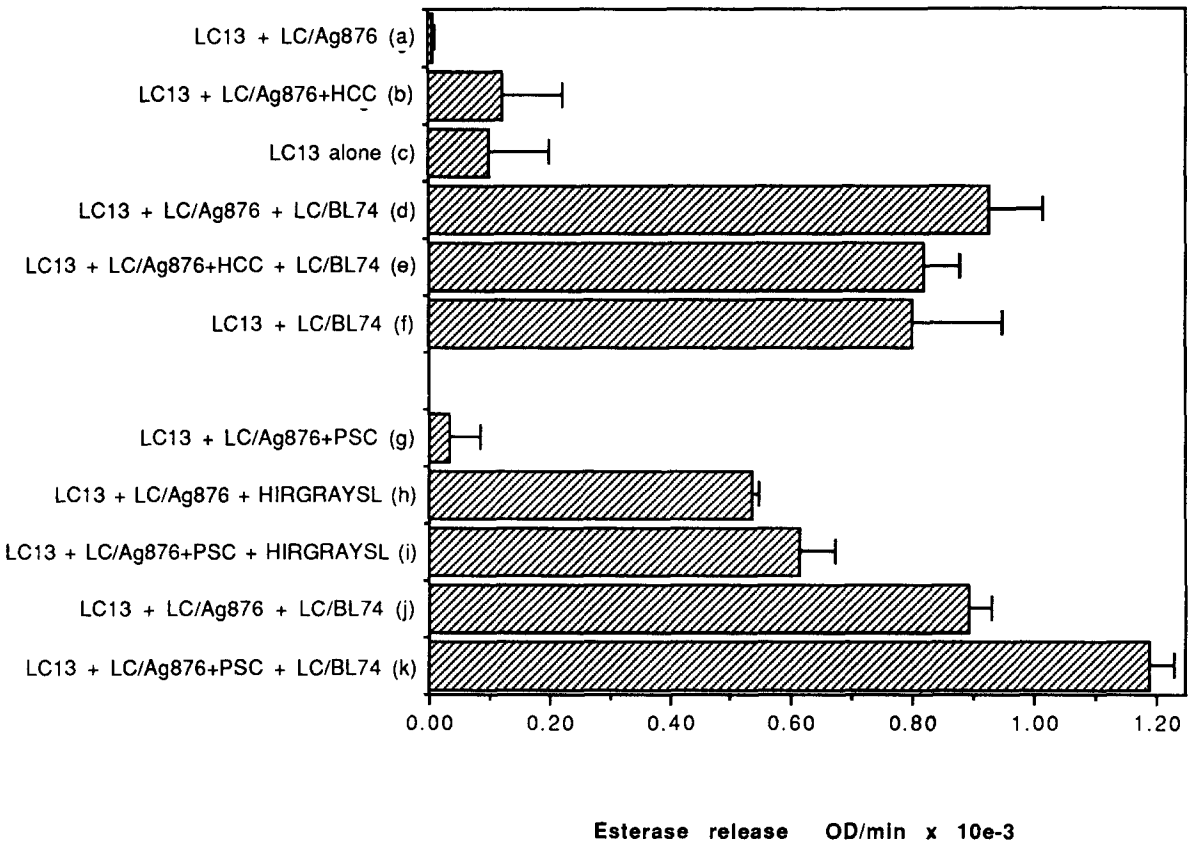

Fig. 3. Toxicity controls. Fixed LC/Ag876 and/or competitor peptides used at the highest concentration (HCC) did not induce BLT activity (a – c). HCC also did not inhibit LC/BL74 (a cell line recognised by LC13) induced BLT activity (d – f). A 1 mM mixture of all the chemicals used in peptide synthesis (PSC) did not induce BLT activity (g), nor did it inhibit HIRGRAYSL (h, i) or LC/BL74 (j, k) induced BLT activity. (b) and (e) are averages from 16 wells, (c) from 6 wells and all others from duplicate wells. Error bars show standard deviations. Values of ΔA/min obtained from medium alone were subtracted from all the above values (a – f and g – k are from separate experiments). Data for a – f (Fig. 3) and Fig. 1 were obtained during the same experiment.

GRAYSL and addition of LC13 (h and i, Fig. 3). LC/Ag876 + PSC also did not interfere with LC/-BL74 induced BLT activity (j and k, Fig. 3).

Peptides YSY and IET were purified to a single peak by HPLC and tested again as described in section 3.3.1. Purified YSY was also tested as described in 3.3.2. In both cases results comparable to those in Figs. 1 and 2 were obtained (data not shown).

5. Discussion

A panel of peptides representing antibody, class II and class I (including HLA B8) epitopes were all able to inhibit CTL recognition of the HLA B8-restricted peptide HIRGRAYSL when incubated overnight prior to the addition of the target peptide HIRGRAYSL. Similar levels of competition were obtained when HIRGRAYSL was added first followed by the competitors for the same period. The peptide HIRGRAYSL did not dissociate significantly (see + C1 and + C2, Fig. 2) during the time course of the experiment, indicating that the off rate of HIRGRAYSL in this system is slow. A slow-off rate of epitope from HLA has also been reported for class II [12, 13]. Taken together these data (and perhaps also those presented by others [14] implicate allosteric inhibition, i.e. the competitor peptides bind to different (allosteric) site(s) to the target peptide and occupancy of the allosteric

site(s) by competitor inhibits CTL activation. The putative allosteric site(s) appear able to bind almost any peptide and their binding appears independent of target peptide binding.

It is not clear where such putative allosteric binding site(s) might be located. They may be separate from or present on the cell surface [15] MHC class I molecules, conceivably localised within the peptide binding groove [16], in binding pockets not occupied by HIRGRAYSL [17]. The presence of non-specific allosteric binding sites on cell surface MHC molecules may interfere with attempts to define immunologically relevant binding specificities in a variety of competition and peptide binding assays [13, 18, 19]. These latter studies show, as does this study, largely degenerate specificity of peptide binding to MHC.

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