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Short communication

THE SPECIFIC T-CELL RESPONSE TO ANTIGENIC PEPTIDES IS INFLUENCED BY BYSTANDER PEPTIDES

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Abstract: T lymphocytes recognize antigens in the form of peptides presented by major histocompatibility complex (MHC) molecules on the cell surface. Only a small proportion of MHC class I and class II molecules are loaded with foreign antigenic peptides; the vast majority are loaded with thousands of different self peptides. It was suggested that MHC molecules presenting self peptides may serve either to decrease (antagonistic effect) or increase (synergistic effect) the T cell response to a specific antigen. Here, we present our finding that transfected mouse fibroblasts presenting a single antigenic peptide covalently bound to a class II MHC molecule stimulated specific mouse T cell hybridoma cells to an interleukin-2 response less efficiently than fibroblasts presenting a similar amount of antigenic peptide in the presence of class II molecules loaded with heterogenous bystander peptides.

Key Words: Bystander peptide, T cell response, Transfectants, Mouse model

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Abbreviations used: A^bwt - fibroblasts transfected with wild-type A^b class II MHC molecule; A^bEp - fibroblasts transfected with A^b molecule with linked Ep; A^bwt + A^bEp - double transfectant fibroblasts; Ep - peptide 52-68 from the Eα class II MHC chain; FACS - fluorescein-activated cell sorter; MHC - major histocompatibility complex; MTT - (3-[4,5 dimethylthiazol-2-lyl]-2,5 diphenyl tetrazolium bromide; TCR - T cell receptor.

INTRODUCTION

T lymphocytes perceive antigens by means of antigen-specific receptors (T cell receptors, TCR). The antigens are displayed on the surface of antigen-presenting cells in the form of peptides bound to major histocompatibility complex (MHC) molecules. The overwhelming majority of MHC molecules are occupied by peptides derived from self proteins of the organism [1]. The self peptides presented by the MHC on the periphery overlap with those expressed on the thymic cortical epithelium, and are therefore involved in the positive selection of developing T cells in the thymus [2, 3]; thus, mature T cells must respond to foreign peptide-MHC combinations in the presence of self peptide-MHC combinations for which their receptors have some affinity. Therefore, bystander peptides may positively or negatively influence the response to a specific peptide. Though there is experimental evidence for this effect, it is ambiguous: Wülfing *et al.* [4] observed that the neutral altered peptide ligand had an augmenting effect on the $CD4^+$ T cell response to antigenic peptide presented by an MHC class II molecule, whereas Sporri and Reis e Sousa [5] found naturally bound self peptides had no effect on the response of $CD8^+$ T cells to antigenic peptide presented by an MHC class I molecule. The aim of this study was to test the effect of bystander peptides on the specific T cell response, using, as antigen-presenting cells, mouse fibroblast lines transfected with genes for (i) mouse MHC class II molecules, A^b, loaded exclusively with one covalently linked peptide, Ep (A^bEp molecules), (ii) wild-type A^b loaded with a wide array of peptides other than Ep (A^bwt), or (iii) both A^bwt and A^bEp (A^bwt+A^bEp) [6]. Three independently obtained anti-A^bEp $CD4^+$ T cell hybridomas were used as responders.

MATERIALS AND METHODS

Cell lines

The mouse fibroblast cell line Ltk-/- was transfected as described by Ignatowicz *et al.* [6] with genes for wild-type mouse class II molecule A^b (clone FT2.3A^bwt), for the A^b molecule covalently linked with a peptide, Eα 52-68 (clone LtkA^bEp), or with both A^bwt and A^bEp (clones FT7.1A^bwt+A^bEp and FT2A^bwt+A^bEp). In all the A^bEp transfectants, the cytoplasmic region of the $A^b\beta$ chain was covalently linked with green fluorescent molecule (GFP) for the purpose of experiments that are not described here. The fibroblasts were cultured as a monolayer in complete culture medium, as described [6].

The $CD4^+$ T cell hybridomas BEα16.3, BAR354, and 105-3 were obtained via the immunization of C57BL/6 mice with Ep peptide and the fusion of spleen cells with BW5147-TCR$\alpha^-\beta^-$ cells, as described [7]. These hybridomas specifically recognize Ep presented by A^b, and secrete considerable amounts of interleukin 2 (IL-2) upon specific antigen stimulation.

The cytotoxic T lymphocyte line, CTLL-2 [8], dependent on IL-2, was used for the IL-2 assay (see below). The cell line HT-2 [9], also IL-2-dependent, was used instead of CTLL-2 in some of the IL-2 assays.

IL-2 assay

Hybridoma cells were cultured with nontransfected or transfected fibroblasts in complete culture medium. The supernatants were harvested 24 h later for IL-2 determination, performed with the MTT test as described using HT-2 [7] or CTLL-2 [10] cells as indicators. Recombinant IL-2 (rIL-2, Sigma, Saint Louis, Missouri, USA) in serial dilutions served as a positive control, and IL-2 units in the tested supernatants were calculated based on the standard curve of rIL-2.

Immunofluorescence staining and FACS analysis

Mouse monoclonal antibodies Y3P (IgG_{2a}, anti-$A^{b,f,p,q,r,s,u,v}$) [11] and YAe (IgG_{2b}, anti-A^bEp) [12] were purified and conjugated with phycoerythrin. Cells were stained with these reagents as previously described [13] and their fluorescence was measured using FACScalibur and analysed using CellQuest software (Beckton Dickinson).

Statistical analysis

At least three experiments were performed for each experimental set with the hybridoma BEα16.3, and the data was analyzed using Statistica 6.1 software (www.statsoft.pl). For hybridomas BAR354 and 105-3, only single experiments were performed.

RESULTS AND DISCUSSION

In order to compare the response of T hybridoma cells to A^bEp alone or in the presence of A^b loaded with other peptides, it was necessary to check whether the different transfected fibroblast lines express comparable amounts of A^b and Ep. Flow cytometry results showed that this was the case: anti-A^bwt staining was similar for the A^bwt (FT2.3A^bwt) and A^bwt+A^bEp (FT2A^bwt+A^bEp and FT7.1A^bwt+A^bEp) cell lines (Fig. 1, left panel, and data not shown), whereas anti-A^bEp stained the A^bEp (LtkA^bEp) and A^bwt+A^bEp (FT2A^bwt+A^bEp and FT7.1A^bwt+A^bEp) cell lines to a similar extent (Fig. 1, middle panel). The A^bEp cell line expressed A^bEp but not A^bwt, as shown by inhibition of anti-A^bwt staining by unlabelled anti-A^bEp antibody (Fig. 1, right panel).

Next, we used these fibroblast lines as stimulator cells in culture with cells of the T cell hybridoma BEα16.3. When 10^5 hybridoma cells were cultured for 24 h with 10^5 fibroblasts, the A^bwt+A^bEp cells stimulated the hybridoma to secrete IL-2 much more strongly than the A^bEp cells did (Fig. 2). The higher stimulatory capacity of A^bwt+A^bEp over A^bEp cells was reproducibly observed over a wide range of cell concentrations of both responders (Fig. 3) and stimulators (Fig. 4).

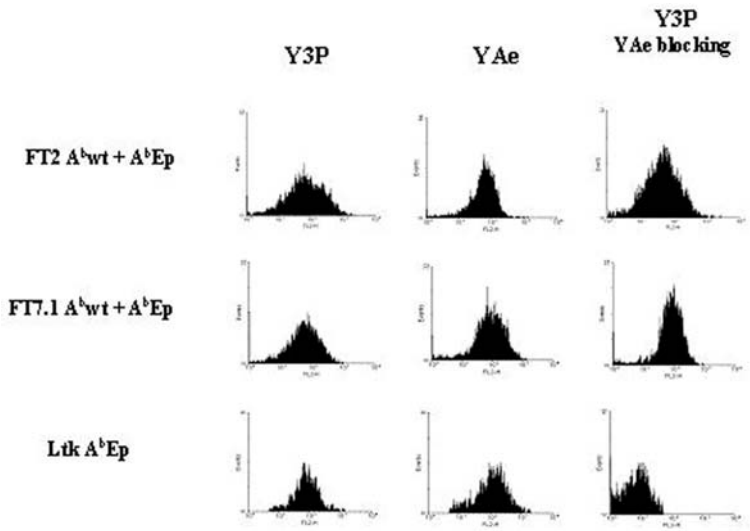

Fig. 1. Expression of wild-type (A^bwt) and Ep-linked (A^bEp) molecules on the cell surface of transfected fibroblasts. Y3P, anti-A^bwt monoclonal antibody. YAe, anti-A^bEp monoclonal antibody. FT2A^bwt+A^bEp and FT7.1A^bwt+A^bEp, two independent A^bwt+A^bEp Ltk-/- fibroblast transfectants. LtkA^bEp, Ltk-/- cells transfected with A^bEp only. Y3P, staining of A^bwt molecules. YAe, staining of A^bEp molecules. Right column, staining of A^bwt molecules after blocking with a saturating amount of anti-A^bEp antibody. Note that LtkA^bEp cells are not stained with anti-A^bwt antibody when covered with unlabelled anti-A^bEp antibody, whereas LtkA^bwt+A^bEp cells are stained by both antibodies, as evidenced by the staining with anti-A^bEp and no blocking of anti-A^bwt staining by unlabelled anti-A^bEp.

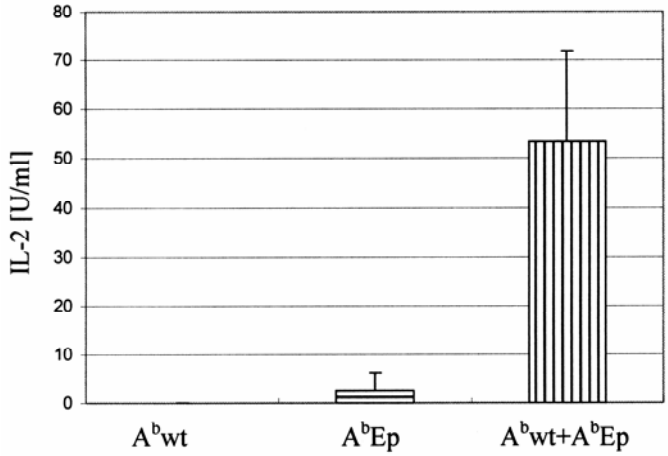

Fig. 2. The interleukin-2 response of hybridoma BEα16.3 to transfected fibroblasts. 10^5 hybridoma cells per well were cultured for 24 h with 10^5 fibroblasts. The supernatants were harvested and tested with CTLL-2 indicator cells in the MTT test. The presented results are the mean ± standard deviation of four independent experiments.

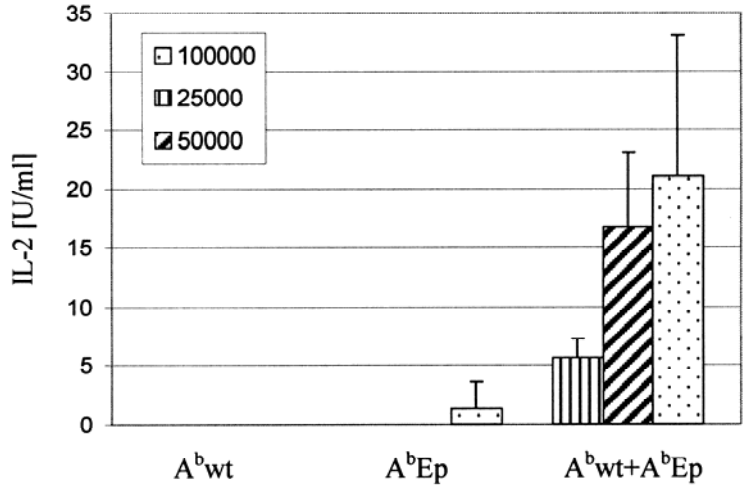

Fig. 3. The interleukin-2 response of hybridoma BEα16.3 to transfected fibroblasts as a function of hybridoma cell concentration. The indicated concentrations of BEα16.3 cells were cultured with 10^5 LtkA^{b}Ep, FT2.3A^{b}wt or FT7.1A^{b}wt+A^{b}Ep fibroblasts, and the supernatants were harvested and tested as in Fig. 2. The presented results are the mean ± standard deviation of three independent experiments.

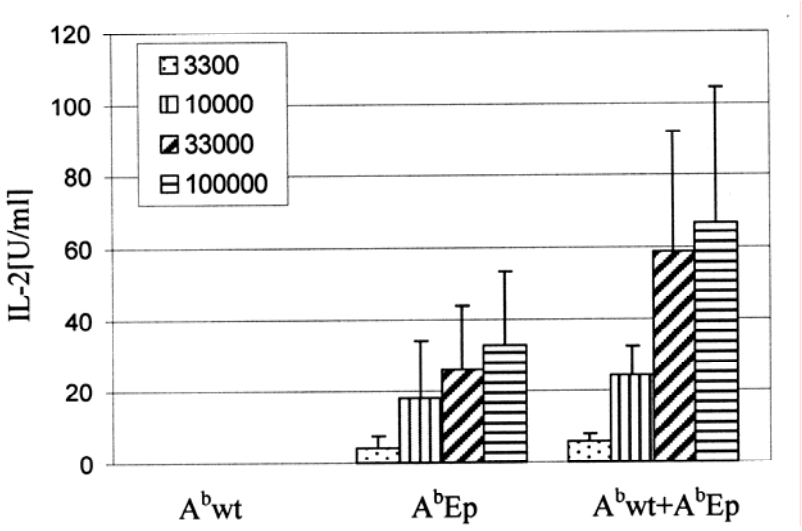

Fig. 4. The interleukin-2 response of hybridoma BEα16.3 to transfected fibroblasts as a function of fibroblast concentration. Hybridoma cells (10^5 per well) were cultured with indicated cell concentrations of transfected LtkA^{b}Ep, FT2.3A^{b}wt or FT7.1A^{b}wt+A^{b}Ep fibroblasts, and after 24 h, the supernatants were harvested and tested as in Fig. 2. The presented results are the mean ± standard deviation of three independent experiments. Parallel cultures with BEα16.3 cells alone gave no IL-2 response (not shown).

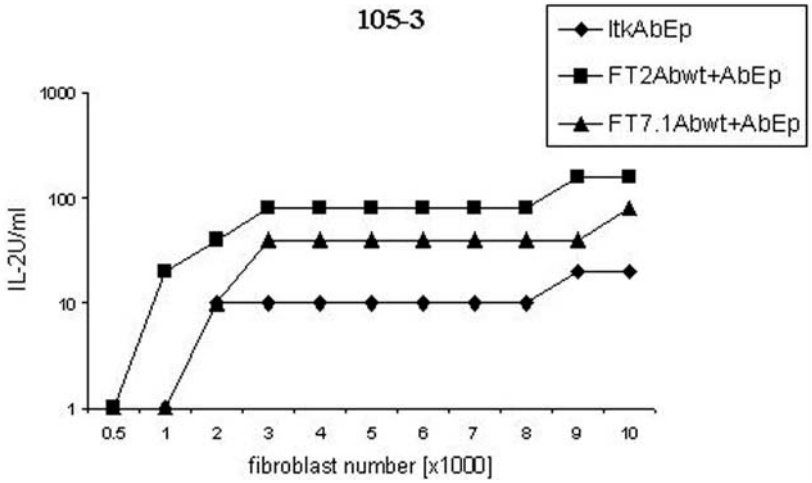

Fig. 5. The interleukin-2 (IL-2) response of hybridoma 105-3 to transfected fibroblasts. Hybridoma cells (25 x 10^3 per well) were cultured with indicated cell concentrations of LtkA^{b}Ep, FT2A^{b}wt+A^{b}Ep or FT7.1A^{b}wt+A^{b}Ep fibroblasts, and after 24 h, the supernatants were harvested and the IL-2 content was measured by proliferation of HT-2 indicator cells in the MTT test.

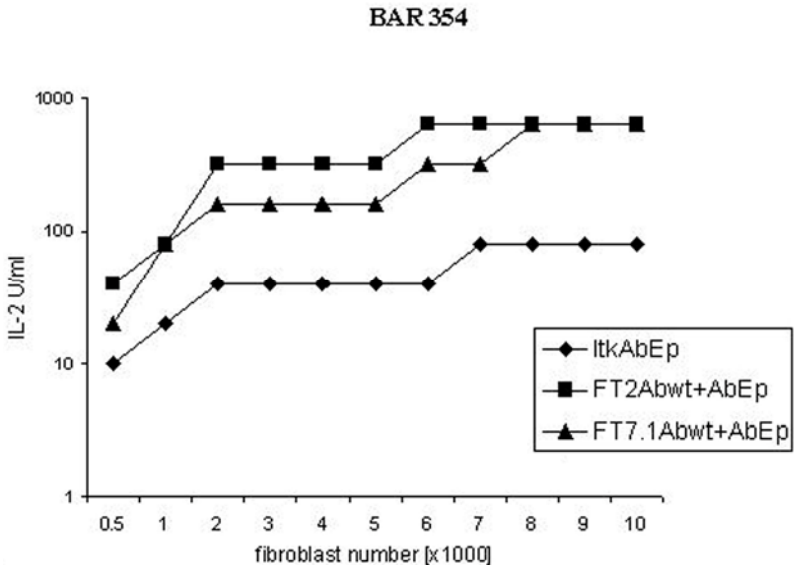

Fig. 6. The interleukin-2 response of hybridoma BAR354 to transfected fibroblasts. Hybridoma cells (25 x 10^3 per well) were cultured with the indicated cell concentrations of LtkA^{b}Ep, FT2A^{b}wt+A^{b}Ep or FT7.1A^{b}wt+A^{b}Ep fibroblasts, and after 24 h, the supernatants were harvested and the IL-2 content was measured by proliferation of HT-2 indicator cells in the MTT test.

Wild-type peptides alone, presented by A^bwt fibroblasts, did not evoke a detectable IL-2 response (Figs 2-4). To confirm these unexpected results, we used two independently produced T cell hybridomas, 105-3 and BAR354. Both hybridomas produced higher amounts of IL-2 when stimulated with A^bwt+A^bEp cells (FT2A^bwt+A^bEp or FT7.1A^bwt+A^bEp) than they did with A^bEp cells (LtkA^bEp) (Figs 5 and 6).

Thus, in our experiments, bystander peptides seemed to enhance the IL-2 response of T cell hybridomas to a specific peptide. Three independently obtained T cell hybridomas responded very similarly. The results described above might have been caused by differences in the expression of some accessory/costimulatory molecule(s), such as CD80, CD86, PD-1L, LICOS, CD45 or CD48, on the transfected fibroblast lines interacting with the T cell coreceptors CD28, CTLA-4, PD-1, ICOS, CD22 or LFA-1, respectively [14, 15]. However, this is unlikely for two reasons. First, such interactions were reported to be much more important for the induction of the antigen response in naive T cells than in effector T cells [16], although distinct functions of the latter seem to depend on accessory molecules to a different extent [17]. Secondly, an effect of possible differences in the expression of accessory molecules between the antigen-presenting fibroblast lines on T cell response seems unlikely in our experimental system because two independently produced A^bwt+A^bEp transfectant cell lines, FT2A^bwt+A^bEp and FT7.1A^bwt+A^bEp, always stimulated our T cell hybridomas more strongly than the A^bEp transfectants presenting Ep only (Figs 2-6).

Therefore, we feel that the explanation of the higher T hybridoma response to A^bwt+A^bEp than to A^bEp cells may lie in a contribution of weak TCR interaction with bystander peptides that are similar or identical to those involved in the positive selection of T lymphocytes from which these hybridomas originated. Several investigators have shown that agonist peptides can promote the positive selection of T lymphocytes *in vivo* [18, 19], especially when presented in low concentrations. Low-affinity self-reactive T cell clones may escape negative selection and peripheral tolerance [20, 21], and such clones might have given rise to our hybridomas. Antigen recognition by T cells is degenerate ([22-25] and other articles in the same issue); therefore, our hybridomas might have recognized not only the strongly agonistic Ep, but also some bystander peptide that was too weak an agonist to stimulate on its own, as seen in the lack of stimulation by A^bwt fibroblasts. Our results are in conflict with data presented by Sporri and Reis e Sousa [5], who did not observe any remarkable effect of self peptides on the T cell response to antigenic peptide. However, their results were obtained in a $CD8^+$ T cell and MHC I/peptide model, whereas our study used $CD4^+$ T cells and MHC II/peptide complexes. $CD8^+$ T cells are stained with anti-TCR or anti-CD3 antibodies more strongly than $CD4^+$ T cells are [26], and the TCR$\alpha\beta$-CD3 cell surface complexes of $CD8^+$ and $CD4^+$ T cells differ biochemically [27]. In addition, $CD4^+$ T cells appear to be more cross-reactive than $CD8^+$ T cells [22-25]. Therefore, these two

cell populations may also behave differently in their interactions with self peptides. Indeed, our results are concordant with those of Wülfing *et al.* [4], who observed an augmentation of the $CD4^+$ T cell hybridoma response to agonist peptide in the presence of an altered agonist peptide, itself unable to stimulate and thus mimicking a self peptide. Complexes of MHC II with bystander peptides, in addition to complexes with a specific antigenic peptide are present in the synapse formed between an antigen-presenting cell and an effector T lymphocyte [28]. A positive signal spreading after binding a few agonist ligands may protect neighboring TCR complexes from binding an inhibitory phosphatase, SHP-1. These protected TCRs, upon binding a self ligand (self peptide + self MHC), might contribute to effective positive downstream signals [29, 30]. In addition, upon contact of the T cell with an antigen-presenting cell, coaggregation of self ligand-bound and agonist-bound TCRs in the immunological synapse might promote a greater average occupancy with the agonist rebinding to those receptors initially recruited into the cluster by bystander ligands, facilitating signal spreading and leading to a stronger signal to the T cell. As pointed out by Štefanová *et al.* [31], "it remains to be seen if self-ligands are truly equivalent to the null (altered agonist) ligands, or if self ligands are too weak to contribute in this positive sense." Our results seem to suggest that self-ligands have sufficient strength in this respect. Interestingly, even in the absence of agonist peptides, self peptides presented by self MHC molecules weakly stimulate agonist-specific T cells, and this interaction seems to be necessary for a proper T cell response upon encounter with an agonist. This was shown by preventing T cells from contacting self peptides before stimulation with an agonist [31] or by prestimulating T cells with an altered peptide ligand [32].

In summary, the fact that three independently obtained hybridomas behaved identically in culture with two independent A^bwt+A^bEp transfectants suggests that we have observed a general phenomenon rather than a cell culture artifact. Thus, our results seem to show that naturally bound bystander peptides have a costimulatory effect on the specific response of effector T cells.

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