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Paolo Dellabona, Bing-Yuan Weia, Nadine Gervois, Christophe Benoist, Diane Mathis. A single amino acid substitution in the Ak molecule fortuitously provokes an alloresponse. *European Journal of Immunology*, 1991, 21 (1), pp.209-13. 10.1002/eji.1830210131 . inserm-03351845

**HAL Id: inserm-03351845**

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Submitted on 22 Sep 2021

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CP-240766

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## A single amino acid substitution in the A<sup>k</sup> molecule fortuitously provokes an alloresponse\*

We discovered by chance that the R28 T cell hybridoma has dual specificity. It responds to a peptide derived from ribonuclease presented by cells displaying A<sup>k</sup> molecules and it reacts, in the absence of added antigen, to cells expressing A<sup>k</sup> complexes with a single amino acid substitution at position 69 of the  $\alpha$  chain. Modelling and functional studies suggest that residue 69 is a peptide contact residue, prompting the hypothesis that R28's alloreactivity is a cross-reactive response to an unknown peptide bound in the 'groove' of the mutant A<sup>k</sup> complex. In this report, we employ a competition assay to confirm that this alloresponse involves a groove-binding peptide, demonstrate that this peptide derives from or depends on fetal calf serum and exploit a panel of antigen-presenting cell lines – each displaying an A<sup>k</sup> complex with a different position 69 substitution – to establish that the alloresponse is not just a heteroclitic response to ribonuclease, itself. We speculate that much of the alloreactivity against murine class II molecules that is revealed *in vitro* may prove to be directed at bovine serum-derived peptides, suggesting that in this context, alloreactivity is a misnomer.

### 1 Introduction

Alloreactivity is both intriguing and important. It is intriguing because as many as one in a hundred T cells is capable of making an alloresponse, *i.e.* can be stimulated by cells displaying a "foreign" MHC molecule in the apparent absence of conventional antigen. It is important because this vigorous response is the basis of both graft rejection and GVH disease, two phenomena that have remained significant medical problems.

An understanding of alloreactivity at the molecular level appears to be on the horizon. It is now well established that MHC class I and II molecules actually serve as peptide receptors [1–6]. During a conventional immune response, foreign antigen is expressed in or is taken up by host cells and is then processed to peptides; these peptides bind to host MHC molecules within a "groove"; and it is these host MHC-foreign peptide complexes that are recognized by T cells. Several independent lines of evidence indicate that

some alloresponses are just a variant of this scenario: cells displaying foreign MHC molecules contain innumerable peptide degradation products, and in addition, can take up host proteins and process them; these peptides bind in the groove of foreign MHC molecules; and it is these foreign MHC-foreign or host peptide complexes that stimulate alloreactive T cells. The most convincing arguments to support this scenario are: (a) those mutations and natural variations of MHC molecules that show the greatest effect on allostimulation are predominantly those which are predicted to influence the binding of peptides within the groove [7–14]. (b) Conventional peptide antigens can modulate an *in vitro* alloresponse, either positively or negatively, when introduced in excess [15]. (c) Most compelling, allostimulatory cells can take up exogenous peptides and subsequently provoke a T cell alloresponse ([16]; Panina-Bordignon, P. et al., submitted for publication). In one case, the exogenous peptide has actually been shown to derive from serum albumin (Panina-Bordignon, P. et al., submitted for publication).

We recently discovered a fortuitous alloresponse that underlines the importance of peptide presentation in allostimulation [17]. The T cell hybridoma R28 responds to a peptide derived from bovine ribonuclease (RNase 41-61) when it is presented by APC displaying the A<sup>k</sup> molecule; it will also react, in the absence of added antigen, to APC expressing an A<sup>k</sup> molecule with a single amino acid substitution at position 69 on the  $\alpha$  chain. Residue 69 appears to be a peptide contact residue according to Brown et al.'s modelling of the antigen-binding site on class II molecules [3] as well as by functional criteria [17]. Thus, we suggested that the mutated I-A<sup>k</sup> molecule can bind a new peptide, or an old peptide in a new way, and that R28 recognizes this mutant MHC-unknown peptide complex cross-reactively. In this report, we further characterize this fortuitous alloresponse, demonstrating that it indeed involves presentation of a groove-binding peptide, that this peptide is derived from FCS or requires FCS for its synthesis, but that it is not RNase 41-61 itself.

[I 8863]

\* This work was supported by institutional funds from the INSERM and the CNRS and by a grant to D. M. and C. B. from the Association pour la Recherche Contre le Cancer (ARC).

□ Recipient of ARC and EMBO fellowships.

△ Recipient of an INSERM fellowship.

○ Recipient of a fellowship from the Groupement des Entreprises Françaises dans la Lutte contre le Cancer.

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**Abbreviations:** RNase: Ribonuclease HEL: Hen egg lysozyme

## 2 Materials and methods

### 2.1 Mutant APC lines

The expressible  $A_{\beta}^k$  cDNA in pKCR3 has been described [18]. Wild-type and mutant  $A_{\alpha}^k$  cDNA were expressed in pKCR7 [19]. The point mutations at position 69 of  $A_{\alpha}^k$  were created by slot-machine mutagenesis essentially according to Landais et al. [19], as will be detailed elsewhere (Wei et al. in preparation).

An expressible  $A_{\alpha}$  cDNA, an expressible  $A_{\beta}^k$  cDNA and the HSV thymidine kinase (tk) gene were triply cotransfected into tk<sup>-</sup> L cells according to Landais et al. [18–20]. Transfectants expressing the tk gene were selected in HAT medium. The 10–100 independent transfectants were pooled and sorted after staining with the pan-Ia reagent 40B. All lines were carried in HAT medium, and were periodically resorted to maintain equivalent I-A<sup>k</sup> expression. At the time of each presentation experiment, an aliquot of cells from each line was stained with an anti- $A_{\beta}^k$  reagent and the level of  $A^k$  complex quantitated by FCM. Lines whose level diverged more than 4-fold from the standard line were excluded from the experiment.

### 2.2 T cell hybridoma

R28 comes from a B10.A(4R) mouse injected with the peptide RNase 41-61. It bears a  $V_{\alpha}1/V_{\beta}6$  TcR (S. Candéias, unpublished).

### 2.3 Antigens

Peptides were synthesized as described [21]. The sequence of RNase 41-61 is KPVNTFVHESLADVQAVCSQK and that of hen egg lysozyme (HEL) 46-61 is NTDGSTDYGILQINSR.

### 2.4 Antigen presentation assays

Antigen presentation assays were conducted by incubating (in duplicate)  $5 \times 10^4$  T hybridoma cells with varying

numbers of APC, or with  $5 \times 10^4$  APC in the presence of varying amounts of antigen (see figure legends for details). After a 24-h incubation, 50  $\mu$ l of SN was harvested and tested for IL2 content, using the IL2-dependent CTLL line, as described [22]. Assays were normally conducted in DMEM supplemented with 10% FCS, but serum-free medium (Gibco, Grand Island, NY) was used in some experiments (Fig. 3).

### 2.5 Competition assay

K69 cells ( $1.5 \times 10^4$ ) or  $1.5 \times 10^4$  KK cells plus a subsaturating amount (200 ng/ml) or RNase 41-61, were incubated with  $5 \times 10^4$  R28 cells. The competitor peptide HEL 46-61 was introduced at the beginning of the 24-h incubation at concentrations varying from 0–32  $\mu$ g/ml. SN were tested as usual by the CTLL assay.

## 3 Results

### 3.1 The R28 alloresponse involves presentation of a groove-binding peptide

R28 is an  $A^k$ -restricted, RNase-specific T cell hybridoma derived from a B10.A(4R) mouse (a kind gift from Dr. P. Allen). We recently discovered that this hybridoma responds, in the absence of added RNase, to APC displaying  $A^k$  molecules with an alanine substitution at position 69 of the  $\alpha$  chain (K69), but not to cells expressing wild-type  $A^k$  molecules (KK). Fig. 1A presents typical APC dose-response curves.

R28's alloresponse does not merely reflect a weak, low-affinity interaction because its stimulation by K69 cells has about the same sensitivity to APC number as its stimulation by KK cells presenting RNase peptide. Fig. 1B shows this comparison.

To determine whether the alloresponse to K69 APC involves presentation of a peptide bound in the groove, we performed the competition experiment depicted in Fig. 2. The R28 alloresponse, just like the anti-RNase response, is inhibited when excess amounts of the high-affinity groove-

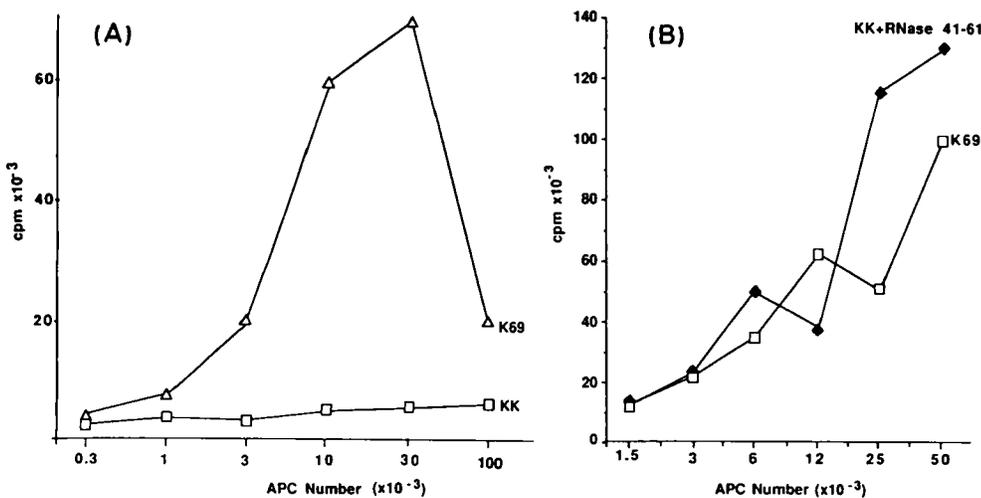
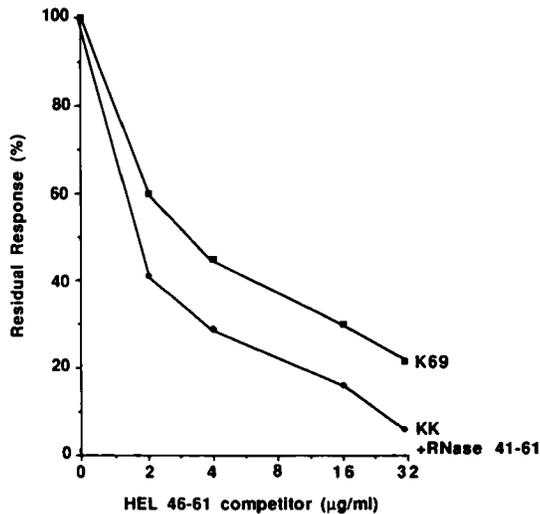


Figure 1. APC dose-response curves for R28 reactivity. (A) Reactivity of R28 against KK (wild-type) or K69 (mutant) cells in the absence of added antigen. (B) Reactivity of R28 to K69 in the absence of antigen or to KK in the presence of RNase 41-61 peptide. Increasing numbers of APC, up to  $5 \times 10^4$ , were added. RNase peptide was at 2  $\mu$ g/ml. IL2 production by R28 was measured by the incorporation of [<sup>3</sup>H]dThd by the CTLL indicator line.



**Figure 2.** Competition by a groove-binding peptide for R28 reactivity. K69 ( $1.5 \times 10^4$ ) or KK cells were incubated with R28 in the absence (K69) or presence (KK) of 200 ng/ml RNase 41-61 peptide and in the presence of increasing amounts, up to 32 µg/ml, of HEL 46-61 competitor peptide. IL2 production by R28 was assessed as in Fig. 1, and the results normalized; 100% corresponds to the response in the absence of competitor.

binding peptide HEL 46-61 are included in the presentation assay.

### 3.2 The R28 alloresponse depends on FCS

In a first attempt to uncover the origin of the allostimulatory peptide, we evaluated the influence of FCS on R28's alloresponse. As indicated by the experiment depicted in Fig. 3A, R28 is stimulated by K69 cells in an incubation medium that contains 10% FCS, but not in serum-free medium. One could argue that the alloresponse requires FCS for a more mundane reason, simply that for physiological reasons the T cell hybridoma is not competent to respond, or the APC are not competent to present, in the absence of growth factors provided by FCS. However, as demonstrated in Fig. 3B, the presentation of RNase pep-

ptide by K69 cells is readily detected, although admittedly less efficiently, in serum-free medium.

### 3.3 The allostimulatory peptide is not RNase 41-61, itself

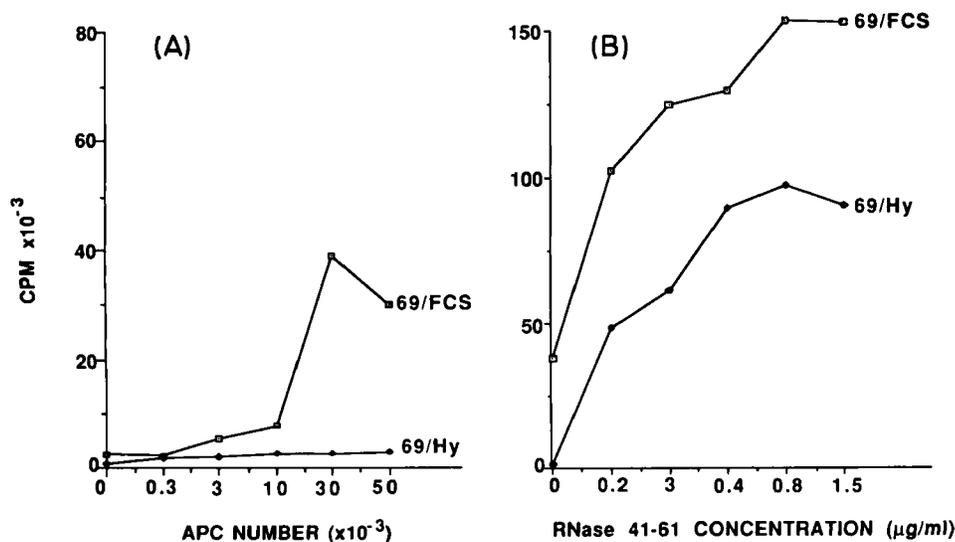
It is entirely possible that the allostimulatory peptide actually derives from RNase in the serum: R28 recognizes a peptide of bovine RNase; the FCS in the incubation medium is of bovine origin and probably contains some amount of RNase. It could be that the mutation at position 69 elicits a heteroclitic response such that low levels of ribonuclease in the incubation medium are now stimulatory. To rule out this possibility, we compared the fine structural requirements at position 69 for RNase vs. allopeptide presentation to R28.

We had on hand a panel of 11 APC lines, each expressing an A<sup>k</sup> complex with a different amino acid at position 69 of the  $\alpha$  chain (B-Y. Wei et al., in preparation). The point mutations are chemically diverse, including most of the different amino acid groups. Fig. 4A compares the ability of these APC lines to stimulate R28 in the presence and absence of RNase 41-61. The profiles are clearly distinguishable: in the presence of RNase peptide, R28 responds to K69<sup>gly</sup>, K69<sup>ala</sup>, K69<sup>ser</sup> and the wild-type K69<sup>thr</sup>; in the absence of RNase, it reacts only to K69<sup>ala</sup>.

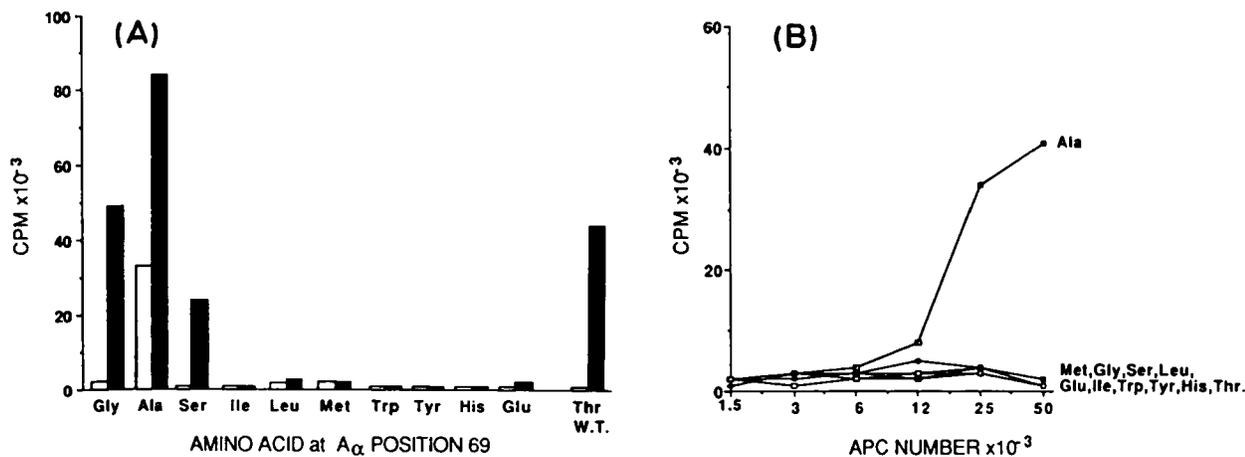
To ensure that the inability of R28 to respond to other APC in the absence of RNase was not merely a quantitative difference, we performed APC dose-response curves with each member of the panel. The results, presented in Fig. 4B, confirm that only K69<sup>ala</sup> cells can act as allostimulators.

## 4 Discussion

Our interest in the R28 alloresponse was originally piqued because there seemed to be strong evidence that residue 69 on the A<sub>α</sub> chain serves as a peptide contact residue. First, Brown et al. have modelled the antigen binding site on a class II complex based on the crystal structure of a class I



**Figure 3.** R28 stimulation in the presence or absence of FCS. (A) APC dose-response curves for K69 cells in serum-free medium or in the standard medium containing 10% FCS. Increasing numbers of APC were added, up to  $5 \times 10^4$ . (B) Antigen dose-response curves for K69 cells in the presence or absence of FCS. K69 cells ( $5 \times 10^4$ ) were added in the presence of increasing amounts of antigen, up to 3 µg/ml RNase 41-61.



**Figure 4.** Fine-structural requirements for R28 reactivity. (A) Alloresponse vs. anti-RNase response to APC expressing A<sup>k</sup> molecules with different point mutations at position 69 of A<sub>α</sub>. Of each APC 5 × 10<sup>4</sup> were incubated in the presence (black bars) or absence (white bars) of 1.5 μg/ml RNase 41-61. (B) Increasing numbers of the different APC, up to 5 × 10<sup>4</sup>, were incubated with R28 in the absence of added RNase.

molecule [3]. According to this model, the side chain of residue 69 clearly points into the groove, in an orientation amenable to direct contact with antigen. Second, we have recently attempted to delineate peptide contact residues on the class II complex by functional studies, using large panels of A<sub>α</sub><sup>k</sup> mutants and peptide analogues [17]. Residue 69 was initially suspect because an alanine substitution at this position had a measurable effect on the presentation of RNase and HEL peptides to all of the hybridomas tested. Perhaps more convincing, non responsiveness of the TS12 hybridoma to the RNase peptide presented by K69 cells could be reversed by a single amino-acid change in the peptide. These various observations suggested that the mutated A<sup>k</sup> molecules on K69 cells may be able to bind a new peptide, or an old peptide in a new way, and that the R28 TcR can fortuitously recognize this complex. By the competition experiment depicted in Fig. 2, we have now demonstrated that this is, indeed, the case. A high-affinity, groove-binding peptide (HEL 46-61) is as able to compete against the R28 alloresponse as against the RNase response.

This cross-reactive response to an unknown peptide requires FCS (Fig. 3), although we have found that simply pulsing the K69 APC for 1 h beforehand is sufficient to elicit the alloresponse from R28 in serum-free medium (data not shown). Several possible explanations come to mind: (a) the unknown peptide derives from FCS; (b) the peptide is synthesized by the APC (or T cell) but only after induction by FCS and (c) the unknown peptide is constitutively synthesized by the APC or T cell but effective presentation of it requires an FCS-inducible accessory molecule.

We do not at present have definitive evidence in favor of one of the three explanations, but arguments can be made for the first and against the last two. Arguing for the idea that the allopeptide derives from FCS is the simple fact that serum proteins are probably the "antigens" in highest concentration in the *in vitro* presentation milieu and, as well, precedent exists for such a finding (Panina-Bordignon et al., submitted). Arguing against the idea that the peptide is synthesized by K69 cells is a large body of data indicating

that, for the most part, intracellular self-proteins are presented by class I rather than class II molecules (see [23, 24] and references therein). Evidence against the hypotheses that the allopeptide is synthesized by R28 cells or that its presentation requires an FCS-induced adhesion molecule is that one can readily detect the alloresponse after "pulsing" the APC with FCS for as little as 1 h (data not shown).

An allopeptide derived from FCS would imply that the R28 "alloreactivity" might more accurately be described as "xenoreactivity". In fact, it seems logical that much of the alloreactivity against murine class II molecules that is revealed *in vitro* by MLR assays will prove to be directed at bovine serum constituents. The normal procedure in the mouse system is to derive alloreactive T cell clones or hybridomas from an MLR culture, and certainly the most concentrated extracellular proteins in such a culture are the serum proteins. In support of this speculation is the finding in several laboratories (*e.g.* [25, 26]) that a large percentage of human "autoreactive" T cells (as revealed in an autologous MLR) actually respond to FCS. It may thus be necessary to study alloreactivity in an *in vivo* context – as graft rejection or GVH disease – in order to arrive at relevant conclusions.

*We are grateful to Dr. P. Allen for the gift of R28, to Caroline Waltzinger for help with the cytofluorimetric analysis, and to Philippe Gerber and Claudine Ebel for technical assistance.*

Received August 29, 1990.

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