

## Cytokine induction by immunomodulatory epitopes in S-antigen and tumor necrosis factor alpha

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### ABSTRACT

Common epitopes on S-antigen (arrestin), a potent autoantigen inducing experimental autoimmune uveoretinitis (EAU), and on human tumor necrosis factor alpha (hTNF $\alpha$ ) are revealed with monoclonal antibodies (mAb) to S-antigen, which inhibit EAU induction. The minimal common sequence for mAb recognition is GVxLxD in the S-antigen/hTNF $\alpha$  amino acid (aa) sequences. Peptides containing this sequence motif exhibit monocyte activating capacity analogous to the autocrine stimulatory capacity of hTNF $\alpha$  itself. In S-antigen this activity is located at epitope S2 (aa residues 40 to 50), corresponding to the peptide PVDGVVLVDPE (peptide S2). In hTNF $\alpha$  the monocyte activating capacity correlates to aa residue 31 to 53, corresponding to the peptide RRANALLANGVELRDNLVVPSE (peptide RRAN). Peptide S2 but not peptide RRAN is competing for mAbs S6H8 and S2D2 binding to S-antigen. Anti-idiotypic antibodies to S2D2 compete with peptide S2 but not peptide RRAN for binding to mAbs S2D2 and S6H8. In human retinal S-antigen epitope S2 is localized at the aa residues 44-54 and is cleaved in the human peptide 4 (aa 31-50). Competition experiments with peptide 4 (aa 31-50) and peptide 5 (aa 41-60) indicate that the C-terminal aa residues VDPD in the epitope S2 play an important role for internal image recognition of the anti-idiotypic antibodies. Peptide S2 and peptide RRAN define common functional structures in the autoantigen and hTNF $\alpha$  molecules. The data suggest regulatory functions of the peptides in cytokine expression, network regulation and in autoimmunity.

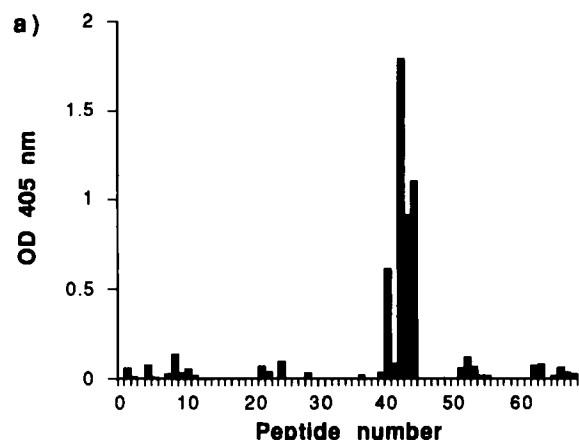
### INTRODUCTION

Experimental autoimmune uveoretinitis (EAU) is a model of ocular autoimmune disease produced in laboratory animals by immunization with retinal S-antigen (S-Ag) (1,2). S-Ag, also named arrestin or 48K protein, is a 404 aa residue soluble protein of retinal photoreceptors involved in regulation of visual transduction (3). Experiments by Y. de Kozak et al. (4-7)

revealed that a particular region of S-Ag is involved in immunoregulation of S-Ag-induced EAU. Some mAbs to S-Ag (S2D2, S6H8 and S7D6) prevent EAU when they are injected to rats at the time of immunization with S-Ag (4). Preimmunization with these mAbs also inhibits EAU induction (5), as do preimmunization with purified rat anti-idiotypic antibodies raised against mAb S2D2 (6). Lewis rats, a strain highly susceptible to S-Ag-induced EAU, have a delayed antibody response against the epitope recognized by mAb S2D2, compared with the refractory BN strain (7). The epitope recognized by these mAbs is phylogenetically conserved in the retinal S-Ag of vertebrates and invertebrates (8) and is present in S-Ag-like proteins from several non ocular cells (9,10) including brain  $\beta$ -arrestin (11).

We localized the epitope(s) (S2) recognized by mAbs S2D2, S6H8, S7D6, S8D8 and S8D1 in the N-terminus of the S-Ag sequence. Epitope S2 displays a common sequence motif with TNF $\alpha$ . In vitro experiments using synthetic peptides from bovine S-Ag (peptide S2) and from hTNF $\alpha$  (peptide RRAN) showed that these peptides stimulate TNF $\alpha$  mRNA expression in human monocytes (12), an effect analogous to the autocrine stimulatory capacity of the hTNF $\alpha$  molecule itself (13), and TNF $\alpha$  secretion. The characteristics of these epitopes were analysed in aa residue exchange experiments (14).

We summarize here the properties of the S-Ag/TNF $\alpha$  common epitopes and present new data concerning competition of anti-idiotypic S2D2 antibodies and peptides with S-Ag for binding to mAb S2D2. Materials and methods used are described in references 12 and 14.



| peptide number   | mAb recognition |
|------------------|-----------------|
| 39 E P V D G V V | —               |
| 40 P V D G V V L | +               |
| 41 V D G V V L V | —               |
| 42 D G V V L V D | +               |
| 43 G V V L V D P | +               |
| 44 V V L V D P E | +               |
| 45 V L V D P E L | —               |

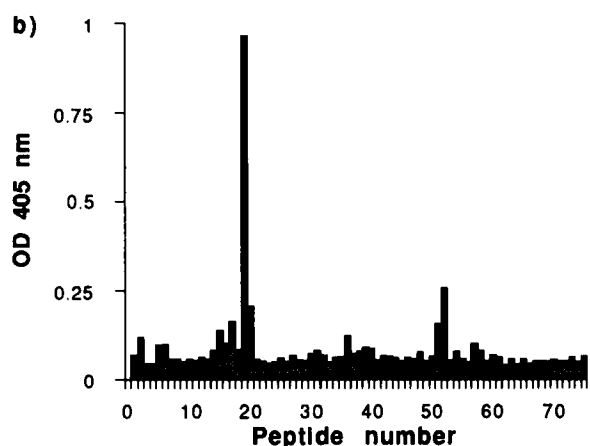


Figure 1: Immunoreactivity profile of a) 68 heptapeptides overlapping by six aa residues from bovine retinal S-Ag (N-terminal residues 1-74) and b) 75 heptapeptides overlapping by five aa residues from the complete mature hTNF $\alpha$  molecule. The S-Ag/hTNF $\alpha$  common epitopes are revealed with mAb S6H8 (1 $\mu$ g/ml) and horseradish peroxidase labeled protein A (Bio-Rad).

#### LOCALIZATION OF CROSSREACTIVE EPITOPES IN S-ANTIGEN AND HUMAN TNF $\alpha$

The phylogenetically conserved epitope(s) S2 recognized by mAbs S2D2, S6H8, S7D6, S8D8 and S8D1 was mapped to the N-terminal CNBr fragment CB 74 of bovine retinal S-Ag by different methods (7). PEPSCAN analysis allowed to compare the reactivity of 68 synthetic heptapeptides overlapping by six aa residues reproducing this 74 aa N-terminal region. Epitope S2 was localized at the aa residues 40 to 50 (PVDGVVLVDPE, peptide S2) as shown for mAb S6H8 in Figure 1 a. The immunoreactivity profile for the other mAbs was nearly identical as for the mAb S6H8. The boundaries of the epitope appear to be proline in position 40 and glutamic acid in position 50. With the disappearance of proline 40, the immunoreactivity was negligible and reappeared with the next overlapping peptide starting with aspartic acid 42 (fig. 1 a). None of the mAbs was interacting with the presently known T-cell stimulating and uveitopathogenic sites which are located in the C-terminal part of S-Ag (CB 123, CB 35, CB 46) (15-21), whereas peptide S2 exhibits no uveitopathogenicity (unpublished result, Y. de Kozak).

PEPSCAN-analysis of the hTNF $\alpha$  molecule with

Table 1: Stimulation of human TNF $\alpha$  release. TNF $\alpha$  release was determined in the supernatant of human monocyte cultures after 20 h incubation with 10  $\mu$ g/ml of the indicated preparations. TNF $\alpha$  concentrations were determined by ELISA (23).

| Preparation tested                   | TNF $\alpha$ (ng / ml) |
|--------------------------------------|------------------------|
| not stimulated                       | <1.0                   |
| S-Ag                                 | 5.4                    |
| peptide S2 (EPVDGVVLVDPE)            | 3.6                    |
| human TNF $\alpha$ peptides:         |                        |
| aa 31 - 42 (RRANALLANGVE)            | 2.5                    |
| aa 37 - 48 (LANGVELRDNQL)            | 1.9                    |
| aa 43 - 54 (LRDNQLVVPSEG)            | 1.7                    |
| aa 49 - 60 (VVPSEGLYLIYS)            | <1.0                   |
| aa 79 - 90 (TISRIVSYQTK)             | <1.0                   |
| aa 25 - 36 (QLQWLNRRANAL)            | <1.0                   |
| aa 31 - 53 (RRANALLANGVELRDNQLVVPSE) | 20.4                   |

these antibodies revealed a crossreactive epitope at the residues 39 to 45, NGVELRD (Figure 1 b), distinct from the epitope recognized by mAbs which inhibit TNF $\alpha$  cytotoxicity. The common motif in the sequences 40-50 of S-Ag (PVDGVVLVDPE) and 39-45 of hTNF $\alpha$  (NGVELRD) is GVxLxD. This crossreaction was confirmed by Western blot analysis of TNF $\alpha$  with mAbs S2D2 and S6H8. The mAbs recognized TNF $\alpha$  in both its monomeric and dimeric forms (12).

#### BIOLOGICAL ACTIVITY OF THE S-ANTIGEN AND TNF $\alpha$ COMMON EPITOPE

The common recognition of the S-Ag and TNF $\alpha$  epitopes by these mAbs suggested that the immunomodulatory properties of the S-Ag epitope S2 deduced from EAU suppression experiments could be related to regulation of TNF $\alpha$  production. TNF $\alpha$  synthesis by monocytes can be stimulated by TNF $\alpha$  itself (autocrine stimulation) (13). We studied the capacity of numerous peptides from hTNF $\alpha$ , peptide S2, recombinant hTNF $\alpha$  and native S-Ag to influence the expression of TNF $\alpha$  mRNA in the adherent fraction of human monocytes in vitro. Stimulation of TNF $\alpha$  mRNA synthesis was achieved by TNF $\alpha$ , S-Ag and peptides containing the common motif and derived from either TNF $\alpha$  (peptide RRAN) or S-Ag (peptide S2), but not by several other TNF $\alpha$  peptides (12). Peptide RRAN (aa residues 31-53, RRANALLANGVELRDNQLVVPSE), which possess the autocrine stimulatory potential of TNF $\alpha$ , is part of a larger TNF $\alpha$  peptide (aa residues 31-68) with fibroblast chemotaxis inducing capacity (22). TNF $\alpha$ , S-Ag or synthetic peptides with TNF $\alpha$  mRNA

inducing capacity were also capable to stimulate IL-1 $\beta$  mRNA expression (unpublished results). In addition, measurable amounts of hTNF $\alpha$  protein were released after stimulation of monocytes with native S-Ag and peptides (Table 1).

#### PEPTIDE INTERACTIONS WITH MONOCLONAL ANTIBODIES TO S-AG AND POLYCLONAL ANTI-IDIOTYPIC ANTIBODIES TO S2D2

We investigated two further questions: (1) Is peptide RRAN blocking the binding of mAbs S6H8 or S2D2 to S-Ag in the same way as peptide S2? (2) Are these peptides competing with purified rat anti-idiotypic antibodies (internal image of mAb S2D2) for their binding to mAb S2D2 and S6H8? Peptides employed in ELISA competition are listed in Table 2.

Figure 2 a demonstrates that only peptide S2 and not peptide RRAN impairs binding of mAb S6H8 to S-Ag. The same result was found for S2D2. Purified anti-idiotypic antibodies compete also with S2 but not with RRAN for their binding to mAbs S6H8 (Figure 2 b) and S2D2. In this type of experiment we tested four other S-Ag peptides, among which human peptide 4 (aa 31-50) is cleaving epitope S2 (localized at the aa 44-54 of human S-Ag) and peptide 5 (aa 41-60) is containing the complete epitope S2. Peptide 4, which contains only the N-terminal residues PVDGVVL of epitope S2, does not compete with the internal image, whereas peptide 5 exhibits the same competing activity as peptide S2. This indicates that the C-terminal residues VDPD of the human and/or VDPE of the bovine epitope S2 play an essential role in the internal image. The sequence of

Table 2: Peptides employed in competition ELISA

| species           | protein      | peptide name | amino acid residues | amino acid sequence   |
|-------------------|--------------|--------------|---------------------|---|
| bovine            | S-Ag         | peptide S2   | 39-50               | <u>E</u> <u>P</u> <u>V</u> <u>D</u> <u>G</u> <u>V</u> <u>V</u> <u>L</u> <u>V</u> <u>D</u> <u>P</u> <u>E</u>   |
| human             | S-Ag         | peptide 4    | 30-50               | GNRDYIDHVSQ <u>V</u> <u>Q</u> <u>P</u> <u>V</u> <u>D</u> <u>G</u> <u>V</u> <u>V</u> <u>L</u>  |
| human             | S-Ag         | peptide 5    | 40-60               | <u>Q</u> <u>V</u> <u>Q</u> <u>P</u> <u>V</u> <u>D</u> <u>G</u> <u>V</u> <u>V</u> <u>L</u> <u>V</u> <u>D</u> <u>P</u> <u>D</u> <u>L</u> <u>V</u> <u>K</u> <u>G</u> <u>K</u> <u>K</u> |
| human             | TNF $\alpha$ | peptide RRAN | 31-53               | RRAN <u>A</u> <u>L</u> <u>L</u> <u>A</u> <u>N</u> <u>G</u> <u>V</u> <u>E</u> <u>L</u> <u>R</u> <u>D</u> <u>N</u> <u>Q</u> <u>L</u> <u>V</u> <u>V</u> <u>P</u> <u>S</u> <u>E</u>     |
| control peptides: |              |              |                     |   |
| human             | S-Ag         | peptide 1    | 1-20                | MASSGKTSKSEPNHVFVKKI  |
| bovine            | S-Ag         | peptide M    | 303-320             | DTNLASSTIIKEGIDKTV  |

Underlined aa residues are identical or conservative substitutions

VDPD can be cleaved by acidic hydrolysis between aspartic acid (D) and proline (P). Two control peptides, the uveitopathogenic peptide M (aa residues 303-320) from bovine S-Ag and human peptide 1 (aa residues 1-20), had no effect in the competition experiment.

## CONCLUSIONS AND PERSPECTIVES

Bovine S-Ag, peptide S2, human TNF $\alpha$  and peptide RRAN stimulate the expression of TNF $\alpha$  and IL-1 $\beta$  mRNA and TNF $\alpha$  production in human monocytes. Thus peptide S2 and peptide RRAN define functional structures in the autoantigen and TNF $\alpha$  molecules, which might regulate cytokine expression.

However the EAU inhibitory mAbs S2D2 and S6H8 had no neutralizing effect on the induction of monocyte activation by S-Ag, peptide S2, peptide RRAN and TNF $\alpha$  for TNF $\alpha$  biosynthesis or on the cytotoxic effect of

TNF $\alpha$  in a bioassay. The idiotypic approach developed for epitope S2 will be a useful tool to characterize the cytokine inducing receptor(s).

S-Ag appears to be a member of a larger protein family. The cytokine inducing function of epitope S2, whose aa sequence is identical in  $\beta$ -arrestin (the only analog whose sequence is known at the present time) could be involved in other autoimmune diseases and/or participate as cofactor in other pathological processes. The possibility of direct cytokine induction by autoantigens deserves investigation in other models of autoimmune diseases, such as EAU and EAP induced by rhodopsin and IRBP or experimental autoimmune encephalomyelitis (EAE) induced by myelin basic protein. The mAbs which recognize the S-Ag and TNF $\alpha$  cytokine-inducing peptides should be evaluated for their influence on these autoimmune diseases and on other TNF $\alpha$ -mediated experimental diseases.

Presently human peptide RRAN, bovine peptide S2, human peptides 4 and 5 are tested for their effects on EAU induction by S-Ag immunization. The activity of these peptides on EAU induction and their influence on autoimmune network and cytokine network modulation could influence therapeutic approaches in autoimmune disease models.

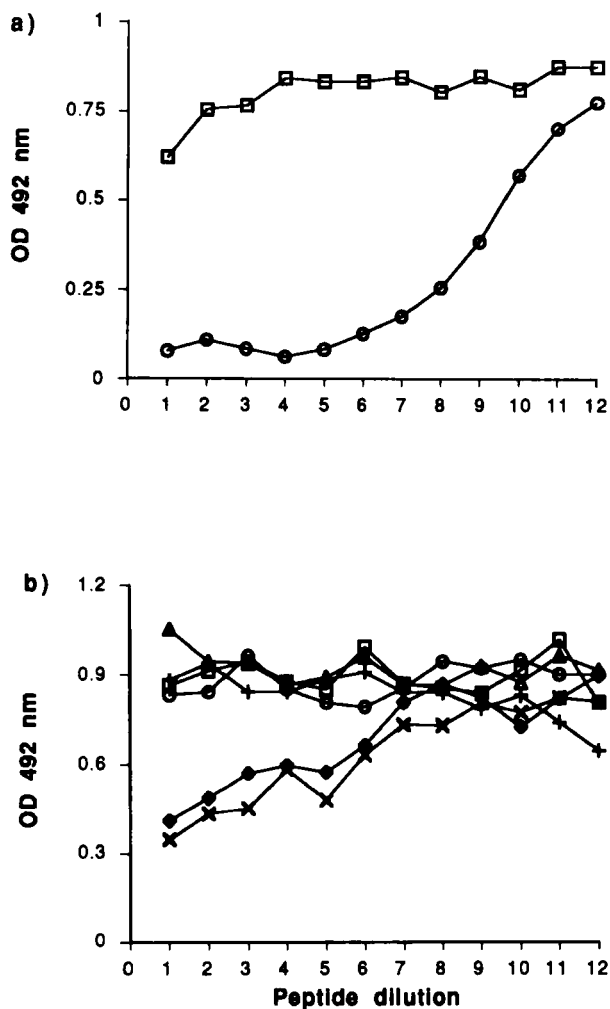


Figure 2: Competition-ELISA

a) on S-Ag coated plates (0.1  $\mu$ g/well) using a defined concentration of affinity purified mAb S6H8. MAb binding was revealed with the anti-mouse biotin/peroxidase-streptavidin-complex system (Amersham).

Competing peptides:

○ Peptide S2 (EPVDGVVLVDPE)

□ Peptide RRAN (RRANALLANGVELRDNQLVVPSE).

b) on affinity purified mAb S6H8 (0.1  $\mu$ g/well) using a defined concentration of rat-anti-idiotypic antibodies.

Anti-Id antibody binding was revealed with a peroxidase-labeled mouse anti-rat kappa chain specific mAb (MARK, Immunotech, Luminy, Marseille, France).

Competing peptides:

x peptide S2 (EPVDGVVLVDPE)

◇ peptide 5 (QVQPVVDGVVLVDPLVKGK)

○ peptide 1 (MASSGKTSKSEPNHVIFKKI)

□ peptide 4 (GNRDYIDHVSQVQPVVDGVVL)

+ peptide RRAN (RRANALLANGVELRDNQLVVPSE)

△ peptide M (DTNLASSTIIKEGIDKTV).

Competing peptides were serially twofold diluted starting with an initial peptide concentration of 1000 molar excess over the coated S-Ag or 10000 molar excess over the coated mAb S6H8.

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