Amino acid sequence requirements in the human IgAl hinge for cleavage by streptococcal IgAl proteases

B. W. Senior*†, M. R. Batten*, M. Killian† and J. M. Woof*

*Department of Molecular and Cellular Pathology, University of Dundee Medical School, Ninewells Hospital, Dundee DD1 9SY, U.K. and †Department of Medical Microbiology and Immunology, Bartholin Building, University of Aarhus, DK-8000, Aarhus C, Denmark

Abstract
All the IgAl proteases of the different pathogenic species of *Streptococcus* cleave the hinge of the α chain of human IgAl only at one proline–threonine peptide bond. In order to study the importance of these amino acids for cleavage, several hinge mutant recombinant IgAl antibodies were constructed. The mutations were found to be without major effect upon the structure or functional abilities of the antibodies. However, they had a major effect upon their sensitivity to cleavage by some of the IgAl proteases.

Introduction
The ability of some important pathogenic bacteria to breach mucosal immune defences is enhanced by their secretion of IgAl proteases. These are highly specific enzymes that cleave at a specific Pro–Ser (type 1 enzymes) or Pro–Thr (type 2 enzymes) peptide bond in one half of a duplicated octapeptide found only in the hinge of the α1 polypeptide of IgAl of humans and higher primates, thereby generating Fab and Fc fragments (see Figure 1). The bacterial pathogens that secrete IgAl proteases are pathogens of mucosal surfaces and the enzymes are believed to act as virulence factors [1–3].

A number of different pathogenic species of *Streptococcus* that are associated with meningitis, lobar pneumonia, endocarditis, dental caries and periodontal disease produce IgAl proteases of a zinc metalloprotease type and all cleave the peptide bond between Pro-227 and Thr-228 (Figure 1) [4–7].

As a preliminary step to designing inhibitors of these enzymes, a number of mutant recombinant IgAl antibodies bearing other amino acids at positions 227 and 228 were prepared (Figure 1). These were examined for the effect of the amino acid substitutions on the sensitivity of the mutant antibodies to cleavage with the different streptococcal IgAl proteases.

Effect of point mutations in the hinge region
Although the amino acid substitutions were shown to be without major effect upon the structure and functional properties of the antibodies as judged by Fc α receptor-mediated rosette formation and the induction of respiratory bursts in neutrophils, they had a major effect upon cleavage by some of the IgAl proteases.

Antibodies P227T and T228P were the most resistant of the mutant antibodies to cleavage by the streptococcal IgAl proteases. P227T was resistant to cleavage by the proteases of all the *Streptococcus sanguis* and *Streptococcus mitis* strains tested and partially resistant to that of *Streptococcus oralis*. However, it was readily cleaved by the protease of *Streptococcus pneumoniae* to give an Fab fragment of mass consistent with cleavage at or close to the 227–228 peptide bond. The mass of the fragment was quite different from the Fab fragment arising from cleavage of the...
The sequence of amino acids in the hinge of the α chain of human IgA1 and the four IgA1 mutants

The wild-type IgA1 hinge contains two identical duplicated halves, one underlined with a solid line, the other with a dotted line. The sites of cleavage of some bacterial IgA1 proteases in the wild-type IgA1 hinge are indicated above. The residues mutated in this study are boxed, and numbered at the bottom:

S. pneumoniae
S. oralis
S. mitis
S. sanguis

N. meningitidis 2
H. influenzae 2

CH1- P S T P P T P S P S T P T P S- CH2

Wildtype IgA1
P227T
T228P
T228V
T228/236V

CH1- P S T P T T P S P S T P T P S- CH2

Mutant antibody and wild-type human IgA1 with Neisseria meningitidis type 2 IgA1 protease, which cleaves at the Pro-235–Thr-236 peptide bond in the latter (Figure 1). The resistance of P227T to cleavage with the protease of S. sanguis appeared to be due to its state of glycosylation.

Antibody T228P reacted in a similar way to P227T when treated with streptococcal IgA1 proteases, except for one strain of S. mitis, which cleaved T228P but not P227T.

Antibody T228V was more sensitive to cleavage than P227T and T228P for it was cleaved not only by the protease of S. pneumoniae but also by that of S. oralis, and was also partially cleaved by those of the S. mitis strains and some S. sanguis strains. The mass of some of the Fab fragments formed indicated cleavage not only at or near Pro-227–Thr-228 but also at or near the Pro-235–Thr-236 site, where the type 2 IgA1 protease of N. meningitidis cleaves wild-type IgA1 (Figure 1).

The doubly mutated antibody T228/236V displayed a similar susceptibility to the streptococcal proteases as antibody T228V. Although it had no Pro–Thr peptide bonds in the hinge (Figure 1), nevertheless all the streptococcal proteases (except one from a strain of S. sanguis) and even the type 2 proteases of N. meningitidis and Haemophilus influenzae, which cleave wild-type IgA1 at such sites, were able to cleave it. The masses of the Fab fragments formed were consistent for the streptococcal IgA1 proteases with cleavage at or near Pro-227–Thr-228 and also, for some of them, at or near Pro-235–Thr-236, the cleavage site in wild-type IgA1 for the type 2 IgA1 proteases of N. meningitidis and H. influenzae (Figure 1).

Conclusions

Differences in the sensitivity of the various streptococcal IgA1 proteases to mutations at residues 227 and 228 in the IgA1 hinge were noted. The IgA1 protease of S. pneumoniae showed no absolute requirement for either proline or threonine at positions 227 and 228, whereas those of S. oralis, S. sanguis and S. mitis strains had an absolute requirement for proline at 227 but not for threonine at 228, which could be substituted with valine. The IgA1 proteases of some strains of S. mitis and S. sanguis differed in the amino acids they required to be present in the hinge for cleavage. However, when valine replaced threonine at position 228, it was found that the IgA1 proteases of some streptococcal strains had the
ability to cleave this mutant antibody not only at 227–228 but also at or close to the Pro-235–Thr-236 peptide bond, which is the specific cleavage site in wild-type IgA1 for type 2 IgA1 proteases of *N. meningitidis* and *H. influenzae*.

References


Received 10 April 2002

Targeted cytokine delivery to neuroblastoma


Medical Oncology, Paterson Institute for Cancer Research, Wilmslow Road, Withington, Manchester M20 4BX, UK.

Abstract

The aim of this study was to construct a fusion protein from the cytokine granulocyte/macrophage colony-stimulating factor (GM-CSF) and a single-chain Fv fragment (scFv D29) and to investigate its potential to activate cells of the immune system against neuroblastoma cells expressing neural cell adhesion molecule (NCAM). Mammalian cell expression of the scFv D29-GM-CSF fusion protein was compared using a number of vectors, including retroviral and adenoviral vectors. The resultant fusion protein, expressed by HeLa cells, was found by ELISA to bind immobilized recombinant NCAM. Moreover, FACS analysis confirmed binding to the human neuroblastoma cell line SKNBE and a murine neuroblastoma cell line engineered to express the glycosylphosphatidylinositol form of human NCAM (N2A-rKNIE). The fusion protein was also found to stimulate the proliferation of the FDC-P1 haemopoietic cell line, which is dependent on GM-CSF (or interleukin 3) for continued growth. *In vitro* clonogenic assays indicated that scFv-GM-CSF could selectively induce growth inhibition of SKNBE cells by murine lymphoid cells.

Introduction

Neuroblastoma is the most common solid malignant tumour in children after brain tumours, accounting for 40% of solid tumours presenting in the first 4 years of life. Neuroblastomas are derived from the neural crest, and have heterogeneous biological, genetic and morphological characteristics. The majority of patients that are diagnosed with neuroblastoma present at stage 4 (of the International Neuroblastoma Staging System), with metastatic disease that may initially respond to therapy, but which has a high probability of recurring. These patients have a low overall survival rate (less than 30%), despite undergoing surgery and moderately aggressive forms of cytotoxic therapy [1].

Recent advances in the fields of antibody engineering and production have led to the design and use of a number of monoclonal antibodies for cancer therapy; for example, Herceptin, Mylotarg, Rituximab and Infliximab. In addition, systemic treatment with cytokines such as interleukin 2 and granulocyte/macrophage colony-stimulating factor (GM-CSF) has been shown to increase the immunogenicity of tumours. However, frequent side effects associated with toxicity limit the effectiveness of cytokine therapy *in vivo*. The conjugation of cytokines with monoclonal antibodies has been demonstrated to concentrate cytokines within the tumour microenvironment, thereby inducing tumour-specific