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Therapeutic cleavage of IgG - new avenues for treating inflammation

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Abstract

Autoantibodies developing in humans contribute to the pathogenesis of several diseases and injected therapeutic antibodies can also trigger adverse side effects. An efficient and rapid elimination of these antibodies are therefore critically needed. Antibody removal by plasmapheresis and immunoadsorption are commonly used methods but have their own limitations. Bacterial enzymes that can cleave IgG molecules or remove carbohydrate moieties to ameliorate their immunogenicity or effector functions *in vivo* offer new avenues for drug development. Recent discoveries highlight the possibility of cleaving or modifying IgG *in vivo* by injection of enzymes. The possibility to modify IgG structures *in vivo* or *ex vivo* opens up new therapeutic possibilities not only for pathogenic antibody-mediated inflammatory diseases but also harmful effects caused by transplantations or treatment with “biologicals”.

Rationale

B cells antibodies and immune complexes are implicated in the pathogenesis of various inflammatory diseases. Similarly, alloantibodies have been shown to contribute to both early and late graft loss [1] and antibodies are increasingly used as therapeutics. Hence treatments targeting antibodies, present new possibilities for the treatment of inflammatory disorders such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), myasthenia gravis (MG), idiopathic thrombocytopenic purpura (ITP), hemophilia, pemphigus vulgaris (PV), Grave's disease (GD) and organ-allograft rejection.

B cell depletion therapy

The importance of B cells in autoimmune diseases has been well documented [2-4]. B cells contribute to the disease process as antigen presenting cells, through costimulatory functions (surface molecules, secreted cytokines and interaction with chemokines) as well as by secreting pathogenic antibodies. Recent success in B cell depletion therapies especially using anti-CD20 antibodies (rituximab) in autoimmune diseases for example RA and SLE is encouraging [5-8]. However, it takes a long time before the antibody levels in serum decreases and an ameliorative effect on disease is seen. Antibodies remains in circulation, probably produced by long-lived plasma cells in the bone marrow [9] where they have to receive survival signals from stromal cells [10]. FcγRIIB was found to be critical for prevention of generation and expansion of autoreactive antibody secreting IgG positive plasma cells [11] and cross-linking this inhibitory receptor on plasma cells in the bone marrow to induce apoptosis has been suggested as a means to reduce the pathogenic antibodies in circulation [12, 13].

Antibody functions and autoimmunity

The antibody (humoral) response in vertebrates is produced against foreign structures and is intended for the protection of hosts against invading pathogenic microorganisms but excessive activation to overcome self-tolerance or misguided immune activation leads to generation of self-reactive antibodies that are potentially pathogenic. It is clear that the presence of autoantibodies precedes the onset of autoimmune diseases in humans, for example in type 1 diabetes and arthritis [6, 14]. Apart from being diagnostic and prognostic tools in several disease conditions, antibodies have several vital functions in the humoral immune and inflammatory response of the host: interacting with Fc receptors (FcR) and complement components, activation of phagocytes, modulation of B cell functions (memory induction, activation and/or feedback inhibition of antibody production), neutralization of toxins, antibody-dependent cellular cytotoxicity (ADCC), activation of natural killer cells and eosinophils, modulation of dendritic cell function, mast cell degranulation and opsonization of antigens leading to enhanced phagocytosis.

The role of antibodies in autoimmune diseases and in biological treatments is the subject of growing interest and has led to the need for understanding the effector functions of antibodies *in vivo*. Antibodies as a constituent of immune complexes play a pivotal role in triggering several inflammatory processes. Multimeric complexes formed by antibodies binding to self-antigens can initiate an inflammatory attack by destabilizing the tissue, possibly by release of tissue degrading enzymes and cytokines by the infiltrating cells [15]. The bound antibodies can trigger and enhance inflammation by optimal activation of the complement cascade and/or FcγR-bearing cells. Complement

fragments binding to immune complexes, target tissue damage, and FcγR cross-linking can activate local mononuclear cells that in turn release proinflammatory cytokines in or near the target tissue inducing neutrophil and macrophage recruitment. These phagocytes can be further activated and create a pro-inflammatory cytokine milieu that can affect the activities of resident cell populations present in the target organs. Release of granules containing many tissue-degrading enzymes and, reactive oxygen and nitrogen radicals by macrophages and neutrophils can amplify their responses thereby forming a vicious cycle damaging the end organs and so cause clinical pathology. Thus, antibodies binding to human self-antigens contribute to the pathogenesis that can lead to severe clinical manifestations.

Antibodies can also directly cause the destruction of their target tissue preceding and independent of disease development and in the absence of any other pathogenic inflammatory factors or the action of immunocytes. In RA, antibodies reactive to type II collagen in the cartilage matrix are directly pathogenic even in the absence of inflammatory mediators [15]. For instance they can impair cartilage formation [16], strongly inhibit collagen fibrillogenesis [17], disrupt collagen fibrils in the extracellular matrix with or without increased matrix synthesis [18], and have deleterious effects on the pre-formed cartilage [19] which all suggest that chondrocyte-reactive antibodies might play an important pathogenic role leading to irreversible cartilage damage in RA. Similarly, IgG autoantibodies from pemphigus patients can exert a direct effect by binding to epidermal cells and this is mediated by steric hindrance and/or by triggering the transduction of a signal to the cell, thereby disrupting structures that maintain cell–

cell or cell–matrix adhesion in the skin [20, 21]. These findings showed that the antibodies alone could initiate the pathogenic events even before the inflammatory phase of the disease.

Similarly, neutralizing IgG antibodies present in up to 50% of hemophilia A patients inactivate and neutralize the therapeutic administration of exogenous factor VIII by sterically hindering its interaction with molecules of the coagulation cascade, or by forming immune complexes leading to accelerated clearance from the circulation [22]. Table 1 shows a list of autoimmune diseases in which IgG plays a major role in mediating the disease processes. Elimination of such pathogenic IgG from circulation in these autoimmune patients becomes all the more vital for remission of the clinical pathology.

Removal of circulating antibodies and limitations of current methods

Antibodies as therapeutic targets gained ground with several clinical and pre-clinical (animal models) observations demonstrating the pathogenic capacity of antibodies to induce a variety of autoimmune diseases. Removal of circulating immune complexes and pathogenic antibodies by therapeutic plasmapheresis or immunoadsorption [23] in many autoimmune diseases proved to be beneficial but are expensive, cumbersome for the patients and takes time. Plasmapheresis is limited by its non-selective removal of all plasma components and requires plasma product replacement such as human albumin or fresh frozen plasma. Although immunoadsorption techniques aim to provide more specific elimination of antibodies with fewer side effects compared to plasmapheresis, extensive immunoadsorption is unavoidable. The number of patients treated by

immunoabsorption also remains small due to a lack of well-defined controlled trials and limited benefits. Moreover the biomaterials used in apheresis, activated complement components and heightened the risk of hemolysis, thrombosis and infections [24, 25]. However, current biocompatible materials (dextran sulfate, protein A – silica or – sepharose, tryptophan, phenylalanine, and Ig – Therasorb columns) seem able to avoid these adverse phenomena. Furthermore, it is important to note that antibodies are more or less evenly distributed both in the intra- and the extravascular compartments and inflammatory processes often occur in the tissue but not in the vasculature. Hence, elimination of antibodies from the circulation alone is not sufficient to control the inflammatory processes [26]. Moreover, repeated and prolonged treatments are absolutely needed to avoid redistribution of pathogenic autoantibodies to the target tissues, although concomitant intravenous immunoglobulin injection might mitigate the effect of Ig adsorption. However, complete elimination of immunoglobulin might lead to immunodeficiency resulting in severe opportunistic infections. Hence, studies focused on developing tools for the transient elimination of antibodies are crucial for treating autoantibody-mediated pathologies.

Exploiting bacterial enzymes for the control of autoimmune diseases

Agents which directly target pathogenic antibodies and so mimic the strategy evolved by infectious microorganisms in trying to avoid the immune system is an interesting therapeutic concept. Diversion of the adaptive immune system by cleaving antibodies is a common mechanism utilized by many pathogenic bacterial species and several microbial extracellular enzymes with immunomodulating activities have been identified [27]. The Gram-positive bacterium *Streptococcus pyogenes* is a common human pathogen, often

causing relatively mild clinical conditions such as pharyngitis, scarlet fever and impetigo. However, invasive strains can penetrate into deeper tissues and cause severe or life-threatening infections such as necrotizing fasciitis, sepsis and streptococcal toxic shock syndrome [28]. The IgG degrading enzyme of *S. pyogenes* (IdeS) (also designated as Mac1) is a cysteine endopeptidase, secreted by group A streptococcal strains during infection. It cleaves the heavy chains of IgG with a unique specificity by binding and cleaving in the hinge region (Fig.1A and 2B), thus generating an Fc and an F(ab')₂ fragment [29, 30] that can be detected by protein G capture and mass spectrometry [31]. By removing the Fc section from the antigen recognizing Fab, immune responses such as complement deposition, and Fc-mediated phagocytosis are blocked. This IgG proteolytic degradation disables opsonophagocytosis and interferes with the killing of group A Streptococcus [28]. Most likely, IdeS bestows a local protective effect for the bacteria. However, the unique specificity and ability of IdeS to cleave all human IgG [28] *in vivo* also provides a possibility to target pathogenic antibodies. Most importantly, IdeS has a higher degree of specificity for IgG than previously described proteinases with proteolytic activity towards immunoglobulins, e.g. papain, pepsin or streptococcal pyrogenic exotoxin B [29, 30] In the mouse, only IgG2a, IgG2c and IgG3 are cleaved and injections of IdeS showed a rapid, specific and efficient cleavage of these subclasses of IgG antibodies [32]. In fact, antibodies inducing arthritis could be inhibited *in vivo* by IdeS treatment (Fig.2A and 2B) in experimental murine models (collagen induced arthritis; CIA and collagen antibody induced arthritis; CAIA) of RA [32]. IdeS treatment reduced the severity of arthritis by cleaving both circulating and cartilage bound antibodies if administered within 24 hours after the onset of clinical arthritis, but did not

block ongoing severe arthritis. IdeS treatment also significantly prevented antibody induced relapse in mice that had chronic arthritis, and delayed the onset and reduced the severity of arthritis in classic CIA. However, cleavage of BCR by “IgG specific” IdeS has not been clearly ascertained, but it should be noted that early B cell progenitors are IgM-expressing and the resurgence of IgG2a antibody levels after IdeS treatment in CIA [32] do however argue against the action of IdeS on BCR. It should also be mentioned that that this IgG cleaving therapy will not directly affect plasma cells as they do not express BCR. However, in these experiments, all of the IgG2a/c and IgG3 antibodies were cleaved and removed, and also replaced after IdeS had been cleared from the circulation, thereby avoiding the permanent elimination of any potentially protective antibodies. Furthermore, it is also possible that IdeS generated Fab fragments of autoreactive antibodies would still bind to and block their antigens but not elicit an effector response to the target. Similarly, the free Fc fragments could block Fc receptors on phagocytes (similar to 2.4G2) and so could prevent inflammation. Streptokinase, another proteolytic enzyme of *S. pyogenes*, has been used as a therapeutic (thrombolytic) agent in humans for decades demonstrating the safety of using bacterial enzymes for therapy. Hence, IdeS treatment could be a new strategy to cleave pathogenic IgG.

Yet another strategy, which targets IgG, is by the removal of carbohydrate moieties with bacterial enzymes. Post-translational modifications, especially glycosylation, have important effects on the structure and biological properties of glycoproteins such as immunoglobulins [33-35]. IgG molecules are mainly glycosylated through covalent attachment at Asn-297 of the CH2 domain within the Fc region with variable

galactosylation but limited sialylation. The remainder of the glycosylation occurs in the hypervariable regions of the Fab. These complex biantennary-type oligosaccharides attached to IgG are essential for effector functions mediated through Fc receptors and complement C1q [36, 37], apart from being critically involved in maintaining the structural integrity of the antibody [38]. X-ray crystallographic studies have revealed multiple non-covalent interactions between the sugar and protein resulting in reciprocal influences on their structural conformation [38, 39]. Modifications in these oligosaccharides affect susceptibility to proteolytic degradation, clearance rate, ADCC and complement dependent cytotoxicity [35, 40]. De-fucosylation on the N297 -linked glycan in the Fc part of the antibody increases its binding capacity significantly to activatory Fc γ RIV in mice and Fc γ RIIA in humans, and also enhanced ADCC activity suggesting the importance of glyco-engineering of antibodies for improved therapy [41-44]. On the other hand, recent studies demonstrated that higher Fc sialylation of IgG leads to its anti-inflammatory [45, 46] as well as reduced pathogenic [47] properties apart from decreased ADCC activity [48]. However, exactly how the oligosaccharides influence the Fc structure and function remains to be elucidated.

Recently, a novel secreted endo- β -*N*-acetylglucosaminidase, a member of the glycosyl hydrolases of family 18 (FGH18) in *S. pyogenes* (EndoS), which specifically hydrolyzes the β -1,4-di-*N*-acetylchitobiose core of the asparagine-linked glycan of human IgG was identified [27]. EndoS has similarities to endo- β -*N*-acetylglucosaminidases that cleave the β 1–4 linkage between the two *N*-acetylglucosamines found in the core of the N-linked glycan of IgG (Fig.1B and 2C). EndoS exclusively hydrolyzes the complex-type

biantennary glycan on the heavy γ -chain of native IgG [27]. Endoglycosidase activity on the IgG molecule by EndoS alters its function through impaired Fc γ R binding as well as decreased activation of the classical pathway of complement, which ultimately leads to increased bacterial survival in human blood. Recent results also demonstrated the effect of specific removal of carbohydrate moieties from IgG by EndoS, which results in reduced binding to Fc γ Rs and formation of less stable immune complexes, and therefore inhibiting experimental arthritis induction [49].

Concluding remarks

The targeted cleavage of pathogenic antibodies with a rapid and efficient destruction of the antibody effector mechanisms would be highly beneficial in several types of disorders and clinical conditions. Patients who have antibodies that are agonistic (e.g. Grave's disease), neutralizing (acquired FVIII deficiency and myasthenia gravis), complement and macrophage activating (Goodpasture's syndrome and autoimmune bullous skin diseases), depleting (autoimmune hemolytic anemia, idiopathic thrombocytopenic purpura) or immune complex associated (RA, systemic lupus erythematosus) could be benefited by treatments targeting elimination of pathogenic IgG. There are also situations created by modern health care in which antibodies may play a pathogenic role. For instance during acute rejection of transplants, antibodies play an important role and there is a need for an immediate reversal of their effect [1, 50, 51]. Likewise, the expanding use of various therapeutic antibodies such as rituximab (anti-CD20) warrants the possibility of immediately blocking their actions in case of unforeseen or severe side effects. A dramatic recent example is the severe adverse effects observed in a phase I clinical trial of an activatory anti-CD28 mAb [52], where the rapid removal of the mAb could have

ameliorated the symptoms. Interestingly, similar to IgG cleaving bacterial enzymes, recent studies with proteases from schistosoma parasites demonstrated human IgE-Fc cleavage [53], which presents possibilities for treating IgE-mediated allergic conditions.

Bacterial enzyme(s) that can cleave IgG transiently or involved in selective removal of carbohydrate moieties thus provide new avenues for blocking pathogenic antibodies *in vivo*. However, antibodies generated against these enzymes could pose problems in treating the patients [54]. Hence, further analyses are required to determine the efficacy of these enzymes in cleaving the substrate in the presence of such neutralizing antibodies. Similarly, construction of fusion proteins with active component of the enzyme that induced negligible amounts of neutralizing antibodies will be useful for clinical applications.

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Figure legends

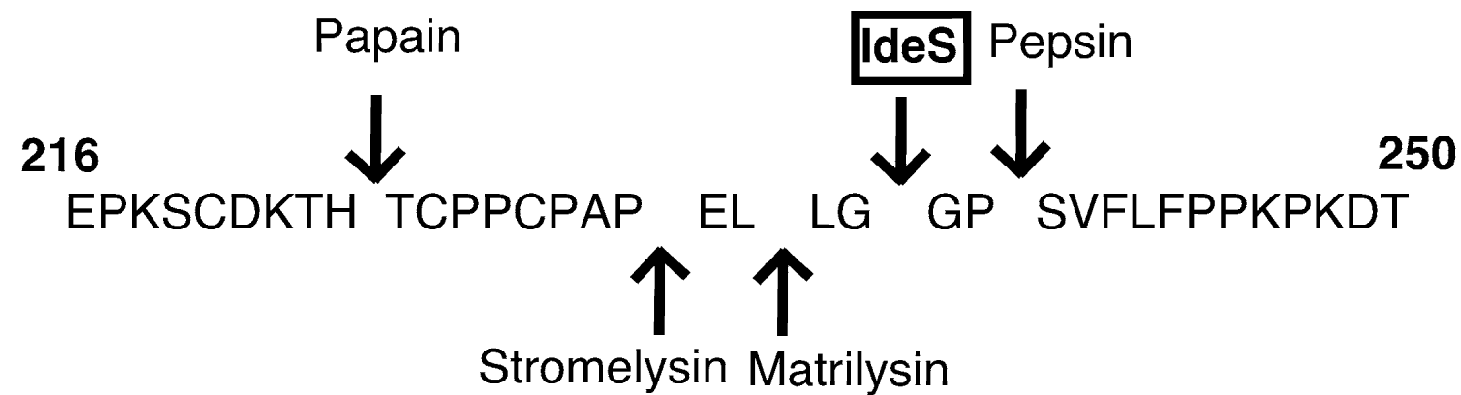
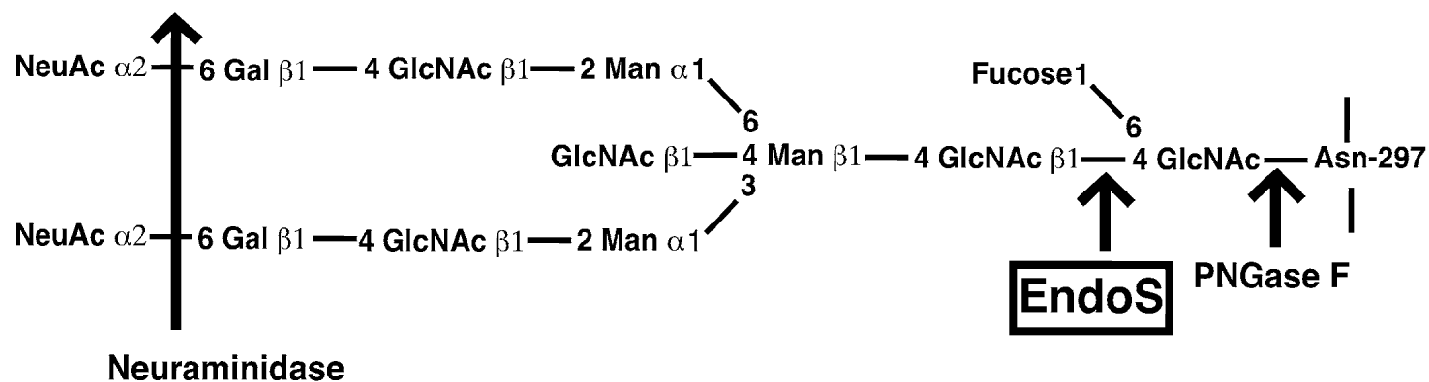
Figure 1: IdeS and EndoS cleavage sites on IgG molecule. (A) Amino acid sequence of the constant region of human IgG1 heavy chain with IdeS cleaving site. Other major protease cleavage sites are also shown for comparison. (B) The oligosaccharide attached to asparagine 297 in the antibody Fc fragment is of the complex biantennary type. EndoS hydrolyses the chitobiose core of the glycan. After EndoS activity, innermost N-acetyl glycosamine is still left attached with Asn-297 with a core fucose moiety. Cleavage sites for peptide-N-glycosidase F (PNGase F) and neuraminidase are also shown. GlcNAc, *N*-acetylglucosamine; Fuc, fucose; Man, mannose; Gal, galactose; NeuAc, sialic acid (source [27, 30, 55]).

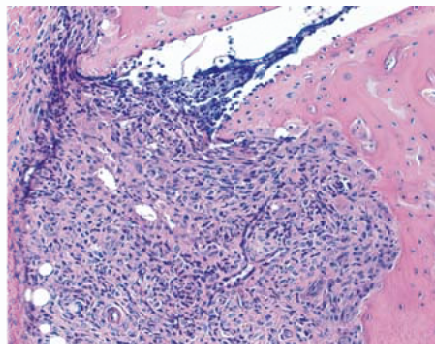
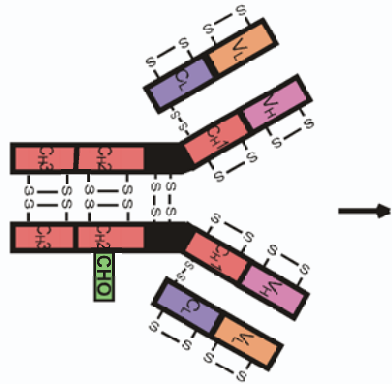
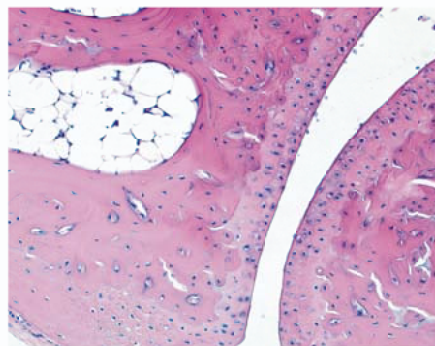
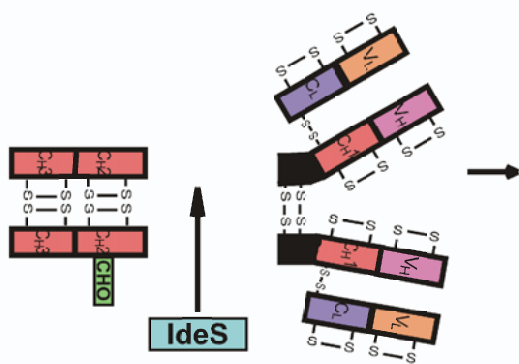
Figure 2: Effect of IdeS and EndoS cleavage on IgG and experimental arthritis (CAIA). (A) Untreated IgG antibodies induced arthritis in naïve mice, (B) IdeS treatment cleaved IgG into an Fc and an F(ab')₂ fragment leading to blocking of arthritis induction and (C) EndoS treatment cleaved carbohydrate moieties after fucose in the CH₂ domain of IgG abrogating IgG arthritogenicity. CHO, carbohydrate. Representative pictures of mouse ankle joint sections stained with hematoxylin and eosin. Original magnification x 10. (source [32, 49]).

Table1: Diseases with IgG mediated pathology

Autoimmune disease	Clinical condition	Target (s)
Graves' or Basedows' disease	Hyperthyroidism	Thyroid gland (TSHR)
Acquired FVIII deficiency	Hemophilia	Factor VIII
Myasthenia gravis	Muscle weakness and fatiguability	Skeletal muscle (AchR)
Goodpasture's syndrome	Glomerulonephritis, Pneumonitis	Lung, Kidney (Collagen type IV)
Autoimmune hemolytic anemia	cytopenia	Red blood cells
Idiopathic thrombocytopenia	cytopenia	Platelets
Autoimmune neutropenias	cytopenia	Neutrophils
Mixed cryoglobulinemia	Purpura, Nephritis, Neuritis	Sometimes associated with hepatitis C infection
Systemic lupus erythematosus	Discoid lupus, Nephritis, Anemia	Systemic (dsDNA, ssDNA)
ANCA associated vasculitis	Purpura, arthritis, Hemoptysis	Vasculature
Rheumatoid arthritis	Inflammation in articular joints	Joints
Sjögren's syndrome	Inflammation in lacrimal glands	Salivary gland. RNP proteins Ro (SSA), La (SSB)
Antiphospholipid syndrome	Blood clotting disorder	Vasculature (coagulation factors)
Pemphigus diseases	Bullous skin diseases	Skin, Mucous membranes, Keratinocytes
Complete congenital heart block	Cardiovascular disease	RNP proteins Ro (SSA), La (SSB)
Primary progressive Multiple Sclerosis	Neurological disorder	Myelin oligodendrocyte glycoprotein
Chronic Urticaria	Cutaneous mast cell degranulation	Alpha-subunit of the IgE receptor

TSHR-Thyroid stimulating hormone receptor; AchR-Acetyl choline receptor; ANCA-Anti-neutrophil cytoplasmic antibodies; RNP- Ribonuclear proteins

A**B**

A**B****C**