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review

The structural requirements for complement activation by IgG: does it hinge on the hinge?

Ole Henrik Brekke, Terje E. Michaelsen and Inger Sandlie

The flexibility of antibody molecules principally derives from the structure of the hinge region. It has generally been accepted that the flexibility of the IgG hinge is necessary for complement activation to occur; however, recent studies dispute this premise. As described here by Ole Henrik Brekke, Terje Michaelsen and Inger Sandlie, it now appears that the only requirement of the hinge region for complement activation is the presence of inter-heavy-chain disulfide bond(s). Furthermore, the structural basis for the differences between IgG subclasses with respect to effector functions appear to be located within the C_H2 domain of the immunoglobulin molecule.

Antibodies are flexible molecules; such flexibility may derive from waving, rotation or 'elbow bending' of the Fab arms, or 'wagging' of the Fc region (Fig. 1). The structure primarily responsible for the internal flexibility of antibodies is located between the first (C_H1) and second (C_H2) domains of the heavy (H) chain constant region, and is termed the hinge. Physiologically, antibody molecules are adaptors that link the target antigen to the effector components and, thereby, mediate elimination of the target in a variety of situations. The hinge-associated flexibility of IgG has generally been considered a prerequisite for this simultaneous binding of antigen and effector molecules. However, recent studies from several investigators now question this view.

The hinge: a three-part structure

The upper hinge

The upper hinge is defined as the peptide region that stretches from the N-terminal end of the C_H1 domain to the first Cys residue in the hinge that forms an inter-H-chain disulfide bond[1] (Table 1). In IgG1, this is formed by Cys226. Another disulfide bond is formed between the H-chain Cys220 residue and the light (L) chain. Residues 221–225 in IgG1 form an open-turn helix, which is a solvent-accessible structure with low structural stability. Thus, this part of the hinge provides flexibility to the Fab arms[2,3]. Domain shuffling experiments have indicated that interactions between the hinge and the C_H1 region can affect this flexibility[4]. Interestingly, recent nuclear magnetic resonance (NMR) studies of the hinge region of intact mouse IgG2a molecules have shown that the flexibility of the upper hinge has a 'mosaic' structure[5]: the N-terminal portion of the upper hinge is rigid, whereas the C-terminal part is flexible. Furthermore, the rigidity of the N-terminal portion was shown to be influenced by the presence of the C_H1 domains, since it became flexible when C_H1 was deleted. The loop formed by residues 131–139 in the C_H1 domain makes several van der Waals contacts with the N-terminal upper hinge[3].

The middle hinge

The middle hinge stretches from the C-terminal end of the upper hinge to Ala231, and contains a variable number of Cys residues that form the inter-H-chain disulfide bonds responsible for the rigidity of the middle hinge. The Cys residues are surrounded by a high number of Pro residues, which form a polyproline helix[2,3]. This rod-shaped structure provides space between the Fab arms and the Fc region[6]. The upper and middle hinge are encoded by separate exons and are termed the 'genetic hinge'.

The lower hinge

The lower hinge is coded by the C_H2 exon. In X-ray crystallographic studies[3] of the IgG1 (λ) human myeloma protein Kol, no significant electron density of the Fc portion is visible beyond Ala231 (see Table 1). Furthermore, in the crystal structure of the Fc fragment of human IgG, no interpretable electron density could be demonstrated for the lower hinge[7]. This is probably due to a significant degree of motion in this region of the molecule, and suggests that the lower hinge mediates flexibility to the Fc as shown by NMR studies[5]. The lower hinge is a solvent-accessible structure and exhibits interaction sites for FcγR (Ref. 8).

Hinge regions of the human IgG isotypes

The most striking difference between the four human IgG isotypes lies in the upper and middle hinge regions. The IgG1, IgG2 and IgG4 genetic hinges are all

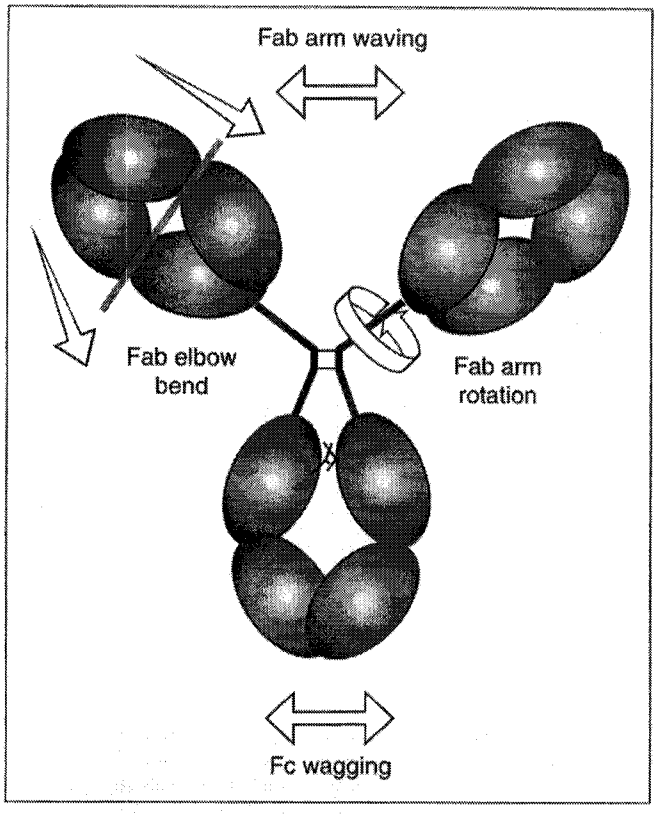

Fig. 1. *Modes of flexibility in the IgG molecule.*

encoded by one exon, yielding either 12 (IgG2 and IgG4) or 15 (IgG1) amino acids (Table 1). By contrast, the genetic hinge of IgG3 is encoded by four separate exons, yielding 62 amino acids. The first exon of the IgG3 hinge encodes 17 amino acids and the second, third and fourth exons yield 15 amino acids each and are identical triplicates of a 45 bp unit[9,10].

The relative ability of isotypes to activate complement

Complement-mediated lysis (CML) of target cells is initiated by the binding of two or more IgGs to the surface of the target cell, followed by multivalent interaction between the IgGs and C1q. C1q is part of C1, the first component of the complement cascade. In order for C1 to be activated, two or more of the globular head regions of C1q have to bind to the C_H2 domains of IgG. C1q binds complexed IgG with a K_a in the order of $10^8 M^{-1}$, while the binding constant for the monovalent binding of C1q with monomeric IgG is in the order of $10^4 M^{-1}$. Thus, the basis of the control mechanism for C1 activation is presumed to be the distortional change that occurs in the C1q structure upon binding complexed IgG, this change leading to activation of the C1 complex[11].

Protein engineering studies of mouse IgG2b suggest that three defined residues comprise the essential binding motif for C1q, and that all IgG isotypes sequenced to date contain this core C1q-binding motif in their C_H2 domain. In most isotypes, the binding motif is Glu318, Lys320 and Lys322; however, mouse IgG1 has Arg322, rat IgG1 has Thr318 and Arg320, and rat IgG2a has Thr318 (Ref. 12). All of these motifs are functional in binding C1q when mutated into mouse IgG2b. Since antibody subclasses show different abilities to bind C1q and activate complement, the context of the motif is of obvious importance. Of the human IgGs, IgG1 and IgG3 are very effective at activating complement, IgG2 is only effective at high concentration and IgG4 is ineffective[13–16].

Table 1. The amino acid sequences of human IgG hinge regions[a]

	Upper hinge 216	Middle hinge	Lower hinge 231
IgG1	EPKSCDKTHT	CPPCP	APELLGGP
IgG2	ERK	CCVECPPCP	APPVA GP
IgG3	ELKTPLGDTTHT	CPRCP (EPKSCDTPPPCPRCP)$_{X3}$	APELLGGP
IgG4	ESKYGPP	CPSCP	APEFLGGP

[a]The upper and middle hinge are encoded by separate exons, whereas the lower hinge is encoded by the C_H2 exon. Numbers 216 and 231 refer to the amino acid sequence position of the beginning of the upper and lower hinge, respectively.

The role of the hinge in complement activation

What is the basis for the inability of human IgG4 molecules to bind and activate complement, and the poor ability of mouse IgG1 to bind C1q? Fluorescence depolarization studies, which measure molecular motion at the hinge, have revealed that the flexibility of a matched set of anti-dansyl antibodies of different isotypes, and from different species, depends on the length of the upper hinge[17]. Human IgG4 and mouse IgG1 both have short upper hinges and were found to have limited movement of their Fab arms. Furthermore, in general, this flexibility was found to correlate with the ability to fix complement[17,18]; that is, naturally occurring IgG molecules with short and rigid hinges show a reduced ability to fix complement. Indeed, the well-characterized human IgG1 myeloma proteins Dob and Mcg, which lack effector functions[19,20], have no hinge region, but still make a tetrameric structure of two H and two L chains due to a disulfide bond between the L chains. This forces these antibodies to adopt a T-shaped conformation, and the Fab arms appear to obstruct the docking of C1q (Ref. 20). Taken together, these results have led to the view that the hinge region might be modulating effector functions for the following reasons: (1) the flexibility of the hinge may be necessary for the Fc to assume a conformation that is favorable for complement fixation by affecting the ability of the Fab arms to 'wave' or the Fc to 'wag'[18]. (2) The mobility of the Fab arms may be necessary to position them relative to the Fc such that they do not cover residues important for the interaction of effector molecules. Thus, restricted hinges may position the Fab arms so as to obstruct this interaction[1]. (3) Similarly, the spacing effect of the middle hinge region may be important in ensuring that the Fab arms do not cover effector molecule interaction sites. However, antibody engineering studies,

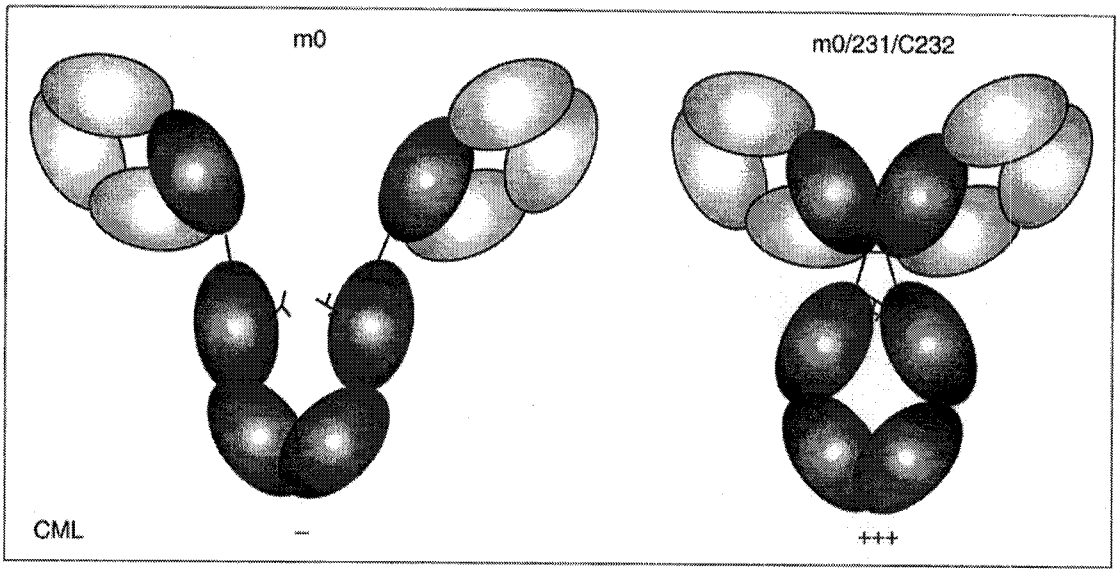

Fig. 2. *Hinge-deleted mutants of IgG. Light-shaded domains are murine and dark-shaded domains are human (IgG3). In mutant m0, the heavy chains are not connected to each other because the genetic hinge has been deleted. In mutant m0/231C232, the heavy chains are covalently linked by a disulfide bond provided by the insertion of a Cys residue as the second amino acid in the lower hinge region. The relative abilities of the mutants to initiate complement-mediated lysis (CML) are shown below.*

carried out by several groups, have questioned this view (see below).

Domain-swapping mutants

Replacement of the hinge region in IgG4 molecules with the IgG3 hinge region results in failure to bind C1q or activate complement, whereas an IgG3 molecule carrying the hinge of IgG4 still binds C1q efficiently[21]. Indeed, an IgG3 molecule with the hinge of IgG4, or the C_H1 domain and the hinge of IgG4, induces CML more efficiently than does normal IgG3 (Ref. 22). Similarly, the replacement of the C_H2 domain of mouse IgG1 with that of mouse IgG2b generates C1q binding and CML at a level comparable with that of IgG2b (Ref. 23). These data strongly suggest that a long and flexible upper hinge is not a prerequisite for complement binding and activation. In support of this, a modified IgG3 molecule, lacking 47 amino acids in the N-terminal end of the hinge region such that the hinge region comprises 15 amino acids and the upper hinge region is reduced from 12 to only 4 amino acids, lost none of its ability to activate complement[24]. Furthermore, a Ser to Cys mutation at amino acid 119 of the C_H1 domain of human IgG1, which introduces a new inter-H-chain disulfide bond, greatly reduces the mobility of the Fab arms without impairing the ability to activate complement[25].

Hinge-deleted mutants

Further dissection of the role of the hinge was accomplished by the construction of a mutant IgG3 molecule from which all four hinge exons were removed, making a hinge-deleted molecule (m0) that did not mediate complement lysis[26] (Fig. 2). When a Cys residue was introduced as the second amino acid in the C_H2 exon, it facilitated formation of a disulfide bond between the two H chains. This mutant, termed m0/231C232 (Fig. 2), lacks the upper and middle hinge altogether and, therefore, is likely to be totally rigid. Furthermore, this mutant has no space between the Fab arms and the Fc. However, m0/231C232 is active in complement activation and demonstrates opsonic activity[26,27]. These findings imply that formation of the tetrameric H_2L_2 IgG molecule, in which two H chains are covalently joined by at least one disulfide bond between the C_H1 and the C_H2 domains, does not require a long and/or flexible hinge region for effector function. The results also confirm that the inter-H-chain disulfide bond is a structural prerequisite for complement activation. This was previously shown in 1964 by Schur and Christian[28], and in 1975 by Isenmann and co-workers[29], who reported that reduction of the inter-H-chain disulfide bridges in rabbit and human antibodies, respectively, destroys their ability to interact with complement.

The mutant m0/231C232 is different from the Dob and Mcg antibodies mentioned above in three important aspects: it is of IgG3 origin, has its L chains bound to Cys131 in the C_H1 domain, and has one disulfide bond between the H chains. Dob and Mcg are IgG1 antibodies and, therefore, should have their L chains bound to Cys220 encoded by the genetic hinge. However, because they lack this region, the two L chains are forced to make an inter-L-chain bond, as has been shown by crystallographic analysis[20]. The Dob and Mcg molecules are rigid, and it has been proposed that this inter-L-chain bond forces the Fab arms to lie at a wider angle than in normal IgG molecules, thus covering the sites usually bound by effector molecules[20]. To test this hypothesis, a series of mutants were made based on the m0 molecule (Fig. 3). The m0/C131S mutant resembles the Mcg and Dob molecules with

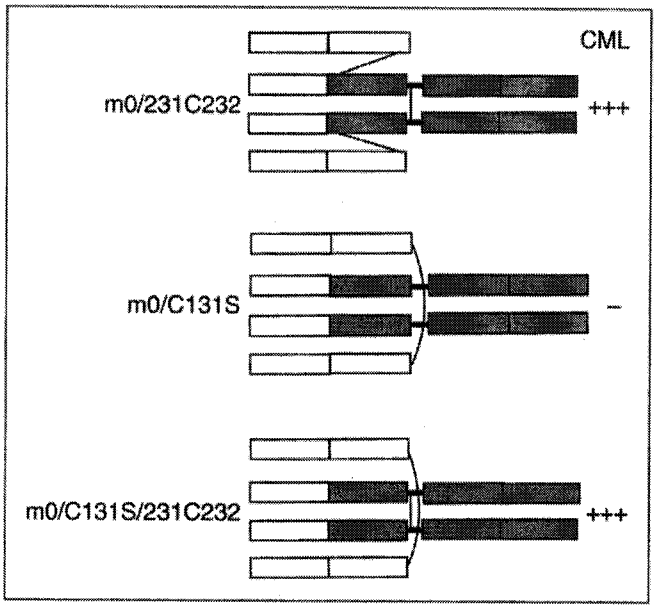

Fig. 3. *Hinge-deleted mutants of IgG3 with novel inter-chain disulfide bonds. The mutants are based on the hinge-deleted mutant m0 (see Fig. 2) as follows: m0/231C232 has a Cys residue introduced into the C_H2 exon, forming an inter-heavy(H)-chain disulfide bond [the molecule also contains a disulfide bond between the H and the light (L) chains]; m0/C131S undergoes an inter-L-chain bond; and m0/C131S/231C232 has both an inter-H- and inter-L-chain bond. The relative abilities of these mutants to mediate complement-mediated lysis (CML) are shown to the right.*

respect to the presence of an inter-L-chain bond, and probably with respect to its overall structure[30]; in common with Dob and Mcg, this molecule has no documented effector functions. The m0/C131S/231C232 mutant has both an inter-H- and an inter-L-chain bond; this molecule is able to mediate effector functions. Thus, it is clear that one disulfide bond between the C_H1 and the C_H2 domains is necessary for effector functions. Moreover, the inter-L-chain bond does not obstruct the binding of C1q when an inter-H-chain disulfide bond is present. However, it may modulate FcγRII and FcγRIII binding, since only low phagocytic activity by FcγRII- and FcγRIII-bearing polymorphonuclear leukocytes could be observed[30].

Entropy

The results described above indicate that mutants of IgG3 in which the hinge region is short and rigid, as well as mutants such as m0/231C232, show an increased ability to activate complement compared with normal IgG3 (Refs 22,24,26,31). It is possible that the long and flexible hinge of IgG3 reduces its potential to activate complement. In chemical interactions, loss of entropy makes an important and unfavorable contribution to the free energy of binding. Because the hinge mutants probably have an overall lower entropy than the flexible IgG3 wild type, one possible explanation for the higher activity of the mutants is that a smaller loss of entropy occurs following the formation of a complex between several antibody molecules and multiple C1q head regions.

The role of the hinge in antigen binding

The observation made by Oi *et al.*[18], that there is a correlation between flexibility of naturally occurring IgGs and their ability to fix complement, applies equally to human, rabbit, rat and mouse antibodies[17,32]. However, although this flexibility is not necessary for complement activation, it allows the antibodies to react with differently spaced antigens. This might be especially important when IgG is membrane bound on B cells. The variation in hinge length and flexibility results in antibodies with different reach and rotational adaptability. Thus, it may be an advantage to the organism that those antibody molecules active in complement activation are also able to reach variably spaced epitopes.

Hinge amino acids responsible for disulfide bond formation

The amino acid sequence in the hinge region varies among isotypes and species but, in humans, it typically contains the sequence Cys-Pro-X-Cys-Pro. A mutant where the genetic hinge comprises the motif Glu-Cys-Pro-Pro-Cys-Pro is observed as 'half molecules' on a nonreducing denaturing gel (O.H. Brekke, T.E. Michaelsen and I. Sandlie, unpublished). Similarly, human IgG4 contains the motif Cys-Pro-Ser-Cys-Pro and is also observed partly as half molecules under denaturing nonreducing conditions[21,22,33]. A mutant with a single amino acid substitution (from Ser to Pro) in this motif leads to the production of covalently linked H_2L_2 antibodies[34]. It appears that the motif, as well as sequences flanking the motif, are important for the ability of Cys residues to locate each other and make the necessary disulfide bond. Indeed, it is possible that, as a consequence of its inability to activate complement, there may have been no evolutionary pressure for IgG4 to keep the hinge sequence and form disulfide bonds efficiently.

C_H2 amino acids necessary for complement activation

The C_H2 domain carries the defined 'core' binding site for C1q, as well as the sites responsible for the isotype variation[12,23,35,36]. Comparing the human IgGs, the C-terminal half of the IgG C_H2 domain (amino acids 292–340) contains residues that are important for C1q binding, since shuffling of this part from the active IgG1 to the inactive IgG4 molecule yielded an IgG4 mutant that is almost as active as IgG1 (Refs 35,36). There are four amino acids unique to IgG4 in C_H2: Phe234, Glu268, Ser330 and Ser331 (these are Leu, His, Ala and Pro, respectively, in active IgG1 and IgG3 molecules). Replacement of Ser331 with Pro improves complement activation of IgG4 (Refs 37,38), but not to the level of IgG1 or IgG3 (Ref. 39), whereas none of the other three replacements were found to have an effect. Ser330 and Ser331 lie close to the hinge link residues in the folded molecule. Substitution of the mouse IgG1 lower hinge region, or three individual hinge-proximal loops, with corresponding IgG2b sequences did not generate a lytic IgG1 molecule (T. Clackson, unpublished). Clearly, more work is needed to elucidate which additional residues in the C_H2 domain are involved in complement activation.

Additional structural requirements

For complement activation, it is crucial that the IgG molecules are glycosylated. Mutants of all human subclasses where Asn297 in the C_H2 domain (to which the main carbohydrate is linked) is mutated to Gln show a change in sensitivity to most proteases, indicating a change in conformation[40]. The carbohydrate normally stabilizes and separates the C_H2 domains, which have no noncovalent interactions[41]. NMR studies comparing native with aglycosylated Fc regions conclude that the overall structures are similar, with no inward 'collapse' of the two C_H2 domains due to the absence of carbohydrate. Nevertheless, a structural perturbation in the vicinity of His268 in a hinge-proximal loop has been detected[42]. Aglycosylated human IgG1 and IgG3 antibodies have both been shown to be deficient in complement activation[40].

The presence of the C_H3 domain is necessary for optimal complement activation[43]; its removal reduces complement activation by 50%. With respect to antigen binding, only one of the Fab arms of each antibody molecule in the antibodies aggregated on an antigenic surface needs to bind antigen in order for C1q to bind and become activated[44]. Furthermore, only one of the C_H2 domains within each individual IgG molecule needs to be active, as hybrid IgGs with Fc regions comprising active mouse IgG2a and inactive mouse IgG1 are still able to carry out this effector function. However, activation is only observed if a threshold in antibody input is overcome, indicating a requirement for minimal density of bound monovalent IgG (Ref. 44).

C1q binding, as such, is not sufficient for complement activation, since several investigators have reported the production of mutant IgG molecules that bind C1q, but do not activate complement[12,40]. IgG isotypes have also been shown to differ in complement activation at the level of complement component C4 (Refs 32,45); the molecular basis for these differences is not known.

Concluding remarks

In summary, provided that antibody aggregation occurs and that there are sufficient numbers of functional C_H2 domains involved, the components required for complement activation include a disulfide bond in the N-terminal end of C_H2 (normally the hinge region), the presence of carbohydrates and paired C_H3 domains. As yet, it is unclear whether these requirements must be met in order to provide the C_H2 domains with a certain orientation or whether they preserve the functional integrity of the C1q-binding site.

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The basis of autoimmunity: Part I Mechanisms of aberrant self-recognition

Argyrios N. Theofilopoulos

In this two-part series, Argyrios N. Theofilopoulos summarizes the current state of affairs in the field of autoimmunity. Part I integrates the collective mechanistic theories of autoimmune diseases. The most straightforward explanation to emerge with regard to organ-specific diseases is the concept that these are caused by inappropriate, yet conventional, immunological responses against self-antigens for which tolerance has never been established. A similar mechanism may be operative in systemic autoimmunity, but other abnormalities such as defects in the apoptosis machinery may also be invoked. Part II will address the genetic contributions predisposing to autoimmune syndromes.

It has long been hypothesized that the development of the immune system is triadic in nature: useless cells are discarded, useful cells are retained and dangerous cells are destroyed or inactivated. In recent years, studies with transgenic and endogenous superantigen (SAg)-expressing mice have strongly suggested that this hypothesis is essentially correct, with the ultimate outcome likely to be dependent on the degree with which antigen receptors react with self-constituents. Specifically, it appears that, as T cells mature in the thymus, those that rearrange and display self major histocompatibility complex (MHC) and peptide-reactive T-cell receptors (TCRs) of a certain affinity/avidity are maintained and propagated within the thymus (positive selection); but, prior to their export to the periphery, those with receptors of dangerously high affinity/avidity are eliminated or inactivated (negative selection)[1–3]. Elimination or inactivation of emerging B cells reactive with membrane-bound self-antigens or soluble self-antigens, respectively, has also been documented[4,5]. Thus, through these editing processes, a state of self-tolerance is achieved. However, despite the recent acquisition of extensive information relating to the mechanisms of self-tolerance, our understanding of the mechanisms leading to pathogenic autoimmunity is still fragmentary and incomplete. Nevertheless, recent advances in this area have begun to assemble the missing pieces of the puzzle. The purpose of this review is to synthesize the widely dispersed concepts and theories of autoimmunity and, thereby, provide a more coherent picture of this complex field.

General issues

A wide spectrum of human and animal diseases are wholly or partially attributable to autoimmunity[6]. This list continually expands as autoimmune mechanisms are implicated in such widely diverse disorders as alopecia, autonomic disorders, certain forms of urticaria, AIDS and even neuropsychological disorders.

As research in autoimmunity progresses, several important issues need to be resolved (detailed in Box 1). The most urgent of these questions concerns the nature of the inciting antigen, and the limited information on this subject is compounded by three areas of ambiguity. First, it is uncertain whether identified autoantibodies and corresponding antigens have anything to do with the primary cause or pathogenesis of a given disease. Second, the immense diversity of autoantibodies observed in some diseases causes considerable difficulty in the identification of the inciting antigen. Third, there is uncertainty as to whether the initial trigger has any relation to the antigen recognized by the autoantibody.

With respect to the third of these ambiguities, complications may arise from the fact that determinants recognized by the autoantibody and the prerequisite T helper (Th) cell may reside on different molecules within a supramolecular complex. For example, for