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Commentary

Cytoplasmic islet cell antibodies (ICA): towards a molecular understanding of the autoantigens

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Cytoplasmic islet cell antibodies (ICA) were first described in Deborah Doniach's laboratory by Bottazzo *et al.* (1974) in a cohort of patients with polyendocrine autoimmune disease. These antibodies were detected by the indirect immunofluorescence test (IFT) and provided the first serological evidence that autoimmunity is involved in type 1 (insulin-dependent) diabetes mellitus. ICA have now been well established as predictive markers to indicate the risk for type 1 diabetes in first-degree relatives of patients with type 1 diabetes (Srikanta *et al.*, 1986; Johnston *et al.*, 1989; Bonifacio *et al.*, 1990; Riley *et al.*, 1990) as well as in the general population (Bruining *et al.*, 1989; Karjalainen, 1990; Boehm *et al.*, 1991; Landin-Olsen *et al.*, 1992). While the IFT has also been the method of choice in the search for unknown antibodies to endocrine organs (Scherbaum *et al.*, 1987) most of the respective autoantigens have now been defined and isolated (Banga *et al.*, 1989). In some of the endocrine antigen–antibody systems, cloning of the autoantigens and the generation of monoclonal autoantibodies and of antigen-specific T cell clones has now allowed investigation of the epitopes with which the autoantibodies or the antigen-specific T cells react.

The nature of the ICA-reactive autoantigen(s) has remained elusive for a long time. According to studies on the sensitivity of the autoantigen to biochemical treatment, Nayak and co-workers (1985) suggested that the antigen is a monosialoganglioside. This assumption was supported by studies showing that monoclonal antibodies to gangliosides react with islet cells (Eisenbarth *et al.*, 1982) and ICA binding to islet cells was reduced by pre-incubation with glycolipid extracts from human pancreas (Colman *et al.*, 1988). In parallel with ICA, another important type 1 diabetes associated antibody specificity, i.e. 64-kDa antibodies, was described which precipitated a 64-kDa protein from islet cell extracts (Baekkeskov *et al.*, 1982). The target antigen of the 64-kDa antibodies was later identified as glutamate decarboxylase (GAD, Baekkeskov *et al.*, 1990). It has been demonstrated that antibodies to the 64-kDa islet cell protein are markers to predict the future development of type 1 diabetes (Baekkeskov *et al.*, 1987; Atkinson *et al.*, 1990; Bärmaier *et al.*, 1991; Thivolet *et al.*, 1992; Seissler *et al.*, 1992). These antibodies, however, seem to have a lower predictive value than ICA and persist for a long time after the onset of type 1 diabetes (Christie *et al.*, 1990a).

While 64-kDa antibodies were shown to be β-cell specific within the islets (Garry *et al.*, 1986; Christie *et al.*, 1990b), ICA-positive sera were initially described as reacting with all endocrine cells of the islet (Bottazzo & Doniach, 1978). It is evident from this historical development that ICA and 64-kDa antibodies have been defined by the methods by which they were detected. Recent evidence has now accumulated from several laboratories, including our own, to suggest that ICA and 64-kDa antibodies are partially identical specificities.

When we produced the first human monoclonal islet cell antibodies (MICA) from the blood of a patient with newly diagnosed type 1 diabetes and used the conventional ICA test for the screening procedure, we were very surprised to find that these monoclonals were also able to precipitate the 64-kDa islet cell antigen which we then identified as GAD (Richter *et al.*, 1992). In contrast to most ICA-positive sera, however, the MICA predominantly stained β-cells within the islets (Richter *et al.*, 1993a) which is consistent with the β-cell specific expression of GAD. Blocking studies with GAD provided clear evidence that the GAD reactivity of ICA was not limited to the single individual from whom the MICA were derived. These observations indicate that ICA-positive sera contain heterogeneous ICA reactivities and they also show that β-cell specific ICA represent a subgroup of ICA.

Using the discrete immunohistochemical analysis of ICA-positive sera, two distinct ICA types could be identified: (i) the well known diffuse cytoplasmic staining pattern, including both β and α-cells, which was found in the sera from patients with type 1 diabetes, and (ii) a granular 'selective' pattern which was found in the sera from non-diabetic patients with endocrine autoimmune diseases (Genovese *et al.*, 1992; Timisit *et al.*, 1992). The 'selective' pattern was completely blocked by pre-incubation with rat brain homogenate indicating that these antibodies recognize an antigen that is also present in the brain (Genovese *et al.*, 1992). In a different approach, Gianini and co-workers (1992) also identified a minor subset of ICA that reacted with human and rat, but not with mouse, islets and showed a β-cell restricted staining pattern within the islets. This restricted

ICA pattern was associated with a markedly lower risk for progression to diabetes.

The latter data have been confirmed by others (Sai *et al.*, 1993) so that it now appears that there exist at least two subsets of ICA in the sera from patients with type 1 diabetes and their first-degree relatives: (i) ICA_{hu+m-} detected on human or rat pancreas, but not on mouse pancreas, (ii) ICA_{hu+m+} which cross-react with mouse pancreas. The systematic search for ICA reacting on mouse pancreas has now shown that ICA may be negative in the conventional test on human tissue but positive when mouse pancreas is used (ICA_{hu-m+}). Preliminary studies indicate that these ICA detected on mouse pancreas are more transient and more closely related to diabetes than ICA detected on human pancreas. Quantification of mouse-reactive ICA may provide a major benefit for the prediction of the future development of diabetes and it may even provide a new tool to calculate the time of diabetes onset in prediabetic individuals.

On the basis of recent work we are now in a position to explain some of these observations. GAD exists in at least two different isoforms with molecular sizes of M_r 65000 (GAD_{65} and M_r 67000 (GAD_{67}) encoded by two distinct genes (Erlander *et al.*, 1991; Karlsen *et al.*, 1991; Kaufman *et al.*, 1991). GAD_{65} corresponds to the 64-kDa antigen and both isoforms are recognized by sera from patients with type 1 diabetes. Recent immunoprecipitation experiments and immunohistochemical analyses have shown that in mouse islets GAD_{65} and GAD_{67} are expressed at a very low level as compared to rat or human islets (Velloso *et al.*, 1993). Both isoforms are expressed in brain neurons (Christgau *et al.*, 1991; Kaufman *et al.*, 1992).

In our recent studies human recombinant GAD_{65} and GAD_{67} were expressed in the baculovirus system (Mauch *et al.*, 1993) and used to determine autoantibodies to GAD_{65} and GAD_{67} in the immunoprecipitation test. When testing the sera from patients with type 1 diabetes and ICA-positive non-diabetic individuals in this system it appeared that GAD_{65} was present in only some sera which were also positive for GAD_{67}, and that antibodies to GAD_{67} are rare in patients with type 1 diabetes and prediabetic individuals (Seissler *et al.*, 1993). Similar results were also obtained by Hagopian and co-workers (1993).

The clear difference in the antibody reactivity to GAD_{65} and GAD_{67} emphasizes the relevance of the diversity of the two isoforms of GAD. The amino acid sequence identity between human GAD_{65} and GAD_{67} is about 65% with the highest diversity for the N-terminal 120 amino acids. This may suggest either that the majority of antibodies to GAD are primarily directed to the N-terminal portion of the protein or that the diversity of the amino acid sequence between GAD_{65} and GAD_{67} leads to conformational changes of the GAD protein. The latter assumption may also be derived from the fact that the sera from patients with type 1 diabetes do not react with denatured GAD_{65} (Baekkeskov *et al.*, 1990).

Our human monoclonal ICAs (MICA), all of them recognizing GAD_{65}, could be used as valuable tools to probe the autoimmune epitopes in the enzyme. Using the MICA and a series of N-terminal and C-terminal regions as well as mutants of GAD_{65}, Dr Wiltrud Richter in our laboratory was able to detect two major conformational epitope areas. It was shown that the intact conformation of the middle and the C-terminal parts of GAD_{65} are crucial to antigen binding. It also became evident that the N-terminal domain of GAD_{65} (which differs most significantly from GAD_{67}) does not harbour the MICA epitopes (Richter *et al.*, 1993b). It may thus be concluded that subtle amino acid differences in the middle and C-terminal domains define the GAD_{65} specific autoimmune epitopes.

Because GAD_{65} and GAD_{67} are the only ICA-reactive antigens known on a molecular basis, further antigen reactivities can be evaluated by subtracting these from total ICA binding. Applying such absorption experiments with human recombinant GAD_{65} and GAD_{67} we were able to show that the vast majority of sera from patients with type 1 diabetes recognize not only GAD_{65} and GAD_{67}, but also additional islet cell autoantigens (Richter *et al.*, 1993c).

ICA have also been detected in the neurological disorder, the stiff-man syndrome, which is associated with type 1 diabetes in a third of the cases (Solimena *et al.*, 1990). These antibodies, however, recognize a spectrum of islet antigens that is different from the usual cases of type 1 diabetes. They recognize linear epitopes on GAD (Baekkeskov *et al.*, 1990; Richter *et al.*, 1993b) and are thus also detected by Western blotting. All ICA positive sera from patients with the stiff-man syndrome studied by us so far reacted with both isoforms of GAD and their immunoreactivity could be completely abolished by preincubation with human recombinant GAD_{65} and GAD_{67} (Richter *et al.*, 1993c), indicating that these are the only antigens responsible for ICA staining in the sera from patients with stiff-man syndrome. The β-cell specific expression of GAD_{65} clearly explains the selective staining pattern seen in all our ICA positive sera from patients with the stiff-man syndrome and in a minority of patients with type 1 diabetes.

Having recognized GAD as a major target B-cell antigen in type 1 diabetes it will now be important to know more about the possible role of GAD as a T-cell antigen, and also about the respective antigenic epitopes which may be studied when human GAD-reactive T-cell clones are available.

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