BOX 1: Methods for measuring (effects of) blocking antibodies

Serological assays

The induction of blocking antibodies in allergic patients during AIT can be studied using serological assays demonstrating their ability to inhibit IgE binding to the allergen[114,151]. The allergen neutralising effect of IgG cannot be measured when the amount of allergen on the solid phase is in excess to allergen-specific antibodies as it occurs for example in the ImmunoCAP system (Thermofisher, Uppsala, Sweden)[152]. However, micro-arrays such as the ImmunoCAP ISAC (Thermofisher, Uppsala, Sweden) and the MeDALL allergen chip[153] contain approximately only 100 fg of allergen per spot. Therefore, it is possible to visualise with such assays the competition of IgG with IgE for allergen binding[112,154-156]Therefore, one can compare allergen-specific IgE binding in serum samples obtained before AIT and after AIT, when blocking allergen-specific IgG has developed. In the case that blocking antibodies have developed the IgE signal will be strongly reduced in the post-treatment samples.

Basophils and mast cells

Measuring the effects of blocking antibodies on allergen-induced basophil activation Shortly after developing the allergen-specific basophil histamine release assay, Lichtenstein and colleagues used this test to study the effects of desensitisation during AIT[157]. The effects of blocking antibodies induced by AIT or even of purified human monoclonal allergen-specific IgG antibodies on allergen-induced basophil degranulation can be visualised by pre-incubation of the allergen before exposure to IgE-loaded basophils from allergic patients when the cells had been isolated and washed to remove serum (i.e., basophil activation with washed cells)[158,159]. Alternatively, basophil activation can be performed in blood samples obtained from patients in the presence of serum and blocking antibodies. In this setting, the effects of blocking antibodies become visible due to addition of allergen to the full blood sample containing already the blocking antibodies (i.e., full blood assay)[160].
More recently, rat basophil and mast cell lines transfected with the human FcεRI can be cultivated and loaded with sera obtained before AIT to represent the patient's sensitivity before the treatment. The cells are then exposed to allergen pre-incubated with serum samples obtained before and after treatment to investigate the development of blocking antibodies in the post-treatment serum[108]. These experiments can be performed with sera as such, purified IgG fractions or sera that had been heat-inactivated at 56°C to remove IgE effects. The advantage of using the transfected cell lines is that the experiments can be conducted with all sera simultaneously with cells having comparable sensitivity. In contrast, experiments performed with fresh basophils from patients at different time points can be subject to variations due to general differences of basophil sensitivity occurring in subjects at different time points.

IgE-facilitated allergen presentation

It is known that IgE-facilitated allergen presentation via CD23 on B cells is a key mechanism in allergen presentation to T cells in allergic patients because it allows tiny amounts of allergens to be presented by an efficient pick-up mechanism. This is of particular relevance in allergy because one has to consider that only minute amounts of allergens can enter the systemic circulation of allergic subjects due to the presence of epithelial barriers. The first study investigated if AIT-induced blocking antibodies can inhibit IgE-facilitated allergen presentation via CD23 was published in 1999[113]. In this study, it could be shown that AIT-induced blocking antibodies inhibited allergen-specific T cell proliferation and secretion of inflammatory cytokine responses. This result was remarkable because it indicated that the reduction of T cell activity during AIT is mediated by blocking antibodies and not or not only by T cell-mediated immunological tolerance mechanisms[161]. To simplify the assay, a CD23-expressing B cell line was developed which can be loaded with serum IgE from a patient allergic to the given allergen and one can then measure the binding of labelled allergen and its inhibition by AIT-induced blocking antibodies[115]. Although this FAB assay[119] can be easily performed for extensive screening of sera it has the disadvantage that the cells are usually loaded only with one IgE-containing serum (i.e., indicator serum) and hence one cannot assess the blocking of allergen binding to CD23-bound IgE of each of the patients to be tested. However, one can also perform this assay with APCs obtained from each patient to be tested and add allergen in the presence of pre-and post-treatment sera to measure the development of blocking antibodies in each of the tested patients[160].

A question which still needs to be investigated is if the inhibition of IgE-facilitated allergen presentation and its effects on subsequent T cell activation is related to a certain type of allergic symptoms. By intuition, one would expect that inhibition of T cell activation by blocking antibodies would be related to a reduction of late-phase allergic reactions. However, to study this, it is not sufficient to measure only the effects of blocking antibodies on IgE-facilitated presentation and their effects on T cell activation and relate the latter parameters with clinical effects such as late-phase skin reactions eventually atopy patch test results. It has been challenging to relate allergen-specific T cell proliferation and cytokine secretion in blood-derived cells with atopy patch test results[162]. When comparing serological assays, basophil activation tests and FAB assays a good correlation was observed among the different assays, and it may therefore be sufficient to perform serological assays for the assessment of AIT-induced blocking antibodies[114].

In vivo methods

The classic experiment demonstration that AIT-induced blocking antibodies inhibit allergen-induced skin test reactions by Prausnitz-Kuestner reaction[25,163] in humans[25] cannot be performed any more due to ethical reasons. One, therefore, can only compare results of *in vivo* provocation testing such as skin testing[164,165], conjunctival provocation testing[166] (and allergen exposure testing [160] with the development of potentially blocking non-IgE antibodies in patients during AIT and calculate correlations. A direct demonstration of the effects of blocking antibodies on *in vivo* test results is hence not possible.