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In vitro methods for quantifying IgE antibodies to betalactams

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The diagnostic methods generally used for studying allergic reactions to betalactams can be performed *in vivo* and *in vitro* (1-3). The *in vivo* method most commonly used is the skin test, which consists of the prick or intradermal application of penicillin molecules, either conjugated or in free form. In the latter case, they then conjugate with an endogenous protein. The subsequent release of histamine or other inflammatory mediators induces an immediate wheal and flare reaction that can be measured. The most widely used *in vitro* test is the measurement of specific IgE antibodies by different methods, the two most common being the radioimmunoassay (RIA) and the enzyme immunoassay. The latter assay is gradually replacing the former. In general, it is assumed that in allergic reactions induced by IgE antibodies, *in vivo* tests are more sensitive than the *in vitro* methods (2), although this is not always the case. In some immediate reactions, serum IgE antibodies are detected which later prove to be negative in the corresponding skin test (4). In this paper, we focus on the evaluation of the *in vitro* tests commonly used for quantifying specific IgE antibodies to penicillins and other betalactams, and show the value of this method as a complement to *in vivo* tests in the evaluation of subjects allergic to betalactams.

Penicillin molecules need to be modified before being used in both *in vitro* and *in vivo* tests to enable them to be recognized by IgE antibodies. However, since allergy to penicillin was recognized many years before the development of *in vitro* tests for detecting IgE antibodies, the initial modifications for these tests were based on derivatives used for skin testing. Thus, although penicillin was used for skin testing as a free drug, diagnostic accuracy was greatly enhanced when penicillin was conjugated to poly-L-lysine (PLL), forming what is called the major determinant of benzylpenicillin (BPO-PLL) (5). Although this conjugate is

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currently known in commercial preparations as PPL, we prefer to identify it as BPO-PLL in order to make comparisons with the *in vitro* findings. This conjugate was soon seen to be more efficient at eliciting positive responses in allergic subjects than free penicillins (6). The advantage of using PLL is that it forms a multivalent penicillin-epitope conjugate of benzylpenicillin, producing a bridging of the IgE molecules located on the surface of the mast cells and basophils, leading to the release of histamine and other inflammatory mediators (1). The same approach in skin testing has been applied until now to *in vitro* evaluation, and in this way the complete antigenic structure can be conjugated to different solid phases by different methods. However, it must be accepted that these conjugates used for recognizing penicillin determinants by IgE antibodies are not totally equivalent to those generated *in vivo*, because the structure of PLL does not exist in living organisms. Thus, we are dealing with a cross-reacting structure, the definitive conjugate of which is not known *in vivo*.

RIA methods represented the second major improvement in the evaluation of allergic responses to betalactams. Thus, after the appearance of assays for quantifying total and specific IgE antibodies to classical allergens (7), a method for determining IgE antibodies to benzylpenicillin using the benzylpenicilloyl conjugated to human serum albumin (BPO-HSA) was developed (8), and for years this technique has been used in many laboratories for both diagnostic and research purposes. Later modifications substituted PLL for HSA, and over the last 12 years our group has dedicated considerable effort to improving this assay and developing new tests for quantifying *in vitro* IgE antibodies to betalactams. Although most of the data we present in this paper have been obtained by RIA, other immunoassays using nonisotopic reagents have similar sensitivity and specificity (9–13).

It is generally accepted that *in vitro* determination of specific IgE antibodies to penicillin (BPO-PLL) corresponds to skin testing with BPO-PLL. The concept of minor determinants was created on the basis of skin test responses to a number of different penicillin metabolites generated from the parental penicillin molecules (3, 14, 15). Later it was established that of all the possible metabolites generated, only benzylpenicillin and benzylpenicilloic were effective (16). The *in vitro* correspondence of these determinants to the skin test results has not been completely clarified; therefore, the concept of minor determinant specificity is based only on skin test responses (14, 15). Different studies, using the classical RAST

approach, have tried to determine how IgE antibodies from subjects allergic to penicillin recognize the different minor determinants, but no additional information has been provided in this respect (4, 9, 17). Previous experience of our group indicates that very high cross-reactivity *in vitro* between major and minor determinants of benzylpenicillin exists and unique specificity to minor determinants cannot be proved.

New methods for quantifying IgE antibodies to betalactams have been developed, but due to methodological differences the studies cannot be compared correctly. Because albumin has a limited capacity for fixing BPO groups, not all of which are exposed to IgE antibodies for recognition (18, 19), the use of PLL was considered to be a reasonable alternative for quantifying IgE antibodies (9). We have carried out extensive studies showing that PLL has a greater capacity than HSA for fixing penicilloyl groups; therefore, *in vitro* tests using the former are more sensitive for detecting IgE antibodies (10). RAST inhibition studies have shown that different molecular weight PLLs have more capacity than HSA for binding BPO groups, with the limitation that those with high molecular weight (greater than 12 kDa) and high concentrations of either hapten or carrier produce a highly nonspecific binding, thereby limiting the specificity of the assay (10). Therefore, the conjugation conditions need to be controlled in order to maintain similar specificities to those obtained with the BPO-HSA conjugate (4, 9, 10). In other studies, the benzylpenicillin molecules have been conjugated to the solid phase with an aliphatic spacer (11) or transferrin (20), or by directly coupling the penicillin G molecule to the surface of microtitre plates (21), although, as stated above, no comparative studies have been done. We carried out studies using different spacers and penicillin molecules, showing that the sensitivity and specificity are similar, the best result being obtained with PLL followed by the amino-spacer and then HSA (10, 12, 13). These data have been established for both benzylpenicilloyl and amoxicilloyl specificities, being confirmed by further studies using other betalactam antibiotics such as penicillin V and ampicillin (22, 23).

Recently, many groups have reported new specificities to penicillins (22, 24–28), cephalosporins (29–31), and other betalactams (32), and this tendency to describe more specificities is likely to increase (33). These data have generated considerable interest and have stimulated the development of new methods for detecting IgE antibodies to different betalactams. Several studies have shown that cephalosporin determinants are also able to produce

selective reactions (29–31, 34), and since the use of these drugs is increasing, it is expected that more cases will be reported. Studies in a large group of subjects with allergic reactions to cephalosporins indicate that several different determinants seem to be recognized by skin testing (31). *In vitro* studies carried out by others support these *in vivo* findings (35, 36). However, the *in vitro* determination of IgE antibodies has a number of limitations that need to be resolved in order to identify the epitopes of cephalosporins.

In our experience, the determination of specific IgE antibodies to BPO-PLL and amoxicilloyl-polylysine (AXO-PLL) *in vitro* has provided very valuable information in subjects with an immediate response to betalactams. We know that at least three groups can be identified: those specific for AXO, those specific for BPO, and a third group that recognizes determinants common to both structures (37). These groups are not only different in terms of antibody recognition, but also differ in their tendency to become negative. The data obtained for skin test survival analysis from the two main groups (selective to amoxicillin vs nonselective) showed differences in their tendency for converting to negative depending on the specificity of the antibody (38), confirming the *in vitro* results.

When we studied patients allergic to betalactams, it was seen that, although in a significant proportion of cases amoxicillin, ampicillin, cephalosporins, or other betalactams were the drugs inducing the reaction, the primary specificity of the antibody was directed to the benzylpenicilloyl determinant, indicating that this was the drug primarily recognized and that the betalactam induced the reaction by cross-reactivity (37).

Another important aspect, mentioned in different parts of this paper, is the establishment of a correlation between *in vivo* and *in vitro* tests. Although the equivalence between these tests is high when comparing BPO specificity, this is not always the case with minor determinants, since we have found subjects with a negative skin test response to BPO and a positive response to minor determinants who were RAST positive to BPO, suggesting that the classical concept of minor determinant specificity needs to be revised (24). Furthermore, in cases of selective IgE responses to amoxicillin, skin tests to AXO-PLL conjugates are negative, minor determinants to amoxicillin are positive, and the

in vitro IgE response to AXO-PLL is also positive. A recent study showing 10% of the subjects to be skin test negative but RAST positive to either penicillin or amoxicillin determinants supports the usefulness of *in vitro* IgE determination to betalactam antibiotics (39).

RAST inhibition is a refinement of the classical RAST assay, enabling confirmation of both the *in vivo* test and the direct RAST assay. It also allows specificities to be studied, cross-reactivity to be evaluated, and study of how the different parts of the betalactam molecule contribute to the epitope binding site (10, 37). Since, as stated above, the final nature of the antigenic determinant involved in allergic reactions to betalactams is not well known, this represents an important limitation when carrying out these types of studies. Although multiple RAST inhibition studies using penicillin molecules have been reported, variations in methodology and the use of sera with different specificities impede direct comparison (28, 35–37, 40, 41). The usual approach we have followed is to employ monomeric rather than polymeric conjugates of the different penicillins with either butylamine or aminocaproic acid as inhibitors, enabling us to control the concentration of the epitopes used for the inhibition (10, 37, 42). This method also allows single parts of the penicillin molecule, including the nuclear portion and side-chains, to be used. The results obtained with this technique support our *in vivo* and direct IgE assays previously reported (33), and are, moreover, supported by clinical data.

We can conclude that the *in vitro* tests for determining IgE antibodies to betalactams are a valuable approach in evaluating subjects with immediate reactions to these antibiotics. They provide additional information that is complementary to *in vivo* studies, and the inhibition studies confirm specificities. Further refinements are required to improve the methodology, based on a better characterization of the betalactam structures involved and the development of new solid-phase assays where the different determinants can be presented, improving the sensitivity for IgE antibody recognition.

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