

Monoclonal antibodies to amoxicillin express different idiotypes determined by anti-idiotypic antibodies production

Penicillins are β -lactam antibiotics able to generate several antigenic determinants that are recognized by the immune system. To study the differences in the antigen binding site of two monoclonal antibodies (Mab) specific to amoxicillin, polyclonal rabbit anti-idiotypic antibodies were produced. One Mab, AO3.2 (IgG2a), specific to a structure formed by the acyl-side chain structure and a part of the nuclear region of amoxicillin. The second one, AO6.2 (IgE), is specific to the side chain of amoxicillin, although it also recognizes the side chain of other penicillins (penicillin G and ampicillin). These antibodies were used to immunize rabbits in order to produce polyclonal anti-idiotypic antibodies, which were purified in several steps by affinity chromatography. The specificity and cross-reactivity studies were made by ELISA and ELISA inhibition. The results suggest that the anti-Id antibodies produced are the internal image of the antigen, since the binding to their specific idio type is blocked mainly by the original hapten (amoxicillin): in 98% of the cases with Anti-Id-1 (induced against AO3.2) and in 59% with Anti-Id-2 (induced against AO6.2). The absence of cross-reactivity of each anti-idiotypic antibody with the different Mabs specific to amoxicillin shows that the idiotypes induced by the same hapten have differences that are reflected by the nonrecognition of these anti-idiotypes. We conclude that such a small molecule as amoxicillin can present several antigenic determinants that induce a panel of antibody specificities especially directed against the side chain.

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Introduction

Penicillins are β -lactam antibiotics widely used in clinical practice (1) and induce many allergic reactions (2), which can be cellular or humoral (3–5). These antibiotics are low molecular weight structures and the study of the immunological response to them is still an exciting area of investigation (6), which has led to an increase in the understanding of the immunological behaviour of low molecular weight substances (7). β -lactams are able to interact covalently with serum proteins, forming immunogenic conjugates (8), and their metabolism can provoke a number of different antigenic determinants that have been implicated in the specificity of the immunological response (6).

In early studies, Levine reported that the major antigenic and allergenic determinant of penicillins was the product resulting from the conjugation of these antibiotics – by the opening of the β -lactam ring – with an ϵ -amino group of a protein, and that the majority of specific antibodies are produced against this structure (9). Later studies have shown that penicillins are able to induce different patterns of antibody specificities that recognize different parts of the antibiotic molecule (8, 10).

Studies with the production of monoclonal antibodies (Mabs) carried out by de Haan et al. (11) showed that at least three antigen determinants are present in the penicillin molecule: the thiazolidine ring, the side chain structure and the new antigenic determinant (NAD), which is the structure that results from the linkage between the carbonyl group of the β -lactam and the amine group of a protein.

Several studies in humans indicate that different parts of the penicillin molecule may contribute in varying degrees to the antigen binding site (12–14). Evidence exists that the side chain contributes in a critical way to the antigenic determinant in the production of specific antibodies against penicillins (3, 15).

The use of hybridoma technology is a powerful tool for identifying the nature and number of antigenic determinants involved in penicillin immunogenicity. We produced a panel of randomly selected Mabs to amoxicillin (AX) (16) and observed that AX conjugated with a protein can induce antibodies with different specificities against several parts of the antibiotic molecule. ELISA inhibition studies confirmed that some of these Mabs were directed exclusively against the side chain structure of AX, with a variable degree of

cross-reactivity with other penicillins, some were specific to the nuclear portion of the antibiotic, and others required the whole antibiotic molecule to have an optimal recognition (16).

Although the anti-idiotypic (anti-Id) antibody does not provide an exact molecular replica of the original antigen, it mimics its binding. This mimicry does not require sequence homology between antigen and anti-Id antibodies (17). Several reports indicate that immunoglobulins with different isotypes can share an idio type (18, 19).

The purpose of our study was to confirm the differences in the specificities of the antibodies to AX with another model. We therefore produced anti-Id antibodies to two of the Mabs that were specific to the AX side-chain (16) and studied whether they were specific to the idio type that induced their production or there was a cross-reactivity between the related idio types because they had similar structures and specificities.

Materials and methods

Preparation of antibiotic conjugates

The amoxicillin-poly L-lysine conjugate (AXO-PLL) was prepared as described (20). Briefly, AX (Beecham, Toledo, Spain) at 200 mg/ml with PLL (Sigma, St. Louis, MO, USA) at 50 mg/ml dissolved in 0.05 M of carbonate-bicarbonate pH 10.2 buffer was incubated at 37°C for 48 h. After the coupling reaction, the conjugates were neutralized and separated from unbound antibiotics by gel filtration chromatography on PBS equilibrated PD-10 columns (Pharmacia, Uppsala, Sweden).

Conjugates of the antibiotics – AX, benzylpenicillin (PG) (CEPA SL, Madrid, Spain), ampicillin (AMP) (Antibiotics SA, León, Spain) – with butylamine (BA) (Sigma) were prepared as described elsewhere (21). PG (1.03 g) was dissolved in 1 ml of water at room temperature. To this solution was added 1.4 ml of BA and it was shaken for 1 h. Water was added (20 ml) and then 30 ml of HCl, slowly. The white pellet was washed and filtered with water. The solution conjugates of AX with BA (AXO-BA) were prepared by slowly dissolving 1.23 g of the antibiotic in a solution of BA (14 M) at room temperature. The solution was stirred for 1 h and then lyophilized. The resulting solids were crystallized from acetonitrile-ether. The conjugate AMPO-BA was prepared the same way.

Monoclonal antibody (Idiotypic) obtention

Two different hybridomae, produced as previously reported by our group (16), were used for anti-Id antibody production. One was AO3.2, whose isotype is IgG_{2a} and the other AO6.2, an IgE antibody. Their specificities are also different: AO3.2 is specific to the AX side chain, but needs the whole antibiotic molecule to achieve an optimal binding, while AO6.2 is also specific to the side chain of AX, although it is able to recognize the side chain of other penicillins such as PG and AMP.

Depending on the Mab isotype (IgG_{2a} or IgE), two solid phases were used for the purification by affinity chromatography: protein A-sepharose 4B column (Sigma) for AO3.2 and an AXO-PLL-sepharose 4B column for AO6.2. The protocol followed was similar for both antibodies. Briefly, the supernatant (culture medium with the Mab) was passed through a column equilibrated with 1.5 M Tris Buffer pH 8.6. The Mabs were eluted with 50 mM diethylamine

pH 10.5 and the pH equilibrated to 7 with 0.1 M Tris buffer pH 6.8. The fractions containing the Mab were detected by a spectrophotometer at 280 nm and later dialysed against PBS.

Rabbit immunization to anti-Id antibody production

Californian rabbits were used to obtain each anti-Id antibody. For the production of anti-Id antibody to AO3.2, the rabbits were given 10 injections of 200 µg of AO3.2 at 2-week intervals. For the first injection monoclonals were emulsified in Freund's Complete Adjuvant (Sigma) and the remaining injections in Freund's Incomplete Adjuvant (Sigma). For the anti-Id antibody to AO6.2, the protocol was the same, using 250 µg of Mab AO6.2 for each injection. In this case six injections were administered. All the injections were made in the animal's back and the blood was collected from the ear artery or by cardiac puncture.

Antibody detection and characterization

ELISA 96-well microtitre plates (Dynatech Laboratory LTD, Sussex, UK) were coated with 100 µl of the Mab inducing the rabbit anti-Id antibodies, at 5 µg/ml in PBS, overnight at 4°C. After saturation with PBS-milk 0.05%, they were incubated with 50 µl of the rabbit sera or purified anti-Id antibodies, 1 h at room temperature. After washing, 50 µl per well of alkaline phosphate goat antirabbit conjugate IgG (Sigma) was added and incubated for 1 h at room temperature. The plates were finally developed with an enzyme substrate (paranitrophenylphosphate) (Sigma) at 2 mg/ml for 1 h at room temperature and in the dark. The optical density (OD) was read at 405–650 nm.

ELISA inhibition To determine the specificity of the anti-Id antibodies, we carried out inhibition studies using in the solid phase the Mab that was used for immunizing the rabbits, either AO3.2 or AO6.2 at 5 µg/ml. In the fluid phase the purified anti-Id antibody was added (the optimal concentration had been determined previously by ELISA) and incubated with 1:2 decreasing concentrations of the different inhibitors (AXO-BA, AMPO-BA and BPO-BA). A control with no inhibitor was included in all cases. This was incubated for 1 h at room temperature. The remaining steps were carried out as indicated above in ELISA. The percentage inhibition was calculated according to the formula:

$$\% \text{ Inhibition} = \frac{[(\text{OD noninhibited} - \text{OD inhibited}) / \text{OD noninhibited}] \times 100}{}$$

To determine the cross-reactivity of the Mab idio types, the two monoclonal antibodies were used in the solid phase, while in the fluid phase different Mabs were added to the anti-Id antibodies in two-fold decreasing concentrations from an initial concentration of 50 µg/ml.

Anti-idiotypic antibody purification

Anti-Id antibody to AO3.2 (Anti-Id-1) These antibodies were produced against a Mab (AO3.2) whose isotype is IgG_{2a}. To purify the Anti-Id-1, several affinity chromatography steps were required. First we obtained the IgG antibodies with protein A-sepharose. Then we eliminated the allospecific and subclass specific IgG antibodies against constant regions of the mouse monoclonal immunoglobulin by passing the sample through a mouse IgG conjugated column and then a mouse IgG_{2a} conjugated column. Finally, the anti-Id antibody (Anti-Id-1) was purified by affinity chromatography in an AO3.2-sepharose 4B column. The protocol followed at each step was the same as described above in idio type obtention.

Anti-Id antibody to AO6.2 (Anti-Id-2) The Mab against which we obtained the Anti-Id-2 was an IgE antibody, so that the sequence of purification took place in a protein A-sepharose 4B column first and then the allospecific and subclass specific antibodies were eliminated with a mouse anti-DNP IgE conjugated column. Finally, the specific anti-idiotype (Anti-Id-2) was purified by affinity chromatography in an AO6.2-sepharose 4B column, which was carried out as described above.

Results

Anti-Id antibody analysis

The antibody to AO3.2 was named Anti-Id-1. Once we observed that the differences in binding were higher with the AO3.2 than with the corresponding isotype (Fig. 1a), we bled the animal and proceeded to the purification as described above. We obtained 420 µg/ml of Anti-Id-1 from 2 ml of rabbit sera.

The antibody to AO6.2 was named Anti-Id-2. For obtaining these Anti-Id-2, only six immunizations were needed to achieve a specific high level of Anti-Id-2 to AO6.2 vs. an anti-DNP IgE antibody that represents the allotypic and subclass specificities (Fig. 1b). After purification we obtained 284 µg/ml of Anti-Id-2 from 2 ml of the initial rabbit sera.

Determination of specificity of anti-Id antibodies

The anti-Id antibodies were specific to the Mab that induced their production in rabbits. Thus, the Anti-Id-1 showed a high binding when the AO3.2 was bound to the solid phase and likewise the Anti-Id-2 to AO6.2. In order to discover the blocking capacity of the hapten in the interaction of the anti-Id with their specific idiotype, we performed ELISA inhibition studies. We used the specific idiotype (AO3.2 or AO6.2) in the solid phase, while in the fluid phase we placed monomeric conjugates of different antibiotics with BA (their structures can be seen in Fig. 2). Figure 3(a) shows the inhibition study made with Anti-Id-1. The maximum inhibition was produced with AXO-BA (98%), with AMPO-BA inhibiting up to 86%. BPO-BA showed the lowest ability to inhibit the Anti-Id-1 union with its idiotype: only 19%.

In Fig. 3(b) we can see the results of the inhibition studies for the Anti-Id-2. The maximum inhibition (56%) was also produced by AXO-BA, followed by AMPO-BA, which inhibited 44% and BPO-BA (27%). Although the inhibition is lower, it represents a high specificity because is very difficult to get a complete blocking with the hapten.

The results showed that the maximum inhibition occurred with AXO-BA for both anti-Ids; therefore, these anti-Id antibodies are specific to the paratope of their respective Mabs, mimicking the antigenic determinant formed by AX. They were also blocked, but to a lower degree, by AMPO-BA, indicating the

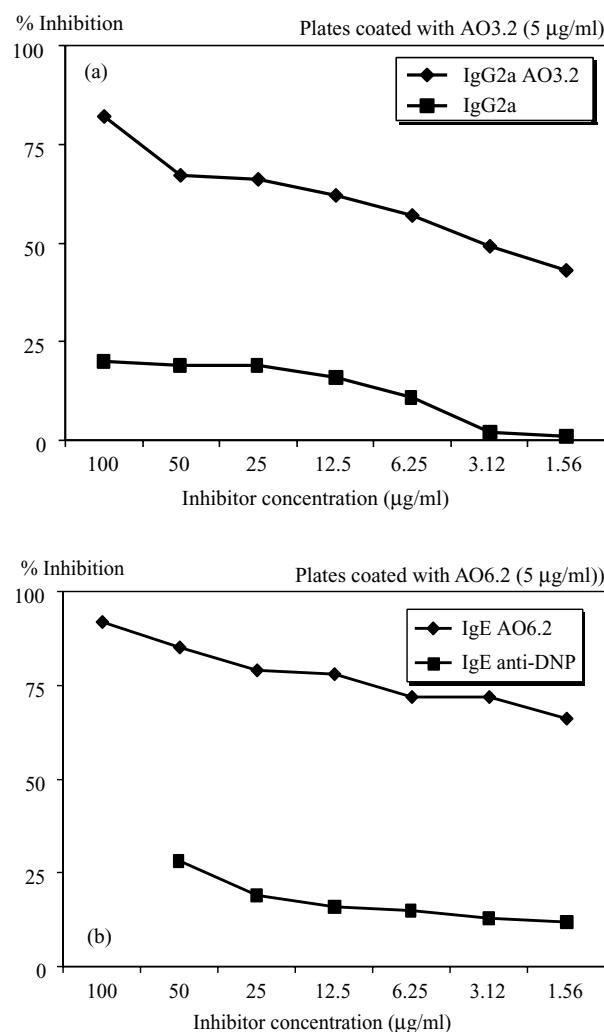


Figure 1. ELISA Inhibition assay. (a) The immunized rabbit serum specificity was tested using in the solid phase the idiotype antibody (AO3.2) at 5 µg/ml. In the fluid phase, we used the serum and, as inhibitors, two-fold dilutions (ranging from 100 µg/ml to 1.56 µg/ml) of both the same AO3.2 (■) to detect the antibodies to the idiotype and a polyclonal IgG_{2a} antibody (◆) to determine the presence of allotypic or subclass specific antibodies. (b) The immunized rabbit serum specificity was tested using in the solid phase the idiotype antibody (AO6.2) at 5 µg/ml. In the fluid phase, we used the serum and, as inhibitors, two-fold dilutions (ranging from 100 µg/ml to 1.56 µg/ml) of both the same AO6.2 (■) to detect the antibodies to the idiotype and a polyclonal IgE antibody (◆) to determine the presence of allotypic or subclass specific antibodies.

cross-reactivity of this conjugate. There was a lower blocking interaction with the other conjugate, BPO-BA, to which the Mabs were also less reactive.

Cross-reactivity of the anti-Id antibodies

Once we proved that the anti-Id antibodies were specific to the antigen binding site of their respective Mabs, we wanted to see if the idiotypes of a large panel of Mabs produced against the same hapten (AXO-HSA) and with differences in their specificities also had

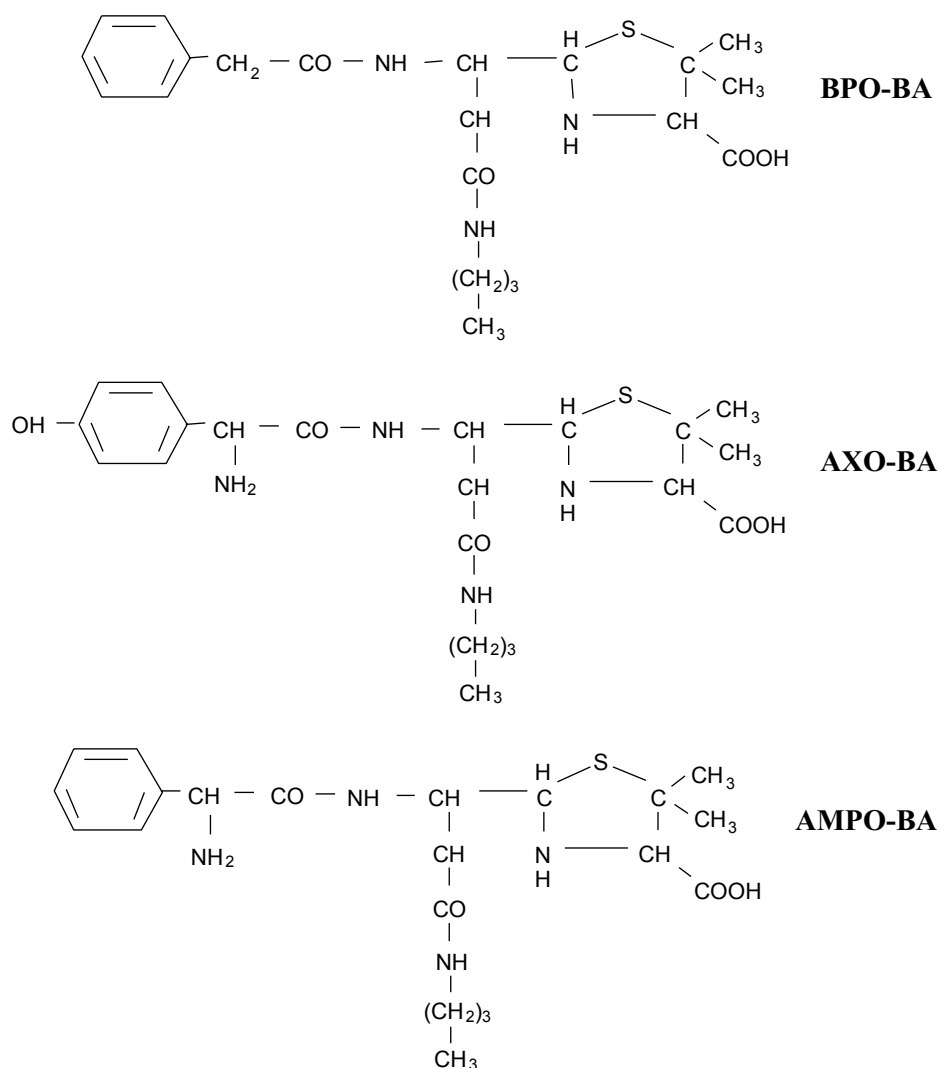


Figure 2. Chemical structure differences in the monomeric conjugates used in the anti-Id specificity studies.

conformational structures. Therefore we performed ELISA inhibition studies, shown in Figs 3(a,b), where in the solid phase we used the respective idiotypes (AO3.2 and AO6.2) and in the fluid phase the different idiotypes to AX that we wanted to compare.

As shown in Fig. 4(a), the Anti-Id-1 only really interacted with its specific idiotypic (AO3.2). The idiotypes present in the other Mabs produced a lower or no inhibition in the union of the Anti-Id-1 with the idiotypic of the solid phase. There was only a slight inhibition (29%) with the Mab AO24.1. This cross-reactivity was not due to a specificity to the constant region of the immunoglobulins, since both AO3.2 and AO24.1 have a different isotype (IgG_{2a} and IgG_{2b} , respectively).

When we studied the Anti-Id-2 (represented in Fig. 4b), we saw that the best inhibitor (69%) was the idiotypic inductor of its production in rabbit (AO6.2). Although in this case two other Mabs produced a significant inhibition – AO9.1 (44%) and AO19.1 (41%)

– their isotype (IgG_1) is not the same as the isotype of the Mab that induced the Anti-Id-2, which is IgE . This degree of blocking capacity means that the idiotypic of these Mabs is similar to the AO6.2 idiotypic, because the Anti-Id-2 cross-reacts with them. Both Mabs (AO9.1 and AO19.1) are specific to the AX side chain, as is AO6.2, which could be the reason for this cross-reactivity, but this does not mean that all Mabs that are specific to the side chain of AX necessarily have the same idiotypic. This is shown by the fact that other Mabs with the same specificity to AO6.2, such as AO7.2 or AO14.1, do not have the ability to cross-react with the Anti-Id-2. Something similar occurs with the Anti-Id-1 that does not cross-react with AO19.1, which belongs to the same group as AO3.2 with respect to its specificity.

Discussion

Although β -lactams are low molecular weight structures, they are able to induce the production of

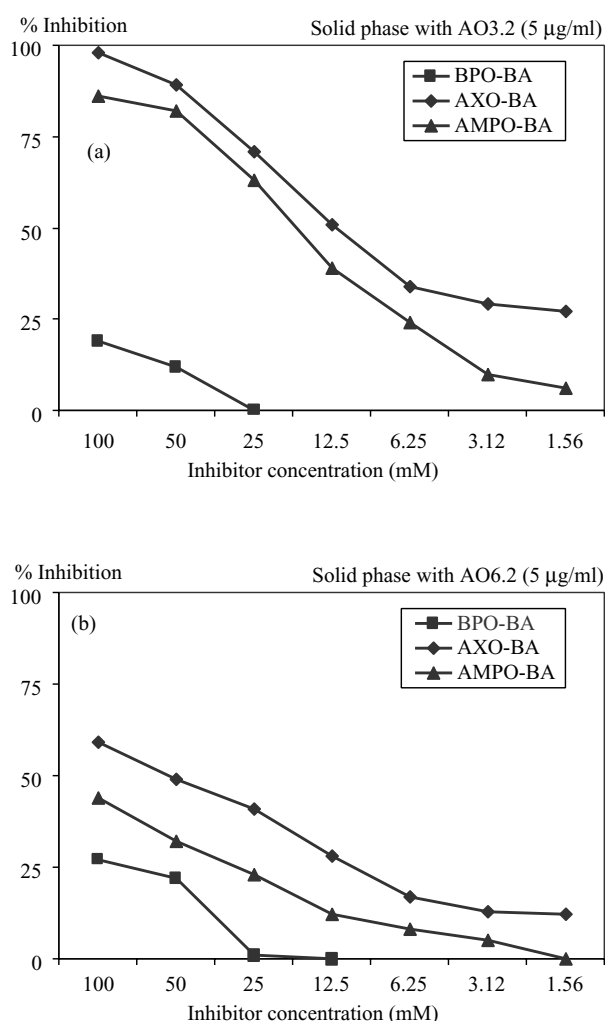


Figure 3. (a) Inhibition curves of the Anti-Id-1 with different monomeric conjugates: in the solid phase AO3.2 at 5 µg/ml and in the fluid phase the purified Anti-Id-1 and 1:2 decreasing concentrations (ranging from 100 to 1.56 mM) of the different inhibitors: AXO-BA (◆), AMPO-BA (■) and BPO-BA (▲). (b) Inhibition curves of the Anti-Id-2 with different monomeric conjugates: in the solid phase AO6.2 at 5 µg/ml and in the fluid phase the purified Anti-Id-2 and 1:2 decreasing concentrations (ranging from 100 to 1.56 mM) of the different inhibitors: AXO-BA (◆), AMPO-BA (■) and BPO-BA (▲).

antibodies with different specificities (8). Many studies have reported that the antigenic determinant inducing specific antibodies in penicillins is the penicilloyl group (10, 22, 23), but evidence exists concerning the importance of other parts of the penicillin molecule in the induction of a specific immune response (24, 25). Some studies show the importance of differences in the recognition of the acyl side chain structures by the specific antibodies (26–28), resulting in low or no cross-reactivity between different penicillins when the antibodies are really directed against these structures. Thus, Blanca et al. showed that there were patients allergic to AX who tolerated the administration of benzylpenicillin, proving that their antibodies recognized the acyl

side chain, which is present only in AX (15). Using Mabs to benzylpenicillin, de Haan et al. demonstrated that at least three well defined epitopes exist in the β -lactam structure: the thiazolidine ring, the acyl side chain, and the NAD (29). In later studies, by producing a large panel of Mabs to AX, we showed that this molecule can induce different specificities and that the main antibody inducing structure was the side chain of AX in 83% of the Mabs produced (16). Some antibodies recognized exclusively this structure, while some Mabs needed the nuclear portion of the antibiotic molecule for maximum recognition and others were only specific to the AX side chain, but could also recognize the side chain structure of other penicillins such as AMP or PG. The remaining antibodies were specific to the nuclear common region of this β -lactam, with a high cross-reactivity with all β -lactam antibiotics. Antibodies that fail to show a concrete specificity can also be produced.

To confirm the variety in the specificity of the antibodies produced against a haptenic molecule and their structural basis in the conformation of the idio type, we induced the production of Anti-Id antibodies against two antibodies specific to the side chain of AX, AO3.2 and AO6.2. The first belongs to the group of Mabs that recognize the side chain of AX and some region of its nuclear structure. The second monoclonal, AO6.2, is specific to the AX side chain, although it has a high cross-reactivity with the side chains of other penicillins (AMP and PG).

The anti-Id antibodies can be grouped mainly in two types according to the idio type region against which they have been produced: anti-Id antibodies that recognize the idiotopes that are outside the paratope and anti-Id antibodies that are specific to the antigen binding site of the idio type. The latter are able to block the link of the idio type with its antigen and are called the internal image of the antigen (30–32). This fact can only be demonstrated when the antigen is small and has the ability to block the union due simply to specific interactions, which is the case of the AXO-BA conjugate (33). A number of studies indicate that it is very difficult to obtain a complete mimicry of the antigen by an anti-Id antibody (17, 34).

From our specificity studies we can see that both Anti-Id-1 and Anti-Id-2 are inhibited in their union with their respective Mabs by AXO-BA. This means that they are anti-Id antibodies directed to the antigen binding site of the Mabs. Moreover, they are blocked by AMPO-BA, with which the antibodies AO3.2 and AO6.2 have a high cross-reactivity. An absence of cross-reactivity has been observed by means of the production of anti-Id antibodies to another haptenic molecule (35). When we analysed the cross-reactivity of the anti-Id antibodies to the idiotypes of the different Mabs produced against AXO-HAS, we also observed its absence. Results reported by other authors indicate

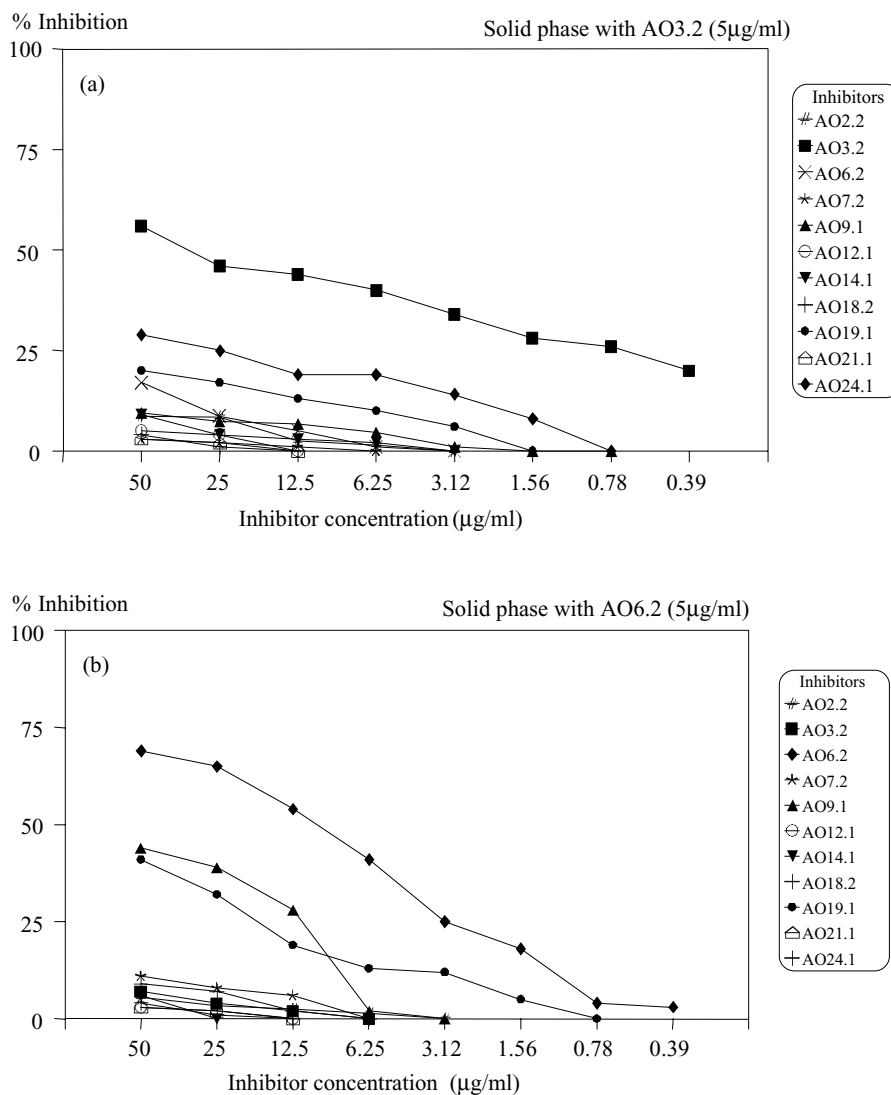


Figure 4. (a) Cross-reactivity study of Anti-Id-1 against different monoclonal antibodies to amoxicillin: in the solid phase AO3.2 at 5 µg/ml and in the fluid phase the purified Anti-Id-1 and, as inhibitors, 1:2 decreasing concentrations (ranging from 50 to 0.39 µg/ml) of the different Mabs. (b) Cross-reactivity study of Anti-Id-2 against different monoclonal antibodies to amoxicillin: in the solid phase AO6.2 at 5 µg/ml and in the fluid phase the purified Anti-Id-2 and, as inhibitors, 1:2 decreasing concentrations (ranging from 50 to 0.39 µg/ml) of the different Mabs.

that there is a high specificity of the anti-Id antibodies to their respective idiotypic (36). In our results we can see that the anti-Ids were blocked only by their homologous idiotypic and that there was no cross-inhibition. These results suggest that the anti-Id antibodies are highly specific to unique variable region determinants. This means that a small hapten such as AX provides several antigenic determinants that induce the production of a great variety of Mabs regarding their specificities. That is demonstrated by the inability of the anti-Id antibody specific to a Mab to recognize the idiotypic of another Mab with the same specific behavior because of differences in the conformational structures of this region of the immunoglobulin.

Summarizing, we conclude that a low haptenic molecule like AX is able to induce the production of antibodies with different specificities because of the formation of many idiotypes that are not all recognized by an anti-Id antibody produced against one of them. This increases our understanding of the recognition of the hapten by the antibody and explains the complexity of the interactions between the antigen and its specific antibodies.

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