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ANTIBODIES AGAINST THE ANTIBIOTICS: AN OVERVIEW

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Summary. - *Antibiotics are small molecules used in the treatment of diseases due to pathogenic microorganisms such as bacteria, viruses, fungi. They have different mechanisms of actions and by exploiting their selective toxicity on the infecting microorganism they do not necessarily damage the host cell. Assay for antibiotic concentrations in blood, urine and other bodyfluids is motivated primarily in conjunction with antibiotic pharmacology and pharmacokinetics to: a) establish the therapeutic levels of the drug; b) monitor antibiotics with narrow toxic/therapeutic ratios; c) detect accumulation of the antibiotic metabolites. The assay used to measure a wide variety of antibiotics is based on the intrinsic capability of these molecules to inhibit the growth of a suitable microorganism. In the last few years other types of tests, e.g. enzyme immunoassays, have been developed based on polyclonal and monoclonal antibodies. Antibodies against non antigenic molecules like antibiotics can be raised by using as antigen the antibiotic (hapten), conjugated with a carrier protein. Polyclonal antibodies containing mixed populations of antibodies (against different antigenic sites of the hapten, the carrier and the site of conjunction between the hapten and the carrier) are largely used to set up enzyme immunoassays for antibiotics. Monoclonal antibodies by recognizing one antigenic site of the hapten have a better specificity but sometimes less affinity than polyclonals. In the case of antibiotics, to raise monoclonal antibodies with good affinity it is important to use targeted immunization and screening strategies that utilize the antibiotic conjugated in different ways to different carriers. With the improvement of conjugation and screening techniques it will be certainly easier to produce good monoclonal antibodies against an increasing number of antibiotics and use them not only for assays but also for other applications as discovery of new molecules of pharmaceutical interest.*

KEY WORDS: monoclonal antibodies, antibiotics, immunoassays.

Riassunto (Anticorpi contro gli antibiotici: una rassegna). - *Gli antibiotici sono piccole molecole usate nel trattamento di patologie causate da microorganismi come batteri, funghi, virus. Agiscono con diversi meccanismi d'azione ed esplicando la loro azione selettiva sul microorganismo infettante, non devono danneggiare la cellula ospite. Il monitoraggio degli antibiotici nei vari fluidi biologici è di estrema importanza per: a) stabilire i livelli terapeutici del farmaco; b) monitorare gli antibiotici con un basso rapporto tra dose tossica e dose terapeutica; c) individuare l'accumulo di metaboliti. Tradizionalmente il saggio usato per l'analisi degli antibiotici si basa sulla capacità di queste molecole di inibire la crescita di un microorganismo usato come tester. Altri tipi di saggi si sono affermati per praticità e rapidità di esecuzione tra cui quelli basati su anticorpi policlonali e monoclonali. Per produrre anticorpi contro gli antibiotici, che di per sé non sono antigenici, occorre coniugare l'antibiotico (aptene), ad una proteina "carrier". Anticorpi policlonali contenenti popolazioni miste di anticorpi (contro i diversi siti antigenici dell'aptene, il carrier e il sito di congiunzione tra aptene e carrier) sono largamente usati negli "enzyme immunoassays" per gli antibiotici. Gli anticorpi monoclonali riconoscendo un solo sito antigenico dell'aptene sono più specifici dei policlonali, ma talvolta meno affini. Nel campo degli antibiotici essi non sono così diffusi come i policlonali poiché per ottenere anticorpi monoclonali con l'affinità desiderata è molto importante avere a disposizione strategie di immunizzazione e screening che utilizzano l'antibiotico coniugato in diversi modi a diversi carriers. Con il miglioramento delle tecniche di coniugazione e di screening sarà certamente più facile produrre anticorpi monoclonali contro un numero sempre maggiore di antibiotici da utilizzare non solo per il monitoraggio ma anche per altre applicazioni come la scoperta di nuove molecole di interesse farmaceutico.*

PAROLE CHIAVE: anticorpi monoclonali, antibiotici, saggi immunologici.

Characteristics of antibiotics

To prevent and treat diseases due to pathogenic microorganisms such as bacteria, viruses, fungi, protozoa, medicine makes use of a large variety of antimicrobial agents like antibiotics and chemiotherapeutic agents. Antibiotics are small molecules which at low concentrations inhibit the growth or lead to the death of microorganisms.

They can be of natural origin, i.e. they are secondary metabolites of bacteria or fungi, (e.g. teicoplanin), of synthetic origin (e.g. quinolones) or semisynthetic derivatives of natural products (e.g. beta-lactams, aminoglycosides, rifampicin). Antibiotics can act in several ways: for example, by inhibiting bacterial cell wall synthesis, damaging the cytoplasmic membranes or interfering with nucleic acid or protein biosynthesis (Table 1).

The central concept in antibiotic use is that of "selective toxicity" against the infecting organism without damage to the host cells. The ideal antibiotic would have no deleterious effect on the patient but would be lethal to the microorganism. The "selective toxicity" is obtained by exploiting differences between the biochemistry of the infecting agent and that of the host. Frequently, even though antibiotics inhibit biochemical processes or structures unique to bacterial cells, when given sistemically, they act on the cells of the host in other ways, and this can make them rather toxic.

Interaction of antibiotics with body compartments

The knowledge of the fate of antibiotics when they interact with the body compartments is needed to develop rational therapeutic regimens. Amongst the many properties that must be exibited by therapeutically useful antibiotics, the ability to achieve effective concentrations and act in the complex environment of the infected lesion is essential. Most antibiotics are given sistemically and the delivery and maintenance of effective concentrations achieved in the blood are in turn determined by the absorption, excretion and metabolism of the drug and the way in which the blood-born drug is distributed to the tissues.

Table 1. - *Sites of action of antibiotics*

Cell wall synthesis		
Cytoplasmic membrane (permeability)	Synthesis of cytoplasmic components	Nucleic acid synthesis
β-lactam antibiotics, Vancomycin, Teicoplanin, Fosfomycin, Cycloserine		
Colistin	Chloramphenicol	Rifampicin
Polymyxin B	Tetracycline	Griseofulvin
Amphotericin B	Macrolides	Fusidic acid
Nystatin	Lincomycin	Gyrase
	Aminoglycosides	inhibitors

From knowledge of the concentration profile of the agent in serum, it is possible to calculate its rate of absorption and elimination and the volume of distribution. The volume of distribution indicates whether the antibiotic is largely confined within the vascular compartments or spreads out into the extracellular fluid, the site of most infections, or penetrate into cells where some organisms, like mycobacteria or brucella, multiply. Antibiotics vary greatly in their absorption, blood concentrations, distribution in the body, tissue diffusion, accumulation, excretion and metabolism. The metabolic products of oxydation, reduction hydrolysis and conjugation are frequently microbiologically inactive or less active than the parent compound. The modified antibiotic may be present in the blood, or excreted in urine, bile or feces. This inactivation is usually associated with detoxification of the antibiotic as observed with chloramphenicol through its conjugation with glucuronic acid. Occasionally however, metabolic products can have enhanced toxicity as in the acetylation of sulfonamides.

Most antibiotics are eliminated predominantly through the kidneys, by glomerular filtration and also, in some cases, tubular secretion. A few antibiotics (e.g. rifampicin, fusidic acid, cefoperazone, ceftriazone) are also excreted in the bile.

Need for antibiotics assays

Assays of antibiotics concentrations in blood, urine, and other body fluids and tissues are essential in studies of pharmacology and pharmacokinetics; examples are:

a) measurement of antibiotics in body fluids to establish the pharmacokinetics behaviour and therefore determine the therapeutic levels of drugs in the various body compartments;

b) monitoring of antibiotics with narrow toxic/therapeutic ratios [1-6];

c) detection of antibiotics (e.g. chloramphenicol) metabolite accumulation in patients with organ disfunction, such as hepatic failure;

d) monitoring of drug absorption through the digestive tract in the case of orally delivered antibiotics [7, 8].

Traditionally the routine analysis of a wide variety of antibiotics, regardless of the structure and matrices involved, has been based on the intrinsic capability of these molecules to inhibit the growth of a suitable microorganism [9]. In this kind of test the level of an antibiotic is determined by comparing a definable microbiological response (e.g. inhibition zone, number of cells, turbidity) to those obtained from the application of a series of standard concentrations to a suitable test organism.

The major advantages of the biological assay are simplicity and versatility. Virtually any antimicrobial agent can be measured, provided that a sensitive test organism and an appropriate culture medium are available.

The microbiological assay, although remaining the basis of the analytical science involved, has the disadvantage of non specificity: the organisms used in the assay

may respond to more than one antibiotic as well as to other non specific inhibitory material. To overcome the problem encountered with microbiological assay, in the past few years a large variety of sensitive analytical techniques using antibodies have been developed [10].

Such immunological procedures beside possessing reasonable sensitivity, accuracy and precision, have the potential of showing a significant degree of specificity since they are based on the recognition of a structural component of the molecule. Immunological assays can detect all moieties, microbiologically active or not, that have a structure that can evoke the antigenic response.

The basis of the immunoassay for antibiotics depends upon the non-covalent reversible binding of the antibiotic by specific antibodies in a reaction that obeys to the law of mass action:

$$Ag + Ab \underset{k2}{\overset{k1}{\leftrightarrow}} Ag - Ab$$

where Ag represents the antigen (e.g. the antibiotic). Ab the specific antibodies and Ag-Ab the bound complex. At any given time some of the free reactant are combining with a rate constant k1, to form the complex. At the same time some of the complex is dissociating, with a rate constant k2, to give free antigen and antibody. The reaction is normally allowed to proceed until equilibrium has been attained, when the amounts of antigen present in the free and in the bound fractions will remain constant.

Antibodies against antibiotics

The most important reactant in an immunoassay is the antibody. Small molecules like antibiotics, with a molecular weight ranging up to 2000, are not ordinarily able to evoke an immune response if injected into animals. This characteristic of non "antigenicity" can be overcome by conjugating the antibiotic (hapten) with carrier proteins or synthetic polypeptides. The studies carried out over the last 20 years on the conjugates made with many different haptens and macromolecules have contributed to the understanding of the antigenic specificity of a given molecules, the requirements for the immunogenicity, the structure of antigenic determinants and the nature of the antibody [11].

The protein carriers are chosen on the basis of criteria such as availability of reactive sites, size, solubility, immunogenicity. The most frequently used carriers are reported in Table 2. Recently a simple method for binding hapten to spacer-modified non immunogenic carrier protein like gelatin has been described [12].

Generally the conjugation reaction is performed by using coupling agents like glutaraldehyde, carbodiimides, bis-diazotized benzidine and m-maleimidobenzoyl-N-hydroxy-succinimide ester. The conjugation approach to be used depends on the chemical groups contained in the hapten as reported in Table 3. The coupling reaction should not affect the configuration of the part of the molecule that constitutes the desired antigenic site. It is therefore important to select a coupling reagent that will crosslink through specific groups situated preferably away from the location of the antigenic site. When a conjugate has been made, a new molecule is formed. Some problems can occur as certain parts of the carrier molecule can become immunogenic after reaction with the coupling agent. This leads to the formation of antibodies that react strongly not only with the same carrier treated with the coupling agent in the absence of any hapten, but also with other unrelated carrier protein treated with the same cou-

Table 2. - *Principal carriers used for coupling haptens to proteins*

Carriers (*)	**Mr, K**
Bovine serum albumin (BSA)	67
Ovalbumin	43
Myoglobin	17
Tetanus toxoid	150
Keyhole limpet haemocianin (KLH)	> 2000
Thyroglobulin	669
Bovine, mouse, human gamma globulin	150

(*) see M.H.V. Van Regenmartel *et al.* [29].

Table 3. - *Conjugation of haptens to proteins*

Haptens	**Reagents (*)**
Haptens containing carboxyl groups	- mixed anhydride - carbodiimides - N-hydroxysuccinimide esters
Haptens with amino groups or reducible nitro groups	- carbodiimides - tolylene-2,4-diisocynate - glutaraldehyde - maleimides
Haptens with sulphydril groups	- maleimides - bromoacetimides groups
Haptens with hydroxyl groups acid	- hemisuccinate - diazotized p-aminobenzoic - p-nitrophenyl glycoside
Haptens with aldehyde or keton groups	- O-(carboxymethyl) oximes - p-hydrazinobenzoic acid

(*) see P. Tijssen *et al.* [30].

pling agent. This means that different conjugates have to be prepared with the same hapten by using different coupling agent in order to have a conjugate for immunization and others, as diverse as possible, for the screening of the antibodies [13, 14].

Monoclonal versus polyclonal antibodies

The antibodies can be polyclonal or monoclonal. A serum of an immunized animal contains antibodies specific for different antigenic sites present on the hapten, on the carrier and on the site of conjunction between the hapten and the carrier. It is possible to separate the hapten specific antibodies by hapten- affinity columns but this operation is not possible for all haptens and is expensive and time consuming. Moreover polyclonal antisera are not reproducible reagents since they always depend on the animal. Nevertheless this mixed populations of antibodies are largely used as base reagents for antibiotic immunoassays since they are relatively easy to make and usually exibit a good affinity for the antibiotic molecule. Radio, enzyme and fluoroimmunoassays using polyclonal antisera have been developed for aminoglycosides [15-18] chloramphenicol [19], macrolides [20, 21], vancomycin [22] and teicoplanin [23, 24].

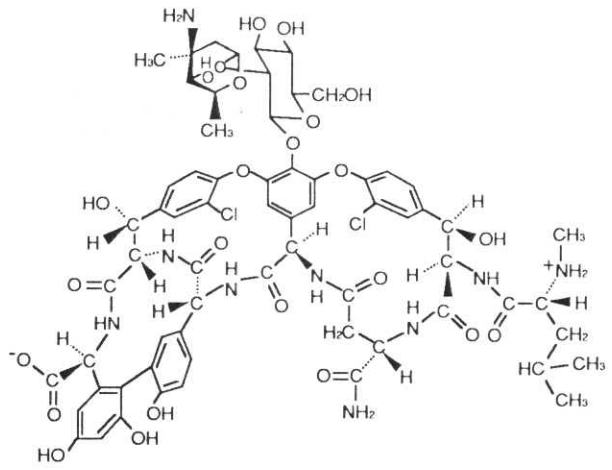
Monoclonal antibodies (MoAbs) are raised by the cell fusion technique by which the antibody-producing cells are immortalized and cloned. The hybrid clone produces a single antibody species that by recognizing only one antigenic site on the molecule dissects the antigenic structures which gave rise to the response. The antibody produced is highly specific. This high specificity is not always accompained by a high affinity. It has often been said that monoclonal antibodies do not have the high affinity achieved by conventional antisera. Today it is believed that MoAbs with the desired affinity can be produced and that the critical point is the choice of appropriate procedures.

In the case of small and sometimes "complicated" molecules like antibiotics (Fig. 1) to obtain MoAbs against the "hapten antibiotic" with good affinity the strategy used is crucial.

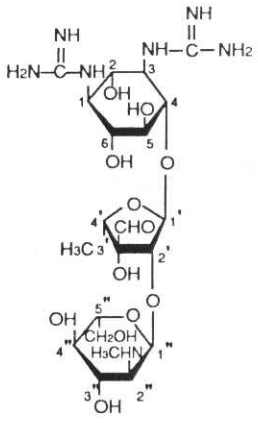
Few papers on the production of MoAbs against antibiotics have been published.

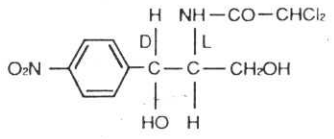
MoAbs against penicillin derivatives have been produced and used to investigate the antigenic nature of the benzylpenicilloyl group and its role in the allergy problem in man [25, 26]. From one of these studies it seems that on the benzylpenicilloyl group there are three antigenic sites one of which is created by the hapten-carrier conjugation [25]. A MoAb was raised against the antibacterial agent chlorhexidine to investigate the IgE-mediated hypersensitivity reactions in humans [27] and one against chloramphenicol has been used to detect the drug in animal tissues [28].

These studies demonstrate that it is very important in raising MoAbs against the "hapten antibiotic" to have targeted immunization and screening strategies that use



Vancomycin
$[C_{66}H_{75}Cl_2N_9O_{24}\,(1{,}449.27)]$





Chloramphenicol
$[C_{11}H_{12}Cl_2N_2O_5\,(323.13)]$

Streptomycin
$[C_{21}H_{39}N_7O_{12}\,(581.58)]$

Fig. 1. - Chemical structures of three antibiotics.

the antibiotic conjugated in different ways to different carriers. That is true in particular for some antibiotics that do not have any available group for conjugation to a carrier protein and that require modifications before coupling, with the risk of obtaining unwanted antibodies.

With the improvement of conjugation and screening techniques it will be certainly easier to raise good MoAbs against an increasing number of antibiotics and utilize them not only for assays but also for other applications in the field of antibiotics.

In particular by utilizing their selective recognition on the antibiotic molecule, it could be possible to project targeted screening for the discovery of molecules of pharmaceutical interest and detect in fermentation broths metabolites with little or no antibacterial activity as valuable starting materials for chemical modification to generate new bioactive molecules.

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