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Molecular recognition in antibodies and its application

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Abstract. The structure and function of immunoglobulins, and the nature of the antibody – antigen interaction are described. Applications of the molecular recognition properties of antibodies are discussed in the areas of immunotherapy, immunoassay, immunotargeting and catalytic antibodies.
Key words. Immunoglobulin; monoclonal antibody; antigen; immunotargeting; cytotoxic agent; immunotoxin; immunoassay; enzyme-linked-immunosorbent-assay (ELISA); catalytic antibody; hapten; conjugate.

Introduction

Ever since Kohler and Milstein [39, 41] developed the cell hybridisation method for producing continuous cell lines secreting a homogeneous (monoclonal) antibody of predefined specificity, there has been a rapid growth in the knowledge and understanding of immunoglobulin and antibody structure, function and production at the molecular level. This has arisen mainly through contributions from X-ray crystallography, NMR, genetic engineering and immunochemical studies, and there is no doubt that the area will continue to develop and expand during the 1990s. As a consequence, society will shortly begin to reap the benefits of newly developed antibody technologies, particularly through the areas of immunotherapy, catalytic antibodies, antibody-targeting and immunodiagnostics.

Immunoglobulin structure and function

Antibody production is induced in vivo when the hosts' lymphoid system comes into contact with a foreign substance, micro-organism, or other infectious agent. Antibodies bind specifically to the antigen that induced their synthesis whereas the term immunoglobulin is used to designate molecules having the same physical characteristics as antibodies but where their antigen specificity is unknown.

In the early part of the 20th century it was widely accepted that the antigen must instruct the specificity of the antibody by providing a template. However, the discovery by Sanger and Thompson [64] in 1953 that proteins had predefined sequences, and the realisation that the primary amino acid sequence was sufficient to specify all the biological activity of a protein [2] led to the demise of this

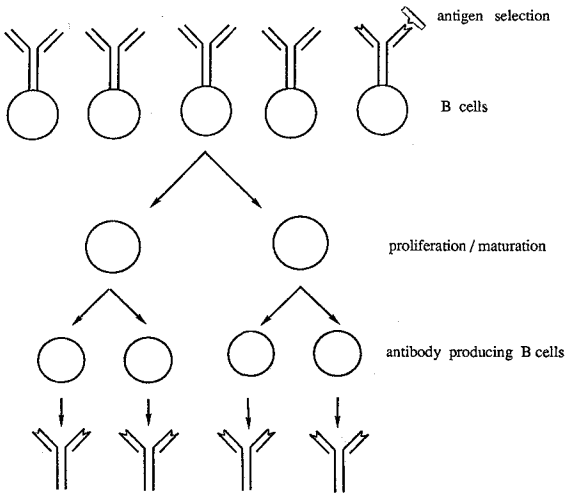

Figure 1. Clonal selection. Antibody producing B cells are each programmed to make only one antibody. The antibody is situated on the surface of the B cell and once antigen binds, the B cells are stimulated to proliferate and mature into antibody producing cells.

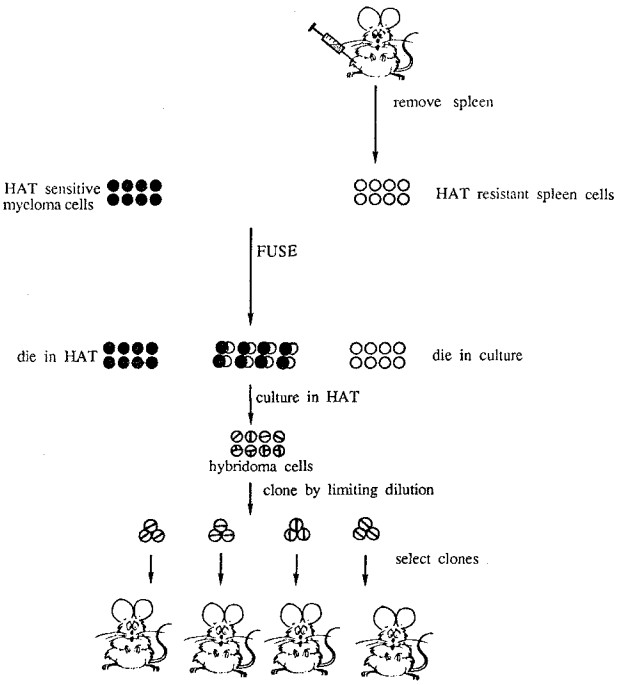

Figure 2. Monoclonal antibody production. Spleen cells from an immunized mouse are fused with HAT-resistant myeloma cells and cultured in HAT medium. Spleen cells die in culture naturally after 1–2 weeks whereas the myeloma cells are killed by the HAT, thus only the fused cells survive. The hybridomas are tested by ELISA for their ability to bind antigen, then cloned by limiting dilution. The monoclonal antibody producing cells are selected by ELISA and injected into mice to produce ascites fluid.

so-called 'Instructionist Theory'. By comparison the 'Clonal Selection Theory' initially put forward by Burnet in 1957[10], readily explains the specificity of the immune response in the framework of modern genetics[9,11]. The theory postulates that each lymphocyte has an unique receptor and is thus precommitted to making only *one* antibody with a characteristic specificity after appropriate stimulation (fig. 1).

The ability of one cell to produce only one antibody was exploited by Kohler and Milstein[39,40] who developed a cell hybridisation technique to produce continuous cell lines secreting antibody of predefined specificity (fig. 2). In the initial studies a HAT-sensitive variant of the MOPC 21 myeloma cell line was fused with splenocytes from mice immunised with sheep red blood cells. The hybrids were selected for growth in HAT (hypoxanthine, aminopterin, thymidine) medium – the spleen cells live only up to 10 days in culture and the myeloma cells die in the presence of HAT medium, thus only the hybrids survive. A number of cloned hybrid cell lines secreting anti-sheep erythrocyte antibodies were obtained. When these cells were injected into mice, ascites tumours were produced and the mouse serum was found to contain large quantities of homogeneous (monoclonal) antibodies.

The availability of monoclonal antibodies using hybridoma technology has revolutionised many areas of research and development and has greatly facilitated studies on the structure-function relationships of antibodies and immunoglobulins.

There are five distinct classes of immunoglobulin molecule which are recognised in most mammals namely IgG, IgA, IgM, IgD, IgE. These differ from each other in terms of size, charge, amino acid composition and carbohydrate content and essentially each immunoglobulin molecule is bifunctional. The Fab (fragment antigen binding) region is concerned with antigen binding, whereas the Fc (fragment complement) portion mediates physiological effector functions such as complement fixation, leucocyte binding and placental transmission. An excellent review on molecular recognition in the Fc portion has recently been published[12].

The polypeptide chains of immunoglobulins are linked together by disulphide bridges and non-covalent forces to give a basic four-chain structure consisting of pairs of identical heavy and identical light chains. IgG, IgD and IgE occur only as monomers of the four chain unit, IgA occurs in both monomeric and polymeric forms whereas humoral IgM occurs only as a pentamer with five four-chain subunits linked together (fig. 3).

IgG is by far the most abundant in serum and its molecular structure is known in some detail. The proteolytic enzymes papain and pepsin have been of enormous value in structure/activity studies on antibodies. These enzymes cleave IgG to form smaller well-defined fragments (fig. 4) permitting extensive structural studies at the molecular level. For example, high resolution crystal structures are available for pooled human Fc[17], rabbit Fc[74], and human Fab[66]. Antibody combining sites have also been examined in detail[16,58]. Diffraction patterns for whole IgG have been characterised by a lack of elec-

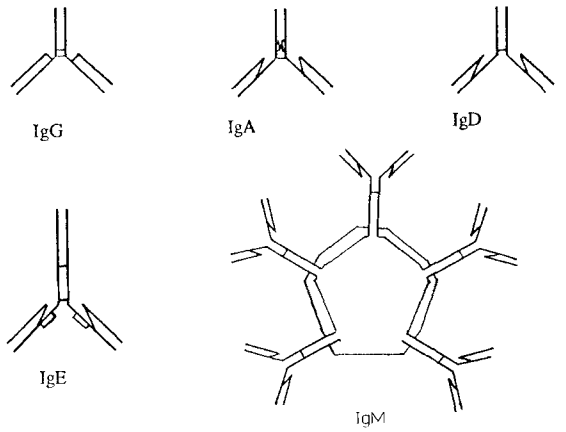

Figure 3. Peptide chain structures of IgG, IgA, IgD, IgE and IgM.

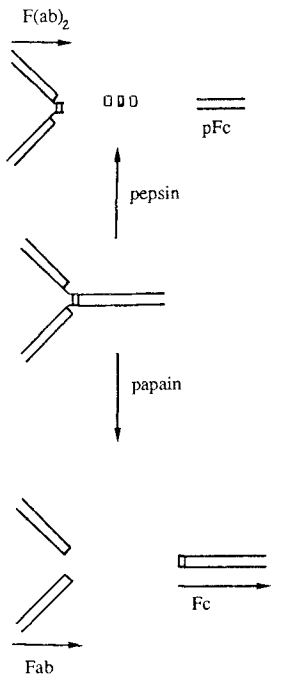

Figure 4. Enzymic cleavage of IgG. The proteases papain and pepsin cleave IgG at different sites. Papain cleaves at the hinge region yielding the Fc fragment and two Fab fragments. Pepsin cleaves the heavy chain producing $F(ab')_2$ and pFc' fragments, and in addition several low molecular weight oligopeptides.

tron density associated with the Fc part of the molecule which has been related to hinge flexibility[22,32].

Human IgG has a molecular weight, of 150,000 and is made up of two identical glycosylated heavy chains (mol.wt 50,000–75,000) and two identical non-glycosylated light chains (mol.wt 25,000). Each molecule has two antigen binding sites and IgG is therefore described as 'divalent'. The heavy chains are joined by disulphide bonds to each other and the light chain is joined by a disulphide bond to one heavy chain. Disulphide bridging between chains is characteristic of immunoglobulins but the exact number and location of such bonds is variable[52]. For example, IgG can be subdivided into 4 subclasses each having different numbers and arrangements of interchain disulphide bonds (fig. 5).

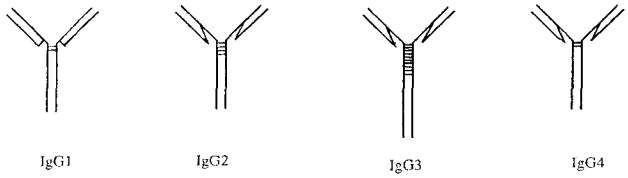

Figure 5. Peptide chain structure of IgG subclasses. The different IgG subclasses have different numbers and arrangements of interchain disulphide bands. For human IgG1 the disulphide band links the light and heavy chain at the hinge region whereas IgG2, IgG3 and IgG4, the band is at the junction between the variable and constant regions.

There are two types of light chain (kappa and lambda) and each immunoglobulin class contains molecules with either kappa or lambda light chains. Individual molecules may possess either kappa or lambda but never both. Each light and heavy chain is made up of a series of homologous units of approximately 110 amino acids. The units characteristically contain one intra-chain disulphide bond between cysteine units which are situated about 20 amino acids from each end, and are folded into a domain. The domains contain large amounts of β-pleated sheet[57] and are compact globular structures resistant to proteolytic attack.

The amino acid sequence of the N-terminal units varies greatly between molecules and is consequently known as the variable region (V). The light and heavy chain of this region are designated V_L and V_H, respectively. The variable domains of an antibody consist of a β-sheet framework with six hypervariable segments which comprise 80 amino acid residues and are attached to a framework of V_L and V_H β-sheet bilayers. Novotny and co-workers[53] analysed refined X-ray crystallographic co-ordinates for three antigen binding fragments (Fab KOL[44], MCPC 603[68] and NEW[66]), and used their results to introduce a general model for the V_L and V_H interface forming the binding region. The region was found to consist of two closely packed β-sheets, and its geometry corresponds to a 9 stranded cylindrical barrel of average radius 0.84 nm and an average angle of $-53°$ between its two constituent β sheets. The barrel forms the bottom sides of the antigen combining site. Their results indicate that the structural variability of the binding site is considerably less than was thought previously (this has recently been confirmed by Tramontano and colleagues[78]).

The nature of the antigen-antibody interaction

The binding of an antigen to an antibody occurs through the formation of multiple non-covalent bonds between the antigen and the amino acid binding site[77]. The attractive forces are mainly hydrogen bonds, electrostatic, Van der Waals and hydrophobic interactions and although individually weak, by comparison with covalent

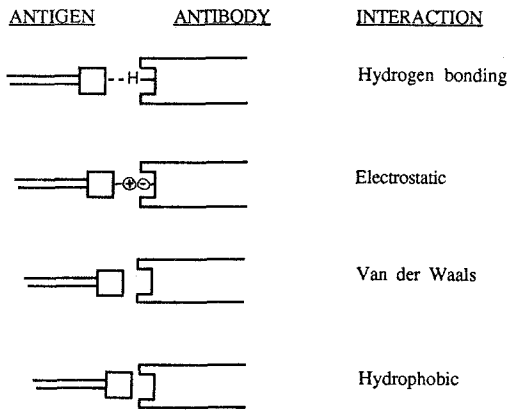

Figure 6. The intermolecular attractive forces between antibody and antigen molecules.

bonds, their multiplicity leads to a considerable binding energy. These non-covalent interactions are critically dependent on the distance between the interacting groups (fig. 6), thus they must be close in molecular terms before the forces become significant. However, if the electron clouds of the antigen and antibody overlap then steric repulsive forces come into play. Although $F \alpha \frac{1}{d^{12}}$ these forces act at very short range, they can be vital in determining the specificity of the antibody for a particular antigen. The sum of the repulsive and attractive forces determine the strength of the antigen-antibody bond and is termed the antibody affinity. For highly specific antibodies affinity constants are typically $> 10^{-9}$ M (kD). The antigen-antibody bond is generally stable from pH 4–9 and over a wide range of salt concentrations. When an antigen with several recognition sites, a multi-epitope antigen, combines with more than one of the antibody combining sites, the binding energy is considerably greater than the sum of the individual sites involved i.e. a bonus effect. The strength with which a polyclonal antiserum binds a multivalent antigen is termed the avidity. Typical avidity constants are 10^7 for IgG, 10^{11} for IgM.

Antibody specificity

The interaction between an antibody and its antigen is often highly specific, that is, the binding sites of antibodies directed against determinants on one antigen are not complementary to determinants on a different antigen. For many practical applications including immunotargeting, and immunodiagnostics, highly specific antibodies are essential. However, there are applications where antibodies of broad specificity or lower affinity can be extremely useful: for example in affinity chromatography, where it is intended to fully recover the antigen, and catalytic antibodies, where tight binding would lead to inhibition of catalysis and where it is desirable to be able to transform a wide range of substrates.

Immunotargeting

Immunotargeting was first suggested as far back as 1906 when Paul Ehrlich proposed using "bodies which possess a particular affinity for a certain organ as a carrier by which to bring therapeutically active groups to the organ in question"[19]. This prospect has fascinated investigators for decades and in modern day terms combines the specificity of antibodies with the toxicity of potent cell poisons (such as toxins, cytotoxic drugs and ionising radiation) to fashion a weapon which is potentially selectively lethal for tumour cells[50].

Early studies in this area utilised typical antitumour drugs such as methotrexate[45] linked to whole antibodies or ionising radiation in the form of radioiodinated molecules[28,62]. However, despite early promise a practical model of cancer therapy has not been forthcoming. Some of the problems that confront this technique[48] include

1. The cytotoxic agent must be capable of acting after delivery to the cell surface.
2. The biological properties of the antibody and the cytotoxic agent must survive the conjugation process.
3. The conjugate must be stable until it is delivered to its site of action (the linker must resist dissociation before reaching its target).

Recent studies have concentrated on utilising potent cytotoxic drugs such as adriamycin[3], daunomycin[3] and chlorambucil[18] conjugated to Fab or $F(ab)_2$ fragments in place of whole antibodies. It was found to be advantageous to remove the Fc portion of the antibody for several reasons: the immunogenicity of the antibody is reduced, the persistence time of any unreacted drug-conjugate in the blood circulation is shortened, the danger of any complement-mediated cytotoxicity affecting the normal cells in the host animal is decreased and the reduced size allowed increased tumour penetration.

The major aim of immunotargeting is to get as much of the cytotoxin to the site of action as possible – the more potent the toxin the less required to be delivered to the tumour. In this respect plant toxins such as abrin and ricin and certain bacterial toxins are extremely valuable[43]. The mode of action of toxins is reviewed elsewhere[14,29,54,55]. Briefly, toxins comprise two types of polypeptide chains (A and B) which may be linked by disulphide bridges. The B component appears to bind to surface receptors on membranes of susceptible cells and the A-chain then penetrates (or is translocated) across the cell membrane into the cytosol where it terminates protein synthesis.

Two main approaches have been adopted for the synthesis of immunotoxin conjugates[75,76]. The first is to link the intact toxin to the antibody and to rely on the specific binding properties of the antibody. Predictably, however,

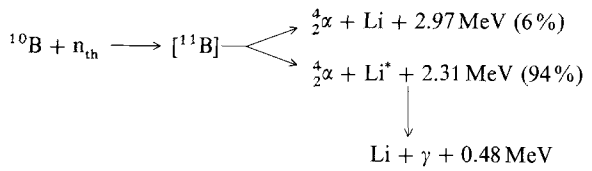

Figure 7. The boron neutron capture reaction.

this method suffers from a lack of complete specificity as the B-chain moiety can bind to non-target cells. A second approach, preferred by most workers, is to link isolated A-chains or ribosome inactivating proteins[26] directly to the antibody. To date many immunotoxins have been made by linking A-chains to antibodies against tumour-associated antigens, immunoglobulin determinants, and lymphocyte antigens[76].

A second method of killing tumour cells involves the use of ionizing radiation. In early studies, the radiation, often ^{131}I, was targeted to the cells as radiolabelled antibodies[28, 62]. This technology has recently been applied to Boron-Neutron-Capture Therapy (BNCT)[6]. BNCT is based on the nuclear reaction that occurs when a stable ^{10}B isotope is irradiated with low energy or thermal neutrons to yield α particles and lithium nuclei (fig. 7). Alpha-particles are relatively slow moving, and produce closely spaced ionizing events consisting of tracks of sharply defined columns. They have a path length of 10 μM, are high LET (linear energy transfer) and can destroy a wide range of biologically active molecules such as DNA, RNA and proteins. Moreover, there is little cellular repair after α particle induced radiation injury. A significant biological event will only be observed if there is a sufficient flux of thermal neutrons and a critical amount of ^{10}B is localised at the target area. Since the radiation produced is extremely localised there is no concomitant injury to normal tissue components.

The main problem encountered by researchers in this area concerns preparing monoclonal antibody – boron complex conjugates which contain sufficient boron to kill the tumour and retain immunoreactivity/selectivity. For example it is necessary to deliver boron at a concentration of approximately 50 μg/g to the tumour so that > 80% of the radiation dose would result from capture. There has been a recent resurgence of interest in BNCT as a means to treat various tumours[25, 31], and research is currently underway worldwide to develop active boronated conjugates.

Mizusawa[47] and Goldenberg[30] conjugated anti-carcinoembryonic antigen (CEA) antibodies with p-[1,2-dicarba-closo-[1-H^3]dodecaboron(12)-2-yl] benzene diazonium ion, and obtained an immunoconjugate estimated to have 30–50 atoms of ^{10}B/molecule of antibody. The conjugate exhibited immunoreactivity and selective in vivo localisation in hamsters carrying human colon xenografts but there was insufficient boron to sustain a lethal ^{10}B (n, α) ^7Li reaction. In an attempt to maximise the number of ^{10}B atoms that could be linked to an antibody molecule Alam[1] and colleagues conjugated a water-soluble polyhedral carborane isocyanate to poly-L-lysine. The resultant macromolecule, which contained 21–28% boron by weight and up to 2000 boron atoms/molecules, was linked to monoclonal antibodies against the B16 melanoma[4, 5]. In vivo distribution studies showed that using such a heavily loaded antibody resulted in an altered distribution. There was a reduction in the amount of boronated antibody localised in human colonic tumours and a corresponding increase in the amount in liver compared to native antibody.

The problems of altered distribution are considerably reduced if antibody therapy is carried out ex vivo. With this in mind Morris and co-workers[49] are investigating tumour therapy through autologous bone marrow rescue – explanted bone marrow is treated with boronated antibody, neutron irradiated to kill cancerous cells, washed and re-implanted.

Immunoassays

Immunological reactions are widely used for assays or detection procedures for antibody or antigen because they offer high levels of specificity and sensitivity. Radioimmunoassay[63] was one of the earliest systems developed and measured the analyte by competition between radiolabelled and unlabelled sample antigen for antibody. The sandwich type solid phase radioimmunoassay (known as the immunoradiometric assay or IMRA) was developed from this system and uses labelled antibody rather than antigen[46, 82]. Both these techniques have a high level of sensitivity and reproducibility and are amenable to automation for large-scale processing. However, the use of isotopes poses problems: the reagents are costly and often have a short shelf-life, complex equipment is required for reading the results and special safety measures have to be imposed for handling and disposing of the reagents. For these reasons isotopic assays have been superseded by ELISA (enzyme linked immunosorbent assays).

ELISA offer several advantages over IRMA: the enzyme labelled reagents can be stored over long periods of time without losing their activity, the reaction can be read visually (without expensive apparatus) and, using multiwell microtitre plates, large numbers of samples can be handled. Further, as many molecules of product can be obtained using one molecule of enzyme, the potential sensitivity of ELISA is greater than that of IMRA[20, 21, 33].

ELISA was pioneered by Engvall and colleagues and by Van Weeman and Schuurs[15, 23, 24, 38, 72, 79, 80] and has since been widely exploited as a detection system: for antigens and antibodies in human and veterinary medicine, in immunology and in the food and agriculture industries.

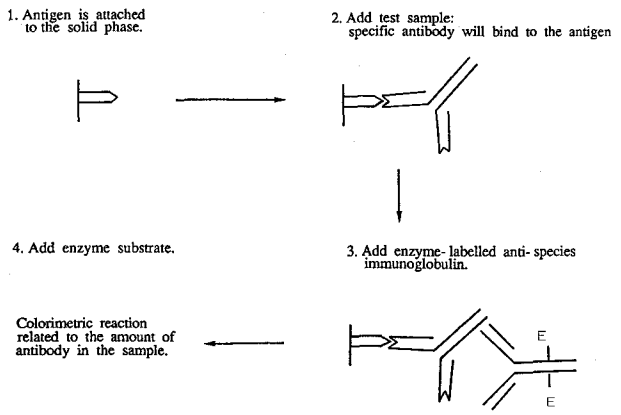

Figure 8. The indirect or sandwich ELISA.

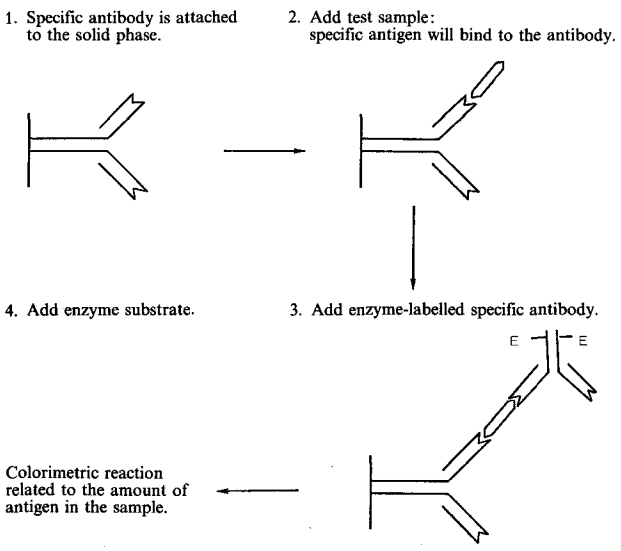

Figure 9a.

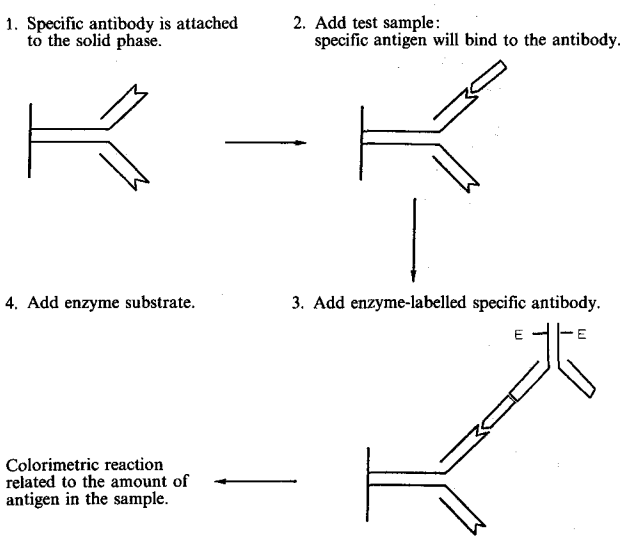

Figure 9b.

Figures 9a and 9b. Two types of two-site ELISA.

Indirect ELISA or the sandwich assay[23] is the simplest form of ELISA. The antigen is bound to the microtitre plate and the antigen solid phase is used to bind specific antibodies in the test sample (fig. 8). Unbound material can be removed by washing and the bound antibody is detected using an enzyme-labelled anti-immunoglobulin. If the enzyme-labelled antibody is specific for a particular class of immunoglobulin then the type of antibody can be determined. In this system the detection of antibody may be limited by the ability of the antibody to adsorb antigen which is directly bound to the solid phase.

The two-site ELISA is a rapid and easy method for antigen detection. In contrast to the indirect ELISA, antibody is bound to the microtitre plate and used in this form to capture the corresponding antigen in the test sample (fig. 9a). Bound antigen is subsequently detected using an enzyme-labelled antibody. Alternatively two monoclonal antibodies that recognize different parts of the antigen can be used (fig. 9b).

Competition ELISA

Competitive binding can also form the basis of ELISA techniques, Firstly, competition for binding to an antigen can be carried out using an antigen-coated microtitre plate, where a fixed level of enzyme-labelled antibody competes with varying levels of unlabelled antibody in the test sample (fig. 10). The sensitivity of the assay is increased when the unlabelled antibody is bound in a sequential fashion before the labelled reagent is added. Alternatively (fig. 11), a specific antibody (or im-

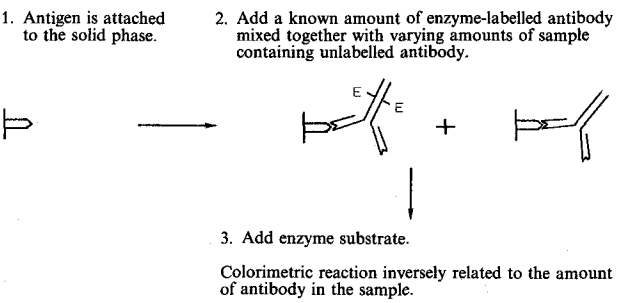

Figure 10. Competition ELISA for binding to an antigen.

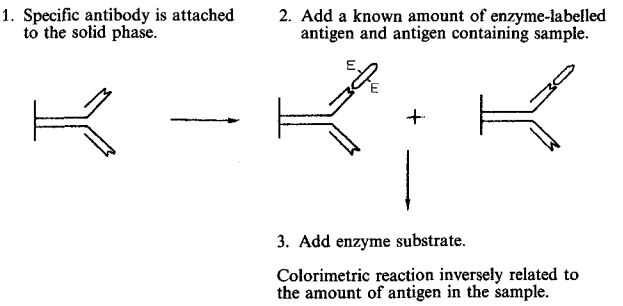

Figure 11. Competition ELISA for binding to an antibody.

munoglobulin containing specific antibody) is attached to the solid phase and the sample, containing the antigen, is added followed by enzyme-labelled antigen. Once enzyme substrate has been added, only the reference wells containing enzyme-labelled antigen alone show full coloration. The inhibition of colour change in the wells is proportional to the amount of antigen in the test samples.

Catalytic antibodies (Abzymes)

The applications of antibodies discussed so far are heavily reliant on the antibody's unique specific binding and recognition properties. Within the last five years, however, antibodies have been developed which exhibit catalytic properties, and in some cases rate enhancements approaching those of enzymes have been observed.

Antibodies and enzymes both achieve binding through the hydrogen bonding, van der Waals, electrostatic and hydrophobic interactions which occur when molecules come together[35, 56]. This binding function has, however, different consequences as enzymes and antibodies have evolved for different purposes. Enzymes have evolved to utilise the binding energy to catalyse a particular reaction which takes place in their active site. On the other hand, antibody molecules are produced in response to the pres-

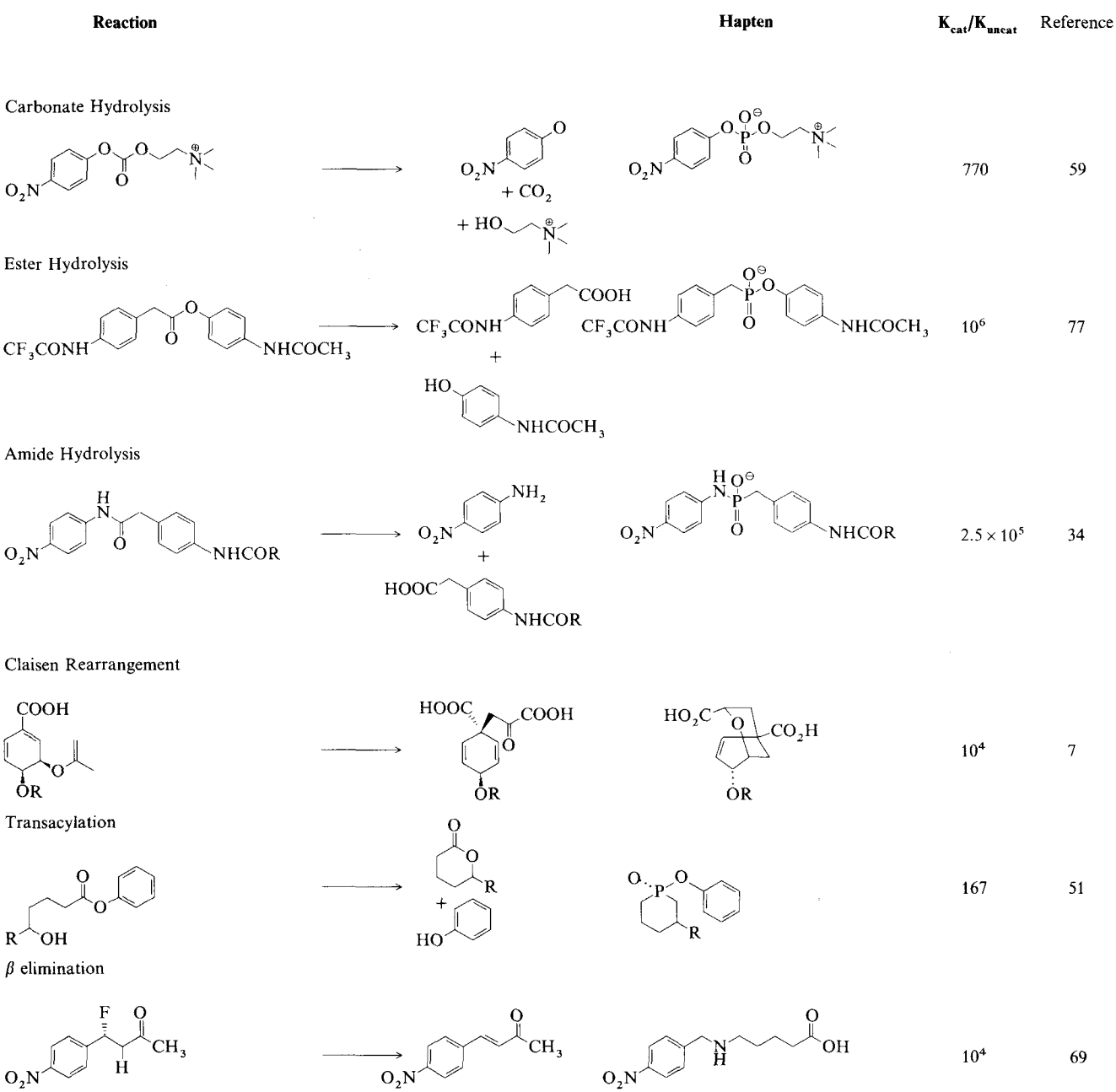

Figure 12. Examples of abzyme-catalyzed chemical transformations.

ence of antigen and their function is to eliminate it. The primary repertoire of antibodies consists of up to 100 million different specificities, each of which can be induced by the encounter of an appropriate B-cell with the appropriate antigen. Moreover, after the initial exposure to antigen, the process of somatic mutation generates a further 10^4 new binding specificities for any antigen. This repertoire has, within the past five years, been shown to include catalytic sites of considerable efficiency[41,42,67,70].

To date, three general strategies have been adopted for generating catalytic antibodies. The most widely used of these exploits the steric and electronic complementarity of the antibody binding site to its corresponding hapten. Standard hybridoma technology (fig. 2) has been used to obtain monoclonal antibodies containing binding sites which:

(1) are complementary to the rate-determining transition state for a given reaction
(2) can reduce the entropic requirements for the reaction by suitably orientating the reactants
(3) incorporate a suitably positioned catalytic amino acid residue
(4) can accommodate the binding of a cofactor.

Using this methodology, monoclonal antibodies have been shown to catalyse a variety of chemical transformations including carbonate, ester and amide hydrolysis, Claisen rearrangement, transacylation and a β-elimination (fig. 12).

An alternative method for generating catalytic antibodies employs the modification of a known antibody through site-directed mutagenesis or chemical modification. Schultz and co-workers have employed site-directed mutagenesis to introduce catalytic activity into the well-characterised antibody MOPC 315 which binds 2,4 dinitrophenyl ligands (K_a characteristically 10^3–10^7 M^{-1})[67]. The F_V fragment of MOPC 315 was expressed in *E.coli* and recombinant DNA technology employed to obtain

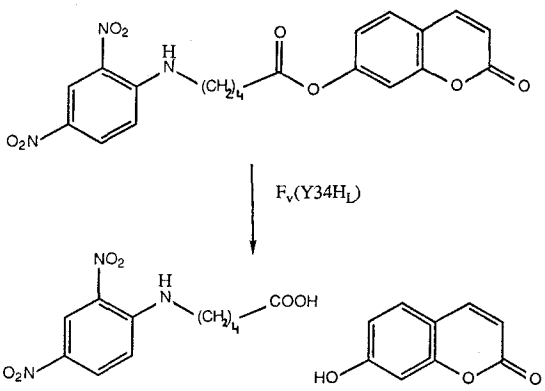

Figure 13. The histidine mutant catalyzed hydrolysis of a dinitrophenyl coumarin.

two mutants in which Tyr[34] of the wild type F_V fragment was replaced by His and Phe. Wild type F_V (F_V (315)) and the Phe mutant (F_V ($Y34F_L$)) bound DNP-L-lysine with similar affinities while the His mutant (F_V ($Y34\ A_L$)) exhibited a six-fold lower affinity. The His mutant catalysed the hydrolysis of a dinitrophenylcoumarin ester (fig. 13) with an initial rate 50 times faster than that of the wild type F_V or the $F_V(Y34F_L)$ mutant. Schultz has suggested that studies of this type will "prove a powerful tool for augmenting the rate enhancements of catalytic antibodies generated via other strategies and for the stepwise evolution of catalytic activity in antibodies to produce efficient selective catalysts"[67].

Chemical modification of proteins has been successfully accomplished by various researchers, in particular Kaiser et al.[37], and Suckling et al.[73]. This approach has recently been extended to make use of the specific binding properties of an available and characterised antibody. For example, Schultz and co-workers have used a cleavable affinity label to selectively modify the binding site of MOPC 315 with thiol and histidine functionalities[60,61] (fig. 14). The modified antibodies catalyzed the hydroly-

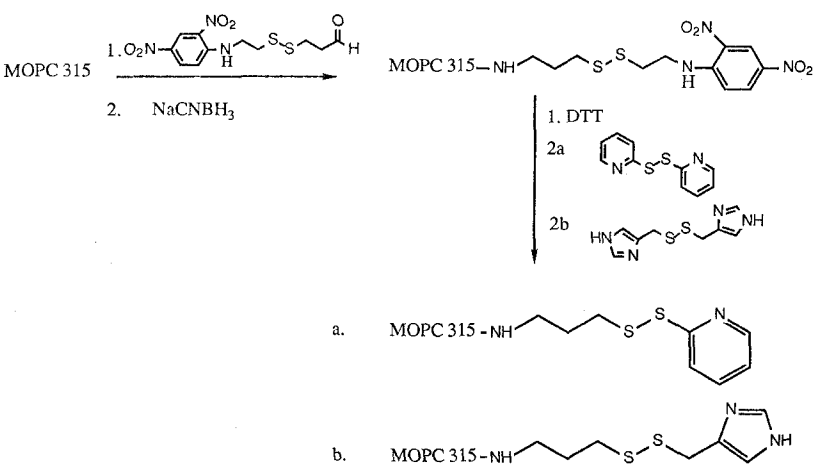

Figure 14. Introduction of a cleavable affinity label into the binding site of MOPC 315.

sis and thiolysis of a dinitrophenylcoumarin ester with rate enhancements of 10^3 and 10^4, respectively. This technique has enormous potential for introducing natural and synthetic catalysts such as transition-metal complexes, cofactors, bases and nucleophiles into an antibody binding site.

The third strategy, involves introducing a complete 'library' of antibody genes from lymphocytes into bacteria in order to produce the binding regions (V_H and V_L) of the antibodies [65]. In theory, it should be possible to clone and express all the antibody specificities form the mRNA of rodent B lymphocytes but unfortunately immunoglobulins are constructed from two chains encoded for by different genes. Nevertheless, studies have shown that the majority of binding energy comes from interactions with the heavy chain and the isolated heavy chain molecules have been reported to retain epitope affinities within two orders of magnitude of the intact antibody.

Expression can be achieved through the creation of bacteriophage libraries by using plasmid vectors in *E. coli* [65, 81]. Thereafter the immunoglobulin variable region genes are amplified and a library is created in *E. coli* which can allow production of considerable quantities of 'antibody' if desired. In this way millions of bacterial clones may be generated which synthesize antibody fragments. These are screened by transferring the proteins onto nitrocellulose filters and using the antigen of interest to identify the specific clone directly. This allows millions of antibody specificities to be screened in a few days and thus it is not expected to be essential to pre-immunise an animal with a transition state analogue – rather, the substrate for the desired reaction is employed to identify which expressed 'antibody' possesses the required catalytic activity. Finally, this technique can be applied to other animal species and so the potential exists to construct human monoclonal catalytic antibodies which could find many applications in the treatment of disease.

Conclusion

In the short period of time since Kohler and Milstein reported the cell hybridisation technique for producing monoclonal antibodies in 1975, a great deal has been learned about the structure and function of antibodies through a combination of chemical and immunological studies. As new antibody-producing technologies develop (in particular ex vivo methods) both animal and human monoclonal antibodies will become more readily available. This will provide antibodies for application in biology, chemistry and medicine.

Acknowledgments. The authors wish to thank Mrs E. A. Quinn for her patient, accurate and cheerful help with typing and word processing.

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