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Phil. Trans. R. Soc. Lond. B 1989 **323**, 487-494
doi: 10.1098/rstb.1989.0026

Phil. Trans. R. Soc. Lond. B **323**, 487–494 (1989)
Printed in Great Britain

Studies of structure and specificity of some antigen–antibody complexes

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By using X-ray diffraction and immunochemical techniques, we have exploited the use of monoclonal antibodies raised against hen egg lysozyme (HEL) to study systematically those factors responsible for the high specificity of antigen–antibody interactions. HEL was chosen for our investigations because its three-dimensional structure and immunochemistry have been well characterized and because naturally occurring sequence variants from different avian species are readily available to test the fine specificity of the antibodies. The X-ray crystal structure of a complex formed between HEL and the Fab D1.3 shows a large complementary surface with close interatomic contacts between antigen and antibody. Thus single amino acid sequence changes in heterologous antigens give antigen–antibody association constants that are several orders of magnitude smaller than that of the homologous antigen. For example, a substitution of His for Glu at position 121 in the antigen is sufficient to diminish significantly the binding between D1.3 and the variant lysozyme. The conformation of HEL when complexed to D1.3 shows no significant difference from that seen in the free molecule, and immunobinding studies with other anti-HEL antibodies suggest that this observation may be generally true for the system of monoclonal antibodies that we have studied.

1. Introduction

Although the immune response to an antigen is a complex and not yet completely understood process, much useful information may be obtained from studies of the antigen–antibody recognition process at the molecular level. The epitopic regions of a foreign molecule are determined by a number of complex factors, which depend not only upon the antigen itself but also upon the immune system of the host (Berzofsky 1985). These factors may be elucidated, in part at least, by three-dimensional structural investigations of the antigen and of its specific antibodies, in both the free and complexed states. Thus we may determine the extent and nature of the antigenic regions, and the degree to which changes in conformation of the antigen occur upon formation of the immune complex. The same considerations concerning the binding site and the conformation of the antibody as a whole, before and after complex formation, hold equal importance. In particular, the nature of the antigen–antibody interface formed by this highly specific recognition should not only extend our understanding of the immune response but also help us to make better use of antibodies as a tool for both basic research and medical applications.

A systematic investigation of specific antibody interactions with specific antigen is now possible through the technique of cellular hybridization (Köhler & Milstein 1975) to produce monoclonal antibodies (MAbs) of pre-defined specificity. Because MAbs can be produced in

large amounts, they are suitable for immunochemical studies and for crystallization trials as the first stage in crystallographic structure determination. To this end we have chosen hen egg lysozyme (HEL) as antigen for our investigations because (i) its three-dimensional structure has been well characterized at high resolution in several different crystalline forms (Blake *et al.* 1967; Berthou *et al.* 1983; M. Ramanadham, L. C. Sieker and L. H. Jensen, personal communication); (ii) its immunogenic properties have been extensively studied (reviewed in Benjamin *et al.* 1984); and (iii) several sequence variants from a variety of avian species are readily available to probe the fine specificity of the anti-HEL MAbs.

We summarize in this paper our results of immunochemical and crystallographic studies, in particular the determination of the three-dimensional structure of a complex formed between the Fab D1.3 and HEL studied at 2.8 Å† resolution. This structure has served as a basis to understand and rationalize the cross reactivity of several anti-HEL antibodies with other avian lysozymes. The patterns of cross reactivity obtained with more than 40 MAbs strongly imply that the whole of the surface of lysozyme that is accessible to antibody is potentially antigenic. The crystal structure of D1.3–HEL and the observation of many ternary complexes (MAb–HEL–MAb′) in immunobinding studies suggest that the conformation of the antigen and, less directly, the conformation of the antibody are essentially conserved, minor adjustments of the sidechains apart. We outline, in addition, crystallographic analyses of other lysozyme–Fab complexes and of a Fab idiotope–Fab anti-idiotope complex obtained by using D1.3 as antigen, which are in progress.

2. Results

The crystal structure of a Fab–lysozyme complex

The preparation and crystallization of the complex formed between the Fab fragment of the monoclonal antibody D1.3 and HEL is described elsewhere (Mariuzza *et al.* 1983). The three-dimensional structure of the complex has been analysed at 2.8 Å resolution from an electron-density map phased by isomorphous replacement (Amit *et al.* 1986). The essential points relating to the antibody–antigen structure are now described.

The interface between the two molecules in the complex is large, having an area of approximately 750 Å^2. A total of 17 residues from the Fab D1.3 make contact with HEL; these are His 30, Tyr 32, Tyr 49, Tyr 50, Phe 91, Trp 92 and Ser 93 from the variable domain of the light (L) chain, and Thr 30, Gly 31, Tyr 32, Trp 52, Gly 53, Asp 54, Arg 99, Asp 100, Tyr 101 and Arg 102 from the variable domain of the heavy (H) chain. Although these residues are distributed between all the six complementarity-determining regions (CDRs), there are more interactions with the H chain, particularly with its CDR3. The CDR3s of both the L and H chain, in fact, occupy the central region of the antigen-binding site and it is probably significant that these reflect the diversity resulting from the somatic recombination process of the V, D and J genetic segments (Tonegawa 1983). The junction points of the V, D and J segments (V_L–J_L, V_H–D and D–J_H) are subject to variability in the position of joining with the additional possibility of deletion or insertion of residues during the maturation of the B-cell lymphocyte. Residues 99–102 of the H chain, which are encoded by the D segment, make direct contact with the antigen. Although the joining segments, J_H and J_L, code for residues mostly outside the region of contact with the antigen, they may contribute to antibody diversity by their influence

† 1 Å = 10^{-10} m = 10^{-1} nm.

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on the conformation of the CDR3s of both chains. In this context it is interesting to note that although residues V_L Tyr 49 and V_H Thr 30 are in direct contact with lysozyme, they do not fall within the generally accepted CDR limits. In murine H and L chains, these two residues are invariant or semi-invariant and are classed as belonging to the framework regions. The three-dimensional structure of D1.3 in the free state has not been determined, but we may suppose that it probably has not changed significantly upon formation of the complex from the fact that the relative arrangements of the domains within the constant and variable dimers do not differ significantly from those found in other known Fab structures (table 1).

TABLE 1. A COMPARISON OF THE VARIABLE DOMAINS OF SOME KNOWN FAB STRUCTURES

(The comparison was made from the optimum superposition of the Ca positions of the same residues used by Colman *et al.* (1987). After optimizing the superposition of the V_L domains of each pair of Fabs from the matrix in turn, the rotation and translation (given in parentheses in angströms) required for optimum superposition of the V_H domains was determined. The Fab structures used were as follows: HyHEL-5 (Sheriff *et al.* 1987), Kol (Marquart *et al.* 1980), New (Saul *et al.* 1978), J539 (Suh *et al.* 1986), McPC603 (Satow *et al.* 1986) and R19.9 (Lascombe *et al.* 1989).)

	HyHEL-5	Kol	New	J539	McPC603	R19.9
D1.3	6.8° (0.39)	2.6° (0.18)	1.9° (0.20)	5.1° (0.49)	4.8° (0.22)	9.5° (0.45)
R19.9	6.9° (1.53)	9.1° (0.27)	8.0° (0.39)	12.8° (0.15)	6.9° (0.17)	
McPC603	4.9° (0.27)	5.4° (0.35)	3.8° (0.68)	7.2° (0.05)		
J539	11.0° (0.61)	4.5° (0.54)	6.7° (0.35)			
New	5.0° (0.58)	3.6° (0.02)				
Kol	8.5° (0.32)					

A total of 16 residues of HEL make contact with the antibody: Asp 18, Asn 19, Arg 21, Gly 22, Tyr 23, Ser 24, Leu 25, Asn 27, Lys 116, Gly 117, Thr 118, Asp 119, Val 120, Gln 121, Ile 124 and Leu 129. Thus the antigenic region consists of two segments of lysozyme which, although separate in primary sequence, form a contiguous area on the surface of the molecule. There is no significant change in the conformation of the antigen upon formation of the complex as indicated by differences in the dihedral angles of the main chain and in the root mean square differences in the C_α positions (0.64 Å).

The antibody-binding site is relatively flat, but with protruding sidechains modulating the contour of the surface. The CDR3s of the H and the L chains together form a small cleft that accepts the protruding residue Gln 121 from lysozyme, reminiscent of antibody–hapten complexes. The sidechain of Gln 121 occupies a large hydrophobic environment inside this cleft but a strong hydrogen bond is formed between $N_\varepsilon 2$ of Gln 121 and the main-chain carbonyl group of V_L Phe 91. This cleft therefore appears to be very specific for Gln, an observation strongly supported by the cross reactivity of D1.3 with other species of lysozyme (see below). The specificity of D1.3 for HEL is greatly enhanced by 11 additional hydrogen bonds, which require a precise juxtaposition between donor and acceptor at the antigen–antibody interface. Six of these hydrogen bonds are formed with three residues from the CDR3 of the H chain that are encoded by the D segment, pointing further to the importance of somatic recombination in generating a functional diversity of antibody specificities. The

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surfaces of the lysozyme and the Fab D1.3 at the binding interface are quite complementary, with a number of aromatic groups from the antibody contributing to the hydrophobic interactions. There are no salt bridges formed between the antibody and the antigen.

Cross reactivity studies

Although we have only solved one crystal structure of a Fab–lysozyme complex at high resolution to date, the specificity of more than 40 IgG1(K) and four IgM monoclonal anti-HEL antibodies has been analysed from their cross reactivities with lysozymes from seven other avian species (partridge, California quail, Bobwhite quail, Japanese quail, turkey, pheasant and guinea-fowls) by using the technique of enzyme-linked immunosorbent assay (ELISA) (Harper *et al.* 1987; M.-M. Riottot, D. Tello, V. Chitarra-Guillon and R. J. Poljak, unpublished results). These lysozymes have between three and ten amino acid changes from HEL, which are scattered over the surface of the molecule. From the pattern of cross reactivity we have been able to classify each MAb into one of 11 groups of similar specificity. Although the definition of this specificity is limited by the relatively small number of variant lysozymes used, the general location of the antigenic regions recognized by each group could be assigned. In particular, the results obtained correlate completely with the crystal structure of D1.3–HEL as all lysozymes where residue 121 is not Gln show no observable binding to this antibody. Thus California quail egg lysozyme, which differs from HEL only at residue 121 in the region of D1.3 binding (His substituted for Glu), shows no observable affinity to D1.3, confirming the very high specificity of this antibody. While model building suggests that a substitution of His for Gln could be accommodated in the cleft of D1.3 by small adjustments in sidechain conformation of the antibody and the antigen, experiment shows that such changes are not tolerated. By contrast, Bobwhite quail egg lysozyme, which has no amino acid differences with HEL in the region recognized by D1.3, binds to D1.3 with equal affinity. Crystallographic analyses of some of these lysozymes are currently under study in our laboratory to provide a firm structural basis for understanding this fine specificity.

Certain monoclonal anti-HEL antibodies form ternary complexes composed of two Fabs and a molecule of lysozyme. The ability or inability to detect ternary complex formation has confirmed the assignments of antigenic determinants by cross-reactivity measurements, because such a complex can only form if the respective binding sites of the two Fabs do not overlap. These ternary complexes are conveniently revealed by gel filtration on high performance liquid chromatography, as additive direct binding assayed by ELISA is sometimes subject to artifacts. The existence of a ternary complex shows that the binding of one Fab to HEL does not significantly distort the binding site of the second Fab. Further support for conservation of antigen conformation upon antibody binding follows from the observation that the catalytic activity of lysozyme towards the hydrolysis of *N*-acetylglucosamine oligomers is not inhibited in the D1.3–HEL complex (Kenett *et al.* 1987). The D1.3 binding site is well separated from the catalytic cleft of HEL so that the bulky Fab fragment does not block the access to small substrates. The presence of bound Fab to HEL, therefore, does not distort the structure of the antigen from its catalytically active conformation.

Further crystallographic investigations

Crystallization trials of other Fab–lysozyme complexes have continued in our laboratory to explore the antigenic properties of lysozyme in further detail, and a number of structural

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studies are currently underway. Table 2 shows preliminary crystallographic data of some of these complexes. Most of the MAbs that we have studied have affinity constants of the order of 10^7 M^{-1}, as determined by the technique of Friguet *et al.* (1985). In an attempt to obtain MAbs with higher affinity constants, BALB/c mice were injected with lower doses of HEL over a longer period. The Fab from one MAb, D10.6.6, obtained in this way has been crystallized and is now undergoing a crystal-structure analysis (Fischmann *et al.* 1988). Cross-reactivity studies of D10.6.6, which has an affinity constant of 0.73×10^9 M^{-1} for HEL, show that it binds to Arg 68. We might therefore expect that this antibody owes its higher affinity towards HEL to the formation of a salt bridge with this residue, as seen in the complex formed between HEL and the monoclonal Fab HyHEL-5 (Sheriff *et al.* 1987). A point of special interest here will be to compare to what extent the epitopes overlap in the two complexes, D10.6.6–HEL and HyHEL-5–HEL.

TABLE 2. CRYSTAL PARAMETERS OF ANTI-HEL FAB–LYSOZYME COMPLEXES

Fab–lysozyme	K_A/M^{-1}	a/Å	b/Å	c/Å	β/deg	space group	M[a]	resolution/Å
D1.3–HEL	4.4×10^7	55.6	143.4	49.1	120.5	$P2_1$	1	2.5
D11.15–PEL	*ca.* 10^8	158.2	49.1	177.6	92.0	C2	2	3.5–3.0
D44.2–HEL	*ca.* 10^7	99.7	167.3	84.7	90	$P2_12_12_1$	2	2.5–2.0
F10.6.6–HEL	*ca.* 10^9	145.6	78.1	63.1	89.05	$P2_1$	2	3.0
		134.0	144.7	98.6		P1	10–12	5

$\alpha = 90.3°$, $\beta = 97.1°$, $\gamma = 90.2°$.

[a] Molecules per asymmetric unit.

Some monoclonal anti-HEL antibodies show higher affinity to different species of lysozyme, forming a heteroclitic antibody–antigen complex. From the crystal structure of the D1.3–HEL complex we see that although the complementarity between antibody and antigen is quite good, there are minor imperfections that lead to small cavities at the interface. Thus a simple explanation for the phenomenon of heteroclitic antibodies would be that a changed residue from a heterologous antigen could fill the space more effectively, leading to a higher affinity. A crystallographic structure analysis of such a complex formed between the anti-HEL Fab D11.15 and pheasant egg lysozyme (PEL), now in progress, should demonstrate whether this explanation is adequate to account for the difference in antibody specificity (Guillon *et al.* 1987).

Idiotope–anti-idiotope interactions

Although antibodies serve to recognize foreign antigens, they themselves may act as antigens within the immune system of the same animal or, as observed in the usual experimental situation, between syngeneic or xenogeneic animals (Oudin & Michel 1963; Kunkel 1963). These antigenic determinants are known as idiotopes and are characteristic of immunoglobulins derived from an individual clone or a set of closely related clones. Idiotypic regions are located on the variable domains, and it has been proposed that these play a role in the regulation of immune responses and in the development of the immune system (Jerne 1974).

A particularly interesting class of idiotopes is formed by those located on the antigen-binding site of an antibody. Where the 'lock and key' hypothesis holds true (i.e. where no significant structural change occurs upon forming the complex), we might expect that anti-idiotypic antibodies recognizing combining-site-associated idiotopes mimic the antigenic region of the

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foreign antigen. There are many documented examples where 'molecular mimicry' by the immune system has been demonstrated (reviewed in Greene & Nisonoff 1984; Bona 1987). In our own laboratory, we have observed anti-anti-idiotope antisera binding to the original foreign antigen, lysozyme.

With a view to characterizing a particular idiotope in precise structural detail, we have begun a crystallographic study of a Fab idiotope–Fab anti-idiotope complex. The Fab fragment of the anti-HEL antibody D1.3 was used as antigen to obtain monoclonal anti-idiotopic antibodies. The Fab fragment from one of these, E225.23, has been crystallized as a complex with D1.3 (Boulot *et al.* 1987). The crystallographic analysis of this complex is now well in progress, using a combination of molecular replacement and isomorphous replacement techniques for the determination of the phases of the X-ray reflections. Although this study has not yet reached the point where we may obtain atomic details of the interaction between the antibody and its anti-idiotope, there is immunochemical evidence that E225.23 recognizes at least some of the CDR regions of D1.3. Thus we have observed binding between E225.23 and a genetically engineered, chimeric variable dimer, Fv46, kindly provided by Dr G. Winter (Riechmann *et al.* 1988), which carries the murine D1.3 CDRs on a framework structure of human origin (V_H from the Fab New and V_L from the Bence-Jones dimer REI). As only the CDRs are shared by D1.3 and the chimeric Fv46, we conclude that E225.23 binds to the grafted CDRs of D1.3, i.e. the idiotope recognized by E225.23 is 'combining-site associated'.

3. Discussion

To date, two other antibody–antigen complexes have been studied at high resolution in other laboratories. The first of these is a complex formed between HEL and the Fab fragment from monoclonal antibody HyHEL-5, providing a useful comparison with the D1.3–HEL complex. Here, the antibody recognizes 14 amino acids on HEL which are also discontinuous in sequence but form an integral surface area on the antigen. The epitope recognized by HyHEL-5 is different to that recognized by D1.3. All six CDR regions participate to form the antigen-binding site, as well as V_H Trp 47 from a framework region. This finding, together with the similar observation in the D1.3–HEL complex, reinforces the fact that a functional division between CDR and framework regions is not rigidly held to. Contrary to D1.3–HEL, V_L CDR1 and V_H CDR2 of the HyHEL-5 complex make the most contacts with the antigen. Furthermore, HEL in the latter complex has undergone a more significant change in structure from its uncomplexed state. The residue Pro 70, in particular, has moved by 1.7 Å. This change might be expected, because the epitope belongs to a region of HEL where the conformation is less restrained by secondary structure and where the temperature factors are higher than average (Sternberg *et al.* 1979). None the less, the overall conformation appears to be generally conserved and the root mean square difference in the backbone atomic positions between free and liganded lysozyme in this complex is only 0.48 Å.

The second example is a complex formed between the avian influenza virus neuraminidase and a Fab of monoclonal origin, NC41 (Colman *et al.* 1987, this symposium). The authors conclude that significant changes in conformation take place both in the antigen and the Fab fragment upon formation of the complex. A comparison between known Fab structures carried out in our laboratory, however, suggests that the reported difference in conformation of the Fab NC41 in the complex with neuraminidase falls within the limits of variability observed (see table 1).

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The structure of the antibody–antigen complex D1.3–HEL shows that IgG immununo-globulins owe their high specificity to a fairly strict complimentarity of Van der Waals interactions and an even more strict pairing of polar interactions (e.g. hydrogen bonds for D1.3–HEL) at the interface between the two molecules. Cross-reactivity measurements between anti-HEL antibodies and heterologous lysozymes, in addition, provide strong evidence for a close fit between antibody and antigen at the site of interaction, but as noted earlier, heteroclitic complex formation shows that the match is not always optimal. This phenomenon may be explained by the presence of imperfections at the antibody–antigen interface in the form of small cavities, which may better accommodate minor changes in shape and chemistry of heterologous antigens. The role of some conformational change in heteroclitic complex formation, however, cannot yet be ruled out. Imperfect complementarity between antibody and antigen may also provide a selection pressure for higher antigen affinity of B-cell clones undergoing somatic mutation during the later stages of the immune response. In conclusion, it is clear that although the three-dimensional structures of the antigen–antibody complexes studied so far have broadened our view of the function of IgG antibodies, there remains much to be learned from further structural studies.

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