Chapter 5. Specificity

I never was attached to that great sect,
Whose doctrine is that each one should select
Out of the crowd a mistress or a friend,
And all the rest, though fair and wise, commend
To cold oblivion
-Percy Bysshe Shelley
Epipsychidion (1821)

Criss-cross specificity

If we immunize an animal with an antigen A, and then test its serum for the presence of antibodies, we may find that it makes antibodies against antigen A but not against a second test antigen that we call B. We say the antibodies produced in response to A are "specific" for the antigen A.

ANTIGEN GIVEN	ANTIBODIES IN SERUM	
A	Anti-A	present
	Anti-B	absent

A more rigorous way of demonstrating immunological specificity is called a "criss-cross specificity" experiment. As before, we immunize an animal with a substance A, and we find that we can detect antibodies that react with A in its serum, and none that react with the control antigen B. In addition, we immunize a second, genetically identical animal with the antigen B, and show that the serum of this animal contains antibodies that react with B but none that react with A.

ANTIGEN GIVEN	ANTIBODIES IN SERUM
A	Anti-A present Anti-B absent
B	Anti-A absent Anti-B present

The anti-B immune response in the second animal is called a positive control for the negative result seen for anti-B antibodies in the first animal, confirming that anti-B antibodies can be made and detected in the experimental system (species, strain, immunization protocol, assay system) being used. Similarly, the negative result seen in line two for A shows that anti-A antibodies are present as the result of immunization with A, and that they are not, for example, always present and

detectable in these animals with the assay system being used. Experiments that include the criss-cross specificity component are thus more rigorous than the experiments using only antigen A, because they contain the built in check that the assays for anti-A and anti-B antibodies are working properly, and that the specificity observed is not an artifact of, for example, A or B being defective antigen preparations. Criss-cross specificity experiments therefore play a key role in experimental immunology. The characteristic structure of criss-cross specificity experiments can be summarized as AA, AB, BA, BB, which in this particular case of a primary immune response means immunize with A, assay for A, immunize with A, assay for B, immunize with B, assay for A and immunize with B, assay with B.

Criss-cross specificity can also be demonstrated in a secondary immune response as follows. Antigen A injected after an earlier injection of the same antigen A, or antigen B after antigen B, evoke stronger ("secondary") immune responses than antigen A after antigen B, or antigen B after antigen A.

Another example is the criss-cross specificity of responses to foreign and self antigens. A mouse makes anti-bovine (cow) albumin antibodies but not anti-mouse albumin antibodies, while a cow makes anti-mouse but not anti-bovine albumin antibodies:

First antigen	Second antigen	Secondary Response?
A	A	yes
A	B	no
B	A	no
B	B	yes

Another example of a criss-cross specificity experiment involves looking at the immune responses to foreign and self antigens using BSA (bovine serum albumin) and MSA (mouse serum albumin). We know that a mouse can make an immune response to BSA, and we expect that the table of responses is as follows:

Antigen	Species	Antibodies to antigen?
MSA	mouse	No
MSA	cow	Yes
BSA	mouse	Yes
BSA	Cow	No

Yet another example is graft rejection. Skin grafts from a mouse of strain A are rejected by a mouse of strain B, but not by a mouse of strain B, and vice versa. The negative control is necessary to confirm that strain B does not have a genetic defect (such as, say, a lack of T cells) that may prevent it from rejecting any grafts.

Graft donor	Graft recipient	Graft rejected?
A	A	No
A	B	Yes
B	A	Yes
B	B	No

In the following we will encounter more examples of ways in which such criss-cross experiments demonstrate immunological specificity. In addition to its role in demonstrating specificity in antibody responses, the criss-cross design will be seen to be important in characterizing T cells and the specific molecules that they secrete.

In some published experiments that aim to demonstrate immunological specificity, criss-cross controls are not included. Such experiments are less rigorous than they could be, and should be accorded less weight than corresponding experiments with the proper controls.

How specific are antibodies?

V region specificity is one of the central concepts of immunology. The V region of a particular antibody may bind to egg lysosyme from a hen but not to egg lysosyme from a pigeon, even though these two proteins resemble each other in their amino acid sequences very closely. The specificity of antibodies is therefore often called "exquisite", but it is not unlimited. The complementarity in shape between an antigen and its antibody is typically not perfect, and there are many ways for a fit to be imperfect. There exists a corresponding large ensemble of antibodies, each of which fits to a given antigen in a different, imperfect way. Similarly, a single antibody can potentially bind to more than one antigen. In practice, specific anti-X antibodies are produced by injecting an animal with an antigen X, and anti-X antibodies are selected to react with X but not with another substance Y that is similar to X. On the other hand, if we look hard enough, we are likely to find that one or more of the anti-X antibodies react (by chance) with another substance Z, possibly using a different part of the antibody's V region. We say that the anti-X antibody *cross-reacts* with Z, and this illustrates that the specificity of antibodies is limited. We have a limited number of antibodies that collectively are able to react with an essentially unlimited number of natural and synthetic antigens. This means that each antibody must be able to react with more than one substance. We therefore say that the V regions of antibodies are *multispecific*.

Multispecificity

For many years each antibody was considered to be specific for just one antigen. The idea was that each antibody bound to the antigen used to produce them, but nothing else. Several counterexamples have eroded this myth. For some antibodies a second antigen has been identified to which an antibody binds, and in some cases the second

antigen binds with an even higher affinity than the affinity to the first antigen. Some antigens, in particular strains of mice, routinely induce such antibodies, which are called "heteroclitic antibodies".[25] Another example of antibodies with complementarity to more than one substance are antibodies to a substance X (anti-X antibodies), to which antiidiotypic antibodies (anti-anti-X) have been produced. Anti-X antibodies then have dual specificity, namely for X and for anti-anti-X.

Dramatic evidence of a high level of multispecificity of antibodies has been observed in studies of idiotypic binding of monoclonal antibodies to each other.[26,27] In these studies the monoclonal antibodies were of fetal or neonatal origin. On average, each monoclonal antibody bound to about 20% of the others. A similar high level of multispecificity for idiotypes is seen among monoclonal antibodies derived following both a primary and a secondary response to a protein antigen.[28]

The mistaken idea of unispecificity of antibodies has several origins. The first was the fact that there is normally a high degree of specificity in antibodies, such that in order to find a second antigen that interacts with a given antibody requires considerable screening. The second origin lies in the structure of the antibody molecule. Small molecules for which an antibody is specific (these are called "haptens") typically bind to a cleft between the antibody heavy and light chains. This became known as "*the* antigen combining site", as if it were a uniquely defined site. Thirdly, the idea of exclusivity was reinforced a perception of an analogy between antibody-antigen and enzyme-substrate binding. Enzymes have a high level of specificity (albeit not unlimited) for their substrates, and the analogy reinforced the idea of a unique site on the V region, a cleft, to which an epitope of an antigen bound. In chapter 9 we will review further evidence that this narrow view of a presumed unispecificity of antibodies, acting as receptors on B cells, is erroneous.

The ratio of the number of antigens that do not bind to a given antibody to the number of antigens that do bind to it is typically large, so one only rarely finds that a randomly selected second antigen binds to a given antibody. There are however a very large number of different antigens, so if we look long enough we will eventually find many antigens that interact with a single antibody, and they do not necessarily interact at the same site. Or most simply, we can demonstrate multispecificity by

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[25] K. Karjalainen and O. Mäkela (1978) A mendalian idiotype is demonstrable in the heteroclitic anti-NP antibodies of the mouse. Eur. J. Immunol. 8, 105-111.

[26] D. Holmberg, S. Forsgen, F. Ivans and A. Coutinho (1984) Reactions amongst IgM antibodies derived from neonatal mice. Eur. J. Immunol. 14, 435-441.

[27] J. F. Kearney, M. Vakil and N. Nicholson (1987) Non-random VH gene expression and idiotype-antiidiotype expression on early B cells. In: Evolution and Vertebrate Immunity: The Antigen Receptor and MHC Gene Families. G. Kelsoe and G. Schulze Eds. Texas University Press, Austin, 175-190.

[28] D-H. Hsu, E. E. Sercarz and A. Miller (1989) Internal connectivity is pervasive among primary and secondary anti-hen egg white lysozyme (HEL) IgG monoclonal antibodies.

generating a large number of different antiidiotypes to the antibody, that can bind to various parts of the V region.

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An attempt to quantify the concept of multispecificity has been made by Inman[32]. The analysis is in terms of haptens. A hapten is a small molecule that is typically attached to a larger molecule such as a protein, to make an antigen large enough to be capable of evoking an immune response to the hapten. Inman estimates that the number of constructable haptens of typical size is at least 10^{16}, which is much greater than the number of antibodies in a mouse, which he estimates to be, at most, 10^{9}. Yet the mouse is able to make antibodies to practically any hapten. This means that each antibody must have a multispecificity of at least 10^{7}, with respect to the universe of all possible haptens. The antibody is then anything but unispecific.

The multispecificity of antibodies is a logical consequence of the cross-linking mechanism by which antibody receptors on lymphocytes function. Anything which is able to cross-link the receptors stimulates the lymphocytes, and this does not entail a need to interact with any particular part of the receptor. The various parts of the V region, including various parts of a the cleft, can bind antigen, ensuring that a single antibody can serve to bind many different antigens.

Non-specific stimuli: mitogens

A mitogen is a substance which, in the context of the immune system, is the opposite of being a specific antigen. Mitogens rather indiscriminately induce the proliferation of a large fraction of T cells or B cells or both; hence they are usually regarded as being non-specific. In order to stimulate non-specifically they must interact with a

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[29] D. Holmberg, S. Forsgen, F. Ivans and A. Coutinho (1984) Reactions amongst IgM antibodies derived from neonatal mice. Eur. J. Immunol. 14, 435-441.

[30] J. F. Kearney, M. Vakil and N. Nicholson (1987) Non-random VH gene expression and idiotype-antiidiotype expression on early B cells. In: Evolution and Vertebrate Immunity: The Antigen Receptor and MHC Gene Families. G. Kelsoe and G. Schulze Eds. Texas University Press, Austin, 175-190.

[31] D-H. Hsu, E. E. Sercarz and A. Miller (1989) Internal connectivity is pervasive among primary and secondary anti-hen egg white lysozyme (HEL) IgG monoclonal antibodies.

[32] J. K. Inman (1978) The antibody combining region: speculations on the hypothesis of general multispecificity. in Theoretical Immunology, (G. I. Bell, A. S. Perelson and G. H. Pimbley, Jr., Eds.), Marcel Dekker, Inc., New York, 243-278.

constant part of the specific receptor, with a common V region determinant, or with some other cell surface molecule that also functions as a receptor. Some mitogens such as lipopolysacharide stimulate B cells, while some, such as conconavalin A, stimulate T cells, and some, such as pokeweed mitogen, stimulate both B cells and T cells. Some mitogens activate T cells that have a particular family of V regions and are known as "superantigens".

Mitogens used to determine the frequency of antigen-specific cells

What fraction of antigens bind to a single antibody? It is a tedious task to experimentally find, by trial and error, antigens that bind to an antibody in addition to the antigen that was used to evoke the production of that antibody. An alternative approach is to non-specifically activate a large fraction of the spleen cells from a mouse using a mitogen, and determine the fraction of the cells that make antibodies that react with a given antigen.[33] Melchers and his collaborators generated a large number of different B cell clones using the mitogens lipopolysaccharide, and determined the fraction that produced antibodies that bound to a single antigen. They found that about one in a thousand of the B cells make antibodies that react with a particular antigen, namely sheep red blood cells. This was a surprisingly high frequency of antigen-reactive B cells, since previous work with immune responses to the antigen itself, rather than to mitogens, had given much lower frequencies of about 1 in 10^5. Let us assume for the moment that sheep red blood cells is a typical antigen.[34] Then the frequency of antigen-reactive lymphocytes for a typical antigen is one in a thousand. Since there are many more than a thousand different antigens (indeed, there are billions of different antigens), each B cell must have a V region that is able to interact with many different antigens; otherwise most antigens would be without corresponding B cells, which is not observed. In other words, we again conclude that the V regions of B cells and antibodies must be highly multispecific. While each V region binds to a large number of antigens, this is nevertheless a small number compared with the number to which each V region does not bind, and in this sense the binding is still highly specific.

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[33] K. Eichmann, A. Coutinho and F. Melchers Absolute frequencies of lipopolysaccharide reactive B cells producing A5A idiotype in unprimed, streptococcal A carbohydrate-primed, anti-A5A-idiotype sensitized and anti-A5A idiotype suppressed A/J mice. J. Exp. Med., **146**, 1436-1449, 1977.

[34] Sheep red blood cells is a complex antigen, with many determinants, but most of the antibodies bind to a single determinant, so it can actually be regarded as effectively a simple (and thus typical) antigen.

Frequency of specific T cells

The frequency of mouse B lymphocytes reactive to an experimental foreign antigen has been determined to be one in 10^5 to one in 10^3. In contrast to this, the frequency of lymphocytes reactive to certain foreign cell surface antigens, namely the major histocompatability antigens (see chapter 12), is known to be as high as a few percent. In fact the frequency of T cells reactive also to other typical antigens has been reported to be as high as one per cent.[35] These frequencies are significantly higher than those observed for B cells.

Measurements of complementarity

The most common measure of complementarity between two substances such as an antigen and an antibody is the affinity K, which is also known as the binding constant or the equilibrium constant. For a reaction A + B <=> C the equilibrium constant K is related to the forward rate constant for the binding reaction, k_f, divided by the dissociation rate constant, k_d:

$$K = k_f / k_d$$

and to the concentrations of A, B and C at equilibrium:

$$K = \frac{[C]}{[A][B]}$$

Accordingly, the affinity can be determined using either equilibrium or kinetic measurements.

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[35] J. Cooper, K. Eichmann, K. Fey, I. Melchers, M. M. Simon and H. U. Weltzien (1984) Network regulation among T cells; qualitative and quantitative studies on suppression in the non-immune state. Immunol. Rev. 79, 64-86.