

IMMUNOLOGY AND MEDICAL MICROBIOLOGY

Antigens and haptens

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Keywords

Antibody; Superantigens; Haptens; T lymphocytes; B lymphocytes; Epitopes; Major histocompatibility complex (MHC); Antigen presenting cell (APC); T dependent (TD) antigens; T independent (TI) antigens; T helper (T_h) cells

Antigens

Substances capable of inducing a specific immune response are called antigens. To be more precise the substances which can generate an immune response (humoral and/or cell mediated) are called immunogens and this ability of a substance is called immunogenicity. While antigens are the substances which react with the products of an immune response, the effector molecules (Ab) / effector cells (CTLs) for elimination of foreign molecules. This property is known as antigenicity. All immunogens are antigens while the reverse is not true. Substances such as proteins, nucleoproteins, polysaccharides and glycolipids can act as antigens. Proteins are known to be the most potent antigens and polysaccharides are second in the list. For cell mediated immunity only proteins and some lipids and glycolipids serve as immunogens. Thus to be antigenic a substance must first be recognized as foreign.

Factors Affecting Immunogenicity

There are various factors which govern the immune response induction by a molecule, such as:

Foreignness

Immunocompetent lymphocytes during their maturation learn not to respond to self antigens, a property known as self tolerance. Any self molecule not exposed to maturing lymphocytes later is recognized as foreign/ non self by the immune system e.g., the antigens sequestered from the immune system as sperm and corneal antigens. These cells also respond to foreign molecules that differ even in minor respects from the self antigens. Upon exposure to a foreign substance, the degree of immunogenicity depends on the degree of its foreignness as Bovine Serum Albumin (BSA) is not immunogenic in cow but is in rabbit. In addition BSA shows more immunogenicity in chicken as compared to a goat.

Molecular size

Most antigens are large, complex molecules with a molecular weight generally greater than about 100,000 daltons. In general large molecules are better immunogens as compared to smaller molecules.

Chemical composition

Highly flexible molecules having no fixed shape eg., gelatin, flagellin are poor antigens. Simple repetitive polysaccharides do not assume a stable configuration hence are poor immunogens. Copolymers instead of homopolymers are found to be good immunogens. Protein folding is also known to affect immunogenicity, the reason that proteins are better immunogens as compared to lipids, carbohydrates and nucleic acids. The addition of aromatic amino acids as tyrosine and phenylalanine further increases the immunogenicity of synthetic polymers, along with a reduction in the required molecular weight of the immunogen.

Degradability

The T helper cells (T_h) cells are required to be activated and help in the development of both the humoral and cell mediated immune response. Since an antigen is to be processed and presented with MHC molecules for development of immune response, the macromolecules that cannot be

degraded and presented are poor immunogens. e.g., Polymers containing D-amino acids cannot be degraded and processed by the degradative enzymes of the antigen presenting cells (APCs) and serve as poor immunogens. Large molecules are readily phagocytosed and processed and hence are more immunogenic.

Infected host and immunogenicity

Apart from the above discussed properties of the molecules the genetic makeup of the host also contributes to the immunogenicity.

Genotype of the recipient individual

The genes that encode MHC, B cell and T cell receptors and various proteins for immune regulation are known to influence the type and the intensity of the immune response.

Dosage and route of administration

An insufficient dose and an excessive high dose act as poor antigens. A lower dose can either fail to activate enough lymphocytes or they induce a nonresponsive state. Too high a dose also fails to induce an immune response because it induces a nonresponsive state in the lymphocytes. Thus a minimal optimal dose and also a repeated administration (booster doses) are required to stimulate a strong immune response by increasing the proliferation of specific T and B cells.

The route of administration influences the cells and organs involved in an immune response. Most experimental immunogens are administered parenterally (around the gut). Other common routes of administration are intravenous, intradermal, subcutaneous, intramuscular and intraperitoneal. It is the route of administration which decides the immune organs and cell populations which will take part in the immune response.

Super-Antigens

When the immune system encounters a conventional T-dependent antigen, only a small fraction ($1 \text{ in } 10^4 - 10^5$) of the T cell population is able to recognize the antigen and become activated (monoclonal/oligoclonal response). However, there are some antigens which polyclonally activate a large fraction of the T cells (up to 25%). These antigens are called superantigens.

The superantigens bind outside the TCR antigen binding cleft. These bind to TCRs with a particular V β sequence and do not depend on antigen specificity. Hence, all t cells with this particular V β sequence can be activated against a superantigen thereby leading to a polyclonal immune response. This response leads to overproduction of Th cytokines which lead to systemic toxicity. Superantigens can be viral proteins (called endogenous superantigens) or they can be bacterial superantigens (called the exogenous superantigens). Examples of superantigens include:

- *Staphylococcal* enterotoxins (causes food poisoning),
- *Staphylococcal* toxic shock toxin (causes toxic shock syndrome),
- *Staphylococcal* exfoliating toxins (causes scalded skin syndrome) and
- *Streptococcal* pyrogenic exotoxins (causes shock).

Although bacterial superantigens are the best studied, there are superantigens associated with viruses and other microorganisms as well.

The diseases associated with exposure to superantigens (Fig.1) are, in part, due to hyper activation of the immune system and subsequent release of large biologically active cytokines by activated T cells.

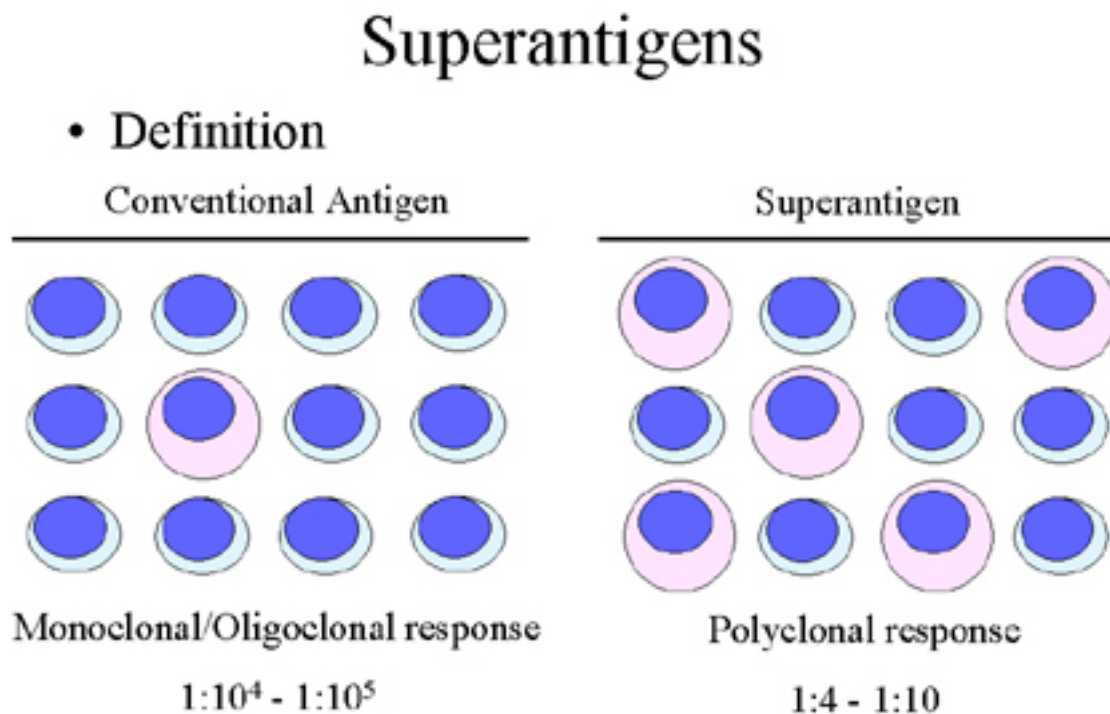


Fig. 1: An antigen upon exposure selects out only one or two cells having specific receptor and leads to their proliferation hence induces mono/oligoclonal response. Superantigens on the other hand induces a polyclonal response involving many cells.

Haptens

(Greek *haptain*, to grasp, to fasten)

The concept of haptens was introduced by Landsteiner in 1923 and these are defined as small molecules which are not immunogenic themselves but when coupled with a carrier protein provide antigenic determinants against which antibodies can be formed. Thus they have the property of antigenicity and not immunogenicity. They are also named as **partial antigens**. These chemical substances which are themselves not immunogenic have the property of reacting specifically with antibodies (Fig. 2).

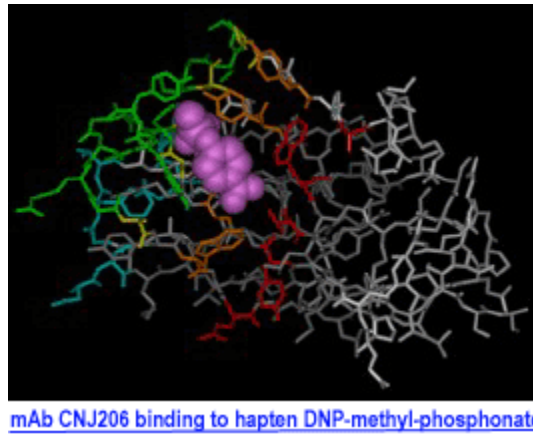


Fig. 2: Binding of monoclonal antibody with hapten (purple colour)

Haptens may be of the following types:

Simple haptens are non precipitating and can inhibit precipitation of specific Ab by the corresponding Ag or complex Ag. They are univalent and hence non precipitating as precipitation require two or more antibody binding sites on the antigen.

Complex haptens on the other hand can precipitate with specific antibodies and are multivalent/ polyvalent.

Hapten-Carrier Conjugate

Chemical coupling of a hapten to a large protein called a carrier yields an immunogenic hapten-carrier conjugate. These conjugates contain multiple copies of the hapten chemically linked to a large protein carrier. They are characterized by having native antigenic determinants of the carrier and hapten as well as new determinants formed by combined parts (adjacent residues of the hapten and the carrier) of both hapten and carrier (Fig.3).

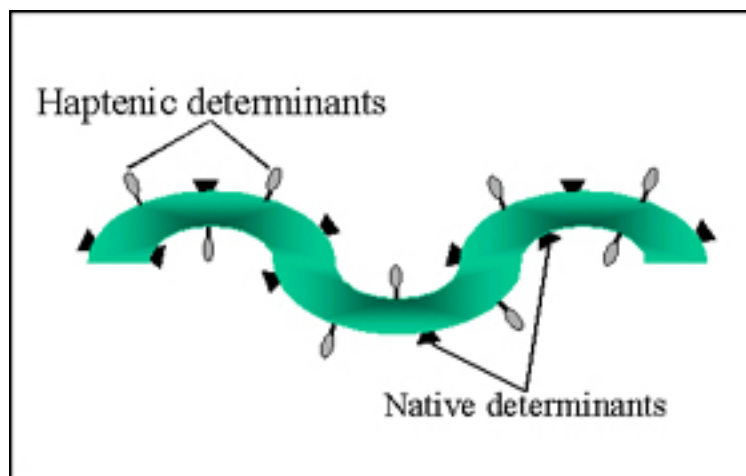


Fig. 3: Hapten carrier conjugates having different determinants

Thus, when animals are immunized with such a conjugate, they produce antibodies specific for the following:

- hapten determinant,
- unaltered epitopes on the carrier protein,
- new epitopes formed by combined parts of hapten and carrier.

Often, amongst all these sets of antibodies produced against the hapten- carrier conjugate, the anti-hapten antibodies are predominant indicating that the hapten is the immunodominant epitope in the complex.

There are important characteristics of anti-hapten antibody responses stimulated by hapten-carrier conjugates:

- such responses require co-operation between hapten specific B cells and carrier specific Th cells,
- Second, in order to stimulate a response, the hapten and the carrier portions have to be physically linked,
- Third, this interaction is MHC restricted.

The explanation for these observations becomes clear when it was appreciated that B cells are efficient APCs. Hapten specific B cells bind the antigen *via* the hapten determinant and present peptides derived from carrier protein. The hapten is responsible for efficient carrier uptake explaining the necessity for physical link between hapten and carrier. Haptens cannot induce T cell response and the reasons are still uncertain.

Simple tests have shown that any alteration in the shape, size or charge of hapten reduces its ability to bind antibodies directed against unmodified hapten. Even minor modifications like the difference between haptens that are stereoisomers usually results in a significant alteration in shape of hapten and thus influence its ability to bind to antibody.

Landsteiner tested whether an anti-hapten antibody could bind to other haptens having a slightly different chemical structure. If a reaction occurs, it is called a cross-reaction. He used various derivatives of aminobenzene as haptens and found that the overall configuration of a hapten plays a major role in determining whether it can react with a given antibody. For example antiserum from rabbits immunized with aminobenzene or one of its carboxyl derivatives (o-aminobenzoic acid, m-aminobenzoic acid or p-aminobenzoic acid) coupled to a carrier protein reacted only with the original immunizing hapten and did not cross react with any of the other haptens.

In contrast, if the overall configuration was kept the same and the hapten was modified in the para position, the antisera showed various degrees of cross-reactivity. By observing which hapten modifications prevented or permitted cross-reactions, he gained insight into the specificity of Ag-Ab reactions. His work with haptens gave a clear insight into the specificity of the immune system and enormous diversity of epitopes that the immune system is capable of recognizing.

Carrier Effect and Carrier Priming

Karl Landsteiner in 1920s conducted experiment which demonstrated the carrier effect. Three groups of mice were immunized (primary antigen) with dinitrophenylated bovine serum albumin (DNP-BSA) and rechallenged (secondary antigen) with either DNP-BSA, BSA or dinitrophenylated ovalbumin (DNP-OA). The antibody response to DNP was obtained with animals immunized twice with the same antigen. The BSA acts as a specific carrier for the antibody response to DNP.

Thus, to obtain an optimum secondary response to a hapten it is usually necessary to immunize the animal with the same antigen in both the primary and the secondary challenge. It is not sufficient that the antigens share a common antigenic determinant but must also be attached to the same carrier molecule. This is known as the carrier effect. The carrier effect thus implies that the cells involved in making the antibody response recognize at least two parts of the antigen.

The requirement for priming lymphocytes to the carrier can be circumvented in the response to a hapten-carrier conjugate if the animal has been previously primed to the carrier alone or receives spleen cells from a donor primed to that carrier.

Different substances can act as haptens e.g., lipids, lipoproteins, polysaccharides, drug molecules like penicillin, paracetamol, aspirin; sulfa drugs as sulfonamides, some anesthetics as succinyl choline, some opiates, picryl chloride, formaldehyde etc.

Examples of Hapten-carrier conjugates of clinical importance

- **Penicillin** is a small molecule and by itself is not immunogenic. Once degraded in body it forms very reactive “penicilloyl” group which can react with proteins (albumin) to form a penicilloyl-protein conjugate (hapten-carrier conjugate). This is recognized as foreign and provokes an immune response. Antibodies (IgE) once generated are bound by IgE receptors on the surface of mast cells and basophils. If in such a condition an individual is treated with penicillin it can cause allergic reactions that can be life threatening. Drug allergies are thus mainly because of the conjugate acting as an antigen.
- Many CD4 T cell mediated reactions/ hypersensitivity reactions (Delayed type of hypersensitivities). Also known as contact sensitivities or contact dermatitis or contact hypersensitivity. It is characterized by eczema at the sites of contact with the allergen. Some examples include:
 - Catechols found in poison ivy, poison-oak and poison sumac
 - Nickel, often used in jewellery
 - Some dyes
- Certain organic molecules/ chemicals used in industry. These chemicals bind spontaneously to normal proteins and so act as haptens.
- Toxin of poison ivy (*Rhus radicans*), The resin of this plant, a mixture of complex catechols known as urushiol, binds to any protein it comes into contact with, including the skin

proteins of anyone who rubs against the plant. The skin cells modified in this way are regarded as foreign and attacked by lymphocytes in a manner similar to the rejection of a skin graft. Destruction of these cells by a cell mediated immune response causes blister formation and the development of a very uncomfortable skin rash known as allergic contact dermatitis.

Commonly used hapten- carrier conjugates are:

- DNP-BGG Dinitrophenol (hapten); Bovine gamma globulin (carrier)
- TNP-BSA Trinitrophenyl (hapten); Bovine serum albumin (carrier)
- ARS-OVA Azophenyl arsenate (hapten); Ovalbumin (carrier)
- LAC-HGG Phenyllactoside (hapten); Human gammaglobulin (carrier)

Uses of Hapten-Carrier Conjugate

- It provides immunologists with a chemically defined determinant that can be subtly modified by chemical means to determine the effect of various chemical structures on immune specificity.
- For diagnosis
Many biologically important substances, including hormones can act as haptens. The hapten specific antibodies generated are useful for measuring the presence of various substances in the body e.g., the original home pregnancy test kit uses antihapten antibodies to determine whether a women's urine sample contains human chorionic gonadotropin, a sign of pregnancy.

The antibodies against the steroid hormones are employed to detect their presence in the blood or urine sample of athletes, weight lifters and body builders. A higher than a particular level of these steroids, if found, can result in the cancellation of the sports person to take part in the event.

- Used in *in situ* hybridization (a technique that allows the visualization of target mRNA or DNA sequences in cells of a mixed population. c DNA or riboprobes can be synthesized and labeled by incorporating a detectable hapten (e.g., biotin, fluorescein, digoxigenin, DNP). Adding multiple detectable haptens to a probe increases its sensitivity.
- Antibody affinity It is used for thermodynamic investigation of the interaction of antigenic site and the antigen. To measure the affinity of a single combining site it is necessary to use a monovalent antigen or even a single isolated antigenic determinant or a hapten. The immunoglobulin affinity can be measured by means of a method known as Equilibrium dialysis.
- Such conjugates also helped in understanding antigen presentation by B lymphocytes and its role in development of humoral immune response.

Epitopes

Macromolecular antigens are much larger than the antigen binding site of their specific receptor. Epitopes also known as antigenic determinants are the regions or sites in the antigen that bind to the antigen binding region of a specific Ab or the T cell receptor (TCR). Chemically determinants include sugars, organic acids and bases, amino acid side chains, hydrocarbons and aromatic groups. There can be more than one epitope in an antigen and the number of antigenic determinants on the surface of an antigen is its valence. If one determinant is present then the antigen is monovalent. Most antigens however have more than one determinant site or more than one copy of the same epitope and these are known as multivalent antigens. Thus there are present a large number of potential antigenic sites on antigens. Larger molecules and hence multivalent antigens generally induce a stronger immune response.

Most intense immune responses are directed against epitopes that are most foreign, such more immunogenic epitopes of an antigen are referred to as immunodominant. Thus, immunodominant epitopes are those which induce a more prominent immune response than other epitopes.

Proteins commonly display different spatially well separated antigenic determinants, each one of which can be bound by two different antibodies, such determinants are called non-overlapping determinants.

If binding of one site to its receptor interferes with the binding of the second then they are called overlapping determinants. The covalent structure of phospholipids and carbohydrates determine the epitopes in these molecules. But in nucleic acids and proteins, non covalent folding of the macromolecule also contributes to the antigenic determinants in these molecules. There are also linear/ sequential determinants which are the epitopes formed by adjacent amino acid residues in the covalent sequence. Conformational determinants/ nonsequential determinants are formed by amino acid residues from separated portions of the linear amino acid sequence which come together only after folding of the molecule. Covalent modifications as phosphorylation or specific proteolysis can produce new antigenic epitopes called neoantigenic determinants which are recognized by specific antibodies.

B Cell and T Cell Epitopes

Most T lymphocytes recognize only peptides, while B cells are capable of recognizing peptides, proteins, nucleic acids, polysaccharides, lipids and small chemicals. Thus B cells and T cells are known to recognize different epitopes on the same antigen molecule.

B Cell Epitopes

B cells recognize soluble antigens with the help of the antibody receptors present on their membrane, leading to a binary complex formation. It is the antigen binding site on the antibody molecules which decides/ selects the antigenic determinant to which it will bind to. The two should have complementary shapes for a strong interaction, and the size of the epitope to be bound to antibody can also be not larger than the antigen binding site of the antibody molecule.

B cells bind to free soluble antigens hence their epitopes are highly accessible sites on the exterior surfaces of the antigen and consist of hydrophilic amino acids.

An epitope on a protein antigen may involve elements of the primary, secondary, tertiary or quaternary structure. Thus B cell epitopes can contain sequential residues along the polypeptide chain or non sequential residues available after the folding of the molecule. This is the reason behind antibodies binding only to native proteins, since denaturation might loose the structure of the epitopes.

Mostly, the B cell epitopes are located in the most mobile regions of the antigen which helps in matching the complementarity with antigen binding site on the antibody molecule. Complex proteins contain multiple overlapping B cell epitopes, some of which are immunodominant.

T Cell Epitopes

T cells do not bind to soluble antigens but are capable of recognizing antigen only after it is processed and presented alongwith Major Histocompatibility Complex (MHC) molecules on APCs. Thus it leads to formation of a trimolecular/ ternary complex. The antigenic site which intracts with MHC molecules is called the epitope and is different from the epitope.

The antigen binding cleft on an MHC molecule determines the size of the peptide(s) which can bind to the MHC molecule.

Before T cells can recognize an antigen, it is to be processed. Endogenous and exogenous antigens are processed by different methods, after which they are presented along with MHC molecules on the APC surface for recognition and activation of T cells.

Processing of antigens reveal internal peptides, hence T cell epitopes are often internal. Denaturation of proteins thus does not alter the T cell epitopes.

MHC molecules expressed in an individual are one of the major deciding factors for the immunodominant epitopes.

T-Dependent and T-Independent Antigens

Immunocompetent B cells for their activation, proliferation and differentiation require interaction with an antigen. Upon exposure to antigen, B cells generate plasma cells and memory B cells. B cells can be activated against two sets of antigens the T dependent type or the T independent type of antigens.

T-dependent (TD) Antigens

These are the antigens that elicit a response with the aid of T-helper cells. With respect to these antigens just the presence of cytokines released by the T-helper cells is not sufficient for B cell activation. Examples of TD antigens are bacteria, foreign red blood cells, proteins, hapten-carrier combinations etc. The triggering of B cells with TD antigens involves three cells namely macrophage, Th cell and a B cell specific for the antigen.

Processing and presentation of antigen to the Th cell provides the first signal for the process (signal 1). Second signal is the costimulation provided by B7-CD28 interaction (signal 2). B7

molecules are present on antigen presenting cells and CD28 molecules are present on T cells. IL-1 and IL-6 stimulate T cells to divide and make IL-2 and IL-4-6, which stimulate T_h cell multiplication. These helper cells directly associate with B cells that display correct antigen-class II MHC complex and secrete cytokines called B cell growth factors (BCGFs; IL-2, IL-4 and IL-5) which lead to B cell multiplication. T cells secrete more cytokines and B cell differentiation factors (BCDFs) which help in differentiation of B cells into Plasma B cells and help secretion of antibodies. B cells also recognize antigens through surface antibody receptors and are triggered to proliferate and differentiate into antibody secreting Plasma B cells. Thus it reveals that B cells and T cells help activate each other. Immune responses that produce IgG, IgA and IgE involve this kind of T dependent antigen triggering. These antigens are associated with isotype switching, affinity maturation and secondary response. These do not induce antibody response in athymic mice and T cell depleted cultures. Paracortex of lymph nodes is referred to as thymus dependent area.

T-Independent (TI) Antigens

These are the antigens which can activate B cells for antibody production in the absence of T cell binding/ cooperation. Examples of such antigens include lipopolysaccharides, flagellin, bacterial cell wall polysaccharides, dextrans, levans, polyglutamic acids, glycolipids, nucleic acids, anti-immunoglobulins, tumor producing agents etc. TI antigens cannot be processed and presented in association with MHC molecules and therefore are not recognized by T_h cells. These can be divided into two types, type 1 (TI-1) and type 2 (TI-2) antigens.

At high concentration, most TI-1 antigens are polyclonal B cell activators (mitogens); they are capable of activating B cells irrespective of their antigenic specificity e.g., lipopolysaccharide, which is also called endotoxin and is a major component of the cell walls of the gram negative bacteria.

TI-2 antigens activate B cells by extensively cross linking the membrane immunoglobulin (mIg) receptors. They are polymeric, bind directly to B cells and cross link several B cell antigen receptors at one time. They expose many identical epitopes to a B cell specific for that determinant. Mostly they lead to formation of IgM antibodies with low affinity and most of the times are not associated with isotype switching, affinity maturation or memory response. Although TI antigens do not require direct T_h cell binding but some can use cytokines derived from T_h cells and thus can lead to isotype switching to IgG.

The practical significance of TI antigens is that many bacterial cell wall polysaccharides are TI antigens and humoral immunity is the major host defense against such bacterial infections. Thus encapsulated bacteria can cause serious infections in individuals with congenital or acquired deficiencies of humoral immunity. Antibody responses to TI antigens may occur at particular anatomic sites in lymphoid tissues. Cortex of the lymph nodes are called the TI area. The TI antigens can stimulate antibody production in athymic mice.

Since TI antigens are generally associated with weaker response, lack of memory cell formation and low class switching, the important role of T_h cells for generating these aspects of humoral response can be easily understood.