Introduction to Diagnostic and Therapeutic Monoclonal Antibodies

Continuing Education for Nuclear Pharmacists
And
Nuclear Medicine Professionals

By

Blaine Templar Smith, R.Ph., Ph.D.

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INTRODUCTION TO DIAGNOSTIC AND THERAPEUTIC MONOCLONAL ANTIBODIES

STATEMENT OF LEARNING OBJECTIVES:

Upon successful completion of this lesson, the reader should be able to:

1. Distinguish between the types of immunity.

2. Describe natural antibody production by the human immune system.

3. Discuss the basic antibody-epitope interaction.

4. Describe the location and functional purpose of antibody structural components.

5. Differentiate among the categories: polyclonal, monoclonal, murine, chimeric, humanized and human antibodies.

6. Discuss the advantages and disadvantages in human use of murine, chimeric, humanized and human antibodies.

7. Discuss the unique physical characteristics of polyclonal and, monoclonal murine chimeric, humanized and human antibodies.

8. Explain the nomenclature scheme used for human use monoclonal antibodies.

9. Discuss factors to be considered when radiolabeling antibodies and fragments.

COURSE OUTLINE

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Introduction to Diagnostic and Therapeutic Monoclonal Antibodies

Blaine Templar Smith, R.Ph., Ph.D.

INTRODUCTION

The promise, for many years, of useful diagnostic and therapeutic monoclonal antibodies has begun to be realized. The applications for use of antibodies, their derivatives and fragments continues to hold even more potential, as common obstacles to their use are resolved. The route that this biotechnology routinely follows is to first be introduced in specialized situations that do not involve radiolabeling. Then, as the safety of each antibody product is established, uses targeting the specific site with radiolabeled diagnostic and therapeutic versions become viable. This has been the case for several monoclonal antibodies. Advances in recombinant DNA technology have also enabled creation of purer, less problematic products. Antibody-related products may find utility in nuclear pharmacy because targets of the original products are useful not only for general medical reasons, but also imaging and therapeutic uses. The most straight-forward scenario is that of a target (epitope) on a human cell or tissue type that, when treated with the original antibody product, results in a therapeutic benefit with appropriate patient safety. After the successful introduction of monoclonal antibodies in general medicine, the migration to imaging and/or therapeutic applications may follow.

It would be helpful if every biological product could find application in radiolabeled diagnostics or therapeutics. However, many antibody-related products that find use in general medicine are either unsuitable or unable to transition to radiolabeled products. Reasons for this can be very simple, such as a product that would not have a use for diagnosis or treatment beyond that of general medicine. Moreover, some promising products may fail to show patient safety, have a tendency to lose a radiolabel in-vivo, have unacceptable cross-reactivity with tissues other than the intended target, or even behave differently with a radiolabel attached.

ANTIBODY PRODUCTION BY THE HUMORAL IMMUNE SYSTEM

Before discussing antibodies themselves, it is helpful to review where they fit into the overall design of the immune system and their natural roles in immunity. This will help the reader place into context the potential uses and limitations of antibodies and antibody-derived products when their utilization for imaging and therapy is considered.

Hematopoiesis is the generation of all circulating cell types from pluripotent hematopoietic stem cells in the bone marrow (Figure 1). Through the process of
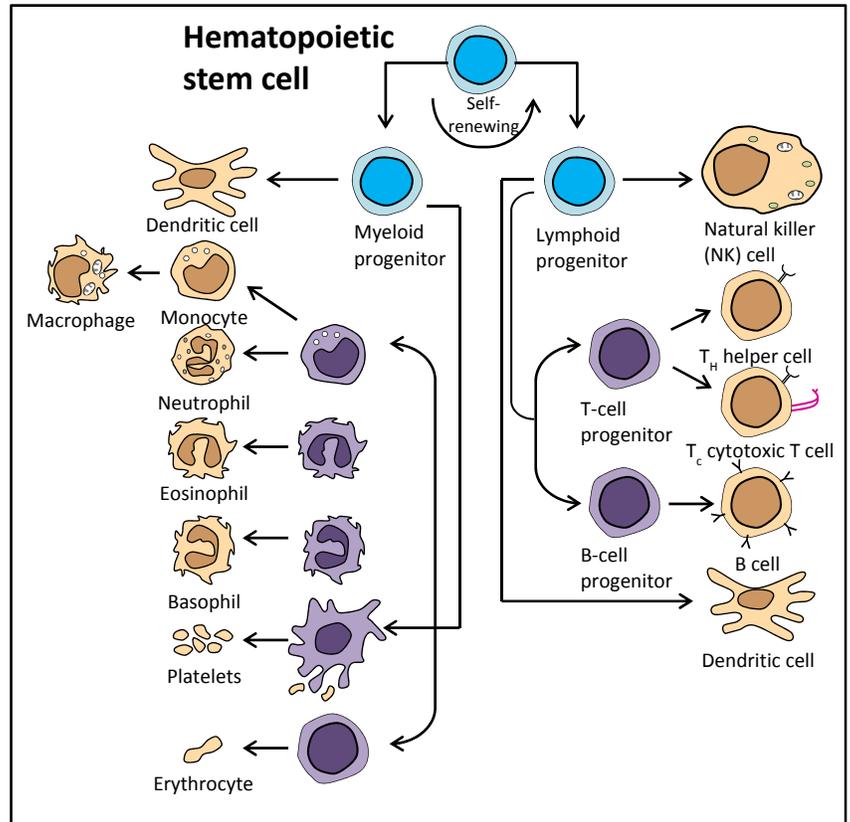

Figure 1. Hematopoiesis

hematopoiesis, important cells and cell products are created: neutrophils, eosinophils, basophils, platelets, erythrocytes, macrophages, natural killer (NK) cells, dendritic cells, and T- and B-cells. These all participate at specific points in the complete immune system and may be variously grouped by differing criteria.

Under normal conditions hematopoiesis is at steady-state, or equilibrium, with elimination. Erythrocytes (red blood cells) last about 120 days in circulation. The body produces 3.7×10^{11} white blood cells (lymphocytes) per day. Some of these live up to 30 years. Left unopposed, hematopoiesis would ultimately produce an overwhelming amount of all of the blood and immune cells which would be devastating to the body, as evidenced by the effects of leukemias and other blood proliferative disorders. One definition of leukemia is the over production, or insufficient elimination, of blood cells. To balance hematopoiesis, the body employs an elegant system known as apoptosis. Apoptosis, programmed cell death, is the orderly process by which a cell brings about its own demise. Changes in the cell that are involved in this process include a decrease in cell volume, modifications of the cytoskeleton, membrane blebbing, chromatin condensation, DNA degradation and fragmentation, and shedding of apoptotic bodies. These changed cells are called apoptotic bodies, intact organelles that

contain cellular debris and potentially damaging enzymes, safely contained from degrading cell's neighbors. Apoptotic bodies and other cellular debris are phagocytized (Figure 2). Necrosis differs, in that cell death is from injury, is not orderly, and often damages neighboring cells. In necrosis, cells swell and burst, releasing damaging and/or inflammatory agents into the external cellular environment. Apoptosis therefore allows an avenue for safe, orderly elimination of older, damaged, or unneeded cells and balances hematopoiesis.
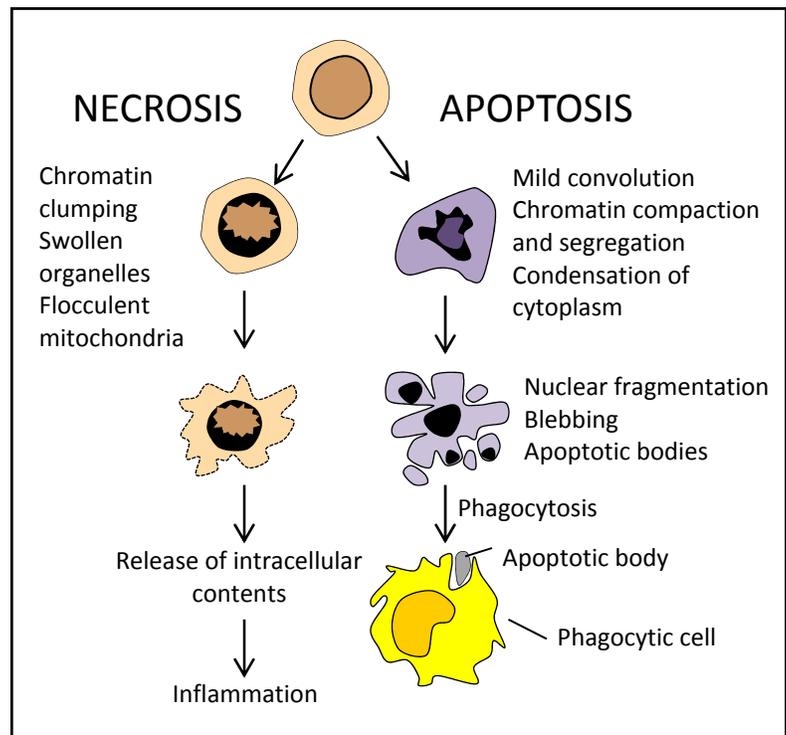

Figure 2. Apoptosis

Classification of Immunity

There are two major methods of classifying human immune system components. One method is based on the characteristic of adaptability: either innate or acquired immunity. The second method consists of dividing components into either cellular or humoral immunity.

Innate versus Acquired Immunity

Innate immunity refers to responses that are fixed, unchanging, and non-evolving (not improving). It is inborn with no requirement of previous exposure to be effective. Innate immunity provides the host with an immediately available, fast-acting response which is crucial to the host survival. It can quell an infection at an early stage or obstruct and slow a foreign organism's development while more deadly and precise responses are marshaled by the immune system. The price of this rapid-deployment is the inability to be flexible – or adaptive – and so innate immunity is comparatively non-specific in its criteria for response and static in its specificity. The innate immune system includes large scale components: anatomic, physiologic, endocytic or phagocytic, and inflammatory barriers; as well as chemical mediators such as cytokines. Anatomic barriers include the skin and mucous membranes. Physiologic barriers include febrile responses, acidic stomach pH, and chemical mediators such as lysozyme, interferons, and complement. Endocytic and phagocytic barriers include cells that

internalize and destroy foreign substances. Inflammatory barriers include phagocytic cells and serum proteins possessing antibacterial activity. The cell members of the innate immune system include macrophages, neutrophils, basophils, eosinophils, dendritic cells, NK cells, mast cells, and others.

Acquired immunity includes immune components that react to new antigens, improve their specificity and effectiveness over time and with re-exposure to antigen. Acquired immunity is often referred to as adaptive immunity. The acquired immune system has the advantages of improving in strength and accuracy, imparting the adaptivity that is lacking in the innate system. These immune abilities are referred to as memory. The compromise is that the ability to improve response requires time; therefore acquired immunity is not available in the initial stages of a foreign intrusion – unless it has been previously exposed to the foreign entity. Once set in motion, acquired immunity provides long-lasting protection for the host. The cells associated with acquired immunity include dendritic cells, B-cells, T-cells and antigen-presenting cells (which include B-cells).

It should be noted there is overlap among the cell types associated with the innate and acquired immune systems, as some cells have functions and capabilities that fall into both categories. The reader is referred to the many immunology textbooks available for a thorough discussion on details of the innate and acquired immune systems and their overlapping functions. A summary of innate and adaptive immunity is provided in Table 1.

Table 1

INNATE VS ADAPTIVE IMMUNITY		
	Innate	**Adaptive**
self / non-self discrimination	present, reaction is against non-self (foreign)	present, reaction is against non-self (foreign)
lag phase	absent, response is immediate	present, response takes at least a few days
specificity	limited, the same response is mounted against a wide variety of agents	high, the response is directed only to the agent(s) that initiated it.
diversity	limited, hence limited specificity	extensive, and resulting in a wide range of antigen receptors.
memory	absent, subsequent exposures to agent generate the same response	present, subsequent exposures to the same agent induce amplified responses

Cellular versus Humoral Immunity

The complete human immune system can also be considered as a division between cellular and humoral classifications. As with the distinctions between the innate and acquired categories, those between the cellular and humoral systems are sometimes blurred by overlapping functions.

Cellular immunity refers those cells of the activated cellular immune system— the effector T-cells (T-helper cells and T-cytotoxic cells). Each of these cells, once stimulated and activated, is considered an effector cell. Effector cells are responsible for direct actions against antigens. Effector T-cells are often referred to via alternative nomenclature: T-helper cells are $CD4^+$ cells; T-cytotoxic cells are $CD8^+$ cells. T-helper cells have overlapping function between cellular and humoral immunities. T-cytotoxic cells, as the name suggests, generally function by destroying irregular cells directly. Thus, the cellular immune system's main function is to survey and remedy *intra*cellular irregularities, such as intracellular bacteria, viruses and cancer.

Humoral immunity pertains to B-cells and their end products— memory B-cells and plasma cells (and their soluble secreted product, antibodies) which circulate in the humor, or extracellular fluids, such as plasma, the lymphatic system, and tissue fluids. B-cells die after about 6 months unless activated by their complementary antigen. If activated by contact with antigen, B-cells differentiate into both memory B-cells and plasma cells. It is the latter that secrete antibodies. Antibodies function by binding soluble antigens or by attaching to targets on the surface of cells. Antibodies promote antigen clearance directly or attract additional immune components to effectively eliminate targets. The memory B-cells are held in reserve, ready for any future re-exposure to the antigen. If re-exposure does occur, the existence of memory B-cells enables a much swifter, stronger, and more accurate antibody response. The plasma cells secrete IgM isotype antibodies for two to three days, then shift to secretion of the higher affinity IgG isotype rather than IgM, and migrate to the bone marrow where they are believed to survive for as long as a year after the humoral response has ended.

The humoral system's major responsibility is protection of the host against *extra*cellular targets. Upon encountering an appropriate foreign entity, very often both the innate and adaptive responses are triggered. This can also be stated as equivalent to stimulation of both the cellular and humoral responses. A summary of both the humoral and cell mediated responses is provided in Table 2. The discussion will focus on the major soluble product of the humoral system, antibodies.

Table 2

	Humoral Immunity	Cellular Immunity
HUMORAL AND CELLULAR IMMUNITY		
Mechanism	Antibody-mediated	Cell mediated
Cell Type	B Lymphocytes: • Memory B cells • Plasma cells • Antibodies	T Lymphocytes • T helper cells CD4$^+$ • T cytotoxic cells CD8$^+$
Mode of action	Antibodies circulating in serum	Direct cell-to-cell contact or secreted soluble products (e.g. cytokines)
Purpose	Primary defense against extracellular pathogens: extracellular bacteria, circulating virus	Primary defense against intracellular pathogens: intracellular bacteria and virus

B-Cells, Plasma Cells and Antibodies

B-cells and their maturation product, plasma cells, are the two effector cells of the humoral branch of the immune system. Antibodies are plasma cells' soluble and mobile effector molecules. Being both soluble and mobile, it is the antibody and its components that have become useful for diagnostic and therapeutic purposes.

The lymphoid progenitor of B-cells produces in the vicinity of 10^6 potential antigen-compatible, preliminary B-cell lineages each day. Variable region genes code for a potential 10^{10} conformations. During the maturation process, B-cells undergo many changes, including the appearance of cell surface-bound immunoglobulin (antibodies) of μ and δ isotypes. These surface immunoglobulins receptors are specific for the programming of the individual B-Cell.
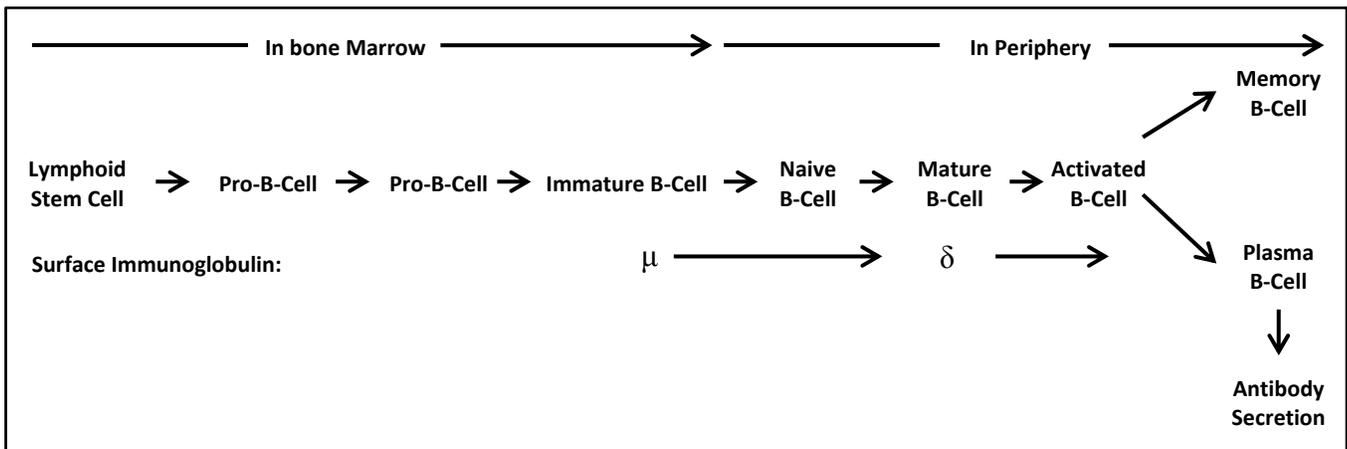
Figure 3. B-Cell Maturation Lifespan

When a B-cell with a particular combining site of surface IgD is selected by virtue of its superior binding specificity, this sets into motion the production of progeny clones, originating from that

particular selected B-cell. As the progeny clone expands, it produces memory B-cells and plasma B-cells. Plasma cells derived from a single clonal lineage of a B-cell can secrete as many as 1.7×10^8 single binding site-specific antibodies per day (Figure 3). However, when considering antibodies for scientific or medical applications, utilizing plasma cells presents a singular, but major obstacle: they survive and secrete antibodies only for a few days. For the natural immune response, this is a good thing as there needs to be an off-switch to antigen responses. If an antigen persists, more effector cells and molecules will be made by the immune system to replace those that depart (usually via apoptosis.) If this did not occur, a response would be never-ending and most likely unnecessary and detrimental to the host. The practical scientific and medical aspects of this will be discussed below.

In a normal immune response, an antigen triggers multiple B-cell lineages, each with differing antibody combining specificities, through a process called clonal selection. This occurs in the bone marrow. (Figure 3) Further refinement in the B-cell selection process continues in the periphery. The benefit of the normal immune response is that multiple clonal lineages of B-cells, each with its unique specificity but targeting the same antigen, are simultaneously stimulated to differentiate into plasma cells or memory cells over a period of four to five days. The result is a variety of antibodies specific for the antigen in the form of a combined polyclonal antibody response. For most scientific and medical applications, polyclonal antibodies are not useful. If antibodies are to be truly useful, they must be exactly the same – from the same clonal line. In other words they must be monoclonal.

Antibody Structure

Each antibody is composed of two identical heavy polypeptide chains and two identical light polypeptide chains. These chains are each held together by interchain disulfide bonds. Each heavy and light chain is made up of folded regions, called domains. Light chains (LC) contain one variable region (denoted V_L for variable light) and one constant region (denoted C_L for constant light). Heavy chains (HC) contain one variable (V_H) and three or four constant regions (C_{H1} - C_{H4}) depending
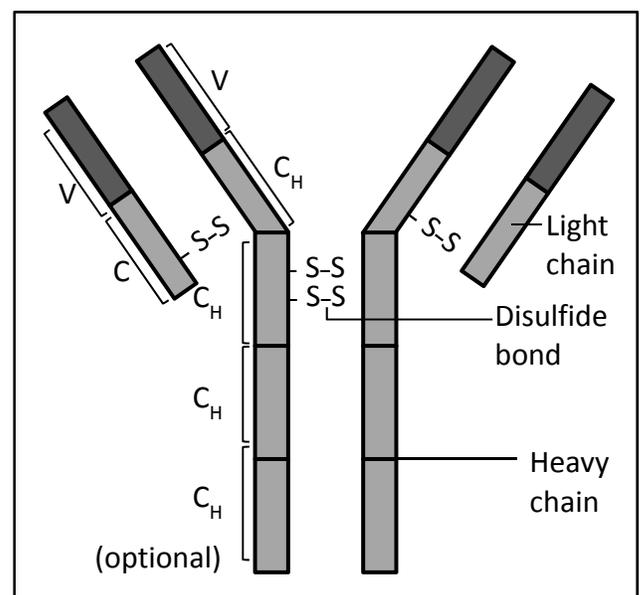

Figure 4. Basic structure of a monoclonal antibody.

on the antibody isotype. Variable regions are named such because the amino acid sequence in this area is variable among antibodies from different B-cell lineages when compared to most of the rest of the antibody structure. At the very ends of the variable regions are the hypervariable regions. It is in the hypervariable regions that the most variability between antibodies from differing lineages occurs. It is this high variability that allows very divergent conformation permutations for antibody-antigen binding. Constant regions are relatively invariant for each class of antibody. The basic antibody structure contains duplicate V_L and V_H regions. These form the antigen-binding site. Therefore, each antibody has two identical antigen binding (ab) sites. All antibody isotypes, except for IgE, are hinged between the C_{H1} and C_{H2} regions to impart flexibility to the main antibody structure. The paired variable and constant regions above this hinge are referred to as the antigen-binding fragment (Fab) of the antibody. The paired constant regions below the hinge create the constant fragment (Fc) of the antibody (Figure 4).

In addition to the basic protein structure of antibodies, there are carbohydrate components. Antibodies are naturally glycosylated (carbohydrate(s) attached) along the heavy chains. The carbohydrate content, especially along the Fc portion of an antibody, profoundly affects many of the actions of the antibody. This includes proper secretion of the antibody from the plasma cell, its kinetics in circulation, the actions of antibody dependent cell mediated cytotoxicity (ADCC) and complement, and the chemistry related to proper radiolabel or linker attachment.

Human antibodies are similar in general structure, but are first divided into five classes, or *isotypes,* (α, δ, ϵ, γ, and μ) based on heavy chain structural differences and then usually into one of two subclasses based on the type of light chain (either κ or λ.) Isotypes provide the general antibody identification— IgA for those composed of α (alpha) heavy chains, IgD for those composed of δ (dela) heavy chains, et cetera. The isotypes differ in their biological properties, functional locations, and ability to deal with different antigens. B-cells undergo affinity maturation (improvement in antibody affinity through a selection process) which includes class (isotype) switching. . Class switching allows daughter cells from the same activated B cell to produce antibodies of different isotypes. Only the constant region of the antibody heavy chain changes during class switching. The variable regions and the antigen specificity remain unchanged. The progeny of a single B cell can produce antibodies, all specific for the same antigen, but with the ability to produce the effector function appropriate for each antigenic challenge. Class switching is triggered by cytokines.

The basic structure of the antibody of any isotype is fundamentally similar. The functional differences of isotypes are to a large extent dictated by structural variations and chemistry that connect the Fc portions of antibodies and impart particular biological functions. It is the fragments of the fundamental antibody structure – the Fab, F(ab')$_2$ and variable/hypervariable regions— that provide the frontier work in diagnosis and therapy. Useful antibody fragments are produced by reduction of hinge-region disulfides or digestion with proteolytic enzymes (Figure 5). Today, these and other antibody products can be produced through use of recombinant DNA technology.
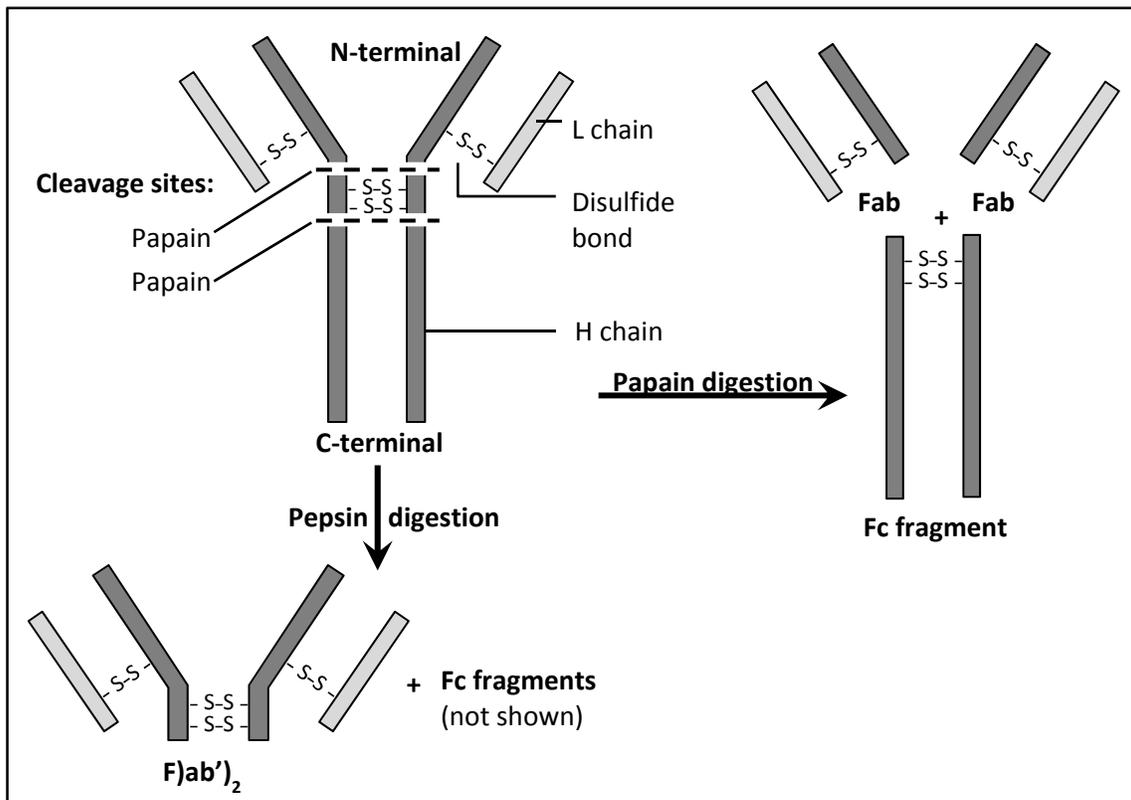
Figure 5. Antibody Enzyme Cleavage Products

THE ANTIBODY-ANTIGEN/EPITOPE INTERACTION

The portion of the antibody responsible for antigen binding is the *complimentarity-determining region* (CDR) located at the end of the variable regions. The CDRs in the V_H and V_L form an antigen-binding pocket that contacts epitopes (antigens) directly (Figure 6). The conformation of this pocket dictates both the extremely fine discrimination antibodies display for proper antigens and the extreme strength with which these antigens are bound.

Affinity (K_a) is the numerical representation of the strength of a ligand-binding site interaction. Antibody binding affinity (K_a values) for low-affinity antigen-antibody complexes is usually between $10^4 – 10^5$ L/mol and improves through the affinity maturation process. For high-affinity antigen-antibody complexes, the binding affinity can be as high as 10^{11} L/mol. This is extremely high and explains why high-affinity antibodies bind antigen very tightly and remain bound for relatively
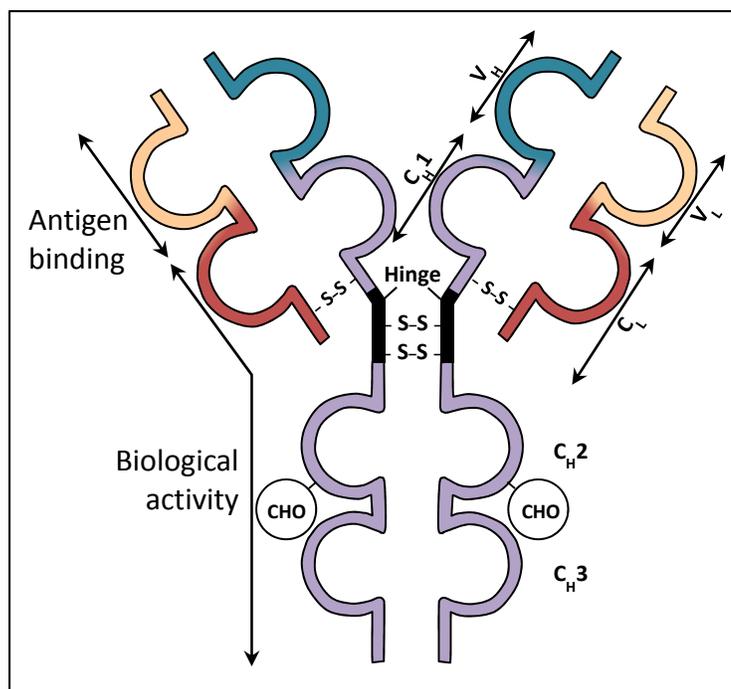

Figure 6. The Antigen Binding Site

long times. Depending on the isotype of the antibody, there are between 2 and 10 antigen binding site per molecule. These affinities are additive and this strength of multiple interactions is the avidity of the antibody. Antibodies usually have both high affinity and avidity—a minimum of 2 antigen binding sites are available for a normal IgG antibody. If the individual binding site affinity is relatively low, the avidity can compensate and provide excellent binding strength. It is this characteristic of high affinity, coupled with typically high avidity that enables monoclonal antibodies to be attractive candidates for radiolabeling. Monoclonal antibodies are extremely desirable in locating an epitope that is very unique or exclusive on a desired target tissue.

The specificity of the antibody-epitope interaction is often superior to that of many chemically derived diagnostic and imaging agents. By their very nature, antibodies – especially those produced after multiple antigen exposures – are extremely discriminating with regard to the tissue or target they bind. This is ideal when radiolabeling antibodies. This accuracy improves target to non-target ratio when attached to imaging isotopes, thus imaging quality is improved. Second, precision delivery of isotopes with particulate emissions is made possible. Finally, there may be a reduction in the amount of radionuclide activity administered to the patient in order to achieve the goal(s) of the procedure, thereby minimizing patient and personnel exposures.

POLYCLONAL AND MONOCLONAL ANTIBODIES

Polyclonal Antibodies

A single plasma cell is capable of secreting over 10^8 antibodies per day. When a plasma cell secretes antibodies, each is exactly the same in all aspects. Their isotype, Fc, and Fab components are identical. However, a normal humoral immune response produces many plasma cells from the selection of many compatible and useful B-cell lineages producing a pooled polyclonal response. This polyclonal response ensures thorough overlap in antibody binding and provides a more effective antibody response to the antigen. This is ideal for disease eradication. For science and medicine, this creates inexactness and imprecision. To provide precision and reproducibility, single clone products – monoclonal antibodies – are required. Until 1975, the inability to reliably create monoclonal antibodies was a major impediment to the application and uses of antibodies. The ability to combine the attributes of a perpetual myeloma clone with those of an antibody-secreting cell with limited life provided the step necessary to propel the areas of science and medicine forward in this front. Propagation and production of characteristically predictable, unlimited amount of monoclonal antibodies began the pioneering into their applications.

Monoclonal Antibodies

The human body secretes antibodies in response to foreign antigens. This phenomenon is manifest through the B-cell lineage with help from T-cells and other components of the immune system. The focus of this discussion is on the humoral response and on the antibody response that results. This antibody response can be said to originate in plasma cells, although the antibody response is much more complex originating much further upstream from the terminally differentiated plasma cell. A normal humoral immune response results in multiple clonal lineages of B-cells which terminally differentiate into antibody-secreting plasma cells resulting in a polyclonal antibody response. For scientific and medical study and use, only one antibody (and so one plasma cell clone) is wanted.

Two basic issues needed to be addressed before practical scientific and medical uses for antibodies could realistically be investigated and applied. First was the issue of the limited lifespan of plasma cells, limiting the supply of any monoclonal antibodies of interest. The second was that only polyclonal antibodies could be obtained from a culture of antigen stimulated B-cells. Monoclonal antibodies could be painstakingly obtained by isolating a single plasma cell, but that was simply a result of both good fortune and hard work.

Murine Monoclonal Antibodies

In 1975, Köhler and Milstein discovered that murine (mouse) antibody-secreting plasma cells and immortal murine myeloma cells could be fused with the benefits of each retained. This discovery propelled science and medicine into the modern monoclonal antibody era. Murine monoclonal antibodies were the obvious product. Murine monoclonal antibodies had a major impact in subsequent years, but the very basis of their origin presented other problems that would need to be overcome.

Köhler and Milstein's method, which is still useful today, involves fusion of a cancerous (immortal) mouse B-cell myeloma with an immunized mouse plasma cell, creating a hybrid cell, or *hybridoma* (Figure 7). The resulting hybrid's immortality is provided by the myeloma cell, and the plasma cell supplies the (monoclonal) antibody secretion function. Hybridomas can be maintained indefinitely in culture, providing a relatively unlimited supply of murine monoclonal antibodies.
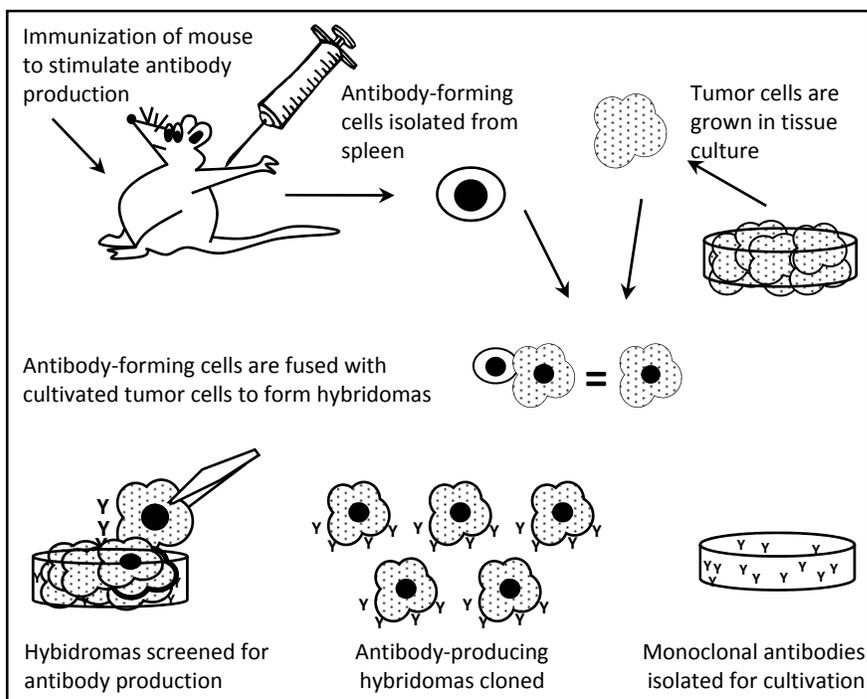
Figure 7. Monoclonal Antibody Production

Mice and humans often differ in the glycosylation of antibodies, a concern for in vivo applications with murine monoclonal antibodies. The role played by glycosylation of antibodies has not been completely explained. However, absent or incorrect placement of carbohydrates on the constant heavy chain (Fc) alters antibody solubility, serum clearance, and the proper interaction between antibodies and Fc receptors. Correct glycosylation of the Fc portion of the antibody is very important if the antibody action depends on complement activation or antibody-dependent cell-mediated cytotoxicity (ADCC). Even if the Fc functions of a murine monoclonal antibody product are not the prime focus of its function, the other issues – of solubility, serum clearance, and general pharmacokinetics – can be perplexing problems to overcome.

The major drawbacks of purely murine monoclonal antibodies are (1) the reduced plasma half-life of murine versus human IgG, and (2) the human against mouse antibody (HAMA) response which further reduces the half-life. A human IgG normally has a half-life of about three weeks. Murine IgG has a half-life of only a few hours. Murine IgG tends to invoke a HAMA response that not only causes faster removal of the mouse IgG, but also can elicit an anaphylactic hypersensitivity response due to its foreign nature. The decreases in circulating half-life necessitate increasing administered doses (which can then lead to increasing HAMA,) or accepting a reduction in effectiveness of the product. Therefore, a high priority goal has been to reduce the content of murine epitopes in monoclonal antibodies while retaining effectiveness. Nevertheless, many completely murine monoclonal antibodies were, and still are, used for imaging and therapeutic applications. Some of these include Muromonab-CD3 (Orthoclone OKT3® an immunosuppressant used in organ transplants) and Y-90 ibritumomab tiuxetan (Zevalin® a radioimmunotherapeutic agent used as an antineoplastic agent in non-Hodgkin's lymphoma).

The incremental progress toward creating a completely human antibody has been an iterative process. Each improved process creates more human-like antibodies. These improvements have included creation of chimeric antibodies, humanized antibodies, and essentially human antibodies prepared through bacteriophage display or transgenic animals.

Chimeric Monoclonal Antibodies

With the emergence of recombinant DNA technology, monoclonal antibodies with greater amounts of human sequences are now being developed and used. The first step in structuring the ideal human monoclonal antibody was the chimeric antibody. Chimeric antibodies are composed of protein sequences from two origins: murine and human. While murine antibodies are 100% murine protein, chimeric antibodies are typically only about 33% murine proteins (Figure 8) with the remainder human protein. In chimeric antibodies, the variable region with the antigenic specificity remains murine. The constant regions which dictate the antibody isotype are replaced with human proteins. Chimeric antibodies are made by melding murine variable region genes with human constant region genes. One recombinant DNA method by which this can be accomplished is by isolating the variable region genes from a murine hybridoma secreting an antibody that binds the desired target, then amplifying these genes using polymerase chain reaction (PCR.) This initial product is a copy DNA (cDNA) of the murine variable region (V-cDNA) The V-cDNA can then be ligated into a plasmid (small circular,

extra chromosomal DNA in certain bacteria).

In a parallel sequence, cDNA for human heavy chain constant regions is also amplified and ligated into a separate plasmid producing heavy chain copy DNA (HC-cDNA). At this point, the V-cDNA and HC-cDNA can be brought together into a common host cell using co-transfection. This elaborate method provides the desired fusion protein, intact chimeric antibody. Because bacteria do not correctly glycosylate human proteins, the antibodies are not usually secreted from the bacteria. Instead, the chimeric antibody is normally expressed in inclusion bodies in the bacteria cells and must be extracted and purified.

Even with this successful and elegant modification to the original murine monoclonal antibody, there can still be a human immune response to the murine peptide sequences in the chimeric antibody structure. The human against chimeric antibody (HACA) is an immune response to the murine portion (variable region) of the antibody

The advancement from murine monoclonal antibodies to chimeric monoclonal antibodies was an important improvement in antibody technology. Useful human-mouse chimeric monoclonal antibodies are summarized in Table 3.

Table 3

CHIMERIC MONOCLONAL ANTIBODIES	
Abciximab (ReoPro®)	Platelet aggregation inhibitor. Used with coronary artery procedures
Infliximab (Remicade®)	Binds tumor necrosis factor. Used to treat autoimmune diseases
Cetuximab (Erbitux®)	Inhibits epidural growth factor. Used to slow growth of metastatic disease
Rituximab (Rituxan®)	Destroys B cells. Used to treat non-Hodgkins lymphoma

Humanized Monoclonal Antibodies

The next iteration designed to reduce murine sequence content was the creation of humanized antibodies. Humanized monoclonal antibodies typically retain only the hypervariable regions, or complementary determining regions (CDRs), of a murine antibody while the remainder of the antibody is human. Thus, humanized antibodies typically contain only 5% to 10% murine composition (Figure 8). The preparation of humanized antibodies follows a path similar to the synthesis of chimeric antibodies. Humanized antibodies generally have less adverse human immunological responses than murine or chimeric monoclonal antibodies.

Humanized monoclonal antibodies typically are synthesized by grafting murine CDRs to a human antibody. Using recombinant DNA technology, human immunoglobulin light and heavy chain genes can be amplified by polymerase chain reaction. The resulting human lymphoid cDNA library can be used as a template for in-vitro synthesis of the entire antibody, except for the CDRs. Murine CDRs are cloned and grown in parallel. The respective genes can then be spliced into vector DNA and incorporated into bacteria for growth. To streamline the process, often both human cDNA - and murine cDNA - containing vectors can be incorporated into the same bacterial cell (co-transfection) and an intact humanized monoclonal antibody can be produced. As with chimeric antibodies, humanized antibodies must be extracted from bacterial cultures and purified.

One problem sometimes encountered with both chimeric and humanized monoclonal antibodies is the retention of the combining region conformational integrity. A single amino acid residue (not related to antigen binding) in the Fc or variable region C-terminal area causes the otherwise ideal CDR to be slightly altered in its three-dimensional conformation. Altering the alignment of a selected CDR residue by even a few degrees can drastically change the antibody's binding affinity and avidity, lowering the binding efficiency of the chimeric or humanized antibody. Minor alterations in the framework residue cDNA are needed to restore or improve the murine antibody-epitope binding. The synthesis process of expressing humanized antibodies may also lead to improper or nonexistent glycosylation. Glycosylation of the Fc is important in solubility, serum clearance, and general pharmacokinetics of antibodies.
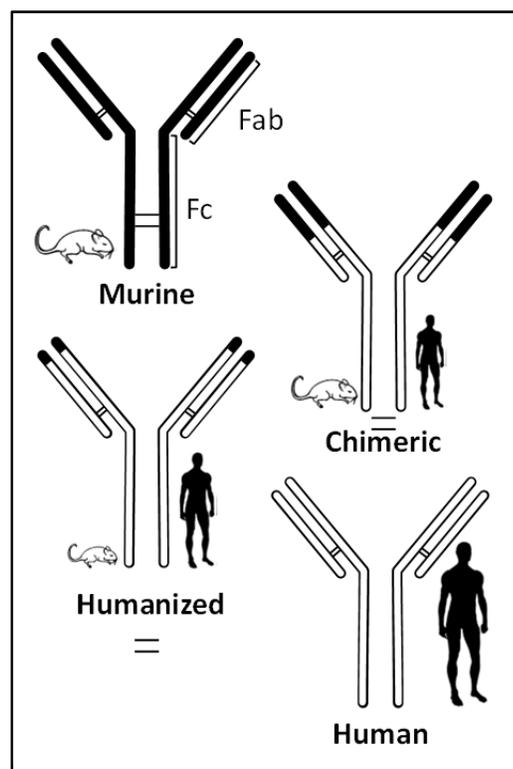
Figure 8. Relative Murine vs Human Content of Various Types of Monoclonal Antibodies

Humanized antibodies are less likely to elicit an immune response than murine or chimeric monoclonal antibodies. The improvement of humanized over both murine and chimeric antibodies has been enthusiastically accepted. The ideal goal is use pure human antibodies for antibody applications, especially those used in in-vivo procedures. Examples of useful humanized monoclonal antibodies are presented in Table 4.

Table 4

HUMANIZED MONOCLONAL ANTIBODIES	
Palivizumab (Synagis®)	Prevention of respiratory syncytial virus (RSV) infections in infants
Trastuzumab (Herceptin®)	Treatment of HER2-positive metastatic breast cancer
Alemtuzumab (Campath®)	Treatment of chronic lymphocytic leukemia (CLL), cutaneous T-cell lymphoma (CTCL) and T-cell lymphoma.

Human (Monoclonal) Antibodies

Human monoclonal antibodies are fully, or nearly 100%, human in composition. The word *monoclonal* is technically not applicable to all synthetic human antibodies because some of the synthesis technologies do not employ hybridoma technology. There are two basic technologies employed to produce human antibodies: (1) genetically engineered, knockout or transgenic mice; and (2) use of phage display libraries.

Knockout mice are developed by harvesting embryonic stem cells from early stage fertilized mouse embryos. An existing gene is inactivated or "knocked out" by replacing it with an artificial piece of DNA. The altered stem cells are then grown into mice with an altered genomic profile. Inactivation of the ability to rearrange germ-line heavy and light chain configurations inhibits the mouse ability to make murine immunoglobulin and the corresponding murine B-cells. Substituting human heavy and light chain germ-line DNA can cause the knockout mouse to produce human B-cells which may produce human monoclonal antibodies. While technically challenging, the use of knockout mice to produce entirely human monoclonal antibodies is an emerging and promising technology.
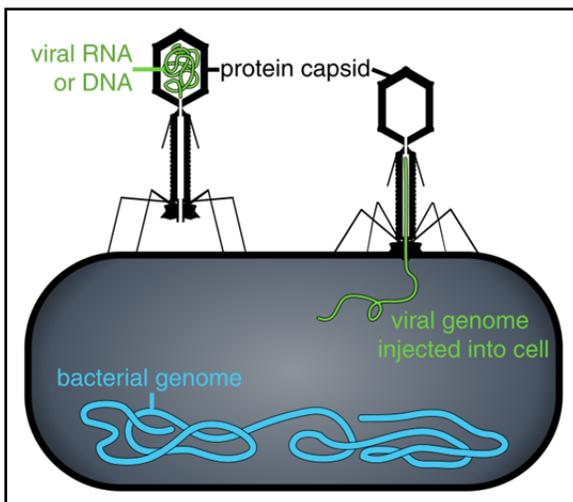
Figure 9: Diagram of how some bacteriophages infect cells: this is not drawn to scale, bacteriophages are about 100 x smaller than bacteria. [CC-BY-SA-3.0; Released under the GNU Free Documentation License]

Human monoclonal antibodies engineered from transgenic mice strains have been reported to: (1) have affinity values toward antigens similar to human antibodies, (2) display pharmacokinetics equivalent to human antibodies, and (3) possess virtually nonexistent hypersensitivity responses compared to murine, chimeric and humanized monoclonal antibodies. As the technology for creating human monoclonal antibodies advances, approved diagnostic and therapeutic products will undoubtedly emerge.

Bacteriophages (or phages) are viruses that infect bacteria. Typical phages have hollow heads where phage DNA or RNA is stored as well as tails which bind to specific molecules on the surface of their target bacteria. The viral DNA is then injected through the tail into the host cell causing the rapid production of identical progeny phages (Figure 9). Eventually the new phages burst from the cell to and infect more bacteria.

Simplistically, specific antibody fragments are "displayed" by fusion into phage DNA structure. The phage display is rapidly reproduced using *E. coli* generating a large population (library) of antibody fragments specific for the target antigen. Each resulting phage has a functional antibody protein on its surface and contains the gene encoding the antibody incorporated into the phage genome. Recent advances using antibody phage display now make it possible to generate human monoclonal antibodies that recognize any desired antigen.

Adalimumab (HUMIRA®) was derived from phage display and was the first fully human monoclonal antibody drug to receive FDA approval in 2002. It is used to treat several conditions including rheumatoid and psoriatic arthritis, Crohn's disease, and ulcerative colitis. Table 5 lists representative human monoclonal antibody therapeutic agents.

Table 5

HUMAN MONOCLONAL ANTIBODY AGENTS	
Panitumumab (Vecitibix®)	Treatment of EGFR expressing, metastatic colorectal cancer
Golimumab (Simponi®)	Blocks TNF-α. Treatment of rheumatoid arthritis
Canakinumab (Ilaris®)	Blocks interleukin-1β. Treatment of Cryopyrin-Associated Periodic Syndrome
Ustekinumab (Stelara®)	Blocks interleukin 12 & 23. Treatment for plaque psoriasis

Nomenclature

Because of the growing prominence of monoclonal antibodies in medicine, the United States Adopted Name (USAN) Council has provided guidelines for nomenclature. In 2011, the USAN Council updated the original guidelines. These are summarized in Table 6. The name for a monoclonal antibody is formatted as

    PREFIX – TARGET - SOURCE SPECIES – SUFFIX
    Prefix: a unique prefix used to identify the product
    Target: usually a three-letter identifier for the disease or target
    Source species: 1 or 2 letter identifier of animal source

Suffix: for monoclonal antibodies is *mab*

For example, the product rituximab (Rituxan) was the first chimeric monoclonal antibody approved in the United States for treatment of malignancy. The name can be translated as:

ri = unique prefix

tu = tumor

xi = chimeric

mab = monoclonal antibody

This is a chimeric (human-mouse) monoclonal antibody that targets CD20 on B cells. It is used to treat NHL and pre-B-Cell acute lymphoblastic leukemia.

If the antibody is radiolabeled, the isotope symbol and mass number are placed before the antibody USAN name and a separate word is used to identify the conjugate between the radioactive label and the monoclonal antibody. An example of a radiolabeled monoclonal antibody ^{90}Y-ibritumomab tiuxetan (Zevalin) is interpreted as

Y-90 labeled = isotope and number

ibri = unique prefix

tum = tumor

o = murine

mab = monoclonal antibody

tiuxetan = the conjugate

This is a murine monoclonal antibody that targets CD20 on B cells radiolabeled with yttrium-90 by chelation of tiuxetan. It is used to treat non-Hodgkin's lymphoma.

ANTIBODY FRAGMENTS

Antibody fragments were originally derived from enzyme digestion of intact antibodies (See Figure 5) producing the Fc, Fab and F (ab')$_2$ products. Production of antibody fragments has become more sophisticated. In some diagnostic and therapeutic situations, antibody fragments, namely Fab and F(ab')$_2$ fragments, are attractive. These fragments can be made from enzyme digests of intact antibodies or synthesized using recombinant DNA technology, circumventing hybridoma and the

drawbacks of whole antibodies. Fragments are easier to manipulate genetically and express in bacterial systems.

The smaller fragments penetrate tissue better and faster, clear the general circulation faster, and are eliminated more completely with less hepatic binding than whole antibodies. These smaller molecules tend to have better penetration into solid tumors than whole antibodies. Generally there is faster systemic clearance than that of intact monoclonal antibodies. While this is a desirable feature, it should be noted that faster clearance sometimes comes with a price, such as increased renal exposure. Fragments are often useful when specific faster tissue penetration and rapid clearance are preferred, as in tumor imaging. Fragments entirely of human peptide sequences present less of an immunogenic target for the patient.

Table 6

NOMENCLATURE FOR NAMING MONOCLONAL ANTIBODY PRODUCTS	
Target	*Source Species*
tu/t = tumors	a = rat
li/l = immunomodulators	axo = rat-mouse chimer
ba/b = bacterial	e = hamster
ci/c = cardiovascular	i = primate
fu/f = antifungal	zu = humanized
ki/k = interleukins	o = mouse
ne/n = Neurons as targets	xi = chimeric
so/s = bone	xizu = chimeric-humanized
vi/v = viruses, antiviral targets	u = fully humanized/human

An early fragment useful in medicine is digoxin immune Fab (Digibind®) used to treat digoxin toxicity. Other non-radiolabled antibody fragments include crotalinae polyvalent immune Fab (CroFab®) an antivenom for four North American snakes and afelimomab (Segard®) under investigation for use in patients with severe sepsis. To date, radiolabeled antibody fragments have been restricted to experimental applications or products that have been withdrawn from the market. However, there is little doubt that fragments will eventually find more accepted uses in nuclear medicine.

RADIOLABELING OF MONOCLONAL ANTIBODIES AND ANTIBODY FRAGMENTS

The exploration of radiolabeled antibodies has made considerable progress since the first reported use of radiolabeled antibody for imaging in 1948. Biotechnology is addressing the obstacles to successful clinical use of radiolabeled antibodies. Techniques have improved uptake in target tissue and made progress in eliminating adverse immune reactions. Radiolabeled site-specific antibodies shows promise in evaluating metastatic disease and monitoring therapeutic outcomes. Labeling with cytotoxic radionuclides can deliver lethal radiation doses to tumors but sparing normal tissue.

Radionuclide Considerations

Gamma and positron-emitting isotopes are useful for radiolabeling antibodies for diagnostic imaging purposes. The half-life of the radionuclide must be matched to the pharmacokinetic properties of the selected antibody. Radionuclides with longer half-lives (e.g., In-111, I-131 or I-124) are suitable for labeling intact antibodies that require several days to reach maximum tumor uptake and be cleared from the circulation. Rapidly cleared fragments may use nuclides with shorter half-lives such as Tc-99m, I-123, Cu-64 or Y-86. Positron-emitters such as F-18 and Ga-68 may be useful for labeling fragments with very rapid uptake and clearance, while radiolabeling with ultra short half-life O-15, N-13 and C-11 is not feasible.

Radionuclides suitable for therapeutic purposes are selected for their damaging particulate emissions. Emissions of alpha-particles, beta-particles or Auger and conversion electrons are weighted on their range in tissue and their linear energy transfer (LET). Alpha particles have the highest LET and travel 50-100 μm (5-10 cell diameters) in tissue. Radionuclides such as At-211, Bi-212, or Ac-225 are useful in treating small clusters of cancer cells or micrometastases. Beta emitters have greater penetration (200-1200 cell diameters) but deliver less energy over the penetration track. The long range allows for greater irradiation of distant nontargeted tumor cells via the crossfire effect. This is advantageous in treating large lesions. Useful beta-emitters include I-131, Y-90, Re-186/Re-188 and Lu-175. Auger electrons travel less than one cell diameter, but deliver high LET. Cell death requires that the antibody be internalized into cells, preferably into the DNA. Auger electron-emitting radionuclides include I-123, In-111 and Ga-67.

Radiolabeling Methods

Halogens

Radiohalogens used to radiolabel antibodies include: I-123, I-124, I-131, Br-76, F-18 and At-211. There are direct and indirect methods of radiohalogenation. Direct radioiodination requires the oxidation of iodine using chloramine-T or Iodo-Gen for electrophilic substitution into tyrosine amino acids in antibodies. Monoclonal antibodies labeled with direct iodination are unstable in vivo due to proteolysis, deiodination, and loss of radioactivity from cells. Attempts to directly label antibodies with other halogens have resulted in similar challenges.

Indirect methods of radiohalogenation involve the use of a bifunctional chelate. The radiohalogen is attached to an intermediate compound that conjugates with the antibody. The principle antibody functional groups used in conjugation reactions are amines, sulfhydryls, and carbohydrates. Radiolabeling with halogens requires multiple steps of manipulation of the radionuclide prior to conjugation with the antibody. The complexity of the labeling process has limited commercial development of radiohalogenated antibodies or antibody fragments. The therapeutic agent I-131 tositumomab (Bexxar®) is an example of a radioiodinated antibody. It is used in treating non-Hodgkin's lymphoma.

Radiometals

Technetium-99m can be attached to antibodies or fragments by either direct or indirect methods. Intact monoclonal antibodies can be labeled directly by first reducing of the disulfide bonds to free thiols. The reduced antibody then labels with transchelation of the Tc-99m from Tc-99m glucoheptonate or Tc-99m MDP. Indirect methods of labeling involve conjugation of a bifunctional chelate (e.g., DTPA) to the antibody. Reduced Tc-99m complexes with the conjugate and is more stable than direct-labeled antibody. An alternative method involves modifying the antibody with an HYNIC (6-hydrazinonicotinic acid) group and labeling it by ligand exchange with Tc-99m glucoheptonate. Both direct and indirect labeling methods have been adapted for commercial kit formulations.

Other radiometals such as In-111, Y-90, Ga-67 and 68, Cu 64, and Lu-177 are successful in radiolabeling intact antibodies as well as antibody fragments through use of a bifunctional chelating agent. A bifunctional chelating agent possesses two functionalities. One portion chelates the metallic radionuclide. The other portion contains a reactive functional group that binds to specific location(s) on the antibody. In general, the antibody or fragment is reacted with the linker and the metallic

radionuclide is then added to form the final conjugate. Effective linkers include ethylenediamine tetraacetic acid (EDTA), diethylenetriamine pentaacetic acid (DTPA) and 1,4,7,10-tetraazacyclodecane-1,4,7,10-tetraacetic acid (DOTA).

The radiolabeling of antibodies and fragments is a careful balance of the physical characteristics of the isotope, the limitations of the labeling method, in vivo stability of the radiolabeled conjugate and the pharmacokinetics of both the conjugate and the radionuclide. Research continues to explore more successful methods for radiolabeling monoclonal antibodies and appropriate fragments.

MONOCLONAL ANTIBODIES AS APPROVED DRUGS

The development of clinically useful radiolabeled monoclonal antibodies has been a slow process challenged by obstacles. Persistent efforts by scientists and clinicians have improved uptake in target tissue, reduced adverse reactions and toxicities, and developed successful radiolabeling techniques. Advances in biotechnology, especially genetic engineering, provide methods to manipulate the antibody molecule to produce human antibodies.

To date only a handful of radiolabeled antibodies have received FDA approval. However, more than 20 non-labeled human antibody therapeutic agents have been approved by the FDA since 2000. This supports the potential of radiolabeled antibodies as a major diagnostic and therapeutic opportunity.

A brief summary of radiolabeled antibodies that have received FDA approval is provided. While many of the agents have been removed from the commercial market, each contributed to the ongoing research in successful radiolabeling.

In-111 capromab pendetide (ProstaScint®) is a murine monoclonal antibody conjugated to a chelator in kit form to which In-111 is added. ProstaScint, approved in 1996, is a diagnostic imaging agent in newly-diagnosed patients with biopsy-proven prostate cancer, thought to be clinically-localized after standard diagnostic evaluation (e.g. chest x-ray, bone scan, CT scan, or MRI), who are at high-risk for pelvic lymph node metastases.

Y-90 ibritumomab tiuxetan (Zevalin™) was the first radioimmunoconjugate approved as an anticancer agent in 2002. Ibritumomab is a murine monoclonal antibody linked to tiuxetan and

available in kit form to which Y-90 is added. The monoclonal antibody targets the CD20 antigen found on the surface of mature B cells and B-cell tumors and delivers cytotoxic radiation directly to malignant cells. Zevalin is indicated for the treatment of relapsed or refractory low grade, follicular, or transformed B-cell non-Hodgkin's lymphoma. This indication includes patients with Rituxan (rituximab)-refractory follicular NHL. Zevalin has been approved as part of a therapeutic regimen involving Rituxan. The requirement for a pre-treatment dosimetry dose using In-111 ibritumomab tiuxetan has been discontinued.

Tc-99m arcitumomab (CEA-Scan) was a murine Fab' (modified Fab with some Fc) fragment available in kit form for direct labeling with Tc-99m. CEA-Scan was approved for diagnostic imaging of colorectal cancer. ^{99m}Tc arcitumomab binds carcinoembryonic antigen (CEA,) an antigen that is expressed on the cell surface of several types of tumors, including colorectal tumors. The manufacturer discontinued commercialization of CEA-Scan.

In-111 satumomab pendetide (OncoScint CR/OV®) was a murine monoclonal antibody specific for tumor-associated glycoprotein (TAG-72), a cell surface antigen associated with almost all colorectal and ovarian adenocarcinomas. The kit form included the antibody conjugated with DTPA as a linker for the added In-111. The manufacturer discontinued commercialization of OncoScint in 2006 stating that the market was negatively affected by positron emission tomography (PET), which had been shown to have similar or higher sensitivity.

Tc-99m sulesomab (LeukoScan®) is a murine Fab′ fragment specific for NCA-90, a surface antigen on activated granulocytes. Available in kit form, Tc-99m is added for direct labeling of the antibody. LeukoScan is indicated for the diagnostic localization of infection and inflammation in bone in patients with suspected osteomyelitis. It has received approval in Europe but is not available in the US.

Tc-99m fanolesomab (NeutroSPEC™) was a murine IgM monoclonal antibody prepared in kit form for direct Tc-99m labeling. The approval indication was for imaging of patients with equivocal signs and symptoms of appendicitis who are five years of age or older. The decision to suspend marketing was based on the life-threatening nature of reported adverse events and the availability of other means to diagnose appendicitis with fewer risks.

I-131 tositumomab (Bexxar®) Tositumomab is a murine IgG2a anti-CD20 antibody indicated for the treatment of patients with CD20-positive relapsed or refractory, low grade, follicular, or transformed non-Hodgkin's lymphoma that have progressed during or after rituximab therapy, including patients with rituximab-refractory non-Hodgkin's lymphoma. Bexxar is produced by direct radioiodination of the antibody. It is available as a ready-to-use product and requires only calculation of the patient dose. A dosimetry image is required before administration of the therapeutic dose. Commercial distribution of the product is limited to specific production dates..

SUMMARY

Radiolabeled tumor-selective monoclonal antibodies have proven to be useful in the localization and assessment of metastatic disease and are emerging as indicators of therapeutic progress. The concept of localizing a cytotoxic radionuclide to a specific cancer cell offers alternatives to conventional forms of therapy. This introduction to monoclonal antibodies and the processes for radiolabeling antibodies for use in detection and treatment of disease provides only a brief overview of the promises and hurdles in creating tumor-selective radiolabeled antibodies. The reader is encouraged to further explore the possible opportunities for nuclear pharmacy in developing and providing such novel products.

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ASSESSMENT QUESTIONS

1. Adaptive immunity differs from innate immunity in that only adaptive immunity
   a. reacts to foreign substances.
   b. has limited specificity in response.
   c. responds immediately.
   d. has capacity for memory.

2. Which of the following is specific to humoral immunity?
   a. T lymphocytes
   b. Cytokines
   c. Antibodies
   d. Intracellular virus

3. Antibody secretion is a direct product of which cell type?
   a. Plasma B-cell
   b. Memory B-cell
   c. Activated B-cell
   d. Pro-B-cell

4. Which region of the antibody determines antigenic specificity?
   a. Variable region
   b. Constant region
   c. Heavy chain(s)
   d. Light chain(s)

5. Isotypes refer to variations in the
   a. Light chain variable region
   b. Light chain constant region
   c. Heavy chain variable region
   d. Heavy chain constant region

6. The carbohydrates along the Fc region affect all of the following except binding to
   a. Antitgen serum clearance
   b. Antigen binding
   c. Antigen solubility
   d. Antigen

7. Cleavage of an antibody molecule by the protease papain produces which of the following:
   a. An antigen binding site and two constant regions
   b. Two heavy-light chain dimmers
   c. Two FAB fragments and one Fc fragment
   d. An antibody without carbohydrates

8. Hybridomas are used to provide ongoing supplies of which antibody type?
   a. Chimeric
   b. Humanized
   c. Murine
   d. Human

9. A major drawback in the human use of murine antibodies is
    a. Difficulty in radiolabeling
    b. Retention of antibody-antigen specificity
    c. Reproducibility of antibody populations
    d. Development of HAMA

10. Which statement about chimeric antibodies is true?
    a. The amount of murine protein is reduced to less than 10%.
    b. The Fc portion of the antibody is murine.
    c. There is a high immune response against chimeric antibodies.
    d. Only the variable regions are murine.

11. Antibody fragments have several advantages over intact monoclonal antibodies including
    a. Prolonged clearance time.
    b. Better tumor penetration.
    c. Improved radiolabeling capacity.
    d. More consistent Fc region.

12. Which of the following methods are used to produce human antibodies?
    a. Hybridoma
    b. Phage display
    c. Biolistic method
    d. Recombineering

13. The nomenclature for monoclonal antibodies is formatted to identify unique factors about the monoclonal antibody. Which of the following is NOT part of the nomenclature?
    a. Disease or cellular target
    b. Conjugate
    c. Radionuclide
    d. Method of cloning

14. Which imaging isotope would be the best selection for radiolabeling an intact monoclonal antibody that requires 2-3 days to localize in the target?
    a. F-18
    b. Y-90
    c. In-111
    d. O-15

15. The crossfire effect provides greater irradiation of large tumors. Which of the following isotopes would have a crossfire effect?
    e. I-123
    f. At-211
    g. Ga-67
    h. Y-90

16. Direct radiolabeling with radiohalogens is problematic due to in vivo
    a. Dehalogenation
    b. Phagocytosis
    c. Cleavage of disulfide bonds
    d. Glycosylation

17. Which of the following radiometals can directly radiolabel antibodies?
    a. Y-90
    b. Tc-99m
    c. Cu-64
    d. Lu-177

18. Bifunctional chelating agents are used to radiolabel antibodies with radiometals. Which of the following is NOT routinely used as a bifunctional chelating agent?
    a. DOTA
    b. DTPA
    c. HYNIC
    d. EDTA

19. Which of the following is an approved In -111 labeled murine monoclonal antibody availiable for imaging?
    a. satumomab pendetide
    b. sulesomab
    c. tositumomab
    d. capromab pendetide

20. Which of the following is a radiolabeled monoclonal antibody approved for radiotherapy?
    a. fanolesomab
    b. sulesomab
    c. ibritumomab tiuxetan
    d. gixuximab pendetide