Chapter 9
TISSUE INJURY

MARKERS OF CELLULAR DAMAGE

Injury to tissue causes a decrease in function or a diminution in the functional capacity of the tissue. It also causes the release of cellular substances from damaged cells. Cellular substances that enter the circulation after their release can be used as plasma markers of tissue injury. The overwhelming majority of tissue injury markers used clinically are intracellular proteins, mostly enzymes.

A simplified model of the disposition of a cellular substance is shown in Figure 9.1 (Lindena *et al.* 1986). Cellular substances are lost by local release. The release of membrane constituents may occur as a normal feature of their turnover but the release of intracellular proteins occurs only with cellular injury. Once released, the substance is either catabolized locally or is taken up in the lymphatic drainage, traverses the lymphatic circulation, and enters the blood. In tissues with highly permeable capillaries and venules, some of the substance enters the plasma directly. From the plasma the substance distributes into the other extracellular body fluids. Systemic catabolism and renal or hepatic clearance leads to elimination of the substance from the plasma and the body fluids.

Due to normal cell turnover, there is a relatively constant release of cellular proteins from the tissues. The long-term, constant release of a protein generates a steady-state concentration of the protein in the body fluids into which it distributes, including the plasma. The magnitude of the concentration will depend upon the balance between the entry of the protein into the fluids and its systemic elimination. Increases in the plasma concentration of a cellular protein above its steady-state concentration result from alterations in either the release or elimination of the protein. The crucial alteration in the case of tissue injury markers is an increased local release of the marker protein as a consequence of cell injury. Examination of Figure 9.1 reveals that, in the presence of normal rates of cell turnover, increased plasma concentrations of an injury marker can also result from increases in the size of the cellular pool of the marker and from decreases in the systemic elimination of the marker. When there is increased cell turnover or marker release due to injury, concomitant increases in the cellular pool or decreases in systemic elimination can cause exaggerated elevations in the plasma concentrations of the markers. The concurrent induction of enzyme synthesis and release of cellular proteins seen as a response to injury in various organs explains the characteristic extreme elevations in the plasma concentration of many injury markers (Schmidt and Schmidt 1987, Pappas 1989).

Injury versus death

An important question concerning the use of intracellular substances as plasma markers of tissue

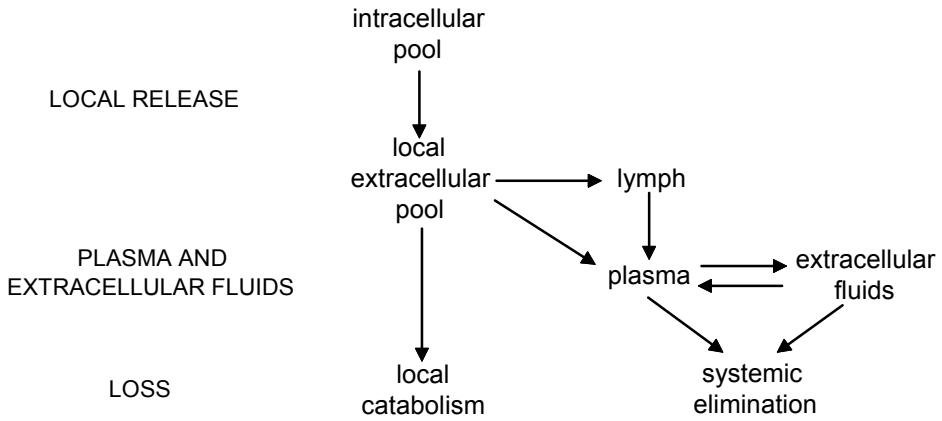

Figure 9.1 A model of the disposition of an intracellular marker of tissue injury.

injury is whether release of the substances can occur when the damage to the cells is reversible or if marker release signifies cell death. In the case of hepatocytes, marker release clearly can occur while cell injury is still reversible (Schmidt and Schmidt 1987). Mild injury causes some release of cytoplasmic substances. The more severe the injury, the greater the amount of cytoplasmic material, including cytoplasmic injury markers, released. With very severe injury, cell death is frequent leading to the release of intracellular substances contained within membrane-bound organelles such as the mitochondria and lysosomes (Karmiike *et al.* 1989). For example, only small amounts of the intramitochondrial enzyme glutamate dehydrogenase are released in the course of typical acute viral hepatitis whereas abundant amounts are liberated in cases of fulminant hepatic necrosis.

Even though various cell and tissue culture studies have shown that cytoplasmic substances can be lost following reversible cellular injury (Schmidt and Schmidt 1987), clinical findings suggest that marker release is highly specific for cell death in some settings. The acute cardiac myocyte response to ischemia is the most important example: elevations in the tissue-specific cardiac injury markers appear to occur only when there is some degree of myocardial necrosis.

Tissue specificity

For a substance to serve as a specific plasma marker of tissue injury, it is necessary that the substance arise predominantly from the cells of the organ or tissue of interest. Otherwise, injury to other tissues containing the substance will cause its release into the circulation. This release will either be misinterpreted as resulting from injury to the tissue of interest when there is none or will interfere with the interpretation of plasma levels of the substance when there is concurrent injury.

Highly specific markers have been identified for a number of tissues. A few are unique cell products, e.g., hemoglobin which is found only in red cells and red cell precursors; some are enzymes found predominantly in the specialized tissue, e.g., lipase most of which arises from the pancreas; and some are tissue-specific isoenzymes of widely distributed enzymes, e.g., the pancreatic isoenzymes of α-amylase. Isoenzymes are proteins that catalyze the same reaction but differ from one another in their structure. In the case of α-amylase, the isoenzymes arise from differences in posttranslational carbohydrate modifications of the enzyme. Even if there is not a high degree of tissue specificity for the isoenzymes of a given enzyme, mathematical techniques of data analysis may be able to quantify the contribution made by a specific tissue to the total plasma pool of the enzyme (Politser *et al.* 1986).

The total concentration of an enzyme can usually be assayed much more quickly and easily than can the concentration of the corresponding tissue-specific isoenzyme. This makes the measurement of total enzyme concentration useful in emergent clinical situations where speed in obtaining a usable result is more important than obtaining a highly specific result. Because total enzyme concentrations are usually sensitive markers of tissue injury, they can also be used to identify those specimens that merit the performance of an expensive and difficult isoenzyme determination. Furthermore, in uncomplicated clinical situations, total enzyme concentrations enjoy quite acceptable diagnostic specificity despite their inferior tissue specificity.

Table 9.1 lists plasma markers of myocardial injury and categorizes the degree of tissue specificity of each (Keffer 1996 and 1997, Bhayana and Henderson 1995). Aspartate aminotransferase was once the standard marker in the diagnosis of acute myocardial infarct but it is widely distributed in body tissues and consequently has low cardiac specificity. It has been supplanted by creatine kinase. Creatine kinase is a two subunit enzyme, the isoenzymes of which are made up of homo- or heterodimers of M and B protein subunits. Cardiac muscle expresses both the M subunit and the B subunit; the M subunit is expressed at a higher level than the B subunit so creatine kinase-MM is the majority intracellular isoenzyme; the ratio of creatine kinase-MM to creatine kinase-MB is approximately 5 to 1. Skeletal muscle expresses the M subunit and the B subunit but the B subunit is expressed at very low levels so that skeletal muscle creatine kinase consists overwhelmingly of creatine kinase-MM. Following muscle injury, the plasma concentration of total creatine kinase increases as a result of the release of creatine kinase-MM; the plasma concentration of creatine kinase-MB increases relatively little except when the muscle injury is massive. Following cardiac injury, the release of creatine kinase-MM and creatine kinase-MB cause the plasma concentrations of both to increase. In the absence of skeletal

Table 9.1
Plasma Markers of Myocardial Injury

Analyte	Tissue specificity	Diagnostic window (myocardial infarct)
Aspartate aminotransferase	low	intermediate
Creatine kinase	medium	intermediate
Creatine kinase-MB	high	early / intermediate
Lactate dehydrogenase	low	late
Lactate dehydrogenase-1	high	late
Myoglobin	medium	early
Cardiac troponin I	absolute	intermediate

muscle injury, an elevation in total creatine kinase concentration indicates cardiac injury. In this sense, total creatine kinase can be considered to be moderately specific for cardiac injury. Because even minor injury to muscle, such as intramuscular injection, can result in the release of significant amounts of creatine kinase-MM into the circulation, the cardiac specificity of total creatine kinase is not equal to that of creatine kinase-MB measured as the plasma isoenzyme concentration. Because of its greater specificity and because it can be measured by immunoassay, which allows for rapid turnaround and 24 h test availability, creatine kinase-MB has become the preferred marker for the routine evaluation of acute cardiac injury. Cardiac troponin I, the form of troponin I expressed in cardiac muscle, is not found in any other tissue and, thus, has an even greater cardiac specificity than creatine kinase-MB. It too can be measured by immunoassay and therefore is very attractive as a cardiac injury marker (Wu *et al*. 1999). However, additional clinical experience must be gained in the use of this marker before it can be recommended that it replace creatine kinase-MB.

The diagnostic window

Tissue injury that is persistent and relatively constant will produce a nearly constant elevation in the plasma concentration of an injury marker. Episodic injury, on the other hand, will cause only a temporary increase in the plasma concentration of an injury marker. For a period of time after the injury, release of the marker will overwhelm the local catabolic capacity of the tissue, and the marker will gain entry to the lymph and the plasma. The marker will accumulate in the plasma, i.e., its concentration will increase, until the amount entering the plasma becomes less than the combined amount moving into the body fluids and undergoing systemic catabolism. When that happens, the plasma concentration will peak and then will fall. The diagnostic window for an injury marker is that interval of time following an episode of injury during which measurement of the plasma concentration of the marker will show elevated values, thereby demonstrating the occurrence of the injury.

The timing of an injury marker's diagnostic window is not constant. It depends, in part, upon the magnitude of the injury experienced. The more severe the injury, the sooner the evidence of injury begins and the longer it lasts. The upper graph in Figure 9.2 depicts plasma marker disposition curves following four different degrees of acute tissue injury. The corresponding diagnostic window for each curve is the interval of time during which the curve is above the critical value for the marker. Note that differences in the magnitude of injury affect the time of termination of the diagnostic window much more than its time of onset. Note also that, in the disposition curve from the least severe tissue injury, the plasma concentration of the marker never exceeds the critical value. This is so even though a typical pattern of marker disposition results from the injury. This illustrates a point concerning the use of critical values derived from population-based criteria: if there is appreciable interindividual variability in the baseline concentrations of an injury marker, small but nonetheless clinically

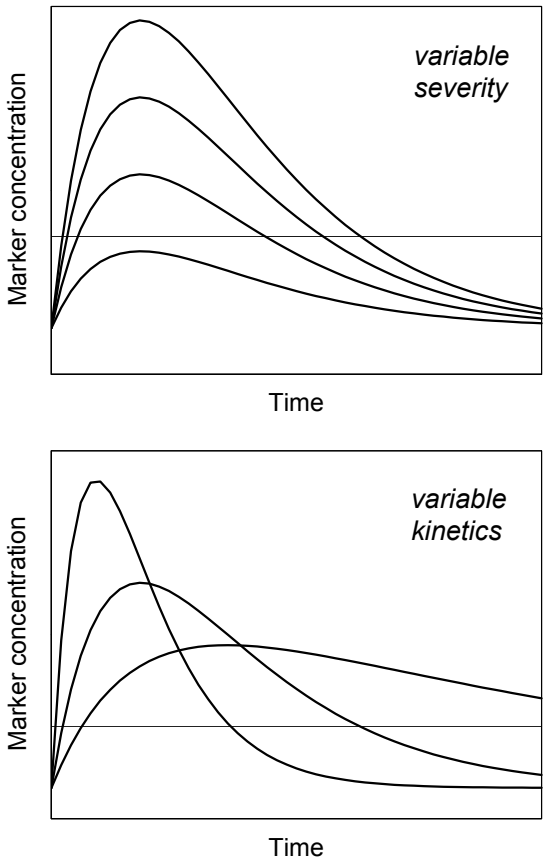

Figure 9.2 The diagnostic window as a function of injury severity and plasma kinetics of the marker. The critical value is indicated by the horizontal line.

meaningful episodes of injury may be missed in individuals with low baseline plasma marker concentrations (e.g., Yusuf *et al.* 1987).

The timing of a marker's diagnostic window can also vary as a result of changes in the disposition of the marker. The most important instances of this are the dispositional changes caused by therapy to limit the extent of the injury. In the case of acute myocardial infarction for example, pharmacologic thrombolysis, if successful, leads to reperfusion of the ischemically injured myocardium and rapid washout of the cardiac injury markers in the injured tissue. Consequently, the diagnostic windows of the markers are shifted to much earlier times (Wei *et al.* 1985). In addition, the increased rapidity of the plasma appearance of the cardiac injury markers can itself be used as a indicator of successful therapy (Laperche *et al.* 1995)

Differences in the timing of the diagnostic window among injury markers arise from differences in the disposition kinetics of the markers (Figure 9.2, lower graph). Injury markers that rapidly enter the circulation following injury have diagnostic windows that begin soon after the onset of the injury. Early markers permit prompt detection of the injurious event and are, therefore, very useful when considering the institution of interventional therapy. The diagnostic windows for the early markers are usually short-lived, however, because the markers tend to be cleared from the plasma rapidly. For example, myoglobin can be used as an early marker of acute myocardial infarction because distinctly elevated plasma concentrations are detectable as soon as two hours after the onset of chest pain (Grenadier *et al.* 1983). Its diagnostic window lasts less than 24 hours, though, because of its rapid clearance by the kidneys. The diagnostic window of a marker will be long-lasting if the local release of the marker is slow, if its rate of synthesis is increased in response to injury, or if it is slowly cleared from the circulation. Lactate dehydrogenase is a long-lasting marker for myocardial infarction; elevations in its plasma concentration appear about 12 h following an infarction and remain elevated one to two weeks. Such markers can be used as diagnostic aids when a patient has delayed seeking medical care.

Estimating the magnitude of an injury

If it is assumed that each cell in a tissue contains the same quantity of an injury marker and that all, or a constant fraction, of the marker eventually enters the circulation, the magnitude of an injury can be estimated by measuring the amount of the marker released into the plasma. Such assumptions are not realistic, of course. The cellular pool of a marker may vary over time, as is true for those markers that are inducible enzymes. Also, an injury marker may not be uniformly distributed in a tissue, as is the case for the hepatic injury markers. Finally, the amount of marker released into the plasma may not be a constant fraction of that liberated, as is probably so for the cardiac injury markers. Consequently, the magnitude of an injury correlates only roughly with the magnitude of the elevation in the plasma concentration of an injury marker.

Test panels

The presence of organ injury is frequently investigated by ordering a combination of injury markers. Combination testing may be used to improve diagnostic specificity when no truly tissue specific marker is available (the "all tests positive" positivity

rule is appropriate for this purpose) or it may serve to increase diagnostic sensitivity (the "any test positive" rule is most applicable for this objective). The increase in diagnostic sensitivity achieved by combination testing is maximized by including markers with dissimilar diagnostic windows.

Organ function markers may also be ordered as a part of an organ test panel. This allows the clinician to look for both of the cardinal signs of injury: decreased organ function and the release of injury markers. When injury is present, its severity can be quantified in two ways, by estimating the magnitude of release of injury markers and by measuring the decrement in organ function.

IMMUNOLOGIC INJURY

There are three general ways in which immunologic injury occurs: (1) complement activation and inflammation due to immune complex deposition, (2) inappropriate immune response to self-antigens (autoimmunity), and (3) appropriate but undesirable immune response to nonself-antigens present on transplanted tissue.

Immune complex deposition

Immune complexes are eliminated from the body by uptake by neutrophils and mononuclear phagocytes. The delivery of soluble immune complexes to these cells is mediated by complement component C3b (Walport and Davies 1996). C3b binds covalently to the N-terminal domains of IgG and interferes with the interactions between the Fc regions of the IgG molecules. It is these interactions that make immune complexes insoluble. Receptors for C3b on red cells bind the complement and associated immune complexes, transporting them primarily to the liver where they are taken up by mononuclear phagocytes which express receptors for C3b, for degradation products of C3b, and for Fc. Genetic and acquired deficiencies of the components of this delivery system, or of the complement components that generate C3b, can lead to reduced efficiency in immune complex clearance and resultant tissue deposition. Tissue deposition of immune complexes can also occur when the production of immune complexes exceeds the capacity for clearing the complexes, usually because of the persistent presence of antigen. This can happen in autoimmune disease, in which the autoantigen is necessarily present persistently, and in long-term infection, such as subacute bacterial endocarditis, in which the failure to clear the infectious agent leads to persistence of the antigen.

Tissue deposition of immune complexes is particularly likely to occur where the exposure to the complexes is greatest—on the endothelial surfaces of blood vessels and in blood vessel walls, particularly in the renal glomeruli. Extravascular deposition of soluble immune complexes probably occurs throughout the body with pathologic consequences occurring where the local tissues and macrophages express proinflammatory Fc receptors. Such sites include the synovial intima, which may explain the propensity for synovitis and arthritis in diseases in which immune complex deposition is present (Edwards and Cambridge 1998).

In addition to being deposited from the circulation, immune complexes can localize in tissues as a result of deposition of antigen with subsequent binding of circulating antibody. This appears to be a mechanism by which immune complexes arise in the glomerulus in certain forms of glomerulonephritis (Hricik *et al.* 1998).

In those diseases in which it is recognized that immune complexes are pathogenic, the laboratory measurement of plasma immune complex concentration has been investigated for a potential role in diagnosis, prognosis, and monitoring. No convincing clinical role for nonspecific immune complex measurement has emerged (Keren and Warren 1992). This may be due to technical difficulties in the assay of the complexes or to the protean nature of the diseases being studied. It may also be due to the fact that serum is assayed even though circulating immune complexes are mostly bound to red cells, suggesting that whole blood determinations might be much more informative.

Autoimmune disease

Autoimmunity is a failure in lymphocyte tolerance to self-antigens (Male *et al.* 1996). This can lead to direct T-cell mediated injury, such as in insulin dependent diabetes mellitus, and to direct antibody mediated injury due to the loss of B-cell self-tolerance, such as in idiopathic thrombocytopenic purpura. Indirect antibody mediated injury can also occur, primarily by immune complex deposition. Indirect injury is sometimes the major injury causing process in the disease. Typically, one or more autoantibodies that are not responsible for direct tissue injury can be demonstrated in the

plasma of patients with autoimmune disease, even when the disease is mediated by direct T-cell cytotoxicity (Naparstek and Plotz 1993). The major reason for the appearance of these autoantibodies is immune system exposure to sequestered or latent self-antigens as a consequence of the primary tissue injury. In patients who suffer a myocardial infarct, for example, the antimyocardial antibodies that are sometimes detectable a few weeks after the infarct are atuoantibodies to latent myocardial self-antigens.

Organ-localized disease. The laboratory evaluation of organ-localized autoimmune diseases consists primarily of studies of organ function, studies of organ injury, and the demonstration of autoantibodies directed against the organ of interest. The most specific autoantibodies to use for this purpose are those directly responsible for the tissue injury, although the responsible autoantibodies are known only in some disorders. One of these disorders is Grave's disease, in which the offending autoantibodies are directed against the extracellular domain of the thyroid stimulating hormone receptor on thyroid cells (Naparstek and Plotz 1993). The binding of the autoantibodies (called thyroid stimulating immunoglobulins, TSIs) causes activation of the receptor, leading to hyperthyroidism. Other autoantibodies are also often found in patients with Grave's disease. These include anti-thyroid peroxidase autoantibodies and anti-thyroglobulin autoantibodies. Neither of these autoantibodies is specific for Grave's disease, being found with appreciable frequency in other forms of thyroiditis including Hashimoto's autoimmune thyroiditis.

Despite being less specific for Grave's disease than the TSIs, anti-thyroid peroxidase autoantibodies are the autoantibodies that are almost always measured in the laboratory evaluation of Grave's disease (Davies *et al*. 1998). This is so because, in contrast to the TSIs, there exists a widely available, highly dependable, and inexpensive commercial assay for anti-thyroid peroxidase autoantibodies (Miles *et al*. 1998). This represents an instance of the superior practicability of one method outweighing the greater diagnostic reliability of another method.

Systemic disease. The systemic autoimmune diseases, which are also called connective tissue diseases, are characterized by immunologic injury to multiple organs. The multifocality of injury in these diseases may be due to a vasculitis that involves various organs, as in Wegener's granulomatosis, or to the deposition of immune complexes in diverse tissues, as in systemic lupus erythematosus. As implied by the pattern of injury, the autoantibodies in systemic disease are not directed against self-antigens in a specific organ but rather to self-antigens that are widely distributed. For instance, antinuclear antibodies, such as those found in systemic lupus erythematosus, bind to the same nuclear antigens in different organs. Rheumatoid factor, the characteristic autoantibody of rheumatoid arthritis, is directed against IgG which is present throughout the extracellular fluids.

The laboratory evaluation of the systemic autoimmune diseases has four components: confirming an immunologic etiology for an illness presenting as a systemic disorder; establishing the diagnosis from among the various systemic diseases; monitoring the activity of the disease; and monitoring organ function in those disorders associated which significant, progressive organ injury.

The immunologic origin of a systemic disorder is revealed by the demonstration of the acute phase response, complement activation, and circulating autoantibodies (McCarty-Farid 1994).

The acute phase response is a nonspecific reaction to tissue injury mediated by cytokines. It is composed of a triad of findings: fever, neutrophilia, and elevation in the concentrations of certain plasma proteins, referred to as acute phase proteins (Gabay and Kushner 1999). All three components of the triad are usually present when injury is caused by bacterial infection. Systemic autoimmune diseases show only the elevation in acute phase protein concentrations (Table 9.2). The acute phase proteins can be divided into two groups. The first group consists of proteins that are normally present in appreciable concentration in the plasma and which experience 1.25 to 3-fold elevations in concentration in an acute phase response. These proteins include fibrinogen. The second group is made up of C-reactive protein and amyloid A protein. These two proteins are normally present in trace quantities but show up to 1000-fold increases in concentration during the acute phase response. Only fibrinogen and C-reactive protein are used clinically as markers of the acute phase response. Mainly for historical reasons, fibrinogen is typically not measured directly but is instead assayed indirectly using the erythrocyte sedimentation rate (ESR) or the plasma viscosity. The ESR is the length of the plasma column that develops when anticoagulated blood sediments

Table 9.2
Expression of the Acute Phase Response According to the Cause of Tissue Injury
(+++, marked elevation; ++, moderate elevation; +, mild elevation; ±, little or no elevation)

Systemic response	Sterile infarct	Systemic autoimmune disease	Bacterial infection	Viral infection
Body temperature	±	+	+++	+++
Neutrophil count	+	+	+++	±
Acute phase protein concentration	+	+++	+++	±

for an hour in a wide-bore hematocrit tube. It depends largely upon the fibrinogen concentration, which strongly influences the propensity for red cells to form rouleaux and sediment, and upon the red cell concentration. Owing to its asymmetric shape, fibrinogen makes a disproportionate contribution to plasma viscosity; it is the major determinant of plasma viscosity except in plasma with very high immunoglobulin concentrations. Because the ESR is easy to perform, is inexpensive, has a rapid turn-around time, and requires no special instrumentation, it is generally used in preference to plasma viscosity even though plasma viscosity correlates more reliably with the fibrinogen concentration (Zlonis 1993). Direct measurement of fibrinogen is the logical alternative to either indirect measure.

Complement activation results in a decrease in the concentration of the components of the complement cascade. If complement activation has been substantial and persistent, there may be a decrease in global complement function as measured by total hemolytic activity (CH50). CH50 is used to screen for deficiencies of the components of the classical complement pathway (AH50 serves a similar role for the alternative pathway). CH50 is, however, relatively insensitive to the smaller reductions in complement component concentrations that typically occur as a consequence of complement activation in systemic autoimmune disease. Individual complement components, in particular C3 and C4, are more sensitive markers for complement activation if they are assayed using variants of the CH50 method. When measured by immunoassay, inactive cleavage products contribute to the signal resulting in spuriously high concentration results and, hence, lower diagnostic sensitivity. Complement fragments (active complement cleavage products) and complement split products (inactive complement cleavage products) appear to be more sensitive markers of complement activation than either CH50 or individual complement components (Rabson 1997). Because these species are normally present in the plasma at very low concentrations, their appearance during complement activation is readily detected. In addition, the degree of complement activation as quantified using these markers correlates with disease activity, making them useful monitoring studies (Peakman *et al.* 1989).

There are two classes of circulating autoantibodies that are screened for in the laboratory evaluation of the systemic autoimmune diseases, rheumatoid factors and anti-nuclear antibodies. Rheumatoid factor are autoantibodies to the Fc portion of IgG. These autoantibodies are sensitive for rheumatoid arthritis, in which they may have a causative role (Edwards and Cambridge 1998), but they are not specific for that disorder as they can be found in patients with other systemic autoimmune diseases, in patients with a variety of infectious diseases, and with increased frequency in the elderly. Nevertheless, their presence (seropositivity) in a patient with a suspected systemic polyarthritis is suggestive of rheumatoid disease. Their absence (seronegativity) helps define the arthritic disorders referred to as the seronegative spondyloarthropathies.

Anti-nuclear antibodies are frequently found in patients with systemic autoimmune diseases, especially patients with systemic lupus erythematosus. They are not, however, completely specific for the systemic autoimmune diseases so they should not be used to screen individuals with a low probability of having one of these diseases. Anti-nuclear antibodies are screened for by indirect immunofluorescence microscopy, most commonly using human epithelial cell line 2 (HEp-2) cultured cells. The cells, which are fixed to a slide, are incubated with patient serum; if autoantibodies to cellular antigens are present, they will bind to the antigens. Bound autoantibodies are revealed by treatment with fluorochrome conjugated anti-human immunoglobulins and

Table 9.3
Autoantibodies of High Specificity in Systemic Autoimmune Diseases (McCarty-Farid 1994, Miles *et al.* 1998, Moder 1998)

Autoantibody	Disease	Sensitivity
anti-dsDNA	systemic lupus erythematosus	low
anti-Sm proteins	systemic lupus erythematosus	low
anti-topoisomerase I	diffuse forms of scleroderma	low
anti-centromere	CREST variant of scleroderma	high
anti-neutrophil proteinase III	Wegener's granulomatosis	high
anti-aminoacyl-tRNA synthetase	polymyositis dermatomyosistis	low

examination under fluorescent illumination. The pattern of fluorescence staining can give some indication of the specificity of the autoantibodies but with the exception of the anti-centromere pattern, which implies anti-centromere autoantibodies, such inferences are not highly reliable. Instead, the specificity of autoantibodies is evaluated by immunoassay with purified antigens.

Unlike the organ-localized autoimmune diseases, autoantibodies of the same specificity develop in many of the systemic autoimmune diseases (Keren and Warren 1993). Table 9.3 lists those autoantibodies that are highly specific markers of particular systemic autoimmune diseases. Detection of these autoantibodies is useful in the diagnostic differentiation of the systemic diseases. Regrettably, the utility of most of these markers is limited by their low sensitivity. Only anti-centromere antibodies and anti-neutrophil proteinase III autoantibodies (also called anti-neutrophil cytoplasmic antibody, ANCA) combine high specificity with high sensitivity.

Laboratory methods for autoantibodies. The laboratory measurement of autoantibodies requires the use of methods that can detect antibodies of a defined specificity. The obvious analytical approach for achieving the separation of the autoantibodies from all the other antibodies present in a test sample is to utilize the inherent specificity of the autoantibody. That means having the autoantibodies bind to their cognate antigen which is present in the test system as a reagent (Miles *et al.* 1998).

If the reagent antigen is immobilized, autoantibodies will become immobilized when they bind to the antigen. All the uninteresting antibodies, which are unbound, can then be washed away and the autoantibodies measured using a signal generating reagent that reacts with immunoglobulins. This is the methodology that underlies autoantibody detection by indirect immunofluorescence microscopy in which histologic tissue sections or tissue culture substrates constitute the immobilized reagent antigen and fluorochrome conjugated anti-human immunoglobulins are the signal generating reagent. It is also the methodology employed in immunoassays, the most popular form of which is enzyme-linked immunosorbent assay (ELISA), in which reagent antigen is bound to the walls of the reaction tube and chromogenic enzyme conjugated anti-human immunoglobulins are the signal generating reagent.

An alternative method for detecting the binding of autoantibodies to the reagent antigen is by particle agglutination. Here the reagent antigen coats small, inert particles. The binding of autoantibodies, which are bivalent in the case of IgG and decavalent in the case of IgM, bridge the particles producing a gel-like lattice. When allowed to settle thoroughly, unbound particles clump together into a button while autoantibody-bound particles remain in a diffuse gel, thereby signaling the presence of autoantibodies. In the investigation of autoantibodies to red cells, reagent red cells serve as both the source of antigen and the agglutinating particle. In contrast to inert particle tests, in red cell tests, clumping indicates the presence of autoantibodies. This is because the outer red cell membrane is negatively charged producing a repulsive force between red cells that keeps them apart. Autoantibody bridging overcomes this repulsion producing red cell clumps.

Tissue transplantation

Transplantation of body tissue from another individual, other than an identical twin, elicits an immune response in the recipient that, if not suppressed, leads to rejection of the allograft. This response, which consists of cellular and humoral components, is primarily to the histocompatibility molecules (HLA antigens) expressed by the donor tissue (Sykes 1996, Vartdal and Thorsby 1999). The HLA antigens, which are the products of a set of linked loci on chromosome 6, show an extreme degree of polymorphism, meaning that there are very many alleles for each of the loci. The intensity of the immune response is roughly proportional to the number of differences between the HLA antigen

Table 9.4
Laboratory Evaluation in Tissue Transplantation

Tissue	Pre-transplant	Post-transplant
Solid organ	histocompatibility testing HLA matching antibody screen lymphocyte crossmatch blood group crossmatch	monitoring for rejection organ function organ injury markers monitoring of immunosuppressive agents
Red cells	compatibility testing blood group matching antibody screen (red cell crossmatch)	if a transfusion reaction is suspected tests of hemoglobin release red cell injury markers direct antiglobulin test evaluation of alloantibody

alleles expressed by the donor and those expressed by the recipient. For this reason, laboratory evaluation for transplantation begins with pre-transplant HLA typing of the recipient and the intended donor (Table 9.4; Milford and Amaç 1997). Clinical experience has shown that the HLA antigens most important in allograft rejection are HLA-A, HLA-B (class I histocompatibility molecules), and HLA-DR (class II histocompatibility molecule) so these are the antigens that are typed.

HLA typing consists of the identification of the allelic form of the HLA antigens of interest. Until recently, this has been done primarily by phenotyping of lymphocytes using the microlymphocytotoxicity assay. In that method, lymphocytes isolated from the blood of the person being studied are distributed among the wells in a tissue typing tray. Each well of the tray contains an antibody of known specificity. After a period of incubation, complement is added and the mixture incubated further. In the wells containing antibody specific for an expressed HLA antigen, the antibody binds the antigen, the complement cascade is activated, and the cells killed. The extent of lymphocyte killing is determined by fluorescence microscopy; viable cells stain one color with a fluorescent dye that is retained only in living cells and killed cells stain another color with a second fluorescent dye that is only taken up by dead cells. The HLA type is assigned based on the specificities of the antibodies in the wells in which lymphocyte killing occurs. More recently, molecular diagnostic methods for HLA typing have become available. These methods are based on determination of the base sequence in the region of DNA coding for the respective HLA antigen. The techniques most commonly applied are allele-specific oligonucleotide hybridization and sequence specific PCR amplification (Bunce *et al*. 1997; see Chapter 10 for a discussion of molecular diagnosis). Molecular diagnostic HLA typing has many advantages over the phenotyping method including better standardization and availability of the typing reagents and much greater ease of study performance and interpretation. Also, phenotyping failures due to down-regulation of HLA expression or lymphocytopenia are avoiding using molecular diagnostic techniques as are uncertainties that arise in the phenotyping of individuals who are homozygous at an HLA locus. For all these reasons, molecular diagnostic HLA typing is supplanting HLA phenotyping.

The presence of pre-existing recipient antibodies to donor HLA antigens is tested using any of a number of variants of the microlymphocytotoxicity assay. Serum from the recipient serves as the antibody source and the donor is the source of the lymphocytes; hence the designation, lymphocyte crossmatch. The detection of anti-HLA antibodies by standard lymphocyte crossmatch is an exclusionary histo-incompatibility because such antibodies cause hyperacute rejection. The clinical significance of anti-HLA antibodies revealed by sensitive variants of the lymphocyte crossmatch (such as the flow cytometry crossmatch) is less clear so their detection is only a relative contraindication to transplantation. Hyperacute rejection can also be caused by naturally occurring antibodies to the A and B antigens of the ABO blood group system so ABO blood group typing of the donor and recipient is performed as part of the pretransplant evaluation.

While a patient is awaiting transplantation, B cell presensitization of a candidate allograft recipient is assessed periodically with an antibody screen. This is especially important for candidates who have

a previous exposure to alloantigens through pregnancy, blood transfusion, or a failed allograft. Using the microlymphocytotoxicity assay, serum from the candidate is tested against a standard panel of lymphocytes that express a wide range of HLA antigens. The percentage of wells in which lymphocyte killing occurs is referred to as the percent panel reactive antibodies. This measure correlates with negative lymphocyte crossmatches and thus is inversely related to the probability of finding a matching donor.

Following transplantation, the function of the transplanted tissue is closely monitored in order to detect surgical complications and to assess the initial functional status of the tissue. For instance, with the transplantation of a cadaver kidney, postoperative oliguria may be due to vascular compromise or a urine leak or obstruction. If there has been a prolonged period of ischemia when obtaining the kidney, the oliguria may be due to preprocurement acute tubular necrosis.

Organ function is also monitored to detect the development of rejection. Rejection always leads to decreased organ function but the laboratory evidence of a decrement in function may appear relatively late in the development of the rejection process. This has lead to the search for early markers of rejection. Histologic findings of rejection appear early and are highly reliable. Periodic biopsy of the allograft therefore offers a way to detect rejection and to institute anti-rejection therapy before significant tissue injury has occurred. Because of the invasiveness of this monitoring approach, it is only routine in heart transplantation where the consequences of a late recognition of rejection are catastrophic. Numerous laboratory studies of immune function have been proposed as early markers of allograft rejection (Waltzer *et al.* 1994, Suthanthiran *et al.* 1997). Unfortunately, to date, none of these markers has been demonstrated to be reliable enough for clinical application. Markers of organ injury are useful early markers of rejection in transplantation of the liver and the pancreas. Plasma aminotransferase concentrations are measured in liver allograft recipients and urinary amylase concentrations are monitored in pancreas allograft recipients in whom the allograft is drained through the bladder. Interestingly, the plasma amylase concentrations is not a reliable rejection marker in pancreas transplantation. One would expect that renal injury markers, especially urinary markers of tubular injury, would be useful in kidney transplantation but their clinical utility has not been demonstrated.

Another component of post-transplantation laboratory evaluation relevant to immune injury is the monitoring of immunosuppressive therapy. The plasma concentrations of these agents are measured on a regular basis to assure that the drug concentrations being achieved are high enough to maintain satisfactory levels of immunosuppression but not so high that they place the patient at undue risk for drug-induced toxicity.

Transfusion. Red cells, which are by far the most frequently transplanted tissue, do not express HLA antigens. Other cell surface antigens on red cells can, however, elicit a typical humoral immune response in the recipient. These antigens constitute the various blood group systems. Blood group matching for transfusion consists of ABO and Rh typing (Table 9.4). In the case of the ABO blood group system (Green 1989), naturally occurring antibodies are invariably present: individuals who are type A (AO heterozygous) have anti-B antibodies, type B (BO heterozygous) individuals have anti-A antibodies, and type O (OO homozygous) individuals have anti-A, anti-B, and anti-A,B antibodies. Anti-A,B antibodies are directed against an epitope shared by the A and B antigens. These antibodies, which develop in the first year of life, are predominantly of the IgM class and cause complement-mediated (intravascular) hemolysis. The D antigen of the Rh blood group system (Cartron *et al.* 1998) is a potent immunogen resulting in the production of IgG class antibodies in Rh negative (D^-D^- homozygous) individuals following sensitization by transfusion of red cells expressing D antigen and in Rh negative mothers following fetal-maternal hemorrhage from a fetus that is Rh positive (DD^- heterozygous or DD homozygous). These antibodies do not fix complement but they do induce macrophage uptake of antibody-bearing red cells by binding to macrophage Fc receptors. This form of red cell loss is referred to as extravascular hemolysis.

ABO typing is accomplished by forward and reverse grouping using the hemagglutination assay. In forward grouping, one aliquot of a dilute suspension of test red cells is mixed with reagent anti-A antibody and another aliquot with reagent anti-B antibody. The mixtures are briefly centrifuged at room temperature and then examined for agglutination. Anti-A antibody agglutinates type A and type

Table 9.5 Agglutination Reactions in ABO Blood Group System Typing (+, agglutination; o, no agglutination)

	Type			
	O	A	B	AB
Forward grouping reagent antibody				
anti-A	o	+	o	+
anti-B	o	o	+	+
Reverse grouping reagent red cells				
A	+	o	+	o
B	+	+	o	o

AB red cells and the anti-B antibody agglutinates type B and type AB red cells. Type O red cells do not agglutinate with either antibody. The type assignments are confirmed by reverse typing in which test serum is mixed with reagent red cells (Table 9.5). Owing to the strong reactivity of the reagent antibodies, the results of forward grouping are usually very clear cut. The agglutination reactions in reverse typing can be more difficult to score because of variability in the reactivity of antibodies in patient and donor plasma. The agglutination induced by weak antibodies can be increased by using a prolonged room temperature incubation or by incubating at 4şC.

Typing for the Rh D antigen also uses the hemagglutination assay. Most DD homozygous and DD^{-} heterozygous red cells agglutinate with the reagent antibodies currently in use. There are polymorphic forms of the D antigen which are not revealed by routine typing, however, usually because of reduced levels of expression (Cartron *et al*. 1998) These weak reacting antigens are detected by the use of a sensitive variant of the hemagglutination assay called the antiglobulin test (also called the antihuman globulin test and, formerly, the Coomb's test). In the antiglobulin test, antibody or complement bound to red cells is demonstrated by incubating the cells with a reagent mixture that contains antibodies to human IgG and the complement components C3b and C3d. These antibodies induce agglutination of red cells even when the antibody bound to the red cells does not. When the anti-blood group antibody is a reagent antibody, as in Rh typing, the antiglobulin test is referred to as indirect. The antiglobulin test is called direct when the anti-blood group antibody is made by the patient and the antibody binding reaction occurs *in vivo*.

A number of automated agglutination techniques and solid phase, nonagglutination methods for ABO and Rh typing have been developed and are in use in many large blood centers (Plapp and Rachel 1992). Solid-phase methods and a micro-column agglutination system are replacing the conventional tube method for performance of the antiglobulin test, both for Rh typing and for antibody detection (Knight and de Silva 1996).

Presensitization of a transfusion recipient is evaluated by an antibody screen in which serum from the candidate is tested against a panel of type O red cells that express the blood group antigens giving rise to the most frequently encountered anti-blood group antibodies. Here again, sensitive variants of the hemagglutination assay are used. Typically, the variations include prolonged incubation time and the use of enhancement media such as low ionic strength saline. The enhancement media promote agglutination chiefly by reducing the cationic charge cloud that surrounds the negatively charged red cells. The surface charge of the red cell can also be diminished by proteolytic enzyme treatment which removes sialic acid containing glycoproteins. Importantly, antibody screen incubations are usually carried out at 37şC in order to limit positive results to antibodies that can react at body temperature; if an antibody cannot react at body temperature, it is very unlikely to be clinically significant. Following incubation, the mixtures are centrifuged and examined for hemolysis or agglutination. Non-agglutinating panel red cells are washed with saline and have an indirect antiglobulin test performed. If there is a positive result, the specificity of the antibody is determined (Garratty 1998). Antibody screens are also performed on donor blood to detect unexpected antibodies that could react with recipient red cells.

Additional testing for pre-existing recipient antibodies by the performance of a red cell crossmatch used to be a standard component of pretransfusion testing. A full crossmatch is now no longer required, unless the recipient is known to have made antibodies in the past, as it has become recognized that ABO and Rh typing and modern antibody screens are adequate to assure red cell compatibility in almost all cases (Cordle *et al*. 1990). A red cell crossmatch designed to detect ABO incompatibility is adequate in the usual case. It consists of a hemagglutination assay in which a dilute solution of donor red cells is mixed with serum from the recipient. The mixture is centrifuged immediately, or after a

five minute incubation at room temperature, and then examined for hemolysis or agglutination. This crossmatch serves only as a check on the ABO matching of the donor and recipient. Modern blood bank practices now allow for the use of an electronic crossmatch in which ABO compatibility between donor and recipient is assured by verification by a computerized data system (Judd 1998).

As mentioned, a full red cell crossmatch is called for if there is a history of antibody formation in the recipient. The crossmatch is performed in a manner similar to an antibody screen, including an antiglobulin testing step, only donor red cells rather than panel red cells are used.

Transfusion recipients are monitored closely during and immediately following the transfusion. If ABO incompatibility is the cause of the reaction, intravascular hemolysis will occur during or within an hour following the transfusion. Laboratory findings consistent with a significant release of hemoglobin into the circulation include substantially reduced plasma concentrations of haptogloblin (a hemoglobin binding protein) and hemopexin (a heme binding protein), the appearance of unbound hemoglobin in the plasma and hemoglobin and hemosiderin in the urine, and an increased plasma concentration of bilirubin. Red cell lysis is marked by increased plasma concentrations of red cell injury markers (e.g., lactate dehydrogenase). Hemolysis occurs quickly and the clearance of plasma hemoglobin and its breakdown products is rapid, so the plasma markers of hemolysis peak within hours after the transfusion and dissipate promptly. Therefore, blood should be collected for these laboratory studies soon after the recognition that a transfusion reaction may have occurred. A direct antiglobulin test should also be performed to provide evidence that the hemolysis, if present, is due to antibodies.

Extravascular hemolysis can occur when there is incompatibility of non-ABO blood group systems including the Rh system. If alloantibodies are present at the time of the transfusion, acute extravascular hemolysis will result. The findings of hemoglobin release into the plasma are not present but there is evidence of increased hemoglobin recycling in the form of an elevated plasma concentration of bilirubin. Red cell injury markers also show an increase in their plasma concentration and the direct antiglobulin test is positive. Delayed extravascular hemolysis can be caused by an anamnestic immune response even when there is no detectable antibody present at the time of pretransfusion testing. Typical laboratory findings will only be present if the response is brisk.

Note that it is exceedingly unlikely for ABO or Rh incompatibility to occur if the donor and recipient are typed as being compatible. Transfusions that are ABO or Rh incompatible are almost always due to clerical errors when releasing the red cell units or identification errors when hanging the red cell units. That is why the first step in the investigation of a transfusion reaction, after immediate discontinuation of the transfusion and institution of resuscitative measures, is a check for discrepancies among the compatibility tag, the product bag label, and the patient's hospital identification band.

INFECTION

The laboratory evaluation of infection has two parts, the tentative classification of an illness as infectious and the definitive demonstration, identification, and characterization of the pathogen responsible. Tentative classification rests primarily on the clinical presentation but there are laboratory findings that may contribute. They include characteristic changes in the plasma concentrations of markers of the injury and function of the involved tissues and changes in the plasma concentrations of markers of the acute phase response.

The presence of a pathogen can be demonstrated by microscopic visualization of the organism in material sampled from the patient or by culture of the organism from the material. Some pathogens are, however, extremely difficult or impossible to visualize or culture; infection by these pathogens is demonstrated by the presence of microbial substances or by an active immune response (Figure 9.3).

The identification of a pathogen entails, at a minimum, the elucidation of the genus of the pathogen, as antimicrobial susceptibilities are largely defined at the genus level. Pathogens may be further identified as to species and, sometimes, as to strain if classification at that level is important in making therapeutic decisions and if the technical difficulty of performing the classification is not unreasonable. For most pathogens, identification includes determining the species of the organism. Characterization of a pathogen consists of the determination of the antimicrobial susceptibilities of the organism. Characterization of the organism by high resolution

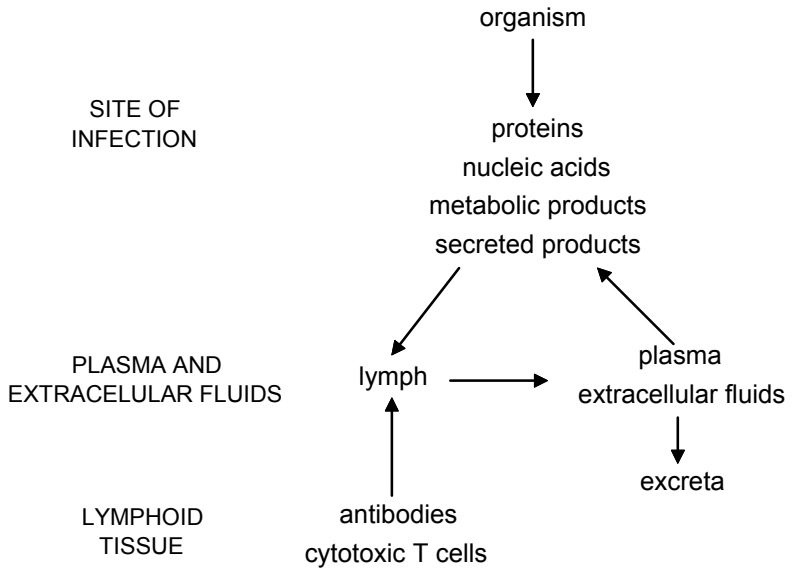

Figure 9.3 A model of the disposition of high specificity markers of infection.

genotyping is sometimes also undertaken for the purposes of clinical epidemiology and hospital infection control (Pfaffer and Herwaldt 1997).

Acute phase response

The acute phase response is a group of nonspecific, cytokine-mediated phenomena that occur in response to inflammation (Gabay and Kushner 1999). As shown in Table 9.2, one or more components of the response may be present as a consequence of infection. Which components are present is determined by the set of cytokines characteristically generated during the infection. Bacterial infections, for instance, typically cause a wide variety of cytokines to be released, resulting in all three of the classical components of the acute phase response: fever, neutrophilia, and increased plasma concentrations of the acute phase proteins.

Both the acute phase protein reaction and neutrophilia have been used as markers for bacterial infection. The acute phase proteins, C-reactive protein and fibrinogen (usually measured using the ESR), are often increased in bacterial infection but the specificity of these markers, as would be predicted, is quite low.

The neutrophilia that occurs as part of the acute phase response is due largely to the release of mature neutrophils held in reserve in the marrow sinusoids, but slightly immature cells, band neutrophils, may also be released. Neutrophilia is demonstrated by an increase in the neutrophil count. The neutrophil count is the concentration of neutrophils in whole blood. The neutrophil count is often called the *absolute* neutrophil count to distinguish it from the neutrophil fraction as reported in a white cell differential. Because neutrophils make up a large fraction of the white cells in circulation, an increase in neutrophil count usually produces an elevation in the white cell count. Increased white cell counts are not inevitable, however, and increased neutrophil counts can be found in some infected patients who have normal white cell counts (Ardron et al. 1994). Neither the neutrophil count nor the white cell count are highly sensitive markers of bacterial infection. This is not surprising as the range of tissue damage and extent of systemic response caused by infections, even infections with the same pathogen, vary so greatly among patients. The neutrophil count may be somewhat more specific than the white cell count in some clinical settings (Gombos *et al.* 1998). Because band neutrophils may be released as part of the acute phase neutrophilic response, the measurement of the band neutrophil count has also been used as a marker of bacterial infection (Novak 1993). The quantification of band neutrophils is even incorporated into most automated blood cell counters. Despite its appeal, the band neutrophil count is no more reliable as a marker of infection than is the neutrophil count (Bentley *et al.* 1987, Ardron *et al.* 1994).

The finding of neutrophils in body fluids being evaluated for bacterial infection quite reliably

indicates the presence of local inflammation but not necessarily infection. For example, high concentrations of neutrophils can be found in joint fluid as a result of crystal-induced arthritis and neutrophils can be found in the urine in glomerulonephritis. In the CSF, a neutrophilic infiltrate is fairly suggestive of a bacterial infection but it can also be caused by viral infection.

Microscopy and culture

If a specimen from the site of infection contains large numbers of the pathogen, the infectious agent may be demonstrated by microscopic examination of the material. Large organisms, especially motile organisms such as Giardia lamblia, can be detected simply by examination of material suspended in saline (wet mount preparation). Fungal hyphae, despite being large, are much more readily visualized after clearing the specimen background by treatment with potassium hydroxide (KOH preparation). Histologic dyes are used to color smaller pathogens in a distinctive fashion, making them relatively easy to detect. Some dyes have a fairly limited scope and thereby achieve considerable diagnostic specificity; for example, Kinyuon carbol-fuchsin stains mycobacteria. Other dyes stain a broad range of pathogens, such as crystal violet, which colors all Gram-positive organisms, and are therefore much less specific. Fluorescence microscopy using fluorochrome-conjugated antibodies directed against species-specific antigens provides high diagnostic specificity. Of equal importance, microscopy using fluorescent reagents is very sensitive, allowing the detection and identification of the pathogen of interest even when it there are very few present in the specimen. The sensitivity of microscopic detection of small numbers of organisms can also be increased in fluid specimens by concentrating the cellular elements of the specimens by centrifugation; this is routinely done in the microbiologic examination of CSF.

The goal of culturing a pathogen is to generate a large enough number of organisms to confirm their presence and to conduct tests for the purpose of identifying and characterizing the pathogen. Culturing consists of the inoculation of a medium in which the pathogen can grow and the maintenance of the medium under conditions that are favorable for growth. This can be a simple task, as in the culture of non-fastidious bacteria, or it can be very demanding, as in the culture of viruses which require living cells as the growth substrate. The composition of the growth medium is often designed to provide a relative growth advantage to the suspected pathogen and to suppress the growth of contaminants or commensal organisms. The advantage may come from the mix of nutrients present in the medium or from the presence of specific suppressive chemicals. For instance, bacterial growth is suppressed in fungal culture by including bacterial antibiotics in the growth medium.

Cultured organisms are identified by morphologic features, growth characteristics, and biochemical properties and, more and more, by immunologic and molecular diagnostic methods. The immunologic methods include particle agglutination (in which the test organisms, usually bacteria, are the particles), direct immunofluorescence microscopy, and immunoassay. These methods employ monoclonal antibodies directed against microbial surface antigens. The specificity of the antibodies may be at the species level, being directed against an antigen shared by all of the strains within the species, or it may be at the strain level, being directed against an antigen expressed only by a particular strain within the species. A wide range of molecular diagnostic methods are used in microbial identification (Tang *et al*. 1997). Because these methods distinguish pathogens based on genotypic differences, they can be exquisitely specific, allowing for organism classification at the substrain level if desired.

Specimen collection. In order for a microbiologic specimen to be useful it must, first of all, actually come from, or arise from, the involved site. This seems an obvious requirement but it is one that not always satisfied. For instance, in pneumonia the site of infection can generally be sampled in the form of expectorated sputum, but some patients are unable to produce a deep cough on demand or else have trouble moving sputum from the back of the throat into the mouth for expectoration. Specimens from such patients generally consist mostly or entirely of saliva not sputum. The problem is compounded by the fact that many clinicians do not inspect specimens closely enough to confirm that they consist of sputum. The end result is that saliva specimens rather than true sputum specimens are frequently submitted to the laboratory. Recognizing this, specimens labeled as sputum are routinely screened by microscopy to confirm that do indeed represent sputum; sputum has neutrophils and only a few squamous epithelial cells (Wilson 1996).

Another requirement of microbiologic specimens is that they must be collected without undue microbial contamination. The avoidance of microbial contamination applies primarily to contamination by organisms on the skin and the mucous membranes. Contamination from the patient's skin is controlled by disinfecting the patient's skin overlying the body site to be sampled. Contamination from the physician's skin is avoided by using aseptic technique. Complete avoidance of contamination from the mucous membranes is not possible if the specimen must pass along the membrane. Expectorated sputum, for instance, will always be contaminated with organisms from the oral cavity. Contaminating organisms from the mucous membranes may be recognizably innocuous but their presence may make the pathogenic species hard to detect. In that case, processing and growth techniques can be used that suppress contaminating organisms and enhance pathogenic organisms. If the contaminating organisms are potential pathogens, it may be possible to determine when they are merely contaminants by quantitative analysis. Contaminating organisms are usually present in low density in a fresh specimen while organisms causing an infection are usually present in high density. In situations in which microbial contamination by the mucous membranes presents a serious impediment to clinical care, it can be avoided altogether by bypassing the membrane route and obtaining the specimen by percutaneous aspiration or by biopsy. Transtracheal aspiration of sputum is one example.

The amount of material contained in a microbiologic specimen must be large enough to contain a detectable number of organisms. The actual amount of material needed will, of course, vary according to the density of the pathogen in the material. For example, only a small volume of pus, just 1 to 2 ml, is needed for the microbiologic study of an abscess because the density of bacteria in pus is very high. In contrast, the density of bacteria in the blood of a patient with septicemia is very low, necessitating that a large volume of blood be obtained, preferably two 10 ml specimens. A particular concern relating to the size of the specimen is the popularity of swabs for specimen collection as swabs absorb only a tiny volume of fluid. Their use is best limited to the sampling of skin and mucous membranes where there is no accumulated fluid to collect.

Finally, microbiologic specimens must be expeditiously placed into the appropriate transport containers and rapidly transferred to the laboratory. Transport containers protect the specimen from microbial contamination during handling and often contain a transport medium that prevents the dehydration of the specimen. Transport media are usually non-nutritive in order to suppress microbial growth but a nutritive medium may be used for the transport of fastidious organisms. Special container systems are available for the transport of specimens to be cultured for anaerobic bacteria.

Specimens that manifestly fail to achieve adequate standards of collection or transport are usually rejected by the laboratory. Explicit criteria for rejection should include: unlabeled or improperly labeled specimens; specimens received in inappropriate, leaking, or broken containers; specimens with obvious contamination; and unpreserved specimens received too long the time of collection (Wilson 1996).

Microbial substances

Microbial substances that are present at the site of infection include proteins, nucleic acids, secreted products, and metabolic products. Their detection in material obtained from the site of infection offers a means of rapidly demonstrating the presence of a pathogen in settings in which microscopy may not always be informative. Tests for these substances can, of course, also be used in combination with microscopy to maximize sensitivity for the rapid demonstration of pathogens.

Microbial proteins, measured using immunologic techniques, are the substances most often studied. Their diagnostic performance as markers of infection can be quite good. As an example, the latex agglutination test for cryptococcal antigen in CSF is both highly sensitive and highly specific for the diagnosis of *Cryptococcus neoformans* meningitis. Such reliability is not a always attained, however. For instance, it has been demonstrated that, using current techniques, bacterial protein detection in CSF has an unacceptably low specificity for the diagnosis of bacterial meningitis (Perkins *et al.* 1995). This study also showed that it is very rare that a true positive result is the sole finding indicative of infection. In almost all cases, routine microbiologic studies also revealed the presence of infection, albeit not always as rapidly. The detection of microbial nucleic acids, measured using molecular diagnostic techniques, is proving to be a consistently reliable method of demonstrating pathogens. Because of the

rapid turn-around time, high sensitivity, and undemanding collection requirements of nucleic acid studies, and because they accomplish identification of a pathogen at the same time they demonstrate its presence, they are likely to have an ever more important clinical role, even when the pathogen is readily visualized or cultured (Tang *et al.* 1997).

Microbial substances not only accumulate at the site of infection but they diffuse locally, enter the lymph, and appear in the plasma (Figure 9.3). Microbial substances that arise from infections that involve blood cells, endothelial cells, or other cells in contact with the blood, have direct access to the plasma. During the period of time that a microbial substance is present in the plasma at a concentration that is measurable, it can be used to demonstrate the presence of the pathogen. This time period is the diagnostic window for the substance.

Hepatitis B is an infection that releases virus particles as well as viral proteins into the plasma (Gitlin 1997). The intact virus consists of an inner core and outer envelope. The inner core contains the double-stranded viral DNA, a viral DNA polymerase, a C protein that gives rise to capsid core antigen, HBcAg, and soluble e-antigen, HBeAg (Conway *et al.* 1998). The outer lipoprotein coat contains the protein that represents the surface antigen, HBsAg. Surface antigen is produced in excess in infected hepatocytes and is in part released as free lipoprotein particles.

Symptoms of hepatitis and laboratory findings of liver injury and dysfunction appear approximately 2 months after exposure to the virus and, in uncomplicated cases, last 2 to 3 months. At that point in the disease, hepatitis virions containing HBV DNA are typically present in the plasma as are detectable concentrations of HBeAg and HBsAg. As shown in Figure 9.4, the diagnostic windows for these markers begin weeks prior to symptomatic disease and extend to the end of the symptomatic period. The diagnostic window for HBsAg is longer, lasting up to months after the disappearance of symptoms. The detection of HBsAg in the plasma is indicative of hepatitis B infection; the finding of HBeAg or HBV DNA indicates disease with a high viral replication rate and high infectivity. HBsAg is also detectable in the plasma of carriers and patients with chronic disease. HBeAg and HBV DNA are present in low, often undetectable, concentrations in these clinical states because of low viral replication rates and correspondingly low virion release rates.

Immune response

An active humoral immune response to infection produces a many-fold increase in the plasma concentration of antibodies specific for the offending pathogen (Keren and Warren 1992). IgM class antibodies appear first, usually within a week of the start of infection and persist for a number of weeks. The delay in the appearance of antibodies is due to the time it takes for activation and maturation of the B cell response. During that period, microbial antigens may be present in the plasma. Once appreciable quantities of antibodies are being produced, the circulating antigen is bound as antigen-antibody complexes and is rapidly removed from circulation. As the IgM antibodies subside, IgG class antibodies appear. These antibodies slowly wax, then wane, with detectable concentrations often persisting in the plasma for very long periods despite resolution of the infective process. IgM antibodies, therefore, mark acute infection. A 4-fold increase in total antibody titer (i.e., roughly a 16-fold increase in plasma antibody concentration) over an interval of 2 or more weeks is also considered indicative of acute infection. IgG antibodies mark prolonged infection or past infection. High concentrations of IgG antibodies suggest that the infection is active or recent.

The specificity of the antibodies produced in response to a pathogen may be to the whole species or only to the particular strain within the species responsible for the infection. In the first case, the dominant immunogenic epitopes are shared by all of the species members; in the second, the immunogenic epitopes are specific at the strain level but not at the species level. If all of the members of a species are comparably virulent, the clinical goal is to identify infection by any species member. If a patient's antibodies are also species-specific, testing can be done using a single species-specific antigen. This is the situation, for example, for *Salmonella typhi*. If, however, the patient's antibodies are strain-specific, testing has to be performed using multiple antigens, one for each relevant strain. If the number of relevant strains is large, such testing is not feasible. This is the situation for most of the other species of *Salmonella*. On the other hand, if only one strain or group in a species is clinically relevant, then antibodies specific for that strain or group should be produced for they can then be used to identify the presence of that particular strain.

The antigens to which antibodies arise in infection may be structural proteins or carbohydrates of

the pathogen or protein products. Many secreted products of b-hemolytic streptococci, for instance, stimulate antibody responses. Anti-streptolysin O and anti-DNase B are used as markers of recent streptococcal infection in the evaluation of Sydenham's chorea and poststreptococcal glomerulonephritis, respectively. Antibodies against structural proteins are used in the evaluation of hepatitis B. The presence of anti-HBc of the IgM class can be used to distinguish acute from chronic hepatitis in patients who are HBsAg positive and who have an ambiguous history. Because anti-HBc first appears weeks after HBsAg is detectable (Figure 9.4), false negative results are possible early in the course of the disease. Anti-HBc of the IgG class is found in acute and chronic hepatitis. The appearance of anti-HBe, and the concomitant disappearance of HBeAg, signals beginning resolution of the disease and is associated with a low viral replication rate and low infectivity. The appearance of anti-HBs indicates recovery from the infection.

Methods in current use for the measurement of antibodies to pathogens include particle agglutination, indirect immunofluorescence microscopy, and various forms of immunoassay. Specificity for IgM or IgG class test antibodies can be incorporated into studies that employ a signal-generating reagent antibody by using an antibody that is immunoglobin class-specific.

In vitro antimicrobial susceptibility testing

The obvious goal of therapy in infectious disease is to eradicate, or at least suppress, the pathogen responsible for the infection. In serious infections, this goal is achieved through the use of an antimicrobial agent. If the pathogen belongs to a species for which the pattern of drug susceptibility is essentially invariant, laboratory identification of the pathogen constitutes adequate information on which to base drug selection. If there is clinically significant variability in the pattern of drug susceptibility of the species, it is desirable to determine the susceptibilities of the organism isolated from the patient so that the most appropriate therapeutic agent can be provided. One way to do this is empirically, by administering the drug, or drug combination, most likely to be effective and seeing if it works. If it does, fine; if it doesn't, the organism can be assumed to have low susceptibility to that drug, or that combination of drugs, and another can be tried, either alone or in combination with the initial drug(s). It must be noted that, even when effective antimicrobial agents are used, the clinical signs of infection may persist for a while. For example, a patient with uncomplicated cystitis may have her dysuria disappear within a few hours of the first dose of an effective antibiotic but a patient with pyelonephritis may continue to have fever and flank pain as long as 2 days after the start of appropriate antibiotic therapy.

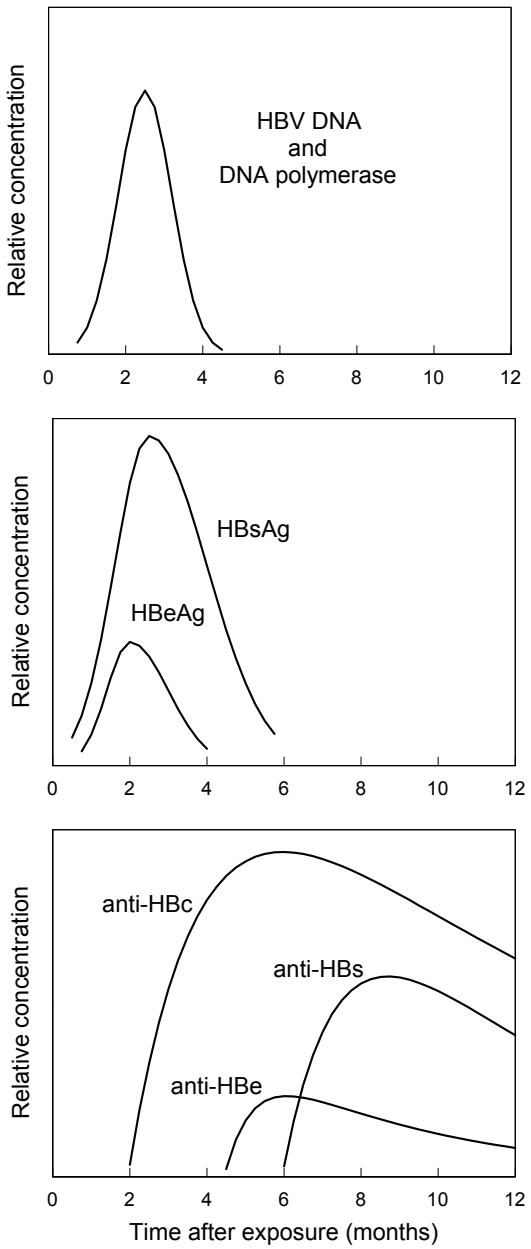

Figure 9.4 The diagnostic windows for markers of acute hepatitis B infection. The top graph shows the window for viral HBV DNA and DNA polymerase, the middle graph shows the windows for viral antigens, and the bottom graph shows the window for HBV-specific antibodies.

For bacteria, the other way to ascertain the drug susceptibilities of an organism is by laboratory testing. Current techniques of laboratory susceptibility testing involve growth of the organism, so the rapidity with which the results are available depends upon the rate of *in vitro* growth of the organism. The results from fast-growing organisms, such as most bacteria, can be known within days. Infection with one of these pathogens can be treated empirically until the susceptibility results are available then, if the results indicate that there is a more appropriate antibiotic, the patient can be switched to that drug. Susceptibility testing of slow-growing pathogens, such as mycobacteria, yields results only after weeks.

The most common laboratory methods for testing antimicrobial susceptibility are the disk diffusion susceptibility test and the microdilution broth susceptibility test (Jorgensen and Ferraro 1998). In the disk diffusion susceptibility test (also called the Bauer-Kirby test) antibiotic impregnated disks of filter paper are placed on the surface of an agar culture plate with defined medium that has previously been uniformly inoculated with a suspension containing a standard number of the organism to be tested. The antibiotics that are studied are those that have an established clinical role in treatment of infections caused by the species of organism being tested. The plate is incubated under ambient air at 35ºC. During the incubation, antibiotic diffuses out of the disk and into the agar, establishing a logarithmic concentration gradient. Bacterial growth is inhibited close to the disk where the concentration of antibiotic exceeds the minimal inhibitory concentration (MIC) for the organism while bacterial growth proceeds at a distance from the disk where the antibiotic concentration is lower. Over time, continuing diffusion of the antibiotic causes the MIC to be located farther and farther from the disk resulting in a larger and larger zone of growth inhibition. At some point, called the critical time, the density of the growing bacteria beyond the MIC is great enough that subsequent exposure to higher concentrations of the antibiotic does not inhibit further growth. Consequently, a discrete interface develops at what was the location of the MIC at the critical time: bacterial growth is present outside, clear agar is present inside. This interface demarcates the definitive zone of inhibition, the width of which is proportional to the MIC for the organism. Because a standard amount of antibiotic is present in the disks used in the test, there is a defined inverse relationship between zone width and MIC. Tables provide critical values for zone widths based on the MICs that correspond to the widths. The critical values define three susceptibility categories: susceptible, resistant, or intermediate. Susceptible organisms have an MIC lower than the antibiotic concentrations that are ordinarily obtained by usual doses of the drug, resistant organisms have an MIC higher than the drug concentrations that are usually achievable clinically, and intermediate organisms have an MIC in the range of drug concentrations usually achieved only by maximal doses of the drug. Note that, for a variety of reasons, the most important of which is that *in vitro* testing is not a perfect model of *in vivo* drug action, *in vitro* susceptibility of an organism does not assure *in vivo* effectiveness of the drug. The clinician must be knowledgeable concerning the drugs, the species of pathogen, and the sites of infection for which there is clinical experience of a disparity between *in vitro* and *in vivo* susceptibility results.

The microdilution broth susceptibility test (or microtube dilution test) provides a direct estimate of the MIC of the organism being tested. A range of clinically meaningful concentrations of antibiotic are added to defined broth culture medium in the wells of a microtiter plate. The wells are then inoculated with a suspension containing a standard number of the organism to be tested. The plate is incubated at 35ºC for 16 to 18 h and then evaluated either visually or by instrumental means for turbidity due to bacterial growth. The MIC is measured as the lowest antibiotic concentration studied that inhibits growth of the organism. The actual MIC is somewhere between that concentration and the next lower concentration studied but exactly where in that range cannot be ascertained without further testing. The measured MIC is used to determine the susceptibility category of the organism. The susceptibility category is reported along with or in place of the MIC.

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