28 CHAPTER 28 Type IV Hypersensitivity: Delayed Hypersensitivity

28.1 KEY POINTS

- Some antigens, when injected into the skin, induce a slowly developing inflammatory response called delayed, or type IV, hypersensitivity.
- Delayed hypersensitivity reactions are mediated by T cells and natural killer cells.
- A good example of a delayed hypersensitivity response is the reaction to intradermal tuberculin in cattle with tuberculosis. This tuberculin test provides a convenient diagnostic test for tuberculosis.
- A different form of type IV hypersensitivity occurs in allergic contact dermatitis. This is a slowly developing inflammatory response that occurs when reactive chemicals bind to skin cells and trigger a T cell response.

Certain antigens, when injected into the skin of sensitized animals, provoke slowly developing inflammation at the injection site. Since this “delayed” hypersensitivity reaction can only be transferred from sensitized to normal animals by lymphocytes, it must be cell mediated. Delayed hypersensitivity reactions are classified as type IV hypersensitivities and result from interactions involving the injected antigen, antigen-presenting cells, and T cells. An important example of a delayed hypersensitivity reaction is the tuberculin response. This is a skin reaction that develops in an animal infected with tuberculosis following an intradermal injection of tuberculin. Delayed hypersensitivity reactions can be considered to be a specialized form of inflammation directed against organisms that are resistant to elimination by conventional inflammatory processes.

28.2 THE TUBERCULIN REACTION

Tuberculin is the name given to extracts of mycobacteria used to skin-test animals in order to identify those suffering from tuberculosis. Several types of tuberculin have been employed for this purpose. The most important is purified protein derivative (PPD) tuberculin, prepared by growing organisms in synthetic medium, killing them with steam, and filtering. The PPD tuberculin is precipitated from this filtrate with trichloroacetic acid, washed, and resuspended in buffer ready for use. Thus PPD tuberculin is a crude antigen mixture. Its major antigenic component is probably the heat-shock protein HSP 65. Many of its proteins are shared among different mycobacterial species, thus ensuring that tests that use PPD tuberculin are relatively nonspecific.

When tuberculin is injected into the skin of a normal animal, there is no apparent response. On the other hand, if it is injected into an animal infected with mycobacteria, a delayed hypersensitivity response occurs. In these animals, a red, indurated (hard) swelling develops at the injection site. The inflammation begins between 12 and 24 hours, reaches its greatest intensity by 24 to 72 hours, and may persist for several weeks before fading gradually. In very severe reactions, tissue destruction and necrosis may occur at the injection site. Histological examination of the lesion shows that it is infiltrated with mononuclear cells (lymphocytes, macrophages), although neutrophils are present in the early hours of the reaction (Figure 28-1).

The tuberculin reaction is mediated by T cells. When an animal is infected with *Mycobacterium tuberculosis,* the organisms are readily phagocytosed by macrophages. Some of this mycobacterial antigen triggers a Th1 response and generates memory cells. These memory T cells will respond to injected mycobacterial antigens such as tuberculin. Since a positive tuberculin test can be elicited many years after exposure to an antigen, some of these memory T cells must be very long lived.

When tuberculin is injected intradermally, it is taken up by Langerhans cells, which then migrate to the draining lymph node (Figure 28-2). Here they present antigen to memory T cells that respond by generating Th1 effector cells. The circulating Th1 cells recognize the antigen when they encounter it in the skin and accumulate around the antigen deposit. By 12 hours in cattle, the injection site is mainly infiltrated with γ/δ^+, $WC1^+$ T cells. (In humans and mice, α/β T cells tend to predominate, whereas in sheep and cattle, γ/δ T cells predominate.) There are no B cells in the lesion.

The γ/δ T cells help to recruit other Th1 lymphocytes and macrophages to the site. The Th1 cells secrete interferon-γ (IFN-γ), interleukin-2 (IL-2), and IL-

FIGURE 28-1 A histological section of a positive tuberculin reaction in bovine skin. Note the perivascular mononuclear cell infiltration as well as the lack of neutrophils or edema. (From Thomson RG: *General veterinary pathology,* Philadelphia, 1978, Saunders.)

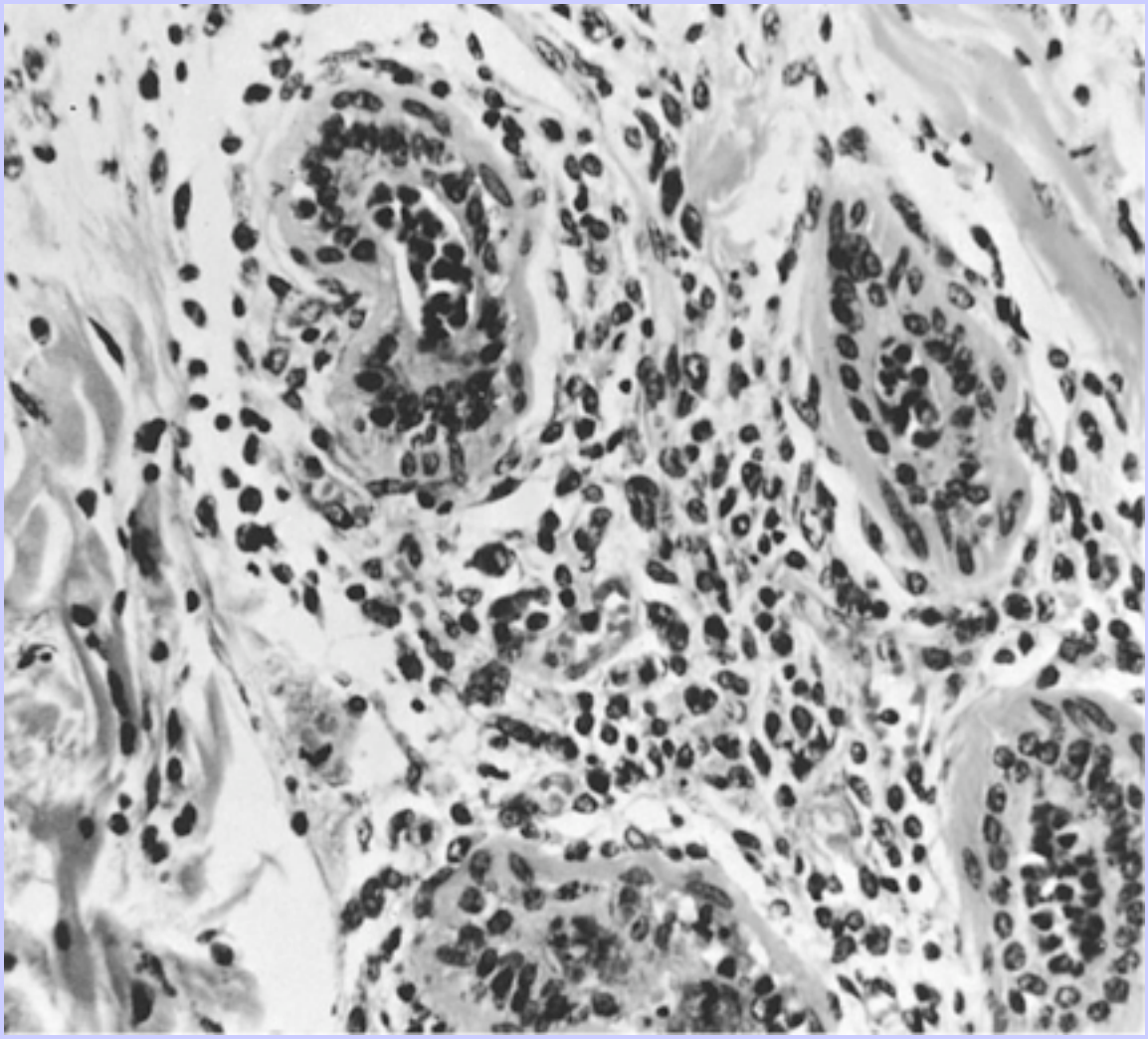

FIGURE 28-2 A schematic diagram depicting the mechanism of a delayed hypersensitivity reaction.

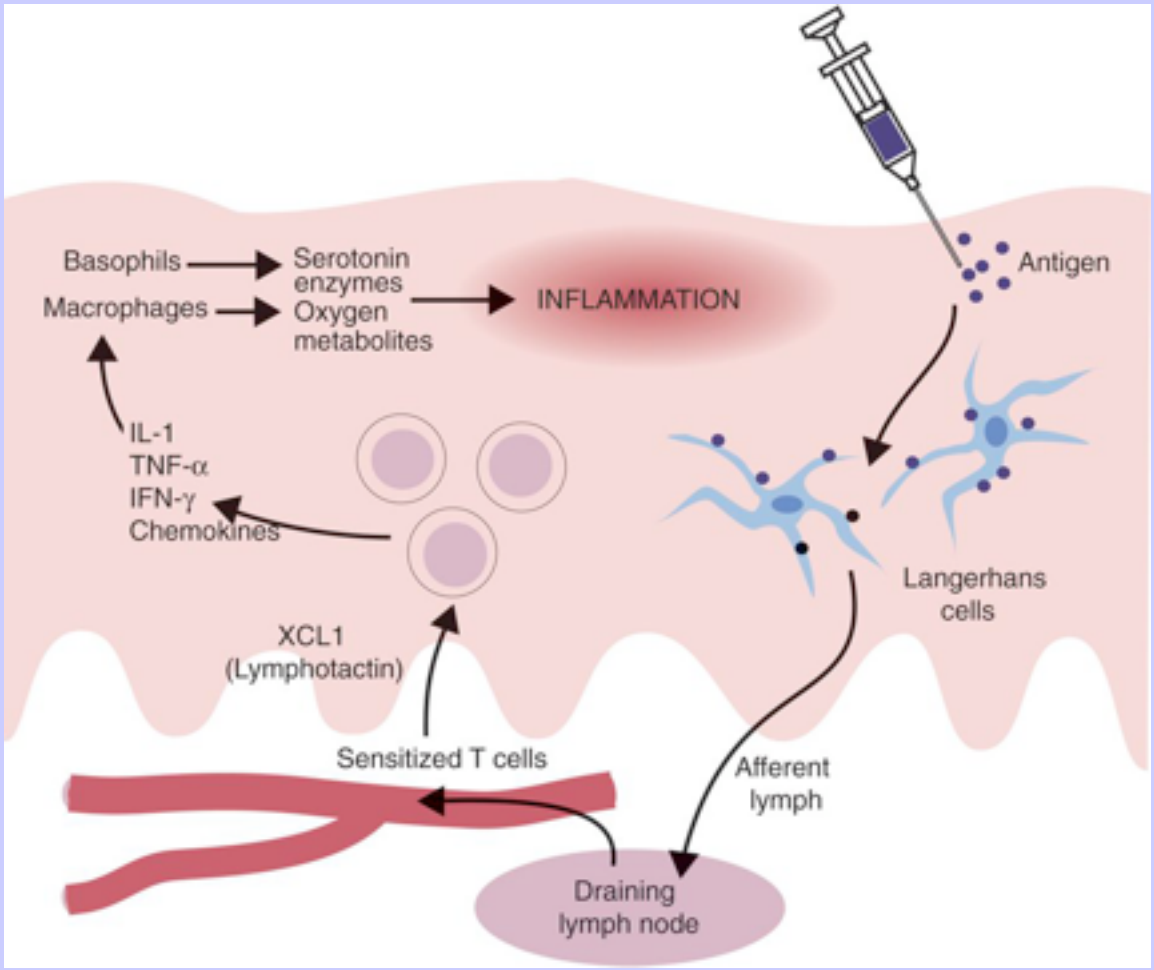

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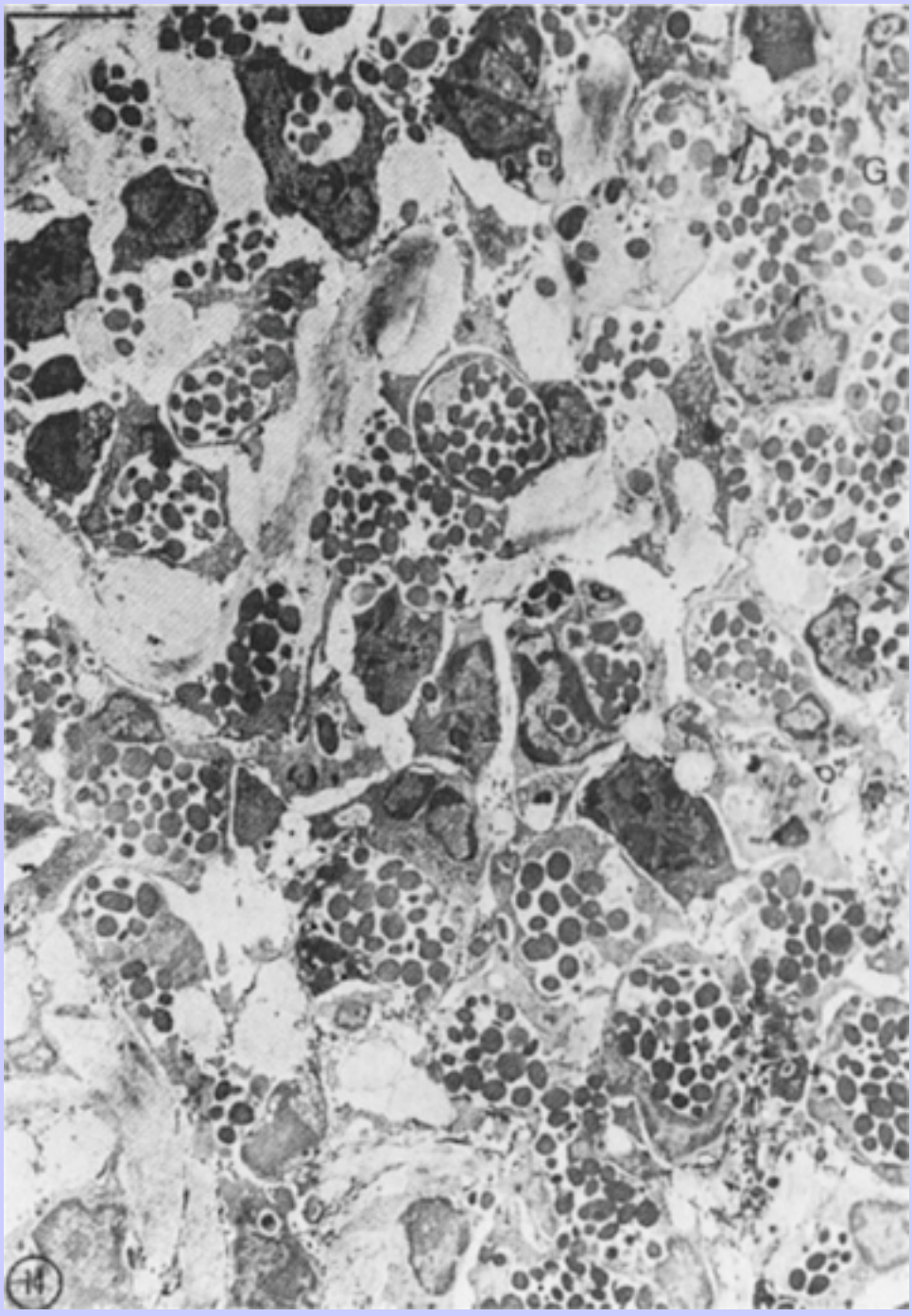
FIGURE 28-3 A section of guinea pig skin 18 hours after attachment of a tick in an animal sensitized by prior infestation with tick larvae. The skin is infiltrated with large numbers of basophils. (From McLaren D, Worms MJ, Askenase PW: *J Pathol* 139:289, 1983.)



16. The first two act on endothelial cells to increase expression of adherence molecules. IL-2 stimulates production of the chemokines CXCL8, CCL5, and XCL1, which attract and activate more T cells. IL-16 attracts $CD4^+$ T cells. The macrophages also release serotonin and chemokines such as CXCL1 and CCL2, which attract basophils. Basophil-derived serotonin (in rodents) or histamine (in humans) causes yet more inflammation and enhances migration of mononuclear cells into the lesion. The T cell–derived chemokines CCL2 and CCL3 can induce mast cell degranulation, whereas some $CD4^+$ T cells can activate mast cells directly through major histocompatibility complex (MHC) II–bound antigen.

T cell–derived chemokines cause inflammation and attract even more T cells. Most of these new T cells are not specifically sensitized for the inducing antigen. Only a very small proportion, perhaps 5%, of the lymphocytes seen in a delayed hypersensitivity reaction are specific for the antigen. The vast majority are attracted nonspecifically by XCL1. By 60 to 72 hours, the predominant lymphocytes are α/β^+, $CD4^+$, and $CD8^+$. Macrophages accumulate in the lesion as a result of the production of CXCL8 and may be activated by IFN-γ. Some of the tissue damage in intense delayed hypersensitivity reactions may be due to the release of proteases and oxidants from these activated macrophages. The macrophages ingest and eventually destroy the injected antigen. This, plus the appearance of regulatory cells in the lesion, permits the tissues to return eventually to normal.

28.2.1 Cutaneous Basophil Hypersensitivity

Under some circumstances, basophils may be the predominant cells in a delayed hypersensitivity reaction (Figure 28-3). This type of reaction, called cutaneous basophil hypersensitivity (CBH), can be transferred between animals with antibody, with purified B cells, or even with T cells. CBH is therefore mediated by several different mechanisms. CBH occurs in chickens in response to intradermal Rous sarcoma virus, in rabbits in response to schistosomes, and in humans with allergic contact dermatitis and renal allograft rejection. CBH reactions may contribute to the development of flea allergy dermatitis in dogs.

28.3 TUBERCULIN REACTIONS IN CATTLE

Because a positive tuberculin reaction occurs only in animals that have, or have had, tuberculosis, skin testing may be used to identify animals affected by this disease. Indeed, the tuberculin test has provided the basis for all tuberculosis eradication schemes that involve the detection and subsequent elimination of infected animals.

Skin testing of cattle may be performed in several ways (Table 28-1). The simplest is the single intradermal (SID) test. In this test, 0.05 ml of PPD tuberculin derived from M. tuberculosis or Mycobacterium bovis is injected into one anal fold and the injection site is examined 72 to 96 hours later. A comparison is easily made between the injected and the uninjected folds, and a positive reaction consisting of a firm lump at the injection site is readily detected.

In the United States, two separate tests are performed. Two injections of tuberculin are made, one into the mucocutaneous junction of the vulva and the other into an anal fold. In other countries, tuberculin is normally injected into the skin on the side of the neck. The neck site is more sensitive than the anal folds, but restraint of the animal may be more difficult and good injection technique is critical.

The advantage of the SID test is its simplicity. Its main disadvantage is that because of cross-reactions it cannot distinguish between tuberculosis and infection by related mycobacteria such as *Mycobacterium avium, M. avium paratuberculosis,* or the *Nocardia* group of organisms. A second disadvantage is that some animals react positively

to the test but on necropsy do not have detectable tuberculosis lesions. The reasons for this are unclear but may be a result of exposure to nonpathogenic mycobacteria such as *Mycobacterium phlei*.

Table 28-1 Tuberculin Tests Used in Cattle

Test	Usage	Advantages	Disadvantages
Single intradermal	Routine testing	Simple	Prone to false positives
			Poor sensitivity
Comparative	When avian TB or Johne's disease is prevalent	More specific than SID	More complex than SID
Short thermal	Use in postpartum animals and in infected animals	High efficiency	Time consuming
			Risk of anaphylaxis
Stormont	Use in postpartum animals and in advanced cases	Very sensitive and accurate	Three visits required
			May sensitize an animal

False-negative SID tests may occur in animals with advanced tuberculosis, in animals with very early infection, in animals that have calved within the preceding 4 to 6 weeks, in very old cows, and in animals tested during the preceding 1 to 10 weeks. The lack of reaction (anergy) seen in advanced cases of tuberculosis is also observed in clinical Johne's disease and appears to be due to the presence of a “blocking factor” in the serum of these animals. This factor may be an antibody that prevents T cells from reacting with antigen. There is also evidence for the involvement of regulatory cells in anergy. Because of these defects in the SID, several modifications of this test have been developed. The comparative test, for example, involves intradermal inoculation of both avian and bovine tuberculins. Each tuberculin is injected into the side of the neck at separate sites, and these sites are examined 72 hours later. In general, if the avian tuberculin site shows the greatest reaction, the animal is considered to be infected with *M. avium* or *M. avium paratuberculosis*. On the other hand, if the M. bovis site shows the greatest reaction, then it is believed that the animal is infected with *M. bovis* or *M. tuberculosis*. This test is useful when a high prevalence of avian tuberculosis or Johne's disease is anticipated. PPD from *M. bovis* is more specific in cattle than *M. tuberculosis,* giving less cross-reaction with *M. avium* as well as being more appropriate for use in cattle and is therefore preferred. In practice, recent evidence suggests that the comparative test has a sensitivity of 90% (10% false negatives) and a specificity of greater than 99% (less than 1% false positives); however, this depends on the criteria used to read the results.

Anther modified tuberculin test is the short thermal test, in which a large volume of tuberculin solution is given subcutaneously and the animal examined for a rise in temperature between 4 and 8 hours later. (Presumably the tuberculin acts on T cells, which then provoke the release of IL-1 and other cytokines from macrophages.) The Stormont test relies on the increased sensitivity of a test site, which occurs after a single injection; it is performed by giving 2 doses of tuberculin at the same injection site 7 days apart. Both tests are relatively sensitive. As a result, they may be used in postpartum cows as well as for the testing of heavily infected animals. Repeated tuberculin testing results in a period of decreased reactivity and the induction of antibodies against *M. bovis* antigen HSP 70.

28.4 TUBERCULIN REACTIONS IN OTHER ANIMALS

Tuberculin skin testing has never been a widely employed procedure in domestic animals other than cattle, so information on these animals is scanty. Nevertheless, it appears that the ability of different species to mount a classic tuberculin reaction varies greatly. In pig and cat, for example, the tuberculin test is unreliable, being positive for only a short period following infection. In pig and dog, the best test is an SID test given in the skin behind the ear, whereas in the cat the short thermal test is probably best. In sheep and goat, the antigen is usually given in the anal fold, but the results are usually unreliable in these species as well. Horses appear to be unusually sensitive to tuberculin, and the dose used must be reduced accordingly. Nevertheless, the results obtained do not always correlate well with the disease status of the animal. In birds, good reactions may be obtained by inoculating tuberculin into the wattle or wing web.

28.5 JOHNIN REACTIONS

Animals infected with *M. avium* var. *paratuberculosis,* the cause of Johne's disease, may develop a delayed hypersensitivity reaction following intradermal inoculation of an extract of this organism called johnin. Johnin can be used in a SID test but, like tuberculin, may give a negative result in animals with clinical disease. An intravenous johnin test is positive in these cases and may be a preferable alternative to the SID test. In this test the antigen is administered intravenously and the animal's temperature is noted 6 hours later. A rise in temperature of 1° C or neutrophilia is considered a positive result. These tests are probably of limited usefulness in individual animals but may help identify infected herds.

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28.6 OTHER SKIN TESTS

Positive delayed hypersensitivity skin reactions may be obtained in any infectious disease in which cell-mediated immunity has a significant role. Thus extracts of *Brucella abortus* have been used from time to time in attempts to diagnose brucellosis. These include brucellin, a filtrate of a 20-day broth culture, and brucellergen, a nucleoprotein extract. Because these preparations may stimulate production of antibodies to brucella, they cannot be employed in areas where eradication is monitored by serological tests. In glanders of horses, a culture filtrate of the organism *Pseudomonas mallei*, termed mallein, is used for skin testing. Mallein can be used in either a short thermal test or an ophthalmic test. An ophthalmic test, also occasionally employed in tuberculosis, is performed by dropping the antigen solution into an eye. Transient conjunctivitis develops if the test is positive. Another method of testing for glanders is the intrapalpebral test. In this test, mallein is injected into the skin of the lower eyelid, where a positive reaction results in swelling and ophthalmia.

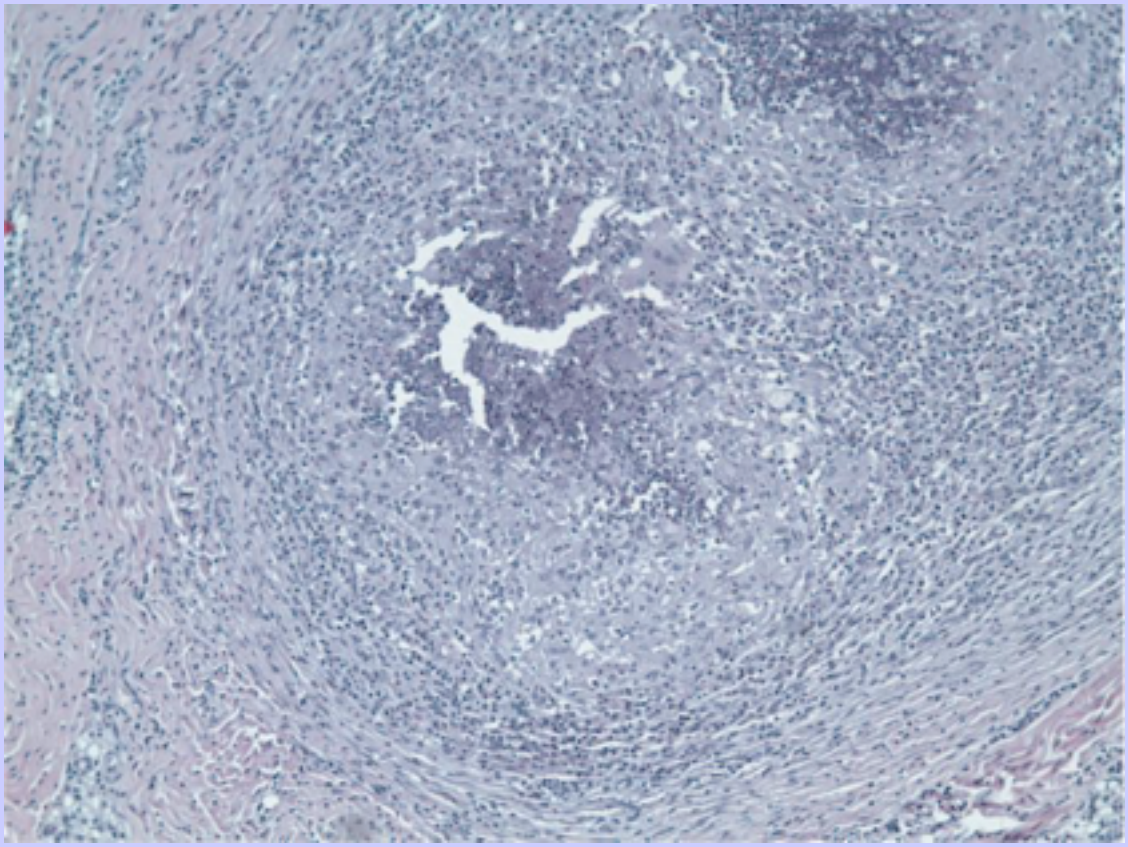
Intradermal skin testing with microbial extracts is also employed in the diagnosis of many fungal diseases; thus histoplasmin is used for histoplasmosis, coccidioidin in coccidioidomycosis, and so on. In these cases, the tests are not very specific, and the test procedure may effectively sensitize the tested animal, causing it to become serologically positive. This problem also arises when toxoplasmin is used in attempts to diagnose toxoplasmosis (see Chapter 24).

28.7 PATHOLOGICAL CONSEQUENCES OF TYPE IV HYPERSENSITIVITY

28.7.1 Tubercle Formation

Although the tuberculin reaction induced by intradermal inoculation is artificial in that antigen is administered by injection, a similar inflammatory response occurs if living tubercle bacilli lodge in tissues and sensitize an animal. However, *M. tuberculosis* is resistant to intracellular destruction until M1 macrophages are activated by Th1 cells (see Chapter 16), and dead organisms are very slowly removed because they contain large quantities of poorly metabolized waxes. As a result, the reaction to whole organisms is prolonged, and macrophages accumulate in very large numbers. Many of these macrophages ingest the bacteria but fail to prevent its growth and so die. Other macrophages fuse to form multinucleated giant cells. After 4 to 5 weeks of infection, microscopic granulomas enlarge and coalesce. The lesion that develops around invading tubercle bacilli therefore consists of a mass of necrotic debris containing both living and dead organisms surrounded by a layer of fibroblasts, lymphocytes, and macrophages, which in this location are called epithelioid cells (see Chapter 4). The entire lesion is called a tubercle (Figure 28-4). The mycobacteria are unable to multiply within the caseous tissue because of its low pH and lack of oxygen. Nevertheless, some bacteria may survive in a dormant state. If the host mounts an adequate immune response of the correct (Th1) type, this may be sufficient to control the infection. However, if immunity is insufficient or inappropriate (Th2), the organisms may escape from the tubercle and spread to local lymph nodes and nearby tissues. When the response is inadequate, the multiplying organisms continue to spread, and the resulting lung damage together with liquefaction of the caseous center of the tubercle leads to rapidly progressive disease. Granuloma formation is also a common result of persistent chronic inflammation. This inflammation may be of immunological origin, as in tuberculosis or brucellosis in some species, or it

FIGURE 28-4 A histological section from the lymph node of a cow infected with *Mycobacterium bovis* showing a small tubercle. The dark central mass is caseous material. It is surrounded by layers of macrophages and lymphocytes and walled off by fibroblasts. (Courtesy Dr. John Edwards.)



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may occur as a result of the presence in tissues of other chronic irritants. For example, granulomas may arise in response to the prolonged irritation caused by talc or asbestos particles. 374

28.7.2 Allergic Contact Dermatitis

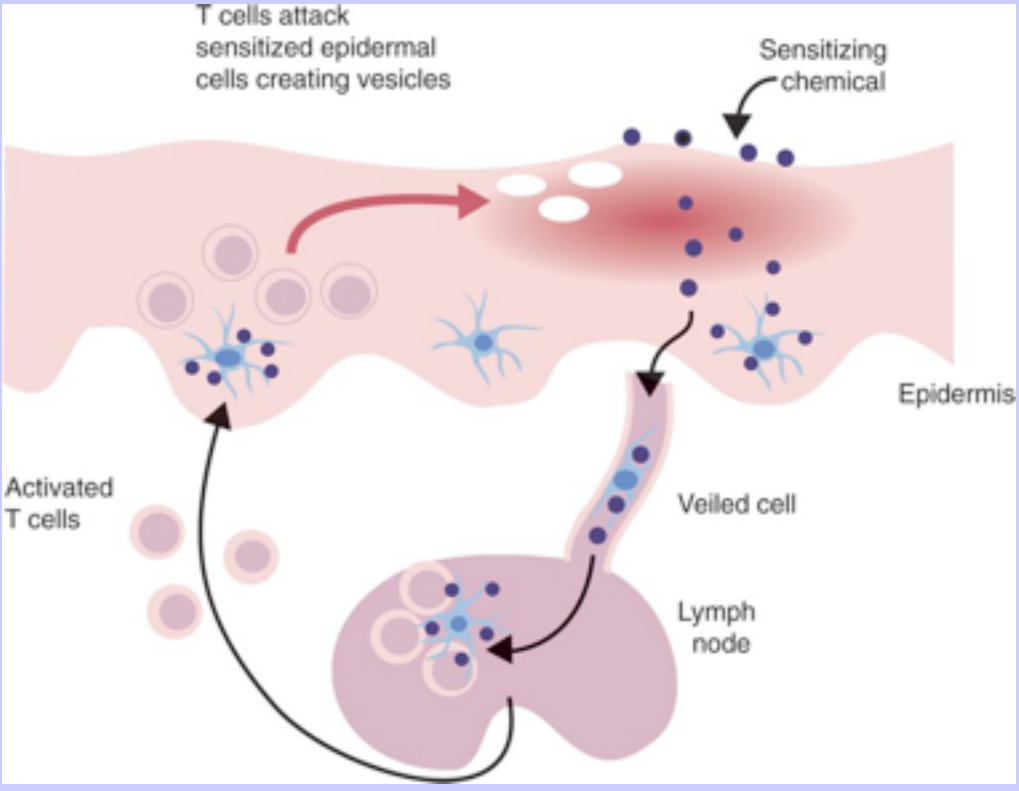
If reactive chemicals are painted onto the skin, they may bind to skin proteins and the resulting complexes are processed by Langerhans cells in the dermis (Figure 28-5). Depending on the antigen, the Langerhans cells may bind the antigen directly to MHC molecules on the cell surface or process the hapten internally into a complete antigen. The Langerhans cells then migrate to draining lymph nodes through afferent lymphatics and present the antigen to T cells. While presenting the antigen, the Langerhans cells secrete large amounts of IL-12 and IL-18, to which Th1 cells respond. These cells in turn produce large amounts of IFN-γ and promote the activities of cytotoxic T cells. Following exposure to an antigen in sensitized animals, macrophages and lymphocytes infiltrate the dermis by 24 hours. Eventually, the cytotoxic T cells destroy and remove the altered cells, resulting in the development of intraepithelial vesicles. This inflammatory reaction presents as an intensely pruritic skin

disease called allergic contact dermatitis. In addition to α/β T cells, other cell types, such as γ/δ T cells, B-1 cells, and natural killer (NK) T cells, may be involved in the reaction.

Recent studies have demonstrated that contact dermatitis can be readily induced in mice that lack all types of lymphocytes except NK cells. In addition, contact dermatitis appears to be antigen-specific insofar as primed animals mount a much stronger response than unprimed animals. This appears to be a property of a subpopulation of NK cells. These NK cells can survive for at least 28 days in mice and so form a memory cell population. These results clearly are at variance with our previous ideas about the antigenic specificity of NK cells and their role in immunity. It is also of interest to note that contact dermatitis will not occur in skin that lacks functional nerve fibers. Clearly allergic dermatitis has a complex and poorly understood etiology.

The chemicals that induce allergic contact dermatitis are usually highly reactive molecules that combine chemically with skin proteins; they include formaldehyde, picric acid, aniline dyes, plant resins and oils, organophosphates, some topical medications such as neomycin, and salts of metals such as nickel and beryllium (Figure 28-6). Thus allergic contact dermatitis can occur on pathologists' fingers as a result of exposure to formaldehyde; on the ears of dogs treated with neomycin for otitis externa; on the foot pads, scrotum, and ventral abdomen of dogs on exposure to some carpet dyes and deodorizers; on parts of the body exposed to the oils (urushiol) of the poison ivy plant *(Rhus radicans)*; and around the neck of animals as a result of exposure to dichlorvos (2,2-dichlorovinyldimethylphosphate) in flea collars (Box 28-1). Severe lesions may develop on the teats of dairy cattle as a result of a contact dermatitis to a component of the rubber in a milking machine (*N*-isopropyl-*N*-phenyl diamine). Allergic contact dermatitis involving the muzzle of dogs has been reported to result from sen

FIGURE 28-5 The pathogenesis of allergic contact dermatitis.



sitivity to components of plastic food bowls. Some dogs, instead of developing the more usual type I hypersensitivity to pollen proteins, experience an allergic contact dermatitis as a result of a type IV hypersensitivity to pollen resins. It is unusual for allergic contact dermatitis to affect the haired areas of the skin unless the allergen is in a liquid. Thus allergic contact dermatitis to shampoo components may result in total-body involvement. The period required for sensitization ranges from 6 months to several years.

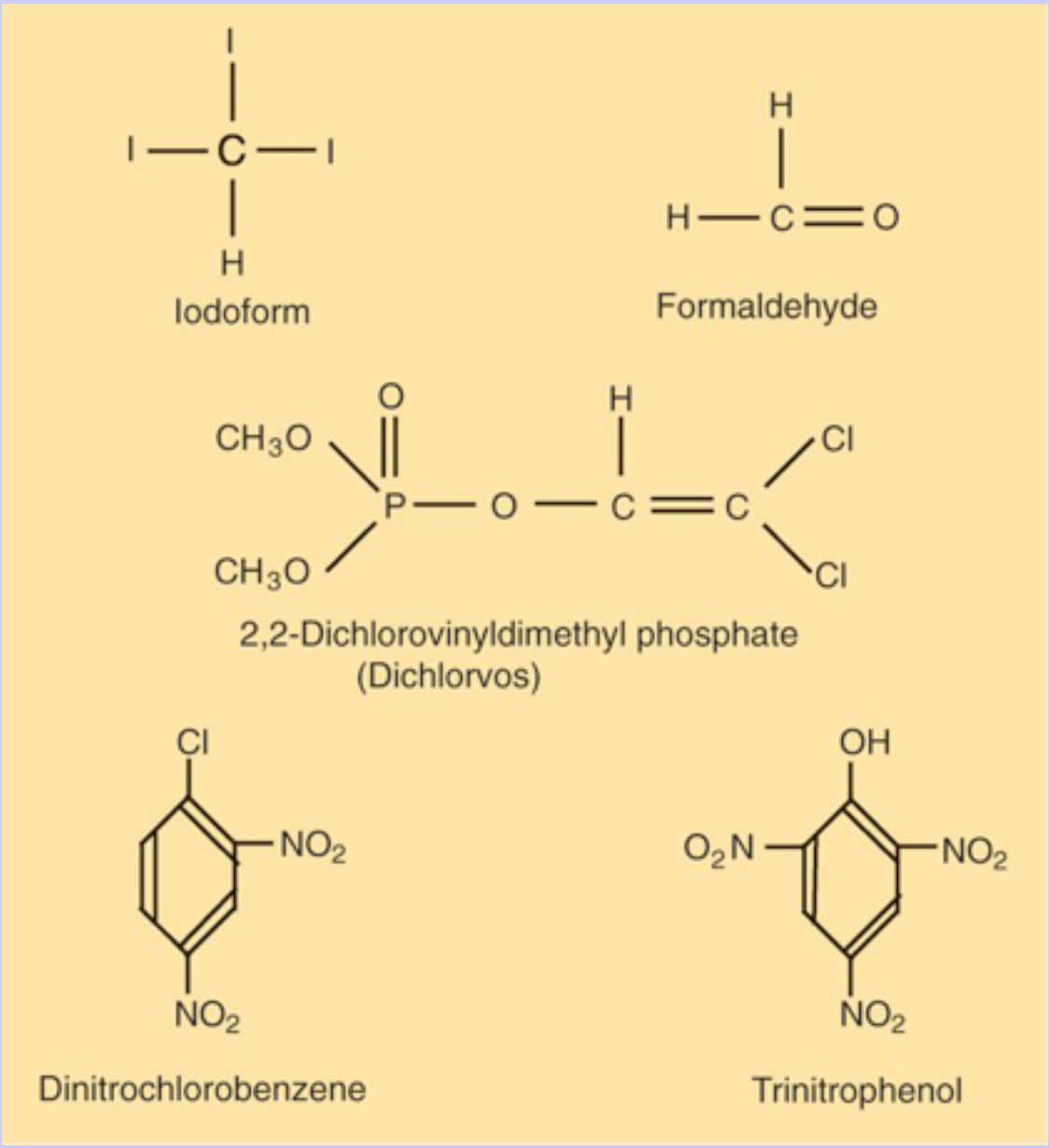
The lesions of allergic contact dermatitis vary in severity, ranging from a mild erythema to a severe erythematous vesiculation. However, because of the intense pruritus, self-trauma, excoriation, ulceration, and secondary staphylococcal pyoderma often mask the true nature of the lesion. If the exposure to the allergen persists, hyperkeratosis, acanthosis, and dermal fibrosis may eventually occur. Histologically, the lesion is marked by a mononuclear cell infiltration and vacuolation of skin cells under attack by cytotoxic T cells (Table 28-2).

Allergic contact dermatitis is diagnosed by removal of the suspected antigen and by patch testing. In “closed” patch tests, suspected allergens are used to impregnate gauze swabs that are then attached to the shaved skin with tape. After 48 to 72 hours the dressing is removed and the areas in contact with the swabs examined. A positive reaction is indicated by local erythema and vesiculation. Closed patch tests may be impractical for some dogs and cats. An “open” patch test may therefore be employed. In this procedure, a solution of the suspected allergen is applied to shaved normal skin and the area examined daily for up to 5 days. Identification of the offending allergen and its avoidance by the animal are the optimal therapies for allergic contact dermatitis. Hyposensitization therapy is not effective. Steroids are used in acute cases, with antibiotics to control secondary infections.

28.7.3 Stevens-Johnson Syndrome

Three related mucocutaneous disorders—erythema multiforme, Stevens-Johnson syndrome, and toxic epi

FIGURE 28-6 Some of the simple chemicals that can cause allergic contact dermatitis.



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dermal necrolysis—are well recognized in humans and have been diagnosed in dogs and cats. The three diseases are characterized by lesions of increasing severity. Erythema multiforme is characterized by patchy skin loss and low morbidity; Stevens-Johnson syndrome is more severe but involves less than 10% of the body surface; toxic epidermal necrolysis is much more serious with affected individuals losing more than 30% of their epidermis. Mortality is high. The three conditions however, overlap considerably. Stevens-Johnson syndrome and toxic epidermal necrolysis are believed to involve a T cell–mediated hypersensitivity to drugs. Erythema multiforme is not associated with drug administration. Affected animals develop vesicles, shed large areas of epidermis, and

develop skin ulcers as a result of widespread apoptosis of their keratinocytes. The apoptosis is believed to result from drugs or their metabolites binding to the epidermal cells, upregulating CD95L expression as well as the production of soluble CD95L, and so triggering their destruction by cytotoxic T cells. Skin lesions are infiltrated mainly by $CD8^+$ T cells and fewer $CD4^+$ cells. Many different drugs may trigger these responses, but common inducers in dogs include trimethoprim-potentiated sulfonamides, beta-lactam antibiotics, penicillin, and cephalexin. Beginning about 14 days after drug exposure, the skin begins to blister and slough. Animals develop generalized illness including dyspnea, vomiting, fever, and weight loss. In dogs, sloughing of the epidermis occurs over the nasal planum, the footpads, and the oral, pharyngeal, nasal, conjunctival and preputial mucosa. Fluid loss leads to electrolyte imbalances while life-threatening secondary infections are common. Biopsies show extensive epidermal cell death.

Table 28-2 Comparison of the Major Forms of Allergic Dermatitis

	Atopic Dermatitis	Allergic Contact Dermatitis
Pathogenesis	Type I hypersensitivity	Type IV hypersensitivity
Clinical signs	Hyperemia, urticaria, pruritus	Hyperemia, vesiculation, alopecia, erythema
Distribution	Face, nose, eyes, feet, perineum	Hairless areas, usually ventral abdomen and feet
Major allergens	Foods and pollens, fleas, inhaled allergens	Reactive chemicals, dyes in contact with skin
Diagnosis	Intradermal testing, immediate response	Delayed response on patch testing
Pathology	Eosinophilic infiltration, edema	Mononuclear cell infiltration, vesiculation
Treatment	Steroids, antihistamines, hyposensitization	Steroids

28.7.3.1

Box 28-1 Sources of Contact Allergens in Animals

- Insecticides in flea collars
    - In sprays
    - In dips
- Wood preservatives
- Floor waxes
- Carpet dyes
- Some pollens
- Dermatological drugs (creams, ointments)
- Leather products
- Paints
- House plants

Treatment involves immediate withdrawal of the offending drug followed by symptomatic treatment including fluid replacement. Corticosteroids should be avoided since they increase the animal's susceptibility to skin infections and worsen the prognosis. Antibiotics should only be administered if skin infections occur. Intravenous administration of high doses of human immunoglobulins have been used successfully to treat this disease in dogs. It is believed that these immunoglobulins block CD95/CD95-ligand interactions and so prevent keratinocyte apoptosis.

28.8 MEASUREMENT OF CELL-MEDIATED IMMUNITY

Although diagnostic immunology is based largely on the detection of serum antibodies, measurement of cell-mediated immune responsiveness in animals may be desirable under some circumstances. For example, in determining the effectiveness of a vaccine, one must take into account that serum antibody levels may not truly reflect the degree of immunity possessed by an animal. Animals without detectable antibodies may possess significant cell-mediated immunity. The term *cell-mediated immunity* encompasses a diverse set of mechanisms that employ T cells and macrophages for protection. Currently, both in vivo and in vitro techniques are used for this purpose.

28.8.1 In Vivo Techniques

The simplest in vivo test of cell-mediated immunity is an intradermal skin test such as the tuberculin test. The inflammation and swelling that occur in response to intradermally injected antigens may be considered cell mediated, provided that it has the characteristic time course and histological features of a type IV reaction. Intradermal skin tests are not always convenient, they are difficult to quantitate, and injection of an antigen may effectively sensitize an animal, thus preventing further testing.

It is sometimes useful to measure the ability of an animal to mount cell-mediated immune responses in general rather than to one specific antigen. One way to do this is to give the animal a small skin allograft and measure its survival time. A much simpler technique is to paint a small area of the animal's skin with a sensitizing chemical such as dinitrochlorobenzene. The intensity of the resulting allergic contact dermatitis provides a rough estimate of the animal's ability to mount a cell-mediated immune response.

If the T cell–stimulating lectin phytohemagglutinin is injected intradermally, it provokes a local tissue reaction with many features of a delayed hypersensitivity response. In pigs, for example, this reaction is characterized by infiltration with γ/δ^{+} $CD4^{-}$ $CD8^{-}$ T cells. This is a very convenient and rapid method of assessing an animal's ability to mount a cell-mediated response without the need for first sensitizing the animal to an antigen. However, the response to phytohemagglutinin is nonspecific and its interpretation may be difficult.

28.8.2 In Vitro Techniques

In vitro tests are designed to measure the antigen-specific activation and proliferation of T cells. These also include their cytotoxic activities and their production of cytokines. All of these tests require that T cells be grown in cell culture; therefore few are useful in the field.

To measure T cell proliferation in response to an antigen, a suspension of purified peripheral blood lymphocytes from the animal to be tested is mixed with the antigen and cultured for 48 to 96 hours. Twelve hours before harvesting, thymidine labeled with the radioactive isotope tritium is added to the cultures. Normal, nondividing lymphocytes do not take up thymidine, but dividing cells do because they are actively synthesizing DNA. Thus,

if the T cells are proliferating, they will take up the tritiated thymidine and their radioactivity will provide a measure of the amount of proliferation. The greater the response of the cells to an antigen, the greater will be their radioactivity. The ratio of the radioactivity in the stimulated cultures to the radioactivity in the controls is called the stimulation index. A related technique is to measure the proliferation of lymphocytes in response to mitogenic lectins (see Chapter 11). The intensity of the lymphocyte proliferative response, as measured by tritiated thymidine uptake, provides an estimate of the reactivity of an animal's lymphocytes.

Radioactive tritium may be replaced in proliferation assays by a simple colorimetric enzyme assay. Methylthiazoldiphenyltetrazolium bromide (MTT) is a pale yellow compound that serves as a substrate for active mitochondrial enzymes. The enzymes change the MTT color to dark blue. The intensity of this color change is a measure of the number of living cells in a culture. Thus in proliferation assays, the number of living cells increases, and this can be measured colorimetrically. The test is sufficiently sensitive to quantify the increase in T cell numbers triggered by antigen or mitogens.

To measure T cell–mediated cytotoxicity it is necessary to have a simple method of measuring cell death. This is usually based on the fact that living cells take up and retain chromium ions but if the cell dies the chromium is released into the extracellular fluid. Radioactive sodium chromate (^{51}Cr) may be used in this way to label target cells. Lymphocytes from an immune animal are mixed in an appropriate ratio with ^{51}Cr-labeled target cells. The mixture is then incubated for 4 to 24 hours at 37° C. At the end of this time, the cell suspension is centrifuged and the presence of ^{51}Cr in the supernatant measured. The amount of chromium released is related directly to the number of target cells killed. The amount of chromium released in the absence of cytotoxic cells must also be measured and subtracted from that released in the presence of cytotoxic cells in order to get a true reading.

A third in vitro assay is the measurement of cytokine release by T cells. One such technique involves assaying the release of IFN-γ by peripheral blood lymphocytes on exposure to tuberculin or to purified mycobacterial proteins. This technique has been developed as an alternative to the tuberculin test for the diagnosis of tuberculosis in cattle and deer. It involves adding tuberculin PPD to heparinized blood and incubating the mixture for 24 to 48 hours at 37° C. The plasma is then removed and assayed for any interferon produced, either by means of a simple bioassay or preferably by use of a sandwich enzyme-linked immunosorbent assay (ELISA) employing monoclonal antibodies. Three “antigens” are used: no antigen (negative control), *M. bovis* PPD, and *M. avium* PPD. The M. avium PPD is used to detect false-positive cross-reactions. Purified, recombinant mycobacterial proteins can reduce the incidence of false-positives even further. This technique has advantages over conventional tuberculin tests in that it does not compromise the immune status of the animal under test by injection of antigen. In addition, the animal does not have to be held for several days for the test to be read. It is also much simpler than other in vitro tests for cell-mediated immunity. The assay is at least as sensitive as the SID test and, if purified recombinant mycobacterial proteins are employed, is highly specific. (Its sensitivity is about 85%, and its specificity is as high as 90% to 99%.) Positive results are obtained earlier than by skin testing. However, it does appear to detect a slightly different population of animals than the skin test. It has also been successfully used to diagnose Johne's disease in sheep.

28.8.2.1

Enzyme-Linked Immunospot Assay

It is possible to use a variation of a sandwich ELISA (see Chapter 38) in order to determine the frequency of cytokine-secreting cells (Figure 28-7). In this assay a capture-antibody directed against the cytokine of interest is coated on the bottom of plastic tissue culture wells. The cells to be tested are cultured on this surface and exposed to the antigen of interest. Any cytokine secreted by these cells will bind to nearby capture-antibodies. Once the culture period is completed, the presence of this bound cytokine is detected by a conventional

sandwich ELISA. This results in the development of a pattern of colored spots that correspond to the location of the cytokine-secreting cells. These spots can be counted and the frequency of specific cytokine-producing cells determined. This assay can also be used to quantitate cytotoxic cells by detecting granzyme or perforin production.

Although all of the assays described above can be used to measure at least some aspects of cell-mediated immunity, none provides a complete picture. The investigator may of course be simply interested in the response to a single antigen or organism. In these cases either a skin test or an in vitro assay may be appropriate. This is best exemplified by the tests available for the diagnosis of tuberculosis. In vitro tests are also useful if the time course of a cell-mediated immune response is to be examined. Repeated testing can be performed simply by obtaining more lymphocytes. If, on the other hand, an investigator wishes to obtain an overview of an animal's abilities in this area, then one of the nonspecific in vivo assays may be

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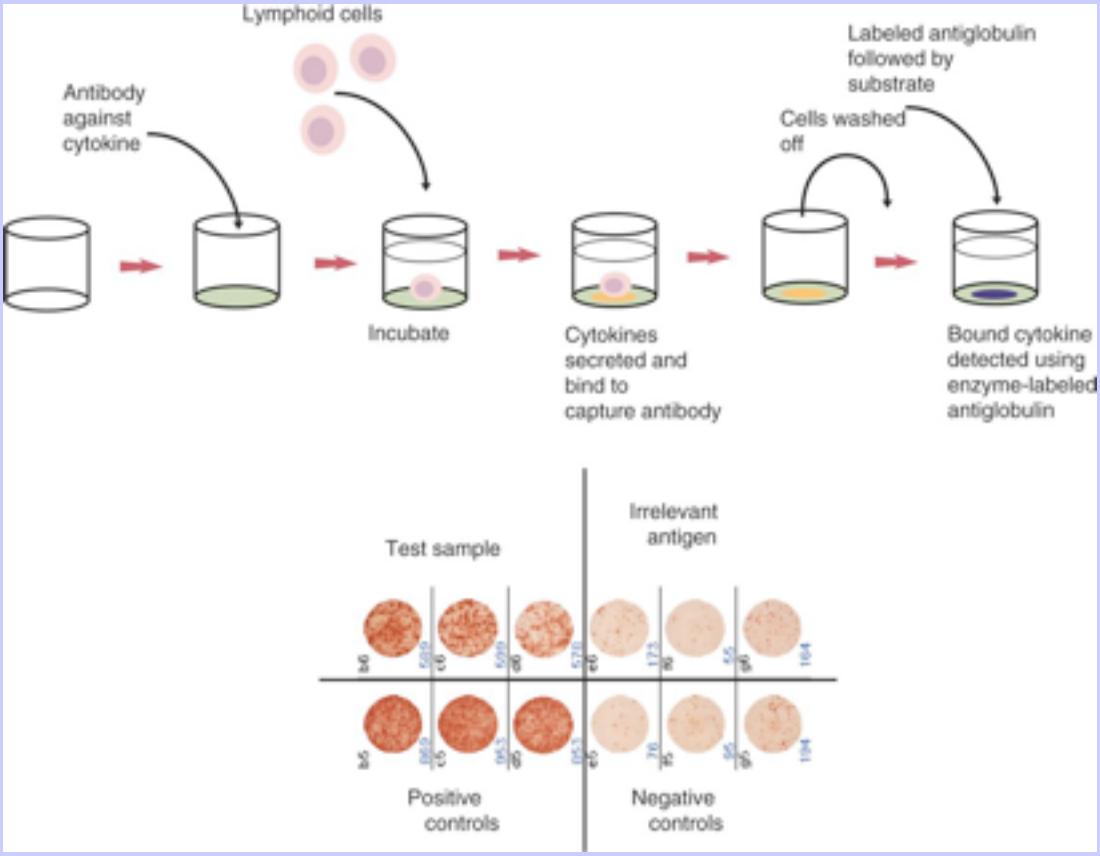
FIGURE 28-7 The principles of the enzyme-linked immunospot assay. The photograph shows the interferon-γ response of bovine peripheral blood mononuclear cells exposed to a defined *Anaplasma marginale* antigen. (Courtesy Dr. W. Mwangi.)



more appropriate. These can be useful, for example, in assessing immune function in young animals thought to be immunodeficient. However, it is important to point out that in these animals a complete hematological examination should be performed before more complex assays are considered. It is also prudent to measure

the important lymphocyte subpopulations by flow cytometry. An animal that has no T cells is unlikely to mount any sort of cell-mediated response.

28.9 SOURCES OF ADDITIONAL INFORMATION

PW Askenase: The role of basophils in health and disease. *Res Staff Phys*. **32**, 1986, 33–41.

PW Askenase, M Van Loveren: Delayed-type hypersensitivity: activation of mast cells by antigen-specific T cell factors initiates the cascade of cellular interactions. *Immunol Today*. **4**, 1983, 259–264.

WH Chambers, PH Klesius: Direct bovine leukocyte migration inhibition assay: standardization and comparison with skin testing. *Vet Immunol Immunopathol*. **5**, 1983, 85–95.

NE Clough, JA Roth: Methods for assessing cell-mediated immunity in infectious disease resistance and in the development of vaccines. *J Am Vet Med Assoc*. **206**, 1995, 1208–1216.

AM Dannenberg, Jr.: Delayed-type hypersensitivity and cell-mediated immunity in the pathogenesis of tuberculosis. *Immunol Today*. **12**, 1991, 228–233.

ML Doherty, ML Monaghan, HF Bassett, et al.: The sequential cellular changes which characterize the tuberculin reaction in cattle. *Vet Immunol Immunopathol*. **35**(Suppl), 1993, 70–71.

MP Godfrey, ME Phillips, PW Askenase: Histopathology of delayed-onset hypersensitivities in contact-sensitive guinea pigs. *Int Arch Allerg Appl Immunol*. **70**, 1983, 50–58.

REW Halliwell, KR Schemmer: The role of basophils in the immunopathogenesis of hypersensitivity to fleas (Ctenocephalis felis) in dogs. *Vet Immunol Immunopathol*. **15**, 1987, 203–213.

M Holzhauer, OC Sampimon, J Sol, et al.: Allergic contact dermatitis of bovine teat skin caused by milking machine cluster rubber. *Vet Rec*. **154**, 2004, 208–209.

GS Kelner, J Kennedy, KB Bacon, et al.: Lymphotactin: a cytokine that represents a new class of chemokine. *Science*. **266**, 1994, 1395–1399.

HE Kennedy, MD Welsh, DG Bryson, et al.: Modulation of immune responses to Mycobacterium bovis in cattle depleted of $WC1^{+}$ gd T cells. *Infect Immun*. **70**, 2002, 1488–1500.

CG Larsen, MK Thomsen, B Gesser, et al.: The delayed type hypersensitivity reaction is dependent on IL-8. *J Immunol*. **155**, 1995, 2151–2157.

JW Lens, HA Drexhage, W Benson, BM Balfour: A study of cells present in lymph draining from a contact allergic reaction in pigs sensitized to DNFB. *Immunology*. **49**, 1983, 415–422.

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WI Morrison, FJ Bourne, DR Cox, et al.: Pathogenesis and diagnosis of infections with Mycobacterium bovis in cattle. *Vet Rec*. **146**, 2000, 236–242.

TJ Nuttall, T Malham: Successful intravenous human immunoglobulin treatment of drug-induced Stevens-Johnson syndrome in a dog. *J Small Anim Pract*. **45**, 2004, 357–361.

P Parham: Adaptable innate killers. *Nature*. **441**, 2006, 415–416.

JM Pollock, RM Girvin, KA Lightbody, et al.: Assessment of defined antigens for the diagnosis of bovine tuberculosis in skin test reactor cattle. *Vet Rec*. **146**, 2000, 659–665.

KT Schultz, HC Maguire: Chemically-induced delayed hypersensitivity in the cat. *Vet Immunol Immunopathol*. **3**, 1982, 585–590.

GB Toews, PR Bergstresser, JW Streilein: Epidermal Langerhans cell density determines whether contact hypersensitivity or unresponsiveness follows skin painting with DNFB. *J Immunol*. **124**, 1980, 445–453.

B Wang, C Feliciani, BG Howell, et al.: Contribution of Langerhans cell–derived IL-18 to contact hypersensitivity. *J Immunol*. **168**, 2002, 3303–3308.

DL Whipple, CA Bolin, AJ Davis, et al.: Comparison of the sensitivity of the caudal fold skin test and a commercial g-interferon assay for diagnosis of bovine tuberculosis. *Am J Vet Res*. **56**, 1995, 415–419.

A Whyte, DO Haskard, RM Binns: Infiltrating gdT cells and selectin endothelial ligands in the cutaneous phytohemagglutinin-induced inflammatory reaction. *Vet Immunol Immunopathol*. **41**, 1994, 31–40.

MM Wong, EN Fish: Chemokines: attractive mediators of the immune response. *Semin Immunol*. **15**, 2003, 5–14.

PR Wood, LA Corner, P Plackett: Development of a simple, rapid in vitro cellular assay for bovine tuberculosis based on the production of g-interferon. *Res Vet Sci*. **49**, 1990, 46–49.

PR Wood, LA Corner, JS Rothel, et al.: Field comparison of the interferon-gamma assay and the single intradermal tuberculin test for the diagnosis of bovine tuberculosis. *Aust Vet J*. **68**, 1991, 286–290.