Methods for assessing cell-mediated immunity in infectious disease resistance and in the development of vaccines

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Immunologic evaluation of vaccines has depended largely on the measurement of an antibody response in the serum of recipient animals. This approach, which worked well for diseases prevented in large part by humoral immunity, ignored the contributions cell-mediated immunity (CMI) or mucosal immunity make to providing protection against many infectious diseases. As a result of this approach, certain vaccines were marketed that produced a satisfactory antibody response but failed to protect vaccinates in the field from disease. In the modern biologics industry, there is increasing pressure for vaccine improvements and new technologic advancements to be based on a sound understanding of the entire basis for immunity, including cell-mediated, mucosal, and humoral immunity that develops after exposure to infectious disease agents.

Historically, the role of antibodies in the development of immunity against most diseases of veterinary importance has been studied more extensively than has the role of CMI; however, our knowledge of cell-mediated immune processes and our technologic capability for study of them has expanded rapidly in recent years. Currently, a considerable amount of research is aimed at characterizing CMI. In a rational immunologic approach to vaccine development, some questions must be addressed when assessing the development of CMI against a specific disease. Does CMI have an important role in providing protection against the disease? What are the CMI responses that develop after vaccination or natural infection with the infectious agent, and how can these responses be measured? Which antigenic components of the infectious agent are targets of the protective CMI responses? Which CMI effector functions provide protection against the disease? What vaccination strategy (antigen delivery system, adjuvant formulation) induces the most protective CMI response against the infectious agent?

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Importance of CMI in Providing Protection Against Specific Diseases

The importance of CMI against a specific disease sometimes is studied by determining that induction of an active antibody response or the acquisition of passively acquired antibody to the causative agent does not correlate well with protection against the disease. With this approach, it is often assumed that if antibodies do not prevent signs of disease, then other immune mechanisms (cell-mediated or mucosal) must be important for protective immunity. In some viral diseases, such as African swine fever,[1] antibodies are nonneutralizing and coexist with virus in the serum. In these cases, CMI responses or other immune mechanisms are assumed to be critical for protective immunity. In diseases such as pseudorabies in swine,[2] neutralizing antibodies may be generated systemically and may be effective against viremia, but a correlation has not been detected between the concentrations of antibodies detected in mucosal or tracheal washings and protection against localized virus challenge-exposure. In such diseases, cell-mediated, mucosal, and humoral immunity may all play important roles in protective immunity.

The correlation between antibody concentrations and disease protection may vary depending on the model used to study specific immunity. Antibodies to bluetongue virus may protect against homotypic, but not heterotypic, virus challenge-exposure[3]; a similar mechanism may exist for equine influenza virus.[4] Antibodies to rotavirus are detected inconsistently in the serum,[5,6] making it difficult to assess the correlation between humoral immunity and disease protection, but suggesting that immune mechanisms other than antibody production may be important. Protective immunity to *Pseudomonas aeruginosa* can be induced in mice that do not have evidence of a concurrent antibody response.[7] Antibodies in the serum appear to play a role in providing protection against *P aeruginosa*, but are not essential for protective immunity.

The importance of CMI in providing protection against specific diseases may also be assessed by suppressing a specific arm of the immune system and evaluating the severity of disease after subsequent

challenge exposure. Pharmacologic suppression of CMI responses may be achieved by treating animals with drugs such as cyclosporine A. Treatment of research subjects with cyclosporine A, which specifically inhibits a key cytokine (ie, interleukin-2) necessary for T-lymphocyte proliferation, has been used to determine the importance of T-lymphocytes in the control of numerous diseases.[8-10] Selective immunosuppression may also be achieved by surgical removal of primary lymphoid tissues. Removal of the thymus at the time of birth eliminates most T-lymphocytes. Humoral immunity may be effectively suppressed in avian species by removal of the bursa of Fabricius, which is the site of B-lymphocyte differentiation, at the time of hatching.[11] Immunologic responses after thymectomy or bursectomy have been compared to determine the relative importance of humoral immunity and CMI against diseases of birds, such as neural lymphomatosis.[12] Although drug administration and surgery can be useful tools in the study of CMI, the immunosuppressive effects associated with their use tend to be broad and are not always well-defined. The T- and B-lymphocytes interact to generate a complete antibody response, so generalized T-cell suppression also is likely to have an impact on antibody production.[13] Immunosuppression induced by anti-lymphocyte antibodies is more specific than that induced by drugs and surgery, because only cells possessing the cell surface marker targeted by the antibodies are eliminated, leaving phenotypically distinct lymphocytes and surrounding tissues intact. Anti-lymphocyte antibodies may be directed against a cell surface marker common to all T-lymphocytes,[14] or they may be directed against specific T-lymphocyte subsets.[15]

Measurement of Antigen-specific CMI Function

Once it has been established that CMI is important in providing protection against a specific disease agent, measurement of CMI can be accomplished by several methods. Some methods are general in nature. That is, in their unmodified forms, they may detect a cellular response to microbial exposure, but may not offer information as to the specific cell populations involved and may not identify the specific microbial antigens that elicited the cellular response. Other methods measure a specific effector function of CMI (cytotoxicity or release of specific cytokines).

Delayed-type hypersensitivity (DTH) assay—One of the first assays of CMI function that was reported is the DTH assay. The best known example of a DTH assay is the official screening test for tuberculosis. Animals previously exposed to tuberculosis-causing *Mycobacterium* organisms develop a localized swelling 48 to 72 hours after intradermal injection with tuberculin antigens.[16] The swelling is composed largely of macrophages and other mononuclear cells attracted to the injection site by soluble substances, called cytokines, that are released from sensitized antigen-specific lymphocytes.

Delayed-type hypersensitivity assays have been used to measure CMI responses to bacteria,[17,18] viruses,[19-21] rickettsiae,[22,23] mycoplasmas,[24] and protozoa.[25] Delayed-type hypersensitivity assays are a popular means for measuring CMI responses, because they are economical to perform and do not require sophisticated laboratory equipment. They do, however, require careful and consistent antigen preparation, intradermal injection technique, and lesion interpretation. Delayed-type hypersensitivity assays can be a qualitative test in that they can differentiate sensitized animals from nonsensitized ones, but they also can be semiquantitative if the magnitude of the CMI response is correlated with the size of the resultant swelling. In a report, lesion size has been correlated with the magnitude of protection[18]; however, other reports have not detected a strong correlation between lesion size and amount of protection.[22,24]

Leukocyte migration inhibition assay—Leukocyte migration inhibition tests are another means of measuring CMI activity. In these assays, preparations of leukocytes are incubated with antigen, which stimulates sensitized T-lymphocytes in the preparations to release cytokines. Certain cytokines released by the lymphocytes inhibit leukocyte motility, a property that, in vivo, serves to enhance deposition of inflammatory cells in areas of infection. The movement of leukocytes under an agarose gel[20,26] or out of a capillary tube[27] is measured and compared with movement of a similar population of cells that were not stimulated with antigen.

Cytokines responsible for inhibiting leukocyte migration probably also play a role in the generation of DTH lesions, but CMI responses detected by the 2 assays do not always correlate well.[26] The leukocyte migration inhibition assay, like the DTH assay, is relatively simple and economical to perform. It has been used successfully with leukocytes obtained from the blood of several mammalian species.[28-31] It is used frequently in studies in birds[26,27,32,33] and may be more sensitive than DTH assays for the detection of CMI in chickens.[34]

Adoptive transfer assays—Adoptive transfer assays measure the ability of sensitized lymphocytes from an immune animal to confer immunity to a naive animal.[35] Just as the administration of serum obtained from an immune animal to a naive animal provides passive immunity to diseases in which humoral immunity plays a substantial role in protection, so can lymphocyte transfer provide passive protection to diseases in which CMI plays an important role. After the lymphocytes are administered to the recipient animal, the effect is usually measured as resistance to challenge exposure,[36,37] but may be assessed through DTH[38] or some other functional assay.

An animal possesses numerous subsets of lymphocytes, each with a unique phenotype. Infectious agents may preferentially stimulate certain lymphocyte subsets. Lymphocyte subsets responsible for protective CMI can be detected by the use of an adoptive transfer assay in which the donor lymphocytes are fractionated prior to transfer. Donor lymphocytes can be enriched to contain specific lymphocyte subsets by lysing contaminating lymphocytes with specific antiserum,[39,40] or flow cytometry can be used to purify lymphocyte subsets expressing differentiating surface molecules.[41]

One of the major complications of adoptive transfer assays is that donor and recipient animals must be matched for major histocompatibility complex (MHC) antigens, or the transfered lymphocytes will be rejected by the recipient animal. This is an easy obstacle to overcome in mice in which highly inbred, MHC-matched strains are available, but it makes studies in conventional animals difficult. Adoptive transfer studies have been performed, using chimeric twin calves[42] and monozygotic twin lambs,[43] but the limited availability of such twins makes this approach impractical for widespread use.

Lymphocyte proliferation assays—Proliferation assays are widely used as a means of measuring CMI. Cellular proliferation is important in vivo for the expansion of reactive lymphocyte populations. In animals that have not developed CMI to a specific antigen, the number of lymphocytes specific for that antigen is low. Animals that have developed CMI against a specific antigen have an increase in the number of circulating lymphocytes that recognize the antigen. Exposure of these lymphocytes to that antigen will cause them to undergo mitosis and secrete cytokines. In proliferation assays, lymphocytes harvested from vaccinated or infected animals are incubated with the antigen that is being studied.[44] After incubating the lymphocytes for several days, radioactive (tritiated) thymidine is added to the lymphocytes being cultured. Lymphocytes that are actively dividing, and thus producing DNA, will incorporate the radioactive thymidine into new DNA molecules. The amount of radioactivity associated with the lymphocytes after nonincorporated thymidine is washed away is proportional to the amount of mitotic activity of the lymphocytes. Results usually are expressed as a ratio (stimulation index) of the radioactivity in antigen-exposed lymphocytes to that in nonantigen-exposed lymphocytes from the same animal. Colorimetric methods also have been developed to detect mitotic activity.[45]

Proliferation assays are technically more complex than the assays discussed previously. Lymphocytes must be cultured for several days, and viability of the cells must remain high during this period. Special safety precautions must be observed when working with radioactive thymidine, and expensive instrumentation is required to measure the radioactivity in lymphocyte samples.

Proliferation assays have been used successfully to measure antigen-specific CMI responses against numerous infectious agents in animals. Although proliferation assays are adaptable to nearly every experimental situation, special attention must be paid to antigens that are intrinsically mitogenic to lymphocytes. Mitogenic antigens stimulate proliferation of lymphocytes, regardless of the lymphocytes' antigenic specificity. Proliferation assays may not be desirable as a means of studying antigen-specific CMI responses to strongly mitogenic antigens because of high nonspecific (background) radioactivity values.

Although proliferation assays are used to measure one feature of the cellular immune response, they are still somewhat nonspecific, because the effector function of the proliferating cells is not determined. Proliferation itself does not imply that a protective immune response is being generated. Clonal expansion of lymphocytes with suppressor activity and of cytotoxic lymphocytes may yield similar proliferation assay results, yet each response has a different influence on the overall immune response.

Cytokine release assays—Cytokine release by activated lymphocytes may be assayed as a measure of CMI. In the assays that were described previously in this report, cytokine release is needed to achieve a measurable response (cutaneous swelling, leukocyte migration inhibition, or lymphocyte proliferation), but the specific cytokines involved are not characterized by the assay. Many cytokines, working alone or in combination with other cytokines, cause specific cellular responses, and cytokine release assays identify those cytokines that are active in CMI responses to a specific infectious agent. Cytokine assays may be bioassays, which measure the effect that specific cytokines have on a defined biologic system. They may measure cytokine concentrations directly, as in ELISA, radioimmunoassays, or precipitation assays, or they may detect cytokine-specific mRNA in lymphocytes.

One form of bioassay uses cytokine-dependent cell lines. These cells have been adapted in the laboratory so that they require a specific cytokine for proliferation. Addition of test fluids containing that specific cytokine to a culture of cytokine-dependent cells causes the cells to proliferate in a dose-dependent manner. Cellular proliferation then can be quantitated by the use of tritiated thymidine or colorimetric methods. Concentrations of cytokine in test fluids can be determined by correlating the amount of cellular proliferation to a value on a standardized dose-response curve. Bioassays are advantageous, because only biologically active cytokines are measured. A limitation of this type of bioassay is the availability of the appropriate cytokine-dependent cell line. Cell lines specific for interleukin-1[46] and interleukin-2[47] of human beings have been developed, as have cell lines specific for a number of cytokines of mice.[48] The

specificity of bioassays must be carefully evaluated; contaminating cytokines may have costimulatory or inhibitory actions on the cell lines of the bioassay system that could affect accurate measurement of the principal cytokine.

Alternate bioassays have been described for the measurement of cytokines that have well-defined, unique actions. Interferon may be assayed by its ability to inhibit virus infection of cells.[49] The ability to lyse certain fibroblast cell lines forms the basis of a bioassay for tumor necrosis factor; this assay has been adapted for use in swine.[50] However, many cytokines have nonexclusive actions that are too generalized to be measured by this type of bioassay.

Direct cytokine assays are more specific and subject to fewer environmental variables than bioassays, but they are currently available for only a few well-characterized cytokines.[48,51] Development and use of direct cytokine assays are limited by the availability of necessary assay reagents. Specific antibodies are needed to detect cytokines in direct assays, and a purified source of cytokine is highly desirable as a control antigen. Most cytokines of human beings and mice have been purified and cloned for large-scale in vitro production, so abundant sources of cytokine and cytokine-specific antibodies are available for these species. However, reagents needed for most direct cytokine assays for animals of veterinary importance are not yet readily available. It is likely that as species-specific reagents for cytokines of domestic animals become more readily available, direct assays will complement bioassays as methods of measuring cytokine release in veterinary applications.

Detection of mRNA molecules that encode cytokine proteins has been gaining in popularity as a method of measuring cytokine production. Cytokine-specific mRNA is produced by activated lymphocytes and has been detected in lymphocytes of ruminants within 4 hours after activation.[52] However, expression of cytokine-specific mRNA by lymphocytes is transient; this must be considered when interpreting assay results. In assays that measure RNA, mRNA specific for the cytokine being studied is detected by use of a nucleic acid probe that is designed to have a sequence of bases complementary to that of the cytokine-specific RNA. The probe is labeled, usually with radioactive phosphorous or biotin, so that cytokine-specific RNA can be detected and quantitated.

Cytotoxicity assays—Cytotoxicity assays measure the ability of lymphocytes to kill target cells. Cytotoxic T-lymphocytes are especially important in the control of viral diseases, although they also play a role in the immunity of hosts to facultative intracellular bacteria.[53] Infected cells that express viral or microbial proteins on their cell surfaces in association with MHC antigens are destroyed by cytotoxic T-lymphocytes.

The most common cytotoxicity assay currently in use is a chromium release assay.[54] In this assay, lymphocytes are incubated with target cells that have been labeled with radioactive chromium. Target cells for antigen-specific CMI responses must be histocompatibility matched with the putative cytotoxic T-lymphocytes, and then are infected with the agent being studied. Radioactive chromium is released into the surrounding cell culture medium as the labeled target cells are lysed by the cytotoxic lymphocytes, and the amount of radioactivity in the medium after cell debris is removed is proportional to the amount of cytotoxic activity. An esterase assay that detects granules released by active cytotoxic lymphocytes has been developed as a nonradioactive alternative to chromium release assays.[55]

Cytotoxicity assays yield valuable information, because the effector function that they measure is usually, but not always, associated with protective immunity. Cytotoxicity assays are prone to the same difficulties as adoptive transfer assays in that the cytotoxic activity of T-lymphocytes is usually MHC restricted, so target cells must be matched with the lymphocytes being tested. Furthermore, the chromium release assay, like the tritiated thymidine proliferation assay, uses radioactive substances; however, radioactive chromium emits γ-rays that are more penetrating than the α-rays emitted by tritium and, therefore, requires additional safety precautions for handling and disposal. Cytotoxicity assays may not be suitable for all infectious agents; for agents that are highly cell-associated, such as herpesvirus of chickens,[56] it may be impossible to harvest enough free virus to infect a sufficient number of target cells.

Comparison of in vivo and in vitro assays—All of the assays reported here provide useful information about CMI function. However, no assay alone provides a complete view of CMI responses. It is important when characterizing the CMI response against a specific infectious agent to look at the results of several assays, each measuring a different aspect of CMI.

In vivo assays (DTH and adoptive transfer) in their simplest forms yield little information regarding specific CMI effector mechanisms that are active in protective immunity; however, they are advantageous, because immune responses are observed in the context of the entire animal. Adoptive transfer assays have an additional advantage, because resistance to challenge exposure, the final determinant of successful immunization, is usually measured directly.

In vitro assays tend to be more specific than in vivo assays. They can be designed so that only a narrow, defined population of cells is assayed and so that only a single effector function is assessed. In vitro assays also are useful to assess the development of CMI responses with time after exposure to an infectious agent. Because actual assays are

performed outside of animals, repeated tests can be performed that use lymphocytes obtained from the same animal. Each animal may be used only once for in vivo assays, because immune responses elicited as a result of the initial assay may influence responses in subsequent assays. Increasingly, in vitro assays are used to define molecular bases of antigen-specific CMI responses, but it is important to recognize that complex cell-cell interactions exist in host animals and that, in vivo, multiple effector functions work concurrently to achieve protective immunity. Conclusions drawn from results of in vitro assays should always be compared with the development of in vivo resistance to challenge exposure.

Cell-mediated Immunity After Natural Infection vs After Vaccination

One cannot assume that the CMI response measured after natural infection is the same as that induced by vaccination. Modified-live vaccines are the most likely to mimic natural infection, because they replicate in the recipient animal. When foreign proteins are actively synthesized during microbial or viral replication in infected host cells, they are processed by a different intracellular pathway than that by which exogenous internalized proteins are degraded. Proteins processed by the endogenous pathway are expressed on infected cell surfaces in conjunction with MHC class I molecules, which makes them the target of MHC class I-restricted cytotoxic effector cells.[57] For certain vaccines, such as varicella-zoster,[58] it has been reported that responder cell frequencies, target antigens, and effector functions are similar between vaccinates and naturally infected human beings; however, in a study of the development of immunity to *Brucella abortus*, vaccination with modified-live vaccine induced weaker proliferation responses in cattle than infection with a low-virulence isolate of *B abortus*.[59]

Killed vaccines and bacterins often are assumed to be less immunogenic than their live counterparts. Diminished CMI responses have been documented for killed vaccines of bluetongue virus,[60] *Salmonella typhimurium*,[49] *Listeria monocytogenes*,[61] and *Rickettsia* sp,[23] compared with natural infection or live vaccines. However, there are a number of other killed vaccines, including those containing rotavirus,[62] equine infectious anemia virus,[63] and foot-and-mouth disease virus,[64] that induce CMI responses similar to natural infection. Some killed vaccines (rabies virus,[19] FeLV,[65] *Brucella* subunit[66]) have been reported to elicit greater CMI responses than natural infection. In some of these cases, however, the enhanced CMI response after vaccination can be attributed to the fact that the organism in a natural infection is immunosuppressive, but the vaccine is not. The adjuvant system used in a killed vaccine can also have a major influence on the development of CMI responses.[17,67]

The duration of vaccine-induced CMI may differ from the duration of CMI induced by natural infection. Typically, the duration of vaccine-induced immunity has not been well-defined. Most veterinary vaccine studies have detected initial vaccine efficacy, but researchers have not monitored immune responses beyond several weeks after vaccination.[68,69] In studies on human beings, data regarding duration of immunity are sometimes gathered indirectly through epidemiologic studies.[70] In nearly all of the reported studies, however, protective immunity over time is determined by challenge-exposure or serologic methods[71,72]; currently, few studies have measured CMI responses over an extended period.

Identification of Microbial Antigens that Induce Protective CMI

Identification of protective microbial antigens is important in implementing a rational immunologic approach to vaccine development. Knowledge of protective antigens facilitates the selection of vaccine strains for whole-cell vaccines; the strain must express the protective antigens and stimulate solid immunity to those antigens. However, vaccination with whole live organisms, in some instances, may not be safe, and products containing whole-cell killed oganisms may not be efficacious. In these cases, subunit vaccines or live recombinant vectored vaccines may be used, both of which contain only selected antigens from the microbial agent in the form of preformed antigen(s) (subunit) or as a cloned gene in a replicating nonpathogenic bacterium or virus (vectored vaccines). Careful selection of the appropriate antigens for inclusion in these vaccines is critical to the efficacy of the final product.

The type of antigen that elicits a protective immune response varies widely among infectious agents. For some organisms, such as cytomegalovirus,[73] one or a few antigens are dominant and are the targets of most of the host's immunoreactivity. With other organisms, such as rotavirus[74] or herpes simplex virus,[75] immunoreactivity is broad-based and directed against several antigens. Surface proteins and glycoproteins are the targets of immune responses for numerous agents, including rabies virus,[76] pseudorabies virus,[77] respiratory syncytial virus,[78] rotavirus,[74] and bordetellae.[75] Antigens located on the surface of extracellular organisms or expressed on the surface of infected host cells are likely candidates to be targets of immune responses, because they are readily accessible to antibodies or lymphocyte antigen receptors; however, sometimes internal proteins, such as those of influenza virus,[79] are processed so that they are expressed on host cell surfaces and are the main targets for cytotoxic T-lymphocytes. Cytotoxic T-lymphocytes that are targeted against nonstructural proteins, such as the secreted gX protein of pseudorabies virus,[77] and against nonconstitutively expressed proteins (proteins not expressed under all conditions), such as

stress-induced catalase II protein of *S typhimurium*,[80] also have been identified.

The assays described previously can be modified to identify proteins that are protective. Instead of vaccinating with a whole-cell preparation, semipurified microbial extracts[49,81,82] or purified proteins[74,83] can be injected, and the CMI response can be evaluated. Alternatively, deletion mutant vaccines can be used, and the CMI response in the absence of the antigen under study can be evaluated. If the physical structures of the antigens of an infectious agent are known, computer analyses of the structures may be helpful to identify potential antigens that may stimulate protective immune responses. Computer programs that are developed on the basis of knowledge of the antigenic structures that stimulate T-cell responses in other organisms can be used to predict potential T-cell reactive sites on antigens in infectious agents being studied.[84]

Different antigens may be targets for different lymphocyte subsets. It has been reported that MHC class I-restricted cytotoxic T-lymphocytes of mice are directed against glycoprotein gC of herpes simplex virus and that glycoprotein gD is the target for MHC class II-restricted cytotoxic cells.[85,86] Cytotoxic T-lymphocytes directed against different antigens may exhibit different reactivities; cytotoxic T-lymphocytes against the G protein of vesicular stomatitis virus are serotype specific, whereas cytotoxic T-lymphocytes against the internal (N) protein are cross-reactive.[79]

Methods of Antigen Delivery that Stimulate CMI

The method of antigen delivery may be as critical to the efficacy of a vaccine as the selection of protective antigens to include in the vaccine. Antigen presentation influences the types of immune responses that are generated in host animals, and the type of immunity favored should correspond to the immune mechanisms that have been identified as protective. By themselves, purified antigens often are poorly immunogenic, which complicates the development of subunit or single antigen vaccines. Areas of research aimed at increasing the CMI response to vaccines include improved adjuvant formulation, generation of vaccine vectors, and the development of bioengineered delivery systems.

Adjuvants—Adjuvants are substances that enhance the immunogenicity of vaccines. They may act to retain the antigen at the injection site (depot effect), or they may serve as irritants to promote a local inflammatory response and recruit antigen-presenting cells (macrophages) to the area of injection. They may improve interactions of the antigen with antigen-presenting cells, or they may enhance interactions between antigen-presenting cells and responding lymphocytes. A number of adjuvants currently are used in the production of veterinary biologicals. Among the most popular are aluminum salts and mineral oil. Although alum and oil emulsions enhance antibody responses to vaccines, they do not necessarily enhance CMI responses.[87] New generation adjuvants, such as block copolymers,[87] specifically enhance CMI responses. Saponins,[53,88,89] muramyl dipeptide,[87,90] and oils complexed to mannides or Freund's incomplete adjuvant[90] also have received renewed interest for their CMI-inducing properties. Immunomodulatory cytokines, such as interleukin-2,[91,92] have been used as adjuvants to enhance CMI responses. Physical and chemical properties of the antigens in a particular vaccine may influence the formulation of the optimal adjuvant for that product.[87] Also, some of the most immunostimulatory adjuvants produce unacceptable side effects in vaccinated animals. There must be a balance between the degree of immunoenhancement an adjuvant affords and the severity and acceptability of the side effects that it produces in vaccinated animals.

Vectored vaccines—Live recombinant vectored vaccines have been developed that stimulate CMI responses to heterologous antigens. In this class of vaccines, a nonpathogenic vector organism (usually a bacterium or virus) is used to produce a specified antigen from another infectious agent. The gene encoding the antigen of interest is inserted in the DNA of the vector organism. As the vector synthesizes protein, antigen is produced from the cloned gene along with the vector's own antigens. Vaccinia virus,[93-96] pseudorabies virus,[97] *S typhimurium*,[98] and Bacillus Calmette-Guerin (*M bovis*)[99,100] have been used successfully as vectors. *S typhimurium*-vectored vaccines have promise as oral vaccines because of the ability of *Salmonella* organisms to invade intestinal tissue and stimulate mucosal immunity.[101] Vectored vaccines have the superior immunogenicity of a live vaccine and can be used to induce immunity to antigens of infectious agents for which vaccination with a live product is not feasible or not desirable. The replication potential of vectored vaccines is especially advantageous for antigens that must be actively synthesized within the host to induce protective immunity.

Biodegradable microspheres—Synthetic antigen delivery systems that stimulate CMI responses also have been developed, including biodegradable microspheres, liposomes, and immunostimulating complexes (ISCOM). Antigens are coated with inert synthetic polymers to form microspheres.[102] Polymers that are used are similar to those found in absorbable suture material. The rate of antigen degradation and, to a certain extent, the anatomic and cellular locations where the particles will be deposited are influenced by the size of the microsphere particle and the chemical structure of the polymer coating. By including particles of different sizes and different polymer densities, sustained, slow release of antigen can be achieved. Pulsed antigen release also can be achieved; this mimics the effect of pri-

mary and booster vaccinations through a single vaccine administration. Microspheres have promise for use in oral vaccines, because they protect the antigen from degradation by digestive enzymes and from inactivation by colostral antibodies, yet they are taken up into lymphoid tissues to effect a local or systemic immune response. Proliferative and cytotoxic CMI responses have been detected after the administration of microsphere vaccines.[103]

Liposomes—Liposomes are created by trapping antigen between lipid bilayers. Like microspheres, the immunoenhancing properties of liposomes may be modified by altering their physical properties.[104] Lipid membranes themselves often have adjuvant properties, but discrete adjuvants also may be incorporated, with the antigen or in separate vesicles, into liposome vaccines. Synergistic effects may exist between adjuvants and liposomal membranes. Liposome vaccines may induce DTH, lymphocyte proliferation, and cytotoxic activity when administered orally or parenterally.[104]

Immunostimulating complexes—An ISCOM is composed of an adjuvant skeleton, such as saponin-glykoalkaloid complexes, into which multiple antigen molecules are inserted.[105,106] By creating a multimeric particle of adjuvant and antigen, the antigenic complexity that is often necessary for adequate immunogenicity is created. Also, the intimate contact between antigen and adjuvant sometimes allows smaller doses of vaccine to be used. An ISCOM can induce antigen-specific lymphocyte proliferation and cytotoxic lymphocyte responses,[107,108] and they have been used in vaccines to stimulate local (mucosal) and systemic immune responses.[109]

Many diseases require that a CMI response develop in the host to provide adequate immune protection. Extensive knowledge of the CMI responses to infectious agents is required when a rational approach to improved vaccine development for these diseases is attempted. The study of CMI is not as straightforward as the study of humoral immunity. Several functional assays, some of which are technically complex, may be performed. Results of the assays must be compared with one another and must be correlated to protection of the host to enable researchers to derive meaningful conclusions regarding antigen-specific CMI responses against an infectious agent. Once gathered, this knowledge can be used to develop and validate vaccines for diseases in which CMI responses are required for protection.

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