Review Articles

Mechanisms of Disease

FRANKLIN H. EPSTEIN, M.D., *Editor*

LYMPHOCYTE-MEDIATED CYTOLYSIS AND DISEASE

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LYMPHOCYTES are equipped to eradicate noxious agents (microbes, cancer cells, and grafts) that disturb the body's equilibrium, but when their cellular activity is excessive, the results are harmful. The list of abnormalities known to be caused by excessive lymphocyte activity is extensive. We review two distinct pathways that account for most of the cellular injury induced by lymphocytes, with an emphasis on their clinical implications.

THE MOLECULAR BASIS OF LYMPHOCYTE-MEDIATED CYTOLYSIS

Unlike humoral immune responses, which are mediated through antibodies and complement and can be transferred in serum to unimmunized subjects, cellular immune responses require the direct participation of effector cells such as T lymphocytes. The functional activities of both helper T lymphocytes (predominantly CD4 cells) and cytotoxic T lymphocytes (predominantly CD8 cells) are initiated by the binding of specific antigen presented in association with the major histocompatibility complex (MHC) on the target cell to T-cell–antigen receptors (Fig. 1). Therefore, the actions of T lymphocytes are considered antigen-specific and MHC-restricted. On activation by target cells, helper T lymphocytes secrete cytokines that promote the recruitment and activation of other cells, such as macrophages, to execute their effector functions. In contrast, activation of cytotoxic T lymphocytes results in direct killing of the target cell. Another class of lymphocytes, known as natural killer cells, do not express classic markers for T or B lymphocytes and yet are capable of lysing a variety of target cells without antigenic stimulation. Unlike cytotoxic T lymphocytes, natural killer cells recognize their target cells in a non–MHC-restricted manner.

Lymphocyte-mediated cell killing involves a sequence of biologic events beginning with binding of the antigen-presenting (target) cell to the killer lymphocyte, by means of the recognition process described above. After the cell–cell interaction, the killer lymphocyte kills the target cell through the action of soluble cytolytic mediators (perforin and granzymes) stored in the cytoplasmic granules in the T cell (Fig. 2A) and a killer-lymphocyte surface molecule (Fas ligand)[1-3] (Fig. 2B). After this cytolytic attack, target cells may die by necrosis (characterized by membrane disruption and organelle destruction) or apoptosis (characterized by chromatin condensation, DNA fragmentation, and membrane blebbing).[4]

Perforin–Granzyme-Dependent Cell Killing

Killer lymphocytes have many lysosome-like cytoplasmic granules that contain electron-dense cores surrounded by vesicular material. When the cells are activated, these granules move toward the plasma membrane of the cell, fuse with it, and then discharge their contents toward the target cell.[5,6]

Perforin (also known as cytolysin), so named because it can form pores that perforate the plasma membrane of target cells,[7,8] is a prominent component of the granules. It is a 70-kd glycoprotein that is produced exclusively by activated killer lymphocytes.[9,10] The pores are formed by calcium-induced aggregation of perforin molecules that have entered the membrane[7,8] (Fig. 3A). Through these pores, which range from 5 to 20 nm in internal diameter and function as high-conductance, nonselective ion channels, water and low-molecular-weight solutes may freely enter the target cells, resulting in their death by "colloid–osmotic lysis" — that is, the cells literally burst (Fig. 3B). This mechanism of action of perforin is reminiscent of that mediated by the membrane-attack complex of complement.[11] In vitro, purified perforin can efficiently lyse a wide variety of nucleated and nonnucleated cells,[7,8] and transfection studies indicate that the expression of perforin in non-killer cells confers the ability to kill.[12] These results, taken together, suggest a paramount role for perforin in the cell killing mediated by killer lymphocytes.

The lymphocyte granules also contain several distinct but related proteases collectively called granzymes. To date, seven mouse and three human granzymes have been identified.[13] Among them,

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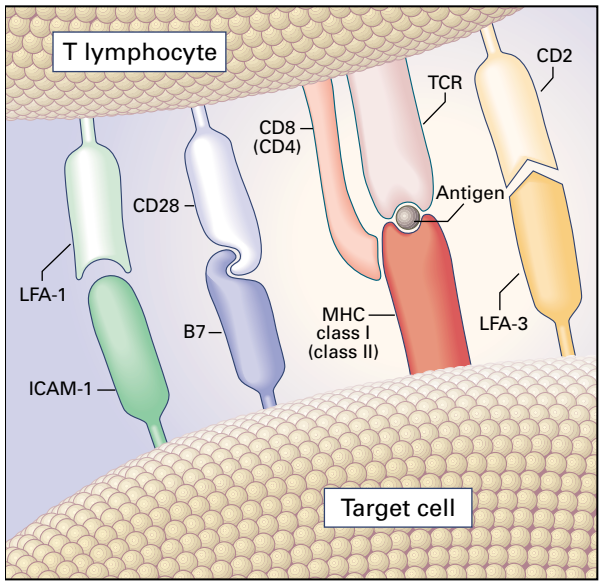


Figure 1. MHC-Restricted Interaction between T Lymphocytes and Target Cells.

The interaction begins with the binding of antigen in conjunction with an MHC molecule (class I for CD8 T cells and class II for CD4 T cells) on the target cell by the T-cell–antigen receptor (TCR). Accessory molecules, such as lymphocyte function antigens (LFA) 1 and 3, intercellular adhesion molecule 1 (ICAM-1), CD2, CD28, and B7 (T-cell costimulatory factor), are involved in this interaction through their enhancement of cell–cell adhesion or transduction of additional cell-activation signals.

granzyme A (a protease with trypsin-like activity) and granzyme B (a unique protease that is specific for aspartic acid residues) have been extensively characterized. Although granzymes themselves are noncytolytic, they may participate indirectly in the cell-killing process. Inhibition of the enzymatic activity or the cellular expression of granzymes reduces the killing activity of T lymphocytes.[14,15] The expression of granzyme A or B together with perforin in nonkiller cells renders these cells functionally cytolytic and capable of inducing apoptosis of nucleated target cells.[16] Purified granzyme A or B, in the presence of perforin, induces apoptosis in target cells.[17,18] Collectively, these results suggest that granzymes, released along with perforin, exert their cytolytic (i.e., apoptosis-inducing) effects only after entering the target cells. Perforin may assist granzymes in entering target cells in two ways: perforin pores may serve as conduits for the granzymes, and the formation of perforin pores in the target-cell membrane may trigger an endocytic-repairing action that allows granzymes to enter the cells.

The cooperative action of perforin and granzymes provides a reasonable explanation for several conflicting observations. For example, perforin alone causes only necrotic death of target cells,[19] whereas T lymphocytes can kill target cells by inducing both necrosis and apoptosis.[20] This discrepancy is reconciled by the evidence that granzymes (particularly granzymes A and B) are involved in inducing apoptosis in target cells.[17,18] By virtue of such cooperative action, perforin and granzymes together are fully competent in mimicking the cytolytic effect of killer lymphocytes. The putative involvement of both perforin and granzyme B in lymphocyte-mediated cytolysis has received strong support from studies of mice with deletions of the perforin gene. The cytolytic activity of killer lymphocytes of these mice is markedly depressed.[21-24] The killer lymphocytes of granzyme B–knockout mice, while able to kill target cells in vitro, do not cause rapid DNA fragmentation in the cells.[25] These results indicate that the cytolytic actions of T lymphocytes result from the coordinated action of perforin and granzymes. Granzymes may trigger apoptosis of target cells by prematurely activating kinase Cdc2, which is regulated during the cell-division cycle (cdc),[26] or by activating a cytoplasmic protease named CPP32 (a protease related to interleukin-1β–converting enzyme).[27]

Fas-Dependent Cell Killing

Monoclonal antibodies that recognize proteins designated Fas and APO-1 on the surface of various target cells can trigger the apoptotic death of these cells by binding to the respective proteins.[28,29] Fas and APO-1 appear to be a single 43-kd protein that belongs to the superfamily of tumor-necrosis-factor and nerve-growth-factor receptors.[28-30] The identification of Fas–APO-1 as a receptor-like molecule raised the question of its involvement in lymphocyte-mediated cytolysis and prompted the search for its ligands. Subsequently, the Fas ligand was isolated and cloned; the sequence proved to have some homology to tumor necrosis factor α.[31] The binding of Fas ligand to Fas can trigger the apoptotic death of Fas-expressing cells through intracellular signaling pathways that are as yet unclear (Fig. 2B).

The involvement of the Fas-dependent pathway in lymphocyte-mediated cytolysis has been further substantiated by studies using perforin-knockout mice. Killer lymphocytes derived from these mice retain residual cytolytic activity that is mediated through the Fas-dependent pathway.[22,23,32,33] This pathway accounts for approximately one third of the total cytolytic activity of CD8 cytotoxic T lymphocytes.[2,33] It also contributes to the cell killing mediated by CD4 cytotoxic T lymphocytes, natural killer cells, and lymphokine-activated killer cells[34,35] and has a role in the so-called activation-induced cell death of T lymphocytes[36] and in the peripheral deletion of autoreactive T lymphocytes.[37]

Ineffective removal of these cells resulting from defects in either Fas ligand or Fas, as exemplified by the *lpr* (lymphoproliferation) phenotype (a *Fas* gene

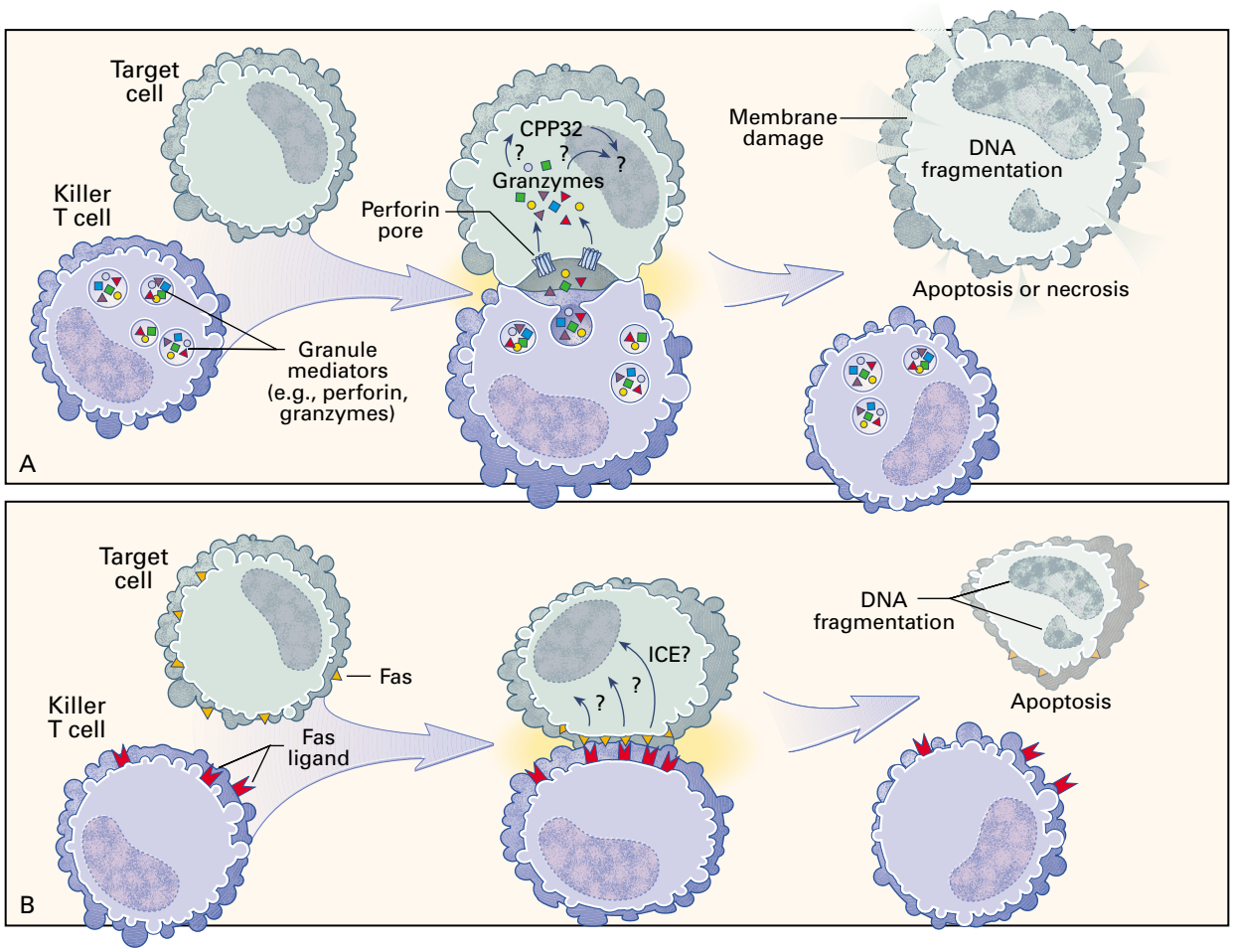


Figure 2. Mechanisms of Lymphocyte-Mediated Cytolysis.

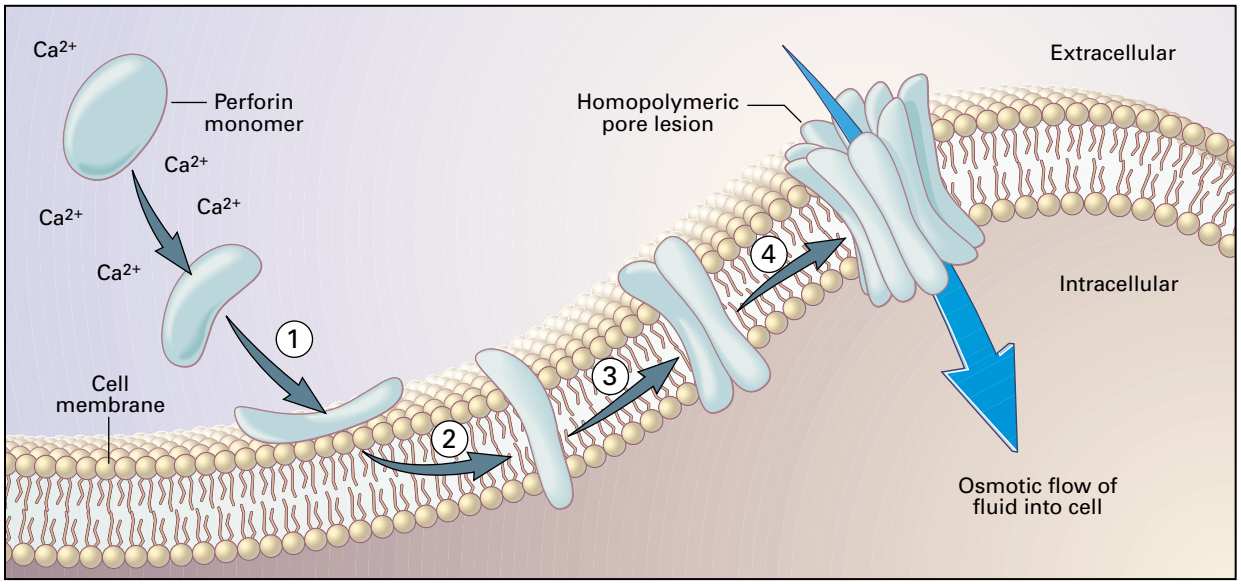
Panel A shows a mechanism dependent on perforin and granzymes. After binding of the target cell to the killer T cell (shown in Fig. 1), cytoplasmic granules in the killer cell are rapidly reoriented toward the target cell in preparation for releasing the contents of the granules, including perforin and granzymes, into the intercellular space. Perforin and granzymes attack the target cell by forming pores in its plasma membrane and entering the cell. The killer cell may detach to attack other targets while the target cell continues to die. Panel B shows a Fas-dependent mechanism. After the target cell binds to the killer cell, the level of expression of Fas ligand on the killer cell rapidly increases. The interaction between Fas ligand and Fas receptor on the target cell leads to apoptosis. Proteases, such as CPP32 and other proteases related to interleukin-1β–converting enzyme (ICE), have been implicated in the transduction of signals for apoptosis.

mutation) and the *gld* (generalized lymphoproliferative disease) phenotype (a Fas-ligand gene mutation) in mice and the Canale–Smith syndrome (a *Fas* gene mutation) in humans,[38] may lead to lymphoproliferation, lymphadenopathy, and autoimmunity. The Fas-dependent pathway may be the chief mechanism by which CD4 cytotoxic T lymphocytes destroy antigen-presenting cells or CD8 cytotoxic T lymphocytes, thereby turning off immune responses.[39,40]

On the basis of these findings, it has been proposed that the Fas-dependent pathway may have primarily an immunoregulatory role and, to a lesser extent, an immune effector role.[41] Recently, Fas-mediated apoptotic death of target cells was found to involve proteases related to interleukin-1β–converting enzyme.[42] The participation of these proteases in both killing dependent on perforin and granzymes and Fas-dependent killing implies that the two seemingly disparate pathways have a final common mechanism of action.

THE INVOLVEMENT OF KILLER LYMPHOCYTES IN IMMUNE PROTECTION AND IMMUNOPATHOGENESIS

Lymphocyte-mediated cytolysis is important in combating invading pathogens and destroying cells bearing foreign characteristics (e.g., transplanted cells), tumor antigens, or autoantigens. A better un-



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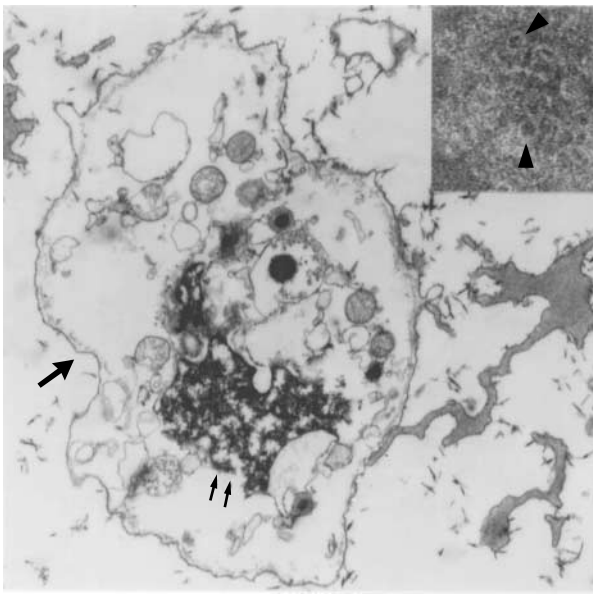

B

Figure 3. The Mechanism of Action of Perforin.

As shown in Panel A, in the presence of calcium ions, perforin monomers undergo conformational changes and bind to the membrane of the target cell (step 1), insert themselves into the membrane (step 2), and subsequently aggregate to form homopolymeric pore structures (steps 3 and 4). These pores may perturb membrane permeability and result in osmotic lysis of the target cell. Panel B shows an electron micrograph of a mouse mastocytoma P815 cell undergoing necrosis mediated by purified mouse perforin (×10,000). Large and small arrows point to the plasma membrane and nucleus of the dying cell, respectively. The inset shows the pore lesions (arrowheads) detected on the membrane of erythrocytes lysed by perforin (×250,000).

derstanding of the actual role of killer lymphocytes in different clinical situations could lead to new therapeutic approaches for more efficiently purging tumor or virus-infected cells or attenuating autoimmune reactions and allograft rejection. In the following sections, we discuss in general the role of killer lymphocytes within the context of some well-known diseases.

Viral Infections

Lysis of target cells mediated by killer lymphocytes has been implicated in the clearance of virus and other intracellular organisms. Natural killer cells are involved in limiting viral replication during the initial stage of an infection, while cytotoxic T lymphocytes undergo clonal selection, expansion, and differentiation to competent effector cells that then are responsible for eliminating the virus.[43] CD8 cytotoxic T lymphocytes have a protective role in murine cytomegalovirus (CMV)[44] and other viral infections.[45,46] In humans, CD8 cytotoxic T lymphocytes also provide immune protection against both initial CMV infection and reactivation of quiescent CMV infection.[47] The latter can cause life-threatening disease in immunodeficient hosts, including recipients of allogeneic bone marrow or other organ transplants and patients with the acquired immunodeficiency syndrome (AIDS).[48] In recipients of allogeneic bone marrow transplants, protection against CMV pneumonia correlated with the appearance of CD8 CMV-specific cytotoxic T lymphocytes.[49,50] Furthermore, adoptive transfer of CMV-specific CD8 cytotoxic T lymphocytes from an immunocompetent bone marrow donor to an immunosuppressed recipient selectively reconstitutes immunity against CMV in the latter and thus protects the recipient from complica-

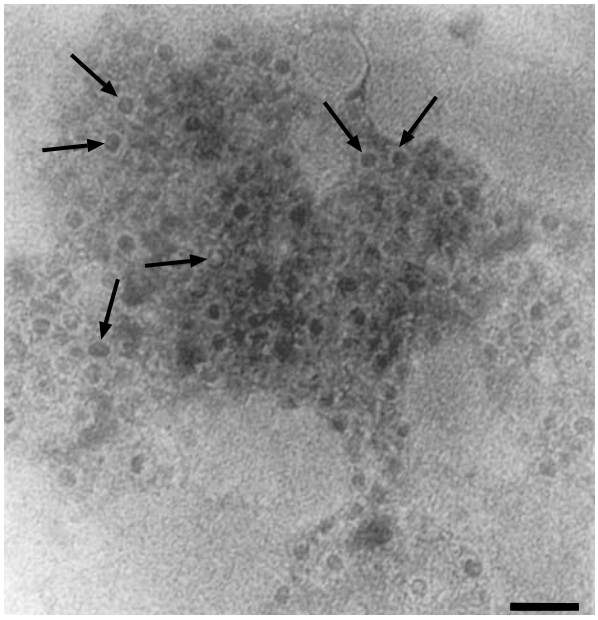

Figure 4. Electron Micrograph of Myocardial Tissue from a Patient with Postviral Myocarditis, Showing Pore Lesions.

Representative perforin-mediated pore lesions are indicated by arrows. The bar represents 30 nm.

tions of CMV infection (e.g., CMV pneumonia).[51] Mice with impaired CD8-mediated cytolysis clear infections with influenzavirus,[52] lymphocytic choriomeningitis virus,[53] and Sendai virus[54] poorly, providing further evidence that these cells have an important role in protective immune reactions.

In infections with certain noncytopathic viruses, killer lymphocytes may both clear virus and contribute to the pathologic process caused by the viral infection. For example, in mice with lymphocytic choriomeningitis virus (LCMV) infection, LCMV-specific cytotoxic T lymphocytes are responsible for both the eradication of virus and the onset of serious illness, depending on the timing of the virus infection, the portal of entry of the virus, and the strain of virus.[55] LCMV-specific cytotoxic T lymphocytes exert their protective effects by either directly lysing virus-infected cells or secreting cytokines (e.g., interferon-γ) that inhibit viral replication, but the cytokines and products of cell lysis can cause inflammation of tissue.[56] The identification of CD8 lymphocytes that express perforin in situ at inflammatory foci in LCMV-infected mice provides evidence of the involvement of activated killer lymphocytes in the pathogenesis and clearance of LCMV infection.[57] Furthermore, the inability of perforin-knockout mice to clear LCMV infection demonstrates that perforin-dependent cytolysis is critical for immune protection against LCMV.[21,23]

Because of the limited availability of animal models of human hepatitis B (HBV) infection, the molecular pathogenesis of HBV-related diseases has remained obscure. However, with the use of recently developed HBV transgenic mice, the immunobiology and pathogenesis of acute and chronic HBV hepatitis have begun to be elucidated.[58] In such mice, MHC-class-I–restricted cytotoxic T lymphocytes can cause apoptosis in hepatocytes with which they are in direct contact and induce widespread liver damage through the actions of cytokines and inflammatory cells recruited into local sites.[59] HBV-infected patients with acute, self-limited hepatitis, but not those with progression to chronic hepatitis, were found to mount vigorous cytotoxic-T-lymphocyte responses against HBV nucleocapsid antigens, envelope antigens, and the viral polymerase.[60,61] These results suggest that an early, efficient immune response involving cytotoxic T lymphocytes may result in the lysis of most HBV-infected hepatocytes and the clearance of virus, and at the same time cause acute but transient hepatitis. A weak response of cytotoxic T lymphocytes, on the other hand, may lead to incomplete lysis of infected hepatocytes and, hence, the continuing replication of HBV in those cells. Persistence of HBV infection may trigger mild but chronic hepatocellular injury mediated by cytotoxic T lymphocytes, manifested clinically as chronic hepatitis. Alternatively, hepatocytes persistently infected by HBV may have abnormal cellular metabolism that causes their spontaneous death, which may trigger secondary inflammation leading to continuous degeneration and regeneration of the liver tissue, culminating in hepatocellular carcinoma.

The role of lymphocyte-mediated immune responses in human immunodeficiency virus (HIV) infection is controversial. Although an HIV-specific cellular immune response may provide immune protection against disease progression,[47,62] cytotoxic T lymphocytes may in fact contribute to the ultimate immunosuppression by continuously lysing infected or uninfected immune cells.[63,64] HIV-infected patients have strong responses of polyclonal cytotoxic T lymphocytes against HIV antigens soon after initial seroconversion, which probably cause the rapid decline of viremia at that time.[65,66] Moreover, HIV-specific cytotoxic T lymphocytes can inhibit the replication of HIV in vitro.[67] These responses, however, usually wane and are insufficient to eradicate HIV, possibly because cytotoxic T lymphocytes fail to combat virus harbored in lymphoid or other reservoirs.[47,68] As a result, AIDS eventually develops in most HIV-infected patients.[62] Nevertheless, in several cohort studies, a small percentage of patients infected with HIV have remained asymptomatic for many years. These patients have detectable and sometimes vigorous responses of CD8 cytotoxic T lymphocytes specific for HIV and extremely low viral loads, as compared with patients with progres-

sive infection.[69,70] Collectively, these studies provide encouraging hints that effective immunity mediated by cytotoxic T lymphocytes may curtail the replication of HIV and thus prevent a catastrophic outcome. The molecular basis of this seeming cessation of HIV replication is likely multifactorial, including the possibility that these patients may have been infected with attenuated strains of the virus.[71,72]

Intracellular Bacterial and Protozoal Infections

In addition to controlling viral infections, cellular immune activity has been shown to be involved in protecting against intracellular bacteria and protozoa. For example, both CD4 and CD8 T lymphocytes are involved in immune protection against infection by *Listeria monocytogenes.* By lysing listeria-infected cells, CD8 cytotoxic T lymphocytes may complement CD4 cells (which secrete interferon-γ) and macrophages (which respond to interferon-γ) to facilitate the resolution of listeriosis. This protective function of CD8 cytotoxic T lymphocytes depends in part on the mechanism mediated by perforin and granzymes, because perforin-deficient mice clear primary listeria infection inefficiently and cannot control a secondary listeria challenge.[73] CD8 cytotoxic T lymphocytes and natural killer cells may control infection with other microorganisms, such as mycobacterium, rickettsia, and theileria, in the same way.[74]

Infections with intracellular protozoal parasites such as *Plasmodium falciparum* are a major public health problem worldwide. In rodents, protective immunity against malaria can be elicited by immunization with radiation-attenuated malaria sporozoites.[75] This protection is mediated by CD8 cytotoxic T lymphocytes capable of recognizing certain sporozoite antigens (e.g., circumsporozoite protein) and of lysing infected hepatocytes.[75] Cytotoxic-T-lymphocyte–dependent immune reactivity against the pre-erythrocyte stage of malaria protozoa may also have a role in protection against *P. falciparum* malaria in humans.[76]

Trypanosoma cruzi, another intracellular parasite, causes Chagas' disease, which is characterized by inflammation and degeneration of cardiac and smooth muscle. The affected muscles are infiltrated predominantly by CD8 cells and to a lesser extent by CD4 cells, suggesting that host immune responses contribute to both the control of the parasites and the progression of clinical disease. Depletion of CD8 or CD4 cells resulted in an increased parasite burden in mice infected with *T. cruzi,*[77] and mice deficient in CD8 cells were very susceptible to infection with *T. cruzi,*[78] indicating the crucial role of these cells in restraining this protozoal infection. It is likely that inflammatory reactions triggered by both the parasites themselves and the specific immune responses against the parasites contribute to the muscle inflammation that occurs in Chagas' disease.[77] A protective role for killer lymphocytes in other intracellular protozoal infections, such as toxoplasmosis, has also been suggested; in a recent study, CD4 cytotoxic T lymphocytes specific for *Toxoplasma gondii* were cloned from a patient with toxoplasmosis,[79] suggesting that lysis of target cells mediated by CD4 cytotoxic T lymphocytes may be partially responsible for the control of this infection.

Autoimmune Diseases

Killer lymphocytes have long been implicated in the pathogenesis of autoimmune diseases. The allergic encephalomyelitis that can be induced by injecting rodents with myelin basic protein or whole-brain homogenates is thought to mimic inflammatory demyelinating disorders such as multiple sclerosis in humans.[80] Experimental allergic encephalomyelitis is characterized by neurologic abnormalities and massive infiltration of lymphocytes into the central nervous system, with injury of astrocytes induced by cytotoxic T lymphocytes specific for myelin basic protein.[81]

Killer lymphocytes have also been implicated in the pathogenesis of other autoimmune disorders. CD8 cytotoxic T lymphocytes and natural killer cells have been identified in the islets of Langerhans in mice with autoimmune nonobese diabetes[82] and the myocardial tissue of mice with myocarditis induced by coxsackievirus B3.[83] In addition, CD4 cytotoxic T lymphocytes have recently been implicated in autoimmune inflammatory colitis in mice.[84] In humans, killer lymphocytes expressing perforin and granzymes have been detected in situ in endocardial tissue obtained from patients with postviral myocarditis,[85] synovial fluid in patients with rheumatoid arthritis,[86] thyroid tissue in patients with Hashimoto's thyroiditis,[87] and aortic tissue in patients with Takayasu's arteritis.[88] Moreover, activated killer lymphocytes have recently been implicated in the pathogenesis of psoriasis.[89] The phenotypes of the killer lymphocytes infiltrating different tissues vary substantially, including CD8 T lymphocytes, CD4 T lymphocytes, γ/δ T cells, and natural killer cells.

The detection of perforin-mediated pore lesions in inflamed human cardiac myocytes[83] (Fig. 4) and of the discharge of perforin from infiltrating cytotoxic T lymphocytes bound to cardiac myocytes[90] provides convincing evidence indicating that killer lymphocytes can lyse target cells in vivo. Furthermore, cytokines secreted by killer lymphocytes and other inflammatory cells infiltrating the islets of Langerhans (e.g., interferon-γ) may contribute to the development of autoimmune diabetes in mice and humans.[91] Taken together, these studies suggest that killer lymphocytes may have a role in inducing and augmenting cell and tissue damage in a variety of autoimmune diseases by lysing autoan-

tigen-expressing cells and secreting cytotoxic cytokines.

Allograft Rejection

Although organ transplantation is now the treatment of choice for some diseases, graft rejection remains the most important factor undermining the success of such treatment. The molecular mechanisms underlying cellular allograft rejection are thought to be cytolytic reactions mediated by killer lymphocytes and delayed-hypersensitivity reactions.[92] Lymphocytes that expressed perforin and granzyme A or B have been detected in situ in both animals and humans who received kidney, heart, or lung transplants.[93,94] Moreover, the extent of the expression of perforin and granzymes in intragraft lymphocytes correlated with the degree of graft rejection,[93,95] and rejection of heart allografts differing by a single MHC class I antigen from the recipient was delayed in perforin-knockout mice.[96]

Tumor Surveillance

Studies demonstrating tumor-specific immune responses have lent support to the possibility of immune surveillance against the formation and growth of tumor.[97] The basis for this immune activity is that immune effector cells can be activated by tumor cells and then become capable of suppressing the growth of or even destroying the tumor cells. The immune cells possibly involved in tumor surveillance include killer lymphocytes.[97] In situ hybridization and immunohistocytochemical techniques were used to detect CD8 cytotoxic T lymphocytes and natural killer cells expressing perforin and granzyme B in tissue sections of follicular lymphomas[98]; these immune cells were apparently present as part of the host immune responses against the tumorous B lymphocytes. Perforin-knockout mice are incapable of rejecting some types of grafted tumor cells,[21] suggesting a role for perforin-mediated lysis in tumor surveillance. Killer lymphocytes may execute the surveillance function not only by directly lysing tumor cells but also by secreting cytostatic or cytocidal cytokines (e.g., tumor necrosis factor α). On the basis of these possibilities, patients with advanced malignant melanoma, renal-cell carcinoma, lung cancer, or malignant glioma refractory to conventional therapies have been treated with lymphokine-activated killer cells or tumor-infiltrating lymphocytes with or without interleukin-2 or other cytokines.[99,100] Although such treatments were effective in several tumor models in mice,[101] their efficiency in the patients was limited.

CONCLUSIONS

Recent advances in the understanding of the basic biology of lymphocyte-mediated cytolysis and its physiologic or clinical relevance have shed light on the development of novel immunotherapeutic approaches to a variety of diseases. Active immunization using vaccines capable of inducing or enhancing immunity mediated by cytotoxic T lymphocytes is being tested for viral and protozoal infections and certain tumors. Adoptive immunotherapy for advanced cancer or CMV infection in immunocompromised patients, although still facing formidable obstacles, has had some preliminary success. In a more specific context, once the structures and mechanisms of action of perforin, granzymes, and Fas ligand are further elucidated, it may be possible to develop novel therapeutic agents based on or targeting these lymphocyte cytotoxins.

Supported in part by grants (CA47307) from the National Cancer Institute, the Hirschl Trust, and the American Heart Association (New York City Affiliate and National Center).

This paper is dedicated to the memory of Dr. Zanvil A. Cohn. We are indebted to Dr. Ralph Steinman for his constant support.

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