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Rapid Communication

Immunohistochemical Localization of Granzyme B Antigen in Cytotoxic Cells in Human Tissues

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Human granzyme B antigen is expressed in cytoplasmic granules of activated cytotoxic T lymphocytes and natural killer cells. Recombinant granzyme B was generated using a prokaryotic expression vector under the control of T7 transcription and translation signals. The 25-kd recombinant protein (granzyme B) was used to develop a rabbit polyclonal antiserum. Purified anti-granzyme B antibodies were used to detect the antigen expression in cytotoxic cells in human tissues. Using the avidin-biotin-complex peroxidase technique, formalin-fixed, paraffin-embedded tissue sections from patients with acute mild or moderate allograft cardiac rejection were stained. A constant cytoplasmic staining of the lymphocytic allograft infiltrate was observed. These results provide a basis for using the anti-granzyme B antibodies for detection of cytotoxic cells in human tissues. The detection and quantitative analysis of the granzyme-associated cytotoxic cells may help to evaluate the significance of these functionally distinct cytotoxic cells in human tissues associated with increased expression of cytoplasmic granule effector molecules. (Am J Pathol 1991, 138:1069–1075)

Human activated cytotoxic T cells and natural killer (NK) cells expressed multicomponent cytoplasmic granules containing cytolytic effector molecules such as perforin,[1–3] proteoglycans,[4] and serine proteinases, alternatively called human granzymes.[5–7] At the protein level, various investigators have isolated and characterized human granzymes from cytotoxic cells.[6,7] Two separate human cDNA clones called Hanunka factor (HuHF) clone and human lymphocyte protease (HLP) clone have been isolated encoding human granzyme A and B, respectively.[8,9] The functions of granzymes in T-cell–mediated reactions are poorly understood. *In vitro* studies have demonstrated 1) the secretion of granzyme A from T cells on T-cell receptor stimulation,[10] 2) the possible involvement of serine proteinases in the lytic process,[11,12] 3) the triggering of initial transmembrane signaling in cytotoxic cells by a serine protease,[13] and DNA degradation by granzyme A in permeabilized target cells.[14]

Until now, *in vivo* expression of cytoplasmic granule proteins in human cytotoxic cells has not been studied, although initial reports using *in situ* hybridization of cellular mRNA with labeled probes for HuHF gene (granzyme A) and HLP gene (granzyme B) described detection of cytotoxic cells in human dermatoses and renal allograft rejection, respectively.[15,16] Using the avidin-biotin-complex (ABC) immunoperoxidase technique, we detected cytotoxic cells in human rejected cardiac allografts using purified polyclonal anti-granzyme B antibody.

Materials and Methods

Production of Polyclonal Anti-granzyme B

Recombinant granzyme B was generated using a prokaryotic expression vector under the control of T7

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transcription and translation signals.[17] Briefly, a Bal I site was introduced by changing nucleotide 17 (of the mature protein) from A to T using the *in vitro* mutagenesis strategy (BioRad, Zurich, Switzerland) resulting in a single amino acid change at residue 6 of mature granzyme B from Glu to Val. The Bal I and BamHI fragment of mutagenized granzyme B was excised and ligated into the Nde I-BamHI sites of the vector pAR 30038 using the synthetic adaptor 5′-ATG ATC ATG GGG GGA CAT GTC-3′ coding for the N terminus Met-Ile-Ile-Gly-Gly-His of mature granzyme B.

Synthesis of recombinant granzyme B was induced in DE3 bacteria, which contain in their genome the T7 polymerase gene under an isopropylthiogalactoside-inducible promoter. The 25-kd recombinant protein was purified from the bacteria by lysing the bacterial pellet (from 100-ml culture) with 20 ml of a solution containing 50 mmol/l (millimolar) TRIS-HCl (pH 7.5), 0.5 mmol/l ethylene diamine tetra acetic acid (EDTA), and 10 mg/ml lysozyme during 2 hours on ice. The bacterial DNA subsequently was sheared by sonication (Branson sonified B12, Danburg, CT) and the protein pelleted in a microfuge (10 minutes at 12,000 rpm). The pellet was washed three times in a solution of 50 mmol/l TRIS-HCl, pH 7.5, 0.5 mmol/l EDTA, and 300 mmol/l NaCl, and dissolved in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer.[18] Recombinant granzyme B was separated from major bacterial proteins by SDS-PAGE (12% polyacrylamide gel) and electroeluted using the Bio-Rad protein elution apparatus. From the 100-ml bacterial culture, approximately 1 to 2 mg recombinant protein was obtained repeatedly.

Recombinant granzyme B (100 μg) was injected subcutaneously into rabbits in complete Freund's adjuvant. At 3-week intervals, rabbits were boosted twice and after the third injection, the rabbits were bled and the sera tested for reaction with purified granzyme B isolated from lymphokine-activated killer (LAK) cells[7] and with recombinant protein. On Western blots of lysates of interleukin-2 (IL-2) activated human peripheral blood lymphocytes, one single protein band with the predicted molecular size of granzyme B was detected.[19] The immunoglobulin G (IgG) fraction of the polyclonal antiserum was purified using a protein G-Sepharose fast-flow column according to the instructions of the manufacturer (Pharmacia, Piscataway, NJ).

Generation of Human Cytotoxic Cells

The human cytotoxic cells for positive control were prepared by cultivation in recombinant IL-2, as described elsewhere.[1,6] The cells were harvested after 10 days in culture and washed three times in phosphate-buffered saline (PBS). Paraffin-embedded cell blocks of LAK cells and unstained peripheral blood mononuclear cells were prepared after fixation in 10% formalin overnight. Some cytocentrifuge preparations were fixed in acetone and 95% alcohol separately and maintained at 4°C.

Human Tissue

Five patients with acute, mild, or moderate cardiac allograft rejection were studied. The allograft biopsies were fixed immediately in 10% buffered formalin for up to 10 hours and were embedded in paraffin. The degree of rejection was assessed using the criteria described by Billingham.[20] The tissue sections of various normal organs were obtained from the autopsy files of The Methodist Hospital.

Immunohistochemical Staining

The tissue sections were deparaffinized in fresh xylene and rehydrated in descending grades of alcohol solution (100% to 70%). The endogenous peroxidase activity was blocked by incubating the sections for 20 minutes in methanol containing 3% H_2O_2. After distilled water and PBS washes, the sections were digested with 0.1% trypsin from bovine pancreas (Sigma Chemical Co., St. Louis, MO) in PBS at 37°C for 30 to 60 minutes. To block nonspecific binding, the sections were covered with protein-blocking solution (Lipshaw Immunon, Detroit, MI). After 20 minutes, the excess blocking solution was removed and the sections were covered with 1:100 to 1:1500 dilutions of granzyme B antibody at room temperature for 90 minutes. After PBS washes, biotinylated goat anti-rabbit IgG (diluted 1:400) was added to the sections for 60 minutes. The tissue sections were washed and incubated for 60 minutes with ABC Elite Kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's recommendations. The immunostaining was developed using diaminobenzidine (100 mg in 200 ml of PBS) plus 200 μl 30% H_2O_2. The slides were counterstained with Mayer's hematoxylin.

In absorption experiments, the granzyme B antibody was absorbed with homogentes of human LAK cells, myometrium, and skeletal muscle. The absorption was carried out at room temperature for 1 hour with equal volumes of the diluted antibody and the homogenates. After absorption, anti-granzyme B binding of human cytotoxic cells and vascular smooth muscle in human cardiac allograft was studied using ABC-peroxidase technique.

Results

Examination of the tissue sections stained by hematoxylin and eosin showed that in acute mild rejections there were

only perivascular aggregates of mononuclear cells with extension into the myocardial interstitium. Moderate rejection was characterized by more increased perivascular and interstitial infiltrate accompanied by degeneration or necrosis of the myocytes.

The study using purified polyclonal anti-granzyme B antibody demonstrated excellent staining of the cytotoxic cells in both positive control slides as well as the paraffin tissue sections of cardiac allograft rejection. Figure 1A shows LAK cells with 98% of the cells staining with granzyme B antibodies at a 1:600 dilution. The staining pattern generally was granular and cytoplasmic. Most LAK cells reacted with CD3, CD4, CD8, and CD16 cell-surface antigens, whereas Leu-7 antigen expression was rare. The normal rabbit serum used as negative control showed no staining of the LAK cells (Figure 1B). In addition, the unstimulated peripheral blood mononuclear cells showed some granzyme B-positive mononuclear cells, probably representing NK cells. The peripheral blood as well as tissue infiltrate of polymorphs, monocytes, or macrophages showed no staining with granzyme B antibody (data not shown).

Figure 1C illustrates the cytoplasmic staining of the lymphocytes infiltrating the myocardium of a patient with moderate acute rejection. The vast majority of cellular infiltrate stained but the intensity of the staining reaction was variable. The staining was strong for the transformed (activated) lymphocytes found in the perivascular, intravascular, and interstitial areas. The staining was less pronounced in the small lymphocytes, which were also present in small numbers in the myocardium. The immune reaction was less pronounced in lymphocytes, which are smaller than those plump lymphoblasts. The antibody reactivity was optimal at a 1:600 dilution because lower dilutions showed strong background staining, whereas higher dilutions led to progressive loss of immunostaining. The best results were obtained after 60 to 90 minutes incubation. Prolonged incubation for 12 to 24 hours at higher dilutions was unsuccessful. A constant immunoreactivity of granzyme B antibody with smooth muscle of blood vessels in the myocardium was observed (Figure 1D). The specificity of granzyme B antibody staining is summarized in Table 1. Absorption experiments (Table 2) confirmed restriction of granzyme B reactivity to an antigenic determinant shared by smooth muscle. The fact that some proteolytic enzymes reduce the nonspecific background and enhance the immune reactivity of the formalin-fixed tissue prompted us to use trypsin.[21] A marked reduction in the nonspecific background and slight improvement in the immunostaining was observed by preincubating the paraffin sections with 0.1% trypsin for 30 minutes. Some foci of unstained lymphocytic infiltrate were present in some sections, especially where the infiltrate was arranged in either ribbons or clumps adjacent to the damaged myocardium. There was no staining of the mononuclear cardiac allograft infiltrate by preimmune rabbit serum used as negative control (data not shown).

Table 1. *Reactivity of Granzyme B Antibody with Various Tissues*

Tissues	Granzyme B antibody reactivity
Gastrointestinal tract	
Intestinal epithelium	–
Kidney	
Tubular epithelium	–
Glomeruli	–
Interstitium	–
Liver	
Hepatocytes	–
Biliary tract epithelium	–
Pancreas	
Pancreatic acini	–
Pancreatic ductal epithelium	–
Lung	
Respiratory epithelium	–
Lung parenchyma	–
Endocrine glands	
Thyroid	–
Adrenal	–
Pituitary	–
Skin	
Epidermis	–
Dermis	–
Muscles	
Cardiac muscle	–
Skeletal muscle	–
Smooth muscle	+
Nervous System	
Cerebrum	–
Cerebellum	–
Spinal cord	–
Peripheral nerve	–

Discussion

Immunohistochemistry has led to a better understanding of the immunology of T cells. The two main advances in immunohistochemical techniques in this area of research include 1) the development of a variety of immunoperox-

Table 2. *Absorption Experiments with Granzyme B Antibody*

Granzyme B antibody	Cardiac allograft infiltrate	Myocardial vascular smooth muscle
Unabsorbed	+	+
Absorbed with LAK* cells	–	–
Absorbed with smooth muscle‡	±†	–
Absorbed with skeletal muscle	+	+

* Lymphokine-activated killer cells.
† Weak residual staining of the cardiac allograft infiltrate.
‡ Myometrium.

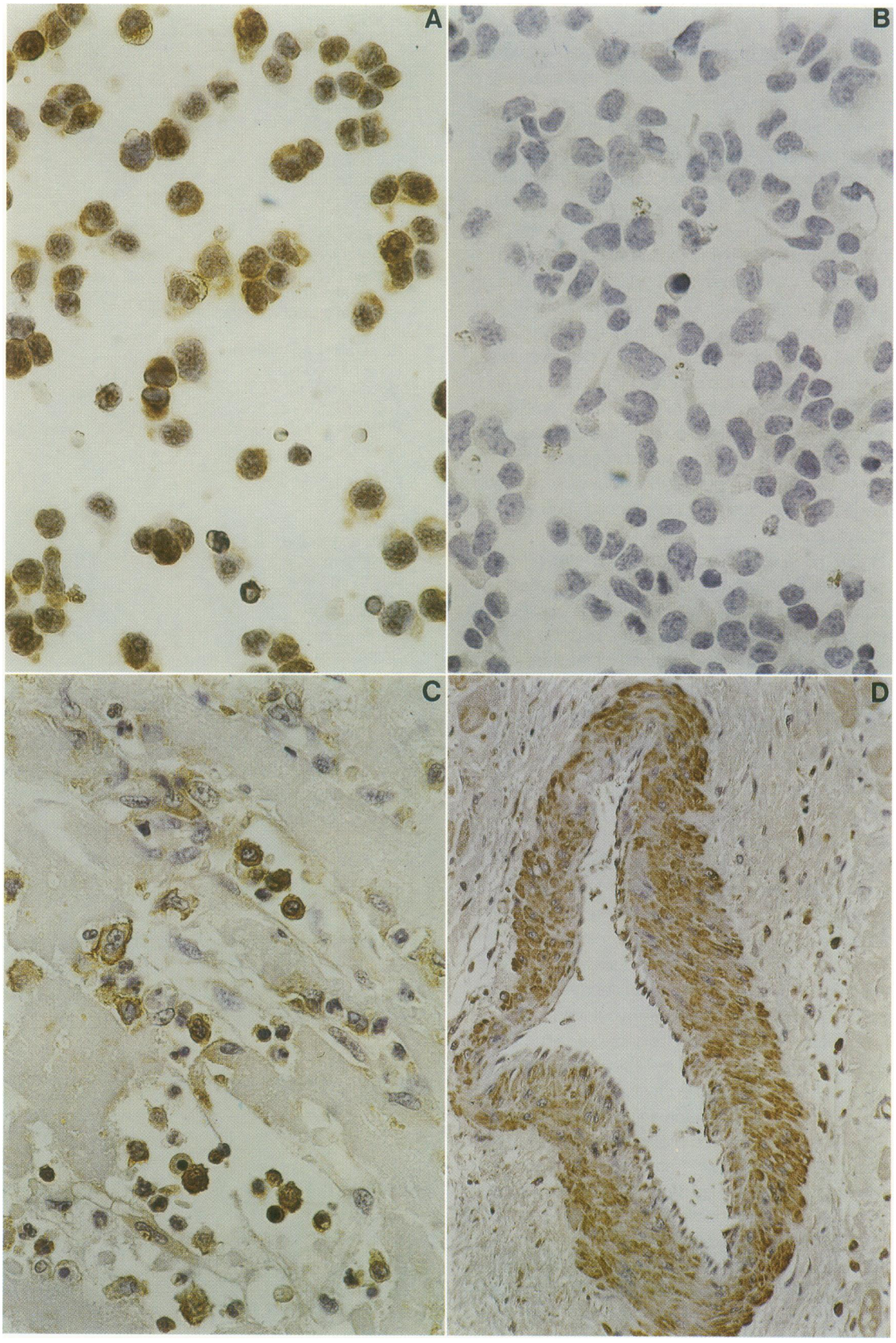
A
B
C
D

Figure 1. *Immunoperoxidase staining.* A: *LAK cells (positive control) using granzyme B antibody (diluted 1:600).* B: *Normal rabbit serum.* C: *Demonstrates immunostaining of the formalin-fixed tissue section from human cardiac allograft rejection by anti-granzyme B antibody (diluted 1:600).* D: *Represents cross-reactivity of anti-granzyme B antibody with vascular smooth muscle.* C and D *were treated with 0.1% trypsin for 30 minutes before immunostaining (original magnification:* A, B, C, ×100; D, ×10).

idase methods and 2) production of a large number of polyclonal and monoclonal antibodies capable of recognizing various T-cell subsets. A large number of antigens generally absent from normal T cells are expressed by activated and/or neoplastic T cells. These include T10 (CD38) and human lymphocyte antigen-DR surface antigens,[22] hairy cell leukemia-associated antigen LeuM5 ($CD11_c$),[23] granulocyte/monocyte-associated antigen LeuM1 (CD15),[24] the Reed–Sternberg cell-associated antigen Ki-1 (CD30),[25–27] and the Tac antigen (CD25/IL-2–R).[20,28] These antigens and the commonly used surface antigens CD4, CD8, CD16, and Leu7, which define cells possessing helper/inducer, cytotoxic/suppressor, or NK activity, obviously do not mark cytoplasmic granules of activated T cells and NK cells implicated in cytolysis by granule exocytosis.[29–32] However a recent study reported a monoclonal antibody, designated TIA-1, that recognizes an intracellular 15-kd cytoplasmic granule-associated protein in murine cytotoxic cells.[33] Another study described identification of pore-forming protein, perforin in cytotoxic cells among inflammatory infiltrate during acute myocarditis.[34] Our study, using tissues from human cardiac allograft rejection, localized granzyme B antigen that is one of the apparently constant components of cytoplasmic granules of human cytotoxic cells. Although the role of granzyme B in cell-mediated cytolysis is unknown, its coexpression with other important cytolytic effector molecules, such as perforin, still makes it a potential cytoplasmic granule marker of cytotoxic cells in human tissues.

Most of the activated T-cell and NK-cell markers react well on frozen sections, which are not always available. The paraffin-embedded human tissues, which are more commonly available, are not always suitable for immunostaining because many of the antigens are inactivated during routine tissue processing. The fact that granzyme B reactivity is well preserved in formalin-fixed tissue provides an excellent means to identify and quantitate cytotoxic cells in formalin-fixed human tissues. The analysis of cell specificity of granzyme B gene expression in various tissues, including smooth muscle (stomach), cardiac, and skeletal muscle, showed no detectable level of granzyme B transcript.[35] The cross-reactivity of granzyme B antibody with smooth muscle, indeed suggests that there may be some unrelated smooth muscle antigen that merely shares a common antigenic determinant with granzyme B antigen. However the immunoreactivity of granzyme B antibody with smooth muscle should not reduce the prospective diagnostic value of the antibody because the smooth muscle cells are quite distinguishable by morphologic criteria. Some foci of unstained mononuclear inflammatory cells among the graft infiltrate were obvious in some sections. A number of possibilities should be considered to explain this phenomenon: 1) The unstained mononuclear infiltrate may not represent cytotoxic cells; 2) lack of immune reaction may be due to relative preservation of cytoplasmic or granule membranes with consequent inaccessibility of the antigen for the antibodies reaction; 3) it may imply *in vivo* degranulation of the cytotoxic cells during lethal hit; 4) because acute rejection takes place within several days, the cellular infiltrate in different areas does not correspond to the same time points. As a result, the cells with poor and/or no staining may represent infiltrate at relatively earlier time points and perhaps with less well-preserved intracytoplasmic antigens.

We emphasize that our study with anti-granzyme B antibody was merely directed toward the *in vivo* detection of granzyme B antigen in cytotoxic cells in human tissues. The presence of predominant granzyme-associated cytotoxic cells in cardiac allograft infiltrates during acute mild or moderate rejection reflects that they are the main cell type engaged in rejection. An *in vivo* study by Mueller et al[36] also demonstrated a predominant T-lymphocytic infiltrate expressing two serine esterase genes in mouse cardiac allograft rejection. However careful quantitative analysis of these cytotoxic cells in human tissue in conjunction with the patient's clinical course is required to understand their role in graft survival. Indeed the production of monoclonal granzyme B antibodies with probable high specificity, high titer, and unlimited supply may make them an invaluable tool for studying human cytotoxic cells among inflammatory infiltrates of multiple organs in various immune reactions.

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References

1. Lowrey DM, Hameed A, Lichtenheld M, Podack ER: Isolation and characterization of cytotoxic granules from human

lymphokine-(IL-2) activated killer cells. Cancer Res 1988, 48:4681–4688

2. Lichtenheld MG, Olsen KJ, Lu P, Lowrey DM, Hameed A, Hengartner H, Podack ER: Human perforin: Structure and function. Nature (London) 1988, 335:448–451
3. Ferguson WS, Verret CR, Reilly EB, Iannini MJ, Eisen HN: Serine esterase and hemolytic activity in human cloned cytotoxic T lymphocytes. J Exp Med 1988, 167:528–540
4. Schmidt RE, MacDermott RP, Bartley G, Bertovich M, Amato DA, Austen KF, Schlossaman SR, Steven RL, Ritz J: Specific release of proteoglycans from human natural killer cells during target lysis. Nature 1985, 318:289–291
5. Fruth U, Sinigaglia F, Schlesier M, Kilgus J, Kramer MD, Simon MD: A novel serine proteinase (HuTSP) isolated from a cloned human $CD8^+$ cytolytic T cell line is expressed and secreted by activated $CD4^+$ and $CD8^+$ lymphocytes. Eru J Immunol 1987, 17:1625–1633
6. Hameed A, Lowrey DM, Lichtenheld M, Podack ER: Characterization of three serine esterases isolated from human IL-2 activated killer cells. J Immunol 1988, 141:3142–3147
7. Krahenbuhl O, Rey C, Jeune D, Lanzavecchia A, Groscurth P, Carrel S, Tschopp J: Characterization of granzymes A and B isolated from granules of cloned human cytotoxic T lymphocytes. J Immunol 1988, 141:3471–3477
8. Gershenfeld HK, Hershberger RJ, Shows TB, Weissmann IL: Cloning and chromosomal assignment of a human cDNA encoding a T cell- and natural killer cell-specific trypsin-like serine protease. Proc Natl Acad Sci USA 1988, 85:1184–1188
9. Schmid J, Weissmann C: Induction of mRNA for a serine protease and a beta-thromboglobulin like protein in mitogen-stimulated human leukocytes. J Immunol 1987, 139:250–256
10. Jenne DE, Tschopp J: Granzymes, a family of serine proteases released from granules of cytolytic T lymphocytes upon T cell receptor stimulation. Immunol Rev 1988, 103:53–68
11. Redelman D, Hudig D: The mechanism of cell-mediated cytotoxicity. III. Protease-specific inhibitors preferentially block later events in cytotoxic T lymphocyte-mediated lysis than do inhibitors of methylation or thiol-reactive agents. Cell Immunol 1983, 81:9–21
12. Redelman D, Hudig D: The mechanism of cell-mediated cytotoxicity. I. Killing by murine cytotoxic T lymphocytes requires cell surface thiols and activated proteases. J Immunol 1980, 124:870–878
13. Utsunomyiya N, Nakanishi M: A serine proteases triggers the initial step of transmembrane signalling in cytotoxic T cells. J Biol Chem 1986, 261(35):16514–16517
14. Hayes MP, Berrebi GA, Henkart PA: Induction of target cell DNA release by the cytotoxic T lymphocyte granule protease granzyme A. J Exp Med 1989, 170:933–946
15. Wood GW, Mueller C, Warnke RA, Weissmann IL: In situ localization of HuHF serine protease mRNA and cytotoxic cell-associated antigen in human dermatoses. Am J Pathol 1988, 133(2):218–225
16. Clement M-V, Haddad P, Ring GH, Pruna A, Sasportes M: Granzyme B gene expression: A marker of human lymphocytes "activated" in vitro or in renal allografts. Human Immunology 1990, 29:159–166
17. Studier FW, Moffatt BA: Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. J Mol Biol 1986, 189:113–130
18. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970, 227:680–685
19. Acha-Orbea H, Scarpellino L, Hertig S, Dupuis M, Tschopp J: Inhibition of lymphocyte-mediated cytotoxicity by perforin antisense oligonucleotides. EMBO 1990, 9(12):3815–3819
20. Billingham ME: Diagnosis of cardiac rejection of endomyocardial biopsy. Heart Transplantation 1982, 1:25–29
21. Ordonez NG, Manning JT, Brooks TE: Effect of trypsinization on the immunostaining of formalin-fixed paraffin-embedded tissues. Am J Surg Pathol 1988, 12(2):121–129
22. Schmidt C, Burman K, Moss DJ, Ryan J, Burrows SR, Misko IS: Interleukin-2 receptors in infectious mononucleosis. Immunology Letters 1989, 23:139–142
23. Chadburn A, Inghirami G, Knowles DM: Hairy cell leukemia-associated antigen LeuM5 ($CD11_c$) is preferentially expressed by benign activated and neoplastic CD8 T cells. Am J Pathol 1990, 136:29–37
24. Pinkus GS, Thomas P, Said JW: Leu-M1—A marker for Reed-Sternberg cells in Hodgkin's disease: An immunoperoxidase study of paraffin-embedded tissues. Am J Pathol 1985, 119:244–252
25. Schwab U, Stein H, Gerdes J, Lemke H, Kirchner H, Schaadt M, Diehl V: Production of a monoclonal antibody specific for Hodgkin and Reed–Sternberg cells of Hodgkin's disease and a subset of normal lymphoid cells. Nature 1982, 299:65–67
26. Turbitt ML, Mackie RM: An assessment of the diagnostic value of the monoclonal antibodies Leu 8, OKT9, OKT10 and Ki67 in cutaneous lymphocytic infiltrates. Br J Dermatol 1986, 115:151–158
27. Gerdes J, Lemke H, Baisch H, Wacker HH, Schwab U, Stein H: Cell cycle analysis of a cell proliferation human nuclear antigen defined by the monoclonal antibody Ki-67. J Immunol 1984, 133:1710–1715
28. Waldmann TA: The structure, function and expression of interleukin 2 receptor on normal and malignant lymphocytes. Science 1986, 232:727–732
29. Henkart PA: Mechanism of lymphocyte-mediated cytotoxicity. Ann Rev Immunol 1985, 3:31–58
30. Podack ER: The molecular mechanism of lymphocyte-mediated cytotoxicity. Immunol Today 1985, 6:21–27
31. Young JDE, Liu CC: Multiple mechanism of lymphocyte-mediated killing. Immunol Today 1988, 9:140–143
32. Tschopp J, Jongeneel CV: Cytotoxic T lymphocyte mediated lysis. Biochemistry 1988, 27:2641–2646
33. Anderson P, Nagler-Anderson C, O'Brien C, Levine H, Wat-

kins S, Slayter HS, Blue M, Schlossman SF: A monoclonal antibody reactive with a 15-kDa cytoplasmic granule-associated protein defines a subpopulation of $CD8^+$ T lymphocytes. J Immunol 1990, 144:574–582

34. Lucy HYY, Joag SV, Zheng L-M, Lee C-P, Lee Y-S, Young JD-E: Perforin mediated myocardial damage in acute myocarditis. Lancet 1990, 336:1019–1021
35. Garcia-Sanz JA, MacDonald HR, Jenne DE, Tschopp J, Nabholz M: Cell specificity of Granzyme B gene expression. J Immunol 1990, 145:3111–3118
36. Mueller C, Gershenfeld HK, Lobe GG, Okada CY, Bleackley RC, Weissman IL: A high proportion of T-lymphocytes that infiltrate H-2 incompatible heart allografts in vivo express genes encoding cytotoxic cell specific serine proteases, but do not express the MEL-14 defined lymph node homing receptor. J Exp Med 1988, 167:1124–1136