Involvement of Granulocyte-Macrophage Colony-Stimulating Factor in Pulmonary Homeostasis

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The in vivo function of murine granulocyte-macrophage colony-stimulating factor (GM-CSF) was investigated in mice, carrying a null allele of the GM-CSF gene, that were generated by gene targeting techniques in embryonic stem cells. Although steady-state hematopoiesis was unimpaired in homozygous mutant animals, all animals developed the progressive accumulation of surfactant lipids and proteins in the alveolar space, the defining characteristic of the idiopathic human disorder pulmonary alveolar proteinosis. Extensive lymphoid hyperplasia associated with lung airways and blood vessels was also found, yet no infectious agents could be detected. These results demonstrate that GM-CSF is not an essential growth factor for basal hematopoiesis and reveal an unexpected, critical role for GM-CSF in pulmonary homeostasis.

Granulocyte-macrophage colony-stimulating factor is a 23-kD glycoprotein initially identified by its ability to promote the in vitro proliferation and differentiation of hematopoietic progenitors to neutrophils and macrophages (*1*). Subsequent studies extended the range of colony-stimulating activity to include the generation of eosinophils, erythrocytes, megakaryocytes, and dendritic cells (*2*, *3*). Administration of GM-CSF to mice, monkeys, and humans (*4*) has demonstrated the ability of the molecule to stimulate hematopoiesis in vivo as well, with the most pronounced effects being on monocyte-macrophages, neutrophils, and eosinophils. In addition to its effects on hematopoiesis, GM-CSF can also modulate the function of mature hematopoietic cells. These effects include enhanced antigen presentation (*5*), increased complement- and antibody-mediated phagocytosis (6), augmented microbicidal capacity (*7*), heightened leukocyte chemotaxis and adhesion (8), and augmented antitumor immunity (9). Other investigations further suggest that GM-CSF can influence endothelial cell activity and placental function (*10*).

The multiplicity of activities described above and the substantial evidence from in vitro studies of redundancy in cytokine function have made delineating the physiologic role of GM-CSF problematic. To understand this role further, we generated mice lacking GM-CSF by homologous recombination in embryonic stem cells (Fig. 1, A and B). Mutant mice were obtained at the expected frequencies, remained clinically healthy throughout 1 year of observation, and were fertile. Supernatants of concanavalin A (Con A)–stimulated splenocytes from mutant animals demonstrated no immunoreactive or bioactive GM-CSF, as revealed by proliferative studies with the FDCP-1 myeloid cell line (Fig. 1C).

Analysis of steady-state hematopoiesis revealed normal numbers of peripheral blood cells, bone marrow progenitors, and tissue hematopoietic populations, including splenic dendritic cells (*11*). This last finding is particularly relevant, as recent studies suggested a critical function for GM-CSF in the generation of this specialized antigen-presenting cell from granulocyte-macrophage committed bone marrow progenitors (*12*). Mutant marrow was also able to reconstitute lethally irradiated mutant recipients (*11*). These findings demonstrate either that redundancy of cytokine function for hematopoiesis exists in vivo or, perhaps, that GM-CSF plays no role at all in basal hematopoiesis. Whichever is the case, the historical notion that GM-CSF is a critical regulator of myelopoiesis in vivo (*13*) must now be modified.

Pathologic analysis revealed abnormalities only in the lungs. By 2 months of age, scattered, amorphous, acellular, eosinophilic material was present in the alveolar spaces of all mutant mice, but not in heterozygote or wild-type mice (Fig. 2, A

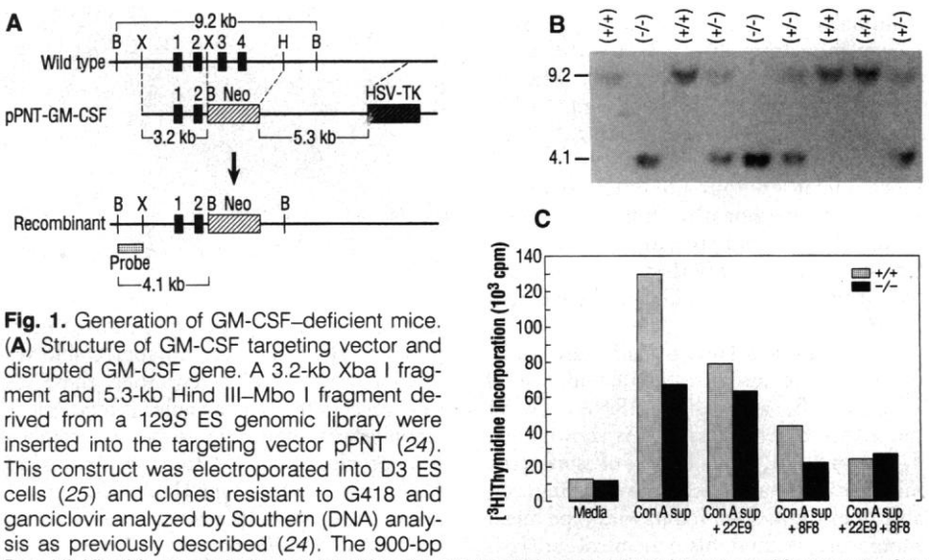

Fig. 1. Generation of GM-CSF–deficient mice. (**A**) Structure of GM-CSF targeting vector and disrupted GM-CSF gene. A 3.2-kb Xba I fragment and 5.3-kb Hind III–Mbo I fragment derived from a 129*S* ES genomic library were inserted into the targeting vector pPNT (*24*). This construct was electroporated into D3 ES cells (*25*) and clones resistant to G418 and ganciclovir analyzed by Southern (DNA) analysis as previously described (*24*). The 900-bp Bam HI–Xba I fragment indicated identifies the 9.2-kb wild-type and 4.1-kb targeted fragments after Bam HI digestion as shown. B, Bam HI; H, Hind III; X, Xba I. (**B**) Genotype of mutant animals. Targeted clones were injected into C57B1/6 blastocysts as described (*26*) to generate chimeric animals transmitting the mutant allele through the germ line. Heterozygous mice were mated to generate mice homozygous for the targeted mutation. Tail DNA (*27*) was digested with Bam HI and probed as above. Molecular sizes are indicated on the left (in kilodaltons). (**C**) Absence of bioactive GM-CSF protein. FDCP-1 cells (5×10^4) (*28*) were stimulated with a 1:100 dilution of Con A (3 μg/ml)–stimulated splenocyte (5×10^6 cells) conditioned medium, and incorporation of [^{3}H]thymidine was determined after 48 hours. Monoclonal antibody 22E9 neutralizes murine GM-CSF and 8F8 neutralizes murine interleukin-3 (*29*).

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and B). The accumulation progressed with time, becoming more widespread (Fig. 2, C and D). Electron microscopy demonstrated that the material had the characteristic morphologic features of surfactant, including the presence of tubular myelin and multilamellated structures (Fig. 3, A and B).

Surfactant is a complex mixture of phospholipid (predominantly dipalmitoylphosphatidylcholine and phosphatidylglycerol) and protein (surfactant proteins A, B, C, and D), which functions in the alveolus primarily to reduce surface tension at the air-liquid interface, although it may also contribute to lung defense (*14*). Surfactant proteins and lipids are synthesized by type II pneumocytes and cleared from the alveolar space by recycling and degradation by type II cells and, to a lesser extent, alveolar macrophages (*15*).

Bronchoalveolar lavage obtained from the mutant animals was highly enriched in pulmonary surfactant lipid and proteins. Protein immunoblot analysis of surfactant proteins SP-A, -B, and -C demonstrated a marked increase in the abundance of each of the proteins, as well as an increased abundance of nonreducing oligomers of SP-A (*16*). Enzyme-linked immunosorbent assay (ELISA) also showed a marked elevation in SP-A and SP-B in lung lavage fluid, confirming the immunoblot analysis (*17*). Total protein content in the lavage fluid was increased approximately eightfold in the mutant mice, from 0.33 ± 0.05 to 2.53 ± 0.35 mg/ml (mean ± SEM, n = 3 animals). SP-A content was increased in lavage fluid from 41 ± 27 to 218 ± 24 U/ml (mean ± SEM, n = 3), whereas SP-B content was increased from 0.12 ± 0.05 to 12.8 ± 2.8 U/ml (mean ± SEM, n = 3). Collectively, the morphologic and biochemical findings presented here are typical of an idiopathic human disorder, pulmonary alveolar proteinosis (*18*). Studies to determine whether some patients with this disorder are GM-CSF–deficient are in progress.

To clarify the basis of surfactant accumulation, we assessed the abundance of SP-A, SP-B, and SP-C mRNAs by S1 nuclease protection assays. As shown (Fig. 4, A and B), the levels of surfactant mRNAs in mutant mice were indistinguishable from those observed in wild-type mice. Moreover, immunohistochemical analysis of sections of mutant lung indicated no accumulation of either mature surfactant protein A or its precursor within the type II pneumocyte (Fig. 4, C and D). In contrast, alveolar macrophages from GM-CSF–deficient mice demonstrated a marked increase of surfactant protein and lipid (Fig. 4, C, D, E, and F). Although quantitation of alveolar macrophages in the mutant mice was complicated by the spectrum of morphologic abnormalities, no appreciable reduction was observed. Together, these results suggest that the alveolar proteinosis is secondary to deranged surfactant catabolism or clearance. Because recent studies have indicated that type II and other respiratory epithelial cells constitutively synthesize GM-CSF (*19*), defective alveolar macrophage processing of surfactant, as a result of absence of GM-CSF in the mutant animals, is strongly implicated.

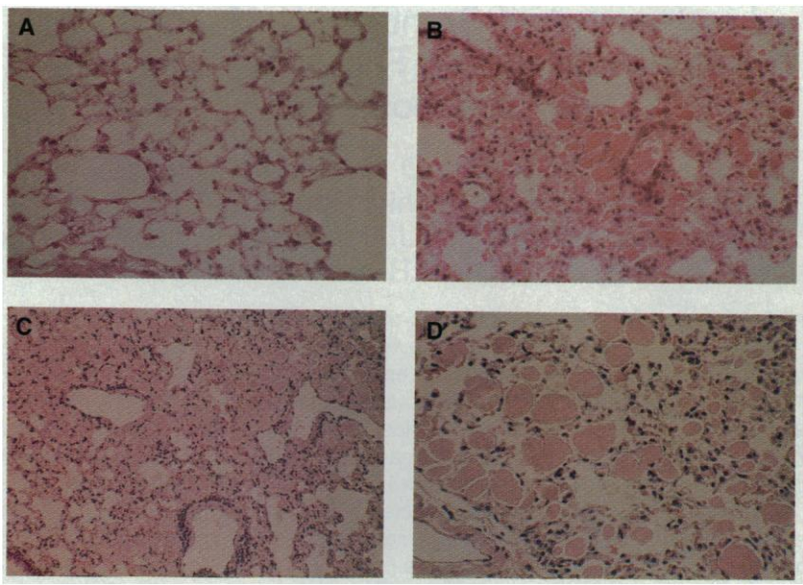

Fig. 2. GM-CSF–deficient mice develop pulmonary alveolar proteinosis. (**A**) Normal lung from a wild-type animal. (**B**) Early alveolar proteinosis in a 2-month-old mutant mouse. Note the acellular eosinophilic material in the alveolar spaces. (**C**) Extensive proteinosis in a 7-month-old mutant mouse. (**D**) Higher magnification of the proteinosis. Tissues were fixed in either Bouin's solution (Sigma) or 10% formalin, embedded in paraffin, and stained with hematoxylin and eosin.

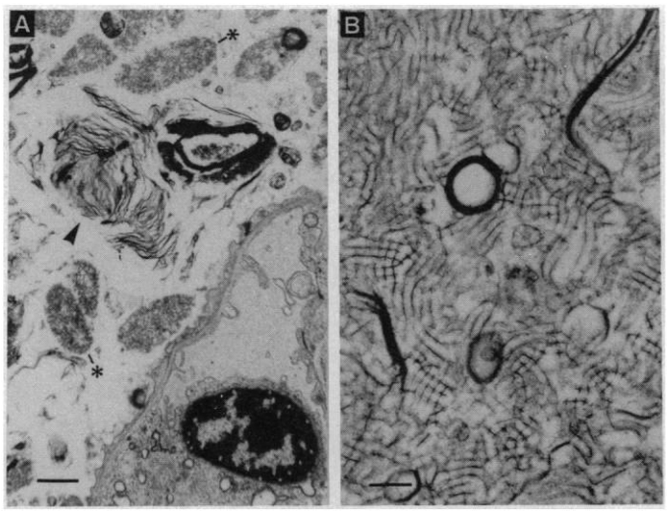

Fig. 3. Surfactant accumulation. (**A**) An alveolar space contains both multilamellated substance (arrow) and tubular myelin (*). Bar, 1.754 × 10^3 nm. (**B**) Higher magnification of tubular myelin discloses a characteristic pattern of intersecting membranes. Bar, 2.174 × 10^2 nm. Tissues were processed in Karnovsky's solution, osmium tetroxide, and Epon and stained with lead citrate.

In addition to the accumulation of surfactant, mutant mice demonstrated an extensive lymphoid hyperplasia around both airways and veins (Fig. 5, B and C), in contrast to littermates, which showed minimal if any lymphoid proliferation (Fig. 5A). Immunohistochemical analysis revealed the presence of B220, CD4, and CD8 positive cells in the hyperplasia (*16*). Cultures of lung washings, special histologic stains for microorganisms, and serologic studies did not reveal an underlying infection. It is possible that the lymphoid hyperplasia represents an excessive response to innocuous inhaled antigens. Alveolar macrophages are known to inhibit mitogen-induced lymphocyte proliferation in vitro (*20*) and perhaps function to buffer the lung from toxicities associated with inflammation.

In light of the recent findings that a number of members of the "collectin" family of proteins (*21*), including surfactant protein A, complement protein Clq, man-

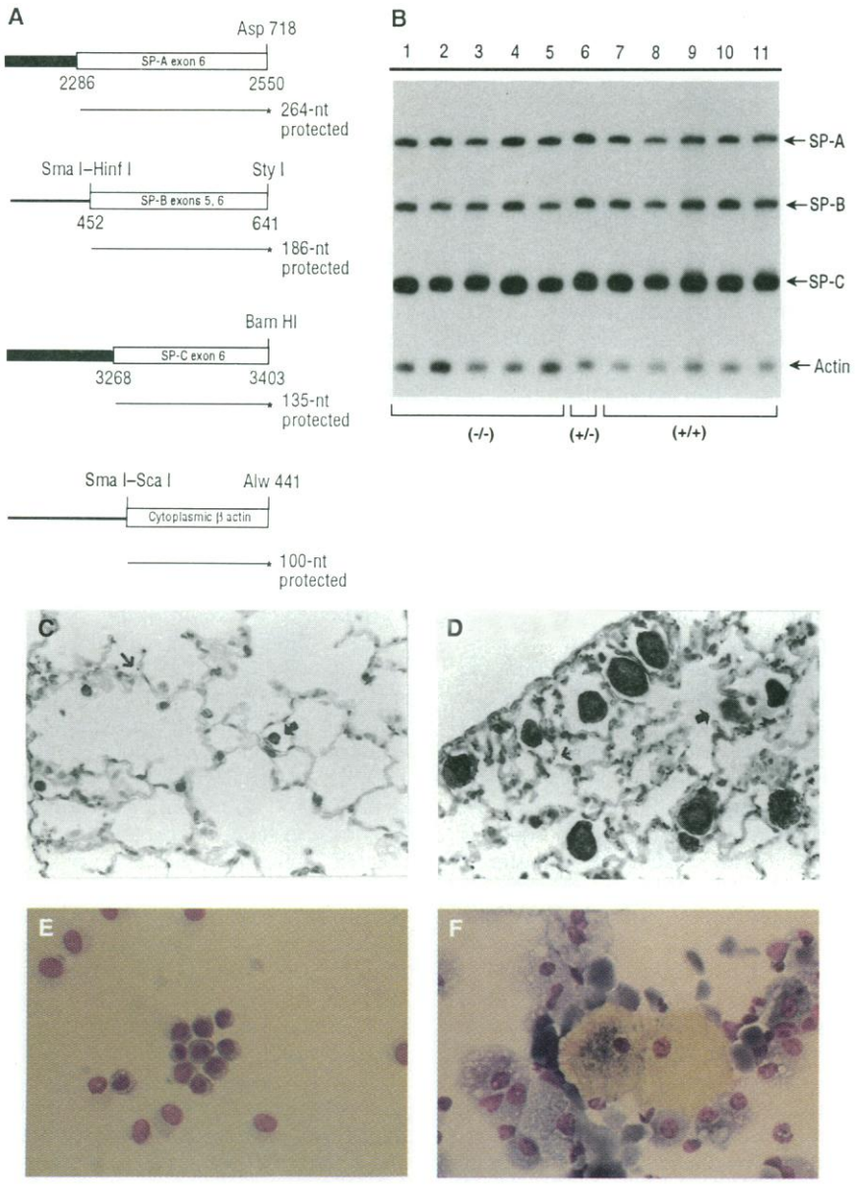

Fig. 4. Surfactant metabolism in the GM-CSF–deficient mice. (**A**) Schematic diagram of the S1 nuclease probes (*30*) and protected fragment sizes for mouse surfactant protein mRNAs and cytoplasmic β-actin. (**B**) Pulmonary surfactant protein mRNA abundance is not altered in mutant mice. S1 nuclease protection analysis of lung surfactant protein mRNAs. Solution hybridization was done at 56°C for 18 hours with an excess of ^{32}P-labeled linearized probes and 2 μg of lung total RNA. After treatment with S1 nuclease, the protected fragments were resolved on a 6% polyacrylamide–8 M urea gel and visualized by autoradiography (2-hour exposure). Lanes 1 to 5, lung RNA from GM-CSF–deficient mice (−/−); lane 6, heterozygous animal (+/−); and lanes 7 to 11, wild-type animals (+/+). (**C**) Immunohistochemistry of surfactant protein A in wild-type and mutant (**D**) lung. Thin arrows denote type II cells and thick arrows indicate alveolar macrophages. Lungs were fixed in 4% paraformaldehyde, paraffin embedded, and stained with the primary antibody at a 1:16,000 dilution after pretreatment with 10% skim milk. Biotinylated goat antibody to guinea pig immunoglobulin G and Vector ABC kit were used to visualize the horseradish peroxidase staining. (**E**) Normal alveolar macrophages from a wild-type mouse. (**F**) Abnormal macrophages from the mutant mouse, appearing stuffed with surfactant. Alveolar macrophages were harvested by repetitive lavage of lungs with cold phosphate-buffered saline containing 1 mM EDTA after cannulation of the trachea with a 25-guage butterfly needle. Cytospin preparations were stained with Wrights and Giemsa.

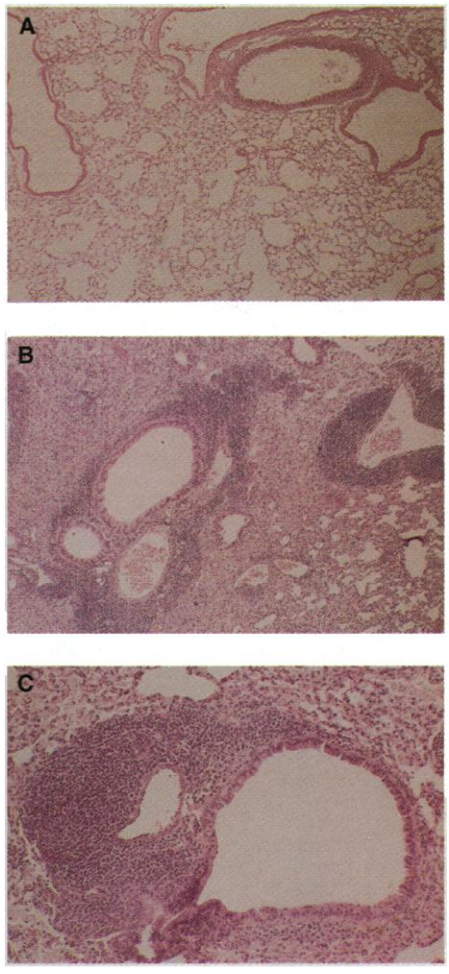

Fig. 5. GM-CSF–deficient mice develop pulmonary lymphoid hyperplasia. (**A**) Normal lung from wild-type mouse. (**B**) Extensive lymphoid hyperplasia surrounding airways and veins in a 7-month-old mutant animal. (**C**) Higher magnification of the hyperplasia.

nan-binding protein, and bovine conglutinin, bind to the same receptor (*22*), it is tempting to speculate that GM-CSF may generally be involved in the regulation of collectin receptors, whose function appears to be related to the uptake and processing of particulate or opsonized material (*23*). This idea could help explain the diversity of biological activities previously attributed to GM-CSF, particularly those related to its antimicrobial properties and ability to enhance antigen presentation, as well as those involved with surfactant homeostasis as revealed by the current studies.

REFERENCES AND NOTES

1. A. Burgess, J. Camakaris, D. Metcalf, *J. Biol. Chem.* **252**, 1998 (1977).
2. G. Johnson and D. Metcalf, *Exp. Hematol.* **8**, 549 (1980); D. Metcalf, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 5327 (1980); D. Metcalf *et al.*, *J. Cell. Physiol.* **128**, 421 (1986).
3. K. Inaba *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 3038 (1993).
4. D. Metcalf *et al.*, *Exp. Hematol.* **15**, 1 (1987); R. Donahue *et al.*, *Nature* **321**, 872 (1986); J. Groopman *et al.*, *N. Engl. J. Med.* **317**, 593 (1987).
5. P. Morrissey *et al.*, *J. Immunol.* **139**, 1113 (1987); M. Witmer-Pack *et al.*, *J. Exp. Med.* **166**, 1484 (1987).
6. H. Collins and G. Bancroft, *Eur. J. Immunol.* **22**, 1447 (1992); R. Weisbart, A. Kacena, A. Schuh, D. Golde, *Nature* **332**, 647 (1988).
7. J. Fleischmann, D. Golde, R. Weisbart, J. Gasson,

Blood **68**, 708 (1986); D. Silberstein *et al.*, *J. Immunol.* **137**, 3290 (1986); W. Weiser *et al.*, *J. Exp. Med.* **166**, 1436 (1987).
8. M. Arnaout, E. Wang, S. Clark, C. Sieff, *J. Clin. Invest.* **78**, 597 (1986); R. Weisbart, D. Golde, J. Gasson, *J. Immunol.* **137**, 3584 (1986).
9. G. Dranoff *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 3539 (1993).
10. F. Bussolino *et al.*, *Nature* **337**, 471 (1989); S. Robertson, G. Mayrhofer, R. Seamark, *Biol. Reprod.* **46**, 1069 (1992).
11. G. Dranoff *et al.*, in preparation.
12. C. Caux, C. Dezutter-Dambuyant, D. Schmitt, J. Banchereau, *Nature* **360**, 258 (1992).
13. D. Metcalf, *The Molecular Control of Blood Cells* (Harvard Univ. Press, Cambridge, MA, 1988).
14. J. Wright and J. Clements, *Am. Rev. Respir. Dis.* **135**, 426 (1987).
15. J. Wright and L. Dobbs, *Annu. Rev. Physiol.* **53**, 395 (1991).
16. G. Dranoff *et al.*, unpublished observations.
17. For ELISA, human SP-A [G. Pryhuber *et al.*, *Pediatr. Res.* **30**, 597 (1991)] was used as the standard; for SP-B, bovine SP-B [T. Gregory *et al.*, *J. Clin. Invest.* **88**, 1976 (1991)] was used as the standard. Murine SP-A and SP-B are expressed in units because standard curves were generated with xenogeneic reagents.
18. S. Rosen, B. Castleman, A. Liebow, *N. Engl. J. Med.* **258**, 1123 (1958).
19. A. Tazi *et al.*, *J. Clin. Invest.* **91**, 566 (1993).
20. N. Bilyk and P. Holt, *J. Exp. Med.* **177**, 1773 (1993).
21. R. Malhotra, J. Haurum, S. Thiel, R. Sim, *Eur. J. Immunol.* **22**, 1437 (1992).
22. R. Malhotra, S. Thiel, K. Reid, R. Sim, *J. Exp. Med.* **172**, 955 (1990).
23. K. Sastry and R. Ezekowitz, *Curr. Opin. Immunol.* **5**, 59 (1993).
24. V. Tybulewicz *et al.*, *Cell* **65**, 1153 (1991).
25. T. Doetschman *et al.*, *J. Embryol. Exp. Morphol.* **87**, 27 (1985).
26. A. Bradley, in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, E. J. Robertson, Ed. (IRL Press, Oxford, 1987), pp. 113–151.
27. P. Laird *et al.*, *Nucleic Acids Res.* **19**, 4293 (1991).
28. T. Dexter *et al.*, *J. Exp. Med.* **152**, 1036 (1980). An ELISA specific for GM-CSF (Endogen) revealed levels of 480 to 950 pg/ml in the supernatants of wild-type animals, 200 to 510 pg/ml in samples from heterozygotes, but no measurable levels (less than 10 pg/ml) in supernatants from mutant mice.
29. J. Abrams and M. Pearce, *J. Immunol.* **140**, 131 (1988); A. O'Garra *et al.*, *Cell. Immunol.* **123**, 189 (1989).
30. S1 nuclease analysis was done as described in T. Yen, D. Gay, J. Pachter, D. Cleveland [*Mol. Cell. Biol.* **8**, 1224 (1988)]. Probes used were as follows: SP-A from T. Korfhagen *et al.* [*Am. J. Physiol.* **263**, L546 (1992)]; SP-B from M. D'Amore-Bruno *et al.* [*Am. J. Physiol.* **262**, L40 (1992)]; SP-C from S. Glasser *et al.* [*J. Biol. Chem.* **265**, 21986 (1990)]; and actin from S. Alonso, A. Minty, Y. Bourlet, M. Buckingham [*J. Mol. Evol.* **23**, 11 (1986)]. Open boxes represent exons; black boxes, introns; and lines, plasmid sequences. Antigen-adsorbed negative controls showed no background staining in panels (C) and (D).
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Identification of a Peptide Recognized by Five Melanoma-Specific Human Cytotoxic T Cell Lines

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Of several thousand peptides presented by the major histocompatibility molecule HLA-A2.1, at least nine are recognized by melanoma-specific cytotoxic T lymphocytes (CTLs). Tandem mass spectrometry was used to identify and to sequence one of these peptide epitopes. Melanoma-specific CTLs had an exceptionally high affinity for this nine-residue peptide, which reconstituted an epitope for CTL lines from each of five different melanoma patients tested. Recognition by multiple CTL lines suggests that this may be a promising candidate for use in peptide-based melanoma vaccines.

Cytotoxic T lymphocytes that recognize tumor cells can often be isolated from cancer patients (*1*). Their existence suggests that there is an immune response to cancer in these patients and that its augmentation might be therapeutic. Immunotherapy as a treatment for cancer has focused on melanoma, both because melanoma spontaneously regresses more frequently than most human malignancies (*2*) and because melanoma-specific CTLs can be generated consistently in vitro (*3*). However, neither adoptive transfer of melanoma-specific CTLs nor specific active immunotherapy with whole melanoma cells or cell-derived preparations has led to the eradication of melanoma or long-term survival in more than a minority of patients (*4*). For immunotherapy to be improved, epitopes recognized by melanoma-specific CTLs must be identified. CTL epitopes are peptides derived from cellular proteins that are presented on the cell surface by major histocompatibility complex (MHC) class I molecules (*5, 6*). Peptides that are expressed by the tumors of many individuals may be useful for immunotherapy (*7–10*), but the most generally applicable would be those that also are recognized by lymphocytes obtained from a large number of different melanoma patients.

Melanoma-specific CTL lines restricted by HLA-A2.1 recognize epitopes present on most allogeneic A2.1$^+$ melanoma cells (*7, 8, 11*). Individual CTL lines have been shown to recognize at least six peptides common to multiple melanomas (*12, 13*), some of which may be recognized by CTL lines derived from different melanoma patients (*13*). The tyrosinase gene has been reported to encode two HLA-A2.1–restricted, melanoma-specific epitopes, each of which is recognized by a single CTL clone (*14*). Here, we report the direct identification, by tandem mass spectrometry, of a peptide epitope that is recognized by high-affinity CTLs from five different melanoma patients: VMM5, VMM6, LD1, DM169, and DM204.

Peptides associated with the HLA-A2.1 molecules expressed on a melanoma cell line, DM6, were isolated as described (*12*) and fractionated by high-performance liquid chromatography (HPLC). We identified HPLC fractions containing relevant peptides by testing their ability to reconstitute epitopes for melanoma-specific CTL lines from two different patients, VMM5 (*12*) and DM204 (*15*), after incubation with the HLA-A2.1–positive lymphoblastoid cell line T2 (*16*) (Fig. 1). Six peaks of activity were identified for cells from VMM5 and three for cells from DM204 (Fig. 1A). Fractions 2 to 3 and 14 to 15 contained peptides recognized by both CTL lines. Fractions 2 and 3 were pooled and rechromatographed (Fig. 1B). Two distinct peaks of activity were found for each CTL line, one of which contained peptides recognized by both (*17*). The number of peptides in the active fractions (greater than 50) exceeded the number that could be sequenced with available material, and third-dimension separations by standard HPLC methods caused loss of material without reducing the number of candidates.

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