Brief Definitive Report

DEPLETION OF CD8$^+$ CELLS IN HUMAN THYMIC MEDULLA RESULTS IN SELECTIVE IMMUNE DEFICIENCY

By C. M. ROIFMAN,* D. HUMMEL,* H. MARTINEZ-VALDEZ,* P. THORNER,‡ P. J. DOHERTY,* S. PAN,§ F. COHEN,‖ AND A. COHEN*

*From the Divisions of *Immunology/Rheumatology and ‡Pathology, The Hospital for Sick Children, Toronto, Ontario, Canada MSG 1X8; and §Department of Pathology University of Medicine and Dentistry of New Jersey, Piscataway, New Jersey 08854; and ‖Department of Pediatrics, Children's Hospital of Michigan, Detroit, Michigan 48201*

CD8 is a surface glycoprotein expressed on most thymocytes and on a subset of mature T cells. The reactivity of mature CD8$^+$ T cells is restricted by polymorphic determinants on class I MHC molecules present on target cells; CD8 itself binds to a monomorphic region of class I MHC proteins and increases the avidity of the interaction between the T cell and the antigen presenting cells (1, 2). Evidence from experiments with inbred transgenic mice supports the hypothesis that the CD8 molecule participates in the intrathymic selection of class I MHC-restricted T cells and in the tolerization to self antigens presented by class I proteins (3–7). No comparable experimental evidence exists in any outbred species. In this article we describe an immune deficient patient with selective depletion of CD8$^+$ cells. Analysis of this patient reveals the existence of CD8$^+$ cells in the thymic cortex but selective depletion of CD8$^+$ cells in the thymic medulla and peripheral blood. Our results point to a process in the human thymus that targets the CD8$^+$ thymocyte.

Materials and Methods

Flow Cytometry. Viable mononuclear cells from thymocytes were isolated using Ficoll/Hypaque. Isolated thymocytes were resuspended at 2×10^6 cells/ml in PBS containing fluorescein-conjugated mAbs and incubated for 30 min at 4°C. After two washes in PBS, labeled cells were analyzed by a Coulter Epics V flow cytometer (Coulter Electronics, Hialeah, FL).

Immunohistological Analysis of Cryostat Sections. Thymic biopsies from the patient and from a normal control, undergoing cardiac surgery, were snap frozen in liquid nitrogen and stored at −70°C until use. 4-μm serial cryostat sections were mounted on glass slides and air dried. Tissue sections were immunostained with the specified mAbs using a three-stage biotin-avidin-peroxidase staining.

Northern Blot Analysis. RNA was extracted from cells in guanidinum isothiocyanate according to the method of Chomezynski and Sacchi (8). Purified RNA (10 μg) was electrophoresed, subsequently transferred to nitrocellulose, blotted and hybridized with labeled probes as previously described (9).

Amplification of CD8 cDNA. A 353-bp fragment of CD8α cDNA was amplified as described

This work was supported by grants from the Medical Research Council of Canada, the Canadian National Cancer Institute and the Leah Reichmann Immunodeficiency Research Fund. Dr. Amos Cohen is a National Cancer Institute of Canada Scholar and Dr. Hector Martinez-Valdez is a Medical Research Council of Canada Fellow.

Address correspondence to Dr. C. M. Roifman, Division of Immunology/Rheumatology, The Hospital for Sick Children, 555 University Avenue, Toronto, Ontario, Canada M5G 1X8.

(10) from cDNA produced by reverse transcription (MMLV reverse transcriptase; Bethesda Research Laboratories, Gaithersburg, MD) of RNA isolated from a thymus biopsy.

Amplification of TCR Vβ cDNAs. DNA fragments of various sizes were amplified as described (8). RNA was transcribed from a primer common to human TCR β1 and β2 constant regions; the cDNA was amplified using the polymerase chain reaction (PCR) with a nested second primer common to human TCR β1 and β2 constant regions together with a primer corresponding to 1 of 20 Vβ families. Identity of the amplified DNA fragments was established through hybridization to a third nested Cβ oligonucleotide.

Results and Discussion

A female infant with a selective deficiency of cell-mediated immunity presented at 9 mo of age with *Pneumocystis carinii* pneumonia. Subsequently she developed chronic diarrhea, and mini-reovirus was repeatedly isolated from her stool. She failed to respond to the Candida skin test despite the persistence of oral thrush and ulcers caused by this fungus. The apparent susceptibility to parasitic, viral, and fungal infections coupled with the knowledge that the patient's older sibling was previously diagnosed as severe combined immunodeficient (SCID) caused us to investigate further her cellular immunity. At the age of 10 mo an allogeneic skin graft obtained from an unrelated, healthy donor was applied and was not rejected by the patient. In contrast to the typical presentation of SCID, her humoral responses were unexceptional: Her Ig levels (IgM, IgG, and IgA) were within the normal range; she made antibody responses to tetanus toxoid, poliomyelitis, rubella, and measles viruses; and she responded with primary and secondary antibody to the novel antigen φX174.

Whereas specific antibody responses require class II MHC antigen-restricted helper T cells, allogeneic graft rejection and immunity to viral infection involve cytotoxic class I MHC-restricted T cells. The clinical presentation of this patient is suggestive of a deficiency of class I MHC-restricted T cells. To further examine this possibility we carried out cellular and molecular analyses of the patient's lymphoid system.

We determined the expression of T cell–specific surface markers on the patient's PBMC by flow cytometric analysis. The analysis revealed a lack of $CD8^+$ cells but typical numbers of $CD4^+$ cells in the peripheral blood (Fig. 1). Levels of expression

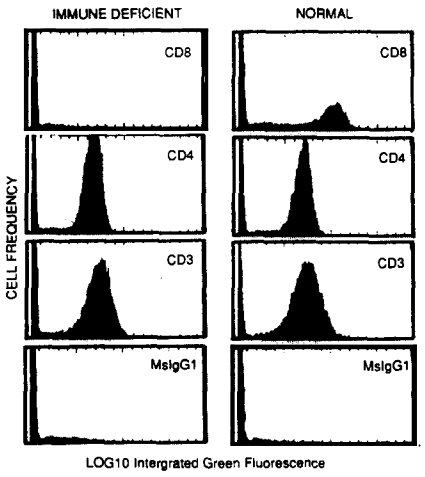

FIGURE 1. Analysis by flow cytometry of T cell surface antigens on peripheral blood cells from the immune-deficient patient and a normal control. Mononuclear cells were separated from white blood cells on a Hypaque-Ficoll gradient, stained with fluorescein-conjugated mAbs against CD8, CD4, and CD3 antigens or control antibodies (MSIgG1), and analyzed using a Coulter Electronics Epics V flow cytometer.

of T cell surface markers CD2, CD3 (Fig. 1), and the T cell antigen receptor (data not shown) were unremarkable. The number of B lymphocytes in the peripheral blood (as judged by the number of $CD20^+$ and sIg^+ expressing cells) was also within the normal range (data not shown). The clinical and immunological findings in this patient are consistent with a selective deficiency of class I MHC antigen-restricted T cell functions.

A structural mutation in the CD8 gene, resulting in a failure to express the CD8 protein, might explain the loss of peripheral $CD8^+$ cells; a lack of positive selection for $CD8^+$ cells or active depletion of $CD8^+$ cells during intrathymic differentiation could also account for the selective depletion. To distinguish among these possibilities, we analyzed the surface expression of CD8 in a thymic biopsy that was obtained at the age of 10 mo. The morphology of the patient's thymus appeared normal, with well-developed Hassall's corpuscles. Both lymphoid and epithelial cells in the thymus stained normally for a monomorphic epitope on class I MHC antigens (data not shown). Both $CD4^+$ and $CD8^+$ cells were present at typical numbers in the thymic cortex. A deficiency unique to the $CD8^+$ cells was apparent, however, in the medulla; no $CD8^+$ cells were detected and almost all medullary thymocytes expressed the $CD4^+$ phenotype (Fig. 2).

The characteristics of this patient's immune system have precedents in those transgenic mice expressing transgenes for the TCR that recognizes the male H-Y antigen in the context of the class I MHC antigens (3). In the male H-Y transgenic mice,

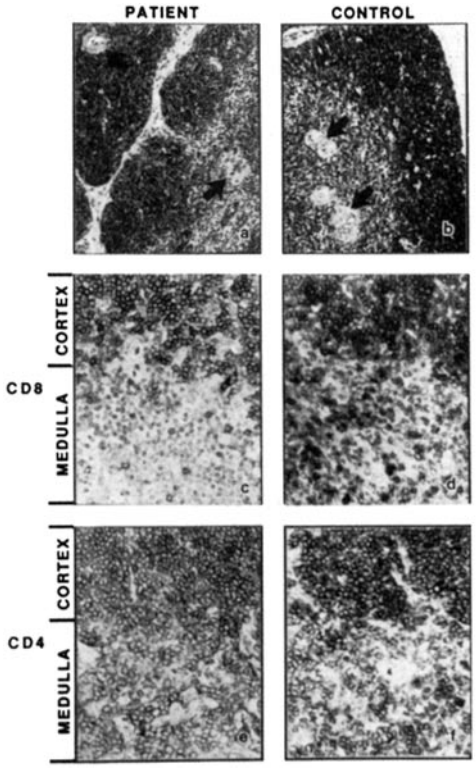

FIGURE 2. Photomicrographs of patient thymus and normal thymus. Comparison of patient's thymus biopsy (*a*) to age-matched control thymus (*b*). Both show a normal architecture with cortical and medullary regions and formation of Hassall's corpuscles (*arrows*) (×110). Immunoperoxidase staining for CD8 shows positive staining of cortical thymocytes in patient (*c*) and control (*d*). Medullary thymocytes in the patient show only rare positive staining (*c*), in contrast to frequent positive cells in the control (*d*) (×280). (*e*, *f*) Immunoperoxidase staining for CD4 shows positive staining of cortical thymocytes as well as most medullary thymocytes in patient (*e*) and control (*f*) (×280).

$CD8^+$ cells are $CD4^+$ $CD8^+$ cortical thymocytes; $CD4^-$ $CD8^+$ cells are depleted from the thymic medulla and peripheral blood. This is attributed to high affinity of the $CD8^+$ cells with class I MHC antigen-bearing cells that leads to intrathymic elimination of $CD8^+$ cells by a process of negative selection (3).

In search of an explanation for the depletion of $CD8^+$ cells, we examined the expression of CD8 mRNA in the patient's thymus and PBL (Fig. 3). Northern blot analysis of RNA extracted from the PBL revealed the presence of normal levels of mRNA coding for several T cell surface proteins including CD3, CD4, and the α and β polypeptides of the TCR, but the complete absence of mRNA for both α and β polypeptides of CD8. Analysis of RNA taken from frozen thymus tissue (taken for histological purposes) detected α CD8 mRNA that was partially degraded (due to the prolonged storage of the tissue). To confirm that the RNA, rescued from the thymic biopsy specimen, indeed contained CD8 mRNA, we transcribed the RNA into cDNA with reverse transcriptase and an oligonucleotide corresponding to the CD8 α V domain (11). The PCR was then used, with two oligonucleotides that frame the CD8α V region, to amplify a 353-bp DNA fragment that was of the predicted size and hybridized to a CD8α probe (Fig. 3 *C*). We therefore conclude that cells transcribing CD8 mRNA are present only in the thymus of this patient.

The surface molecules involved in intrathymic selection of class I MHC antigen-restricted T cells include the CD8 α and β chains, the TCR-α/β heterodimer, and the class I MHC proteins. A mutation in or deletion of any one of these genes could explain the phenotype observed in this immune deficient patient: a defect in the binding site of the CD8 surface protein might impair its affinity toward the monomorphic site of MHC class I molecules resulting in the absence of positive selection of $CD8^+$ cells in the thymus; a deletion of a large portion of the TCR-α or -β V regions may result in elimination of those V region families able to interact with

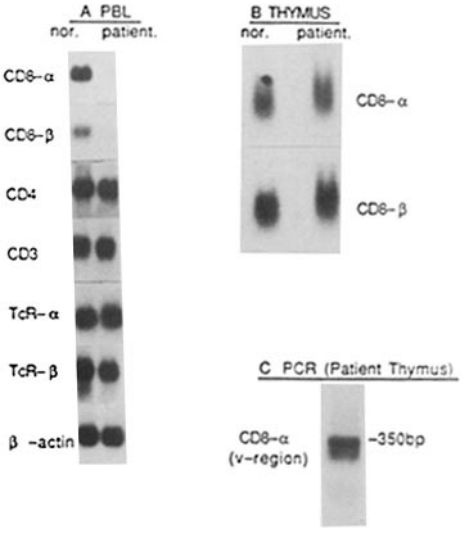

FIGURE 3. Northern blot analysis of PBL and thymocytes from the immune-deficient patient and normal control. RNA was extracted from PBL (*A*) and thymocytes (*B*) with guanidinium isothyocyanate (8). Northern blots were hybridized with the indicated cDNA probes; CD8β cDNA was obtained from Dr. Dan R. Littman (University of California, San Francisco, CA); CD4 and CD8α cDNAs were provided by Dr. Richard Axel (Columbia University, New York, NY); CD3 cDNA was obtained from Dr. Cox Terhorst (Harvard Medical School, Boston, MA). TCR-α and -β cDNAs were obtained from Dr. Tak Mak (Ontario Cancer Institute, Toronto, ON). (*C*) A 353-bp fragment was amplified as described (10) from cDNA produced by reverse transcription (MMLV reverse transcriptase; BRL) of RNA isolated from a thymus biopsy. RNA was transcribed from a primer specific to the CD8α V region (oligo nucleotide CD8V3'-CTGGCAGGAAGACCGGCACGA); the cDNA was amplified using the PCR with oligo nucleotide CD8V3' and a second primer oligo nucleotide CD8V5' (ACGCCGCCAGGCCGAGCCAGT). Identity of the 353-bp fragment was established through hybridization to a CD8α cDNA probe after Southern blotting.

class I MHC molecules of this individual. The deletion of multiple members of class I MHC molecules in this patient is not likely since normal staining of a monomorphic epitope was observed in the patient's thymus (data not shown).

To determine if a major deletion of TCR Vβ genes might explain the phenotype observed in this patient, the expression of all 20 Vβ gene families was determined in her PBLs. RNA from the patient's peripheral blood was reverse transcribed with an oligonucleotide corresponding to sequences common to human Cβ1 and Cβ2. The cDNA was amplified in 20 PCRs using a Cβ primer in all reactions and specific to each reaction an oligonucleotide primer corresponding to a portion of the Vβ region from 1 of 20 Vβ families. The results clearly demonstrate that all 20 Vβ families are expressed in this patient (data not shown). A major deletion of TCR Vβ genes can not explain the lack of $CD8^+$ cells in the patient's peripheral blood.

A more likely explanation for the lack of $CD8^+$ cells in the thymic medulla and periphery of this patient is an abnormality associated with the CD8 molecule. This might involve a mutation in the CD8α or β coding sequences, a mutation in regulatory sequences affecting CD8 expression in the periphery, or impaired signal transduction after binding of ligand to the CD8 molecule.

The phenotype observed in this patient has provided a unique opportunity to examine the differential roles of $CD4^+$ and $CD8^+$ T cell subsets in human immune responses in vivo. The clinical and immunological observations both show that $CD4^+$ cells alone are sufficient for in vivo primary and secondary antibody responses. On the other hand, the lack of $CD8^+$ cells results in failure to respond to an allogeneic skin graft, illustrating the requirement for the $CD8^+$ cells for class I MHC antigen-restricted T cell responses in human.

The selective disappearance of $CD8^+$ cells before their emergence in the thymic medulla is compelling evidence for a process in the thymus targeting $CD8^+$ cells. Whereas the majority of immature cortical thymocytes express the $CD4^+CD8^+$ phenotype, those that survive both positive and negative selection in the thymus differentiate into either $CD4^+CD8^-$ or $CD4^-CD8^+$ cells. In agreement with studies on genetically manipulated and inbred mice (3–7), our studies of an individual with an error in thymic differentiation demonstrate that the process that regulates the selection and amplification of $CD4^-CD8^+$ cells is clearly independent of and separable from the process that governs the differentiation into the $CD4^+CD8^-$ cells. The ability of the TCR on the $CD4^+CD8^+$ cells to interact with thymic peptides presented by class I or class II MHC molecules on undefined thymic cells may regulate these processes of differentiation. Aberrations in the process that targets the $CD8^+$ cells lead to a depletion of these cells in the thymic medulla and peripheral blood.

Summary

CD8 molecules expressed on the surface of a subset of T cells participate in the selection of class I MHC antigen-restricted T cells in the thymus, and in MHC-restricted immune responses of mature class I MHC antigen-restricted T cells. Here we describe an immune-deficient patient with lack of $CD8^+$ peripheral blood cells. The patient presented with *Pneumocystis carinii* pneumonia and was unable to reject an allogeneic skin graft, but had normal primary and secondary antibody responses. Examination of the patient's thymus revealed that the loss of $CD8^+$ cells occurred

during intrathymic differentiation: the patient's immature cortical thymocytes included both $CD4^+$ and $CD8^+$ cells while the mature medullary cells expressed the CD4 but not the CD8 protein on their surface. Northern blot and polymerase chain reaction analyses revealed the presence of CD8 α and β mRNA in the patient's thymus but not in the peripheral blood. Both class I MHC antigen expression and the expressed TCR Vβ repertoire are normal in this patient. These data are consistent with an impaired selection of $CD8^+$ cells in the patient's thymus and support the role of the CD8 surface protein in thymic selection previously characterized in genetically manipulated and inbred mice.

We thank Drs. Dan Littman, Richard Axel, Cox Terhorst, and Tak Mak for supplying cDNA probes.

Received for publication 24 July 1989 and in revised form 14 September 1989.

References

1. Norment, A. M., R. D. Salter, P. Parham, V. H. Engellhard, and D. R. Littman. 1988. Cell-cell adhesion mediated by CD8 and MHC class I molecules. *Nature (Lond.).* 336:79.
2. Dembic, Z., W. Haas, R. Zamoyska, J. Parnes, M. Steinmetz, and H. von Boehmer. 1987. Transfection of the CD8 gene enhances T-cell recognition. *Nature (Lond.).* 326:510.
3. Kisielow, P., H. Bluthmann, M. D. Staerz, M. Steinmetz, and H. von Boehmer. 1988. Tolerance in T-cell receptor transgenic mice involves deletion of nonmature $CD4^+8^+$ thymocytes. *Nature (Lond.).* 333:742.
4. Bluthmann, H., P. Kisielow, Y. Uematsu, M. Malissen, P. Krimpenffort, A. Berns, H. von Boehmer, and M. Steinmetz. 1988. T-cell specific deletion of T-cell receptor transgenes allows functional rearrangement of endogenous α and β-genes. *Nature (Lond.).* 334:156.
5. Kisielow, P., H. Sia Teh, H. Bluthmann, and H. von Boehmer. 1988. Positive and negative selection of antigen-specific T-cells in thymus by restricting MHC molecules. *Nature (Lond.).* 335:730.
6. Sha, W. C., C. A. Nelson, R. D. Newberry, D. M. Kranz, J. H. Russell, and D. Y. Loh. 1988. Positive and negative selection of an antigen receptor on T-cells in transgenic mice. *Nature (Lond.).* 336:73.
7. Teh, H. S., P. Kisielow, B. Scott, H. Kishi, Y. Uematsu, H. Bluthmann, and H. von Boehmer. 1988. Thymic MHC antigenes and the specificity of the $\alpha\beta$ T-cell receptor determines the CD4/CD8 phenotype of T-cells. *Nature (Lond.).* 335:229.
8. Chomezynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol chloroform extraction. *Anal. Biol. Chem.* 162:156.
9. Martinez-Valdez, H., E. Thompson, and A. Chen. 1988. Phorbol esters and calcium ionophores differentially regulate the transcription of Vγ-T-cell antigen receptor gene in human thymocytes. *J. Biol. Chem.* 26:4043.
10. Doherty, P. J., M. Huesca-Contreras, H. M. Dosch, and S. Pan. 1989. Rapid amplification of complementary DNA from small amounts of unfractionated RNA. *Anal. Biochem.* 177:7.
11. Littman, D. R. 1987. The structure of the CD4 and CD8 genes. *Annu. Rev. Immunol.* 5:561.