salt, temperature, and cofactors on triplex formation as well as differences in oligoribonucleotide and oligodeoxyribonucleotide triplex formation can also be investigated. Sequence analysis of a large population of ligands, coupled with detailed biophysical characterization of the complexes, may provide insight into a general recognition motif for duplex DNA. Similarly, peptide and protein libraries may help define the nature of protein-DNA recognition.

REFERENCES AND NOTES

1. R. A. Lerner, S. J. Benkovic, P. G. Schultz, *Science* **252**, 659 (1991); P. G. Schultz, *Angew. Chem. Int. Ed. Engl.* **28**, 1283 (1989).
2. C. Tuerk and L. Gold, *Science* **249**, 505 (1990).
3. A. D. Ellington and J. W. Szostak, *Nature* **346**, 818 (1990).
4. J. K. Scott and G. P. Smith, *Science* **249**, 386 (1990); J. J. Devlin, L. C. Panganiban, P. E. Devlin, *ibid.*, p. 404; S. E. Cwirla *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 6378 (1990).
5. S. P. A. Fodor *et al.*, *Science* **251**, 767 (1991); R. Kaiser *et al.*, *Nature* **350**, 656 (1991).
6. G. Felsenfeld, D. R. Davies, A. Rich, *J. Am. Chem. Soc.* **79**, 2023 (1957); G. Felsenfeld and H. T. Miles, *Annu. Rev. Biochem.* **36**, 407 (1967).
7. M. N. Lipsett, *Biochem. Biophys. Res. Commun.* **11**, 224 (1963); J. H. Miller and H. M. Sobell, *Proc. Natl. Acad. Sci. U.S.A.* **55**, 1201 (1966); A. R. Morgan and R. D. Wells, *J. Mol. Biol.* **37**, 63 (1968); S. Arnott and E. Selsing, *ibid.* **88**, 509 (1974); G. E. Plum *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 9436 (1990); P. Rajagopal and J. Feigon, *Biochemistry* **28**, 7859 (1989); C. de los Santos, M. Rosen, D. Patel, *ibid.*, p. 7282.
8. H. E. Moser and P. B. Dervan, *Science* **238**, 645 (1987).
9. M. N. Lipsett, *J. Biol. Chem.* **239**, 1256 (1964); S. L. Broitman, D. D. Im, J. R. Fresco, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 5120 (1987); Y. Kohwi and T. Kohwi-Shigematsu, *ibid.* **85**, 3781 (1988); P. A. Beal and P. B. Dervan, *Science* **251**, 1360 (1991).
10. L. C. Griffin and P. B. Dervan, *Science* **245**, 967 (1989).
11. S. A. Strobel and P. B. Dervan, *Nature* **350**, 172 (1991).
12. M. Cooney, G. Czernuszewicz, E. H. Postel, S. J. Flint, M. E. Hogan, *Science* **241**, 456 (1988); L. J. Maher III, B. Wold, P. B. Dervan, *ibid.* **245**, 725 (1989).
13. J. F. Milligan, D. R. Groebe, G. W. Witherell, O. C. Uhlenbeck, *Nucleic Acids Res.* **15**, 8783 (1987).
14. The 26-nt pyrimidine-rich strand was synthesized with thiol-derivatized CPG, deprotected, purified by high-performance liquid chromatography (HPLC), and converted to the *S*-thiopyridyl disulfide (*24*). The oligonucleotide was then annealed with a threefold excess of the 25-nt complementary strand, and the resulting duplex DNA was incubated with dithiothreitol (DTT)-treated thiol-Sepharose (Pharmacia) in 50 mM tris·HCl, pH 8.0 buffer at room temperature for 48 hours with shaking. After removel of unreacted DNA, the column loading (3.0 nmol of DNA per milliliter) was determined by reducing an aliquot of the Sepharose support with DTT and assaying DNA concentration.
15. D. S. Pilch, R. Brousseau, R. H. Shafer, *Nucleic Acids Res.* **18**, 5743 (1990).
16. Approximately 18 μg (600 pmol) of the 89-nt RNA transcripts in 200 μl of 50 mM sodium acetate, pH 5.5, 1 mM $MgCl_2$, 2 M NaCl buffer was heated to 65°C for 5 min, allowed to cool to room temperature (RT), and loaded onto a column containing 150 μl of the DNA-derivatized support (~450 pmol of duplex DNA). After incubation at RT for 1 hour, the column was washed with 3.0 ml of the above buffer and bound RNA was eluted with 1.0 ml of 50 mM tris·HCl, pH 8.0, 1 mM EDTA buffer. The RNA was recovered by ethanol precipitation with glycogen (50 μg/ml) as the carrier.
17. Complementary DNAs were prepared from RNAs with M-MuLV reverse transcriptase and a 17-nt primer (5′-GGGCCAAGCTTCTGCAG-3′) as described [J. Sambrook, E. F. Fritsch, T. Maniatis, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989), sections 8.60–8.62]. The cDNAs were then amplified to ~5 μg of double-stranded DNA by PCR with the 33-nt and 17-nt oligonucleotides as primers. The PCR reaction (100 μl) was carried out in a buffer containing 67 mM tris·HCl, pH 8.8, 6.7 mM $MgCl_2$, 10 mM β-mercaptoethanol, 5 μM of each primer, 250 μM of each dNTP, and 2.5 units of Taq polymerase for 15 cycles (1 min at 91°C, 2 min at 52°C, and 1 min at 72°C).
18. A selection carried out with a 25-nt random RNA library, in the absence of the negative selection, failed to generate triplex-forming sequences.
19. Plasmid pDP24 is a derivative of pUC119 [J. Vieira and J. Messing, *Methods Enzymol.* **153**, 3 (1987)] with a 41-bp synthetic DNA fragment containing the T7 promoter sequence (5′-AATTGTAATACGACTCACTATAGGGAGGAATTCCTCGAGGA-3′) inserted into the Eco RI–Hind III fragment. Insertion of a PCR amplified cDNA fragment into the Eco RI–Hind III site of pDP24 places the cDNA under the control of the T7 promoter. Subsequent runoff transcription of Hind III–linearized plasmid generates RNA transcripts identical to those generated from the synthetic 106-nt template.
20. D. Pei, H. D. Ulrich, P. G. Schultz, unpublished results.
21. D. R. Mills, R. L. Peterson, S. Spiegelman, *Proc. Natl. Acad. Sci. U.S.A.* **58**, 217 (1967).
22. J. S. Taylor, P. G. Schultz, P. B. Dervan, *Tetrahedron* **40**, 457 (1984).
23. D. R. Corey, D. Pei, P. G. Schultz, *J. Am. Chem. Soc.* **111**, 8523 (1989); *Biochemistry* **28**, 8277 (1989); D. Pei and P. G. Schultz, *J. Am. Chem. Soc.* **112**, 4579 (1990).
24. D. Pei, D. R. Corey, P. G. Schultz, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 9858 (1990).
25. [1-(Benzyldithio)hexyl]-6-oxy-cyanoethyl-*N*, *N*-diisopropylamino-phosphoramidite (**1**) was synthesized by reacting 1-(benzyldithio)hexan-6-ol (**2**) with two equivalents of 2-cyanoethyl-*N*, *N*-diisopropylchlorophosphoramidite (Aldrich) in anhydrous acetonitrile containing four equivalents of diisopropylethylamine at RT for 30 min. The product was extracted with 10% aqueous sodium bicarbonate solution, dried, and used directly on an automated DNA synthesizer. After the oligoribonucleotide was cleaved from the solid support and deprotected (*26*), the benzylmercaptan group was removed with 20 mM DTT in a 50 mM tris·HCl (pH 8.0), 1 mM EDTA buffer to regenerate the 5′-sulfhydryl. For the synthesis of compound **2**, see T. Muaiyama and K. Takahashi, *Tetrahedron Lett.* **56**, 5907 (1968).
26. T. Wu, K. K. Ogilvie, R. T. Pon, *Nucleic Acids Res.* **17**, 3501 (1989).
27. The cleavage pattern produced by conjugate **5** contains cleavage sites both to the 5′ side of the homopurine target site and within the homopurine binding site. This result suggests that two different binding modes may be possible, one involving the hairpin loop structure and a second that involves 5′-TTCTCTCCC-3′ binding to the 3′ half of the homopurine target.
28. Plasmid pDP26 was constructed by inserting a synthetic DNA fragment (5′-GATCCGACGATCCCTCTCTCTCTCTTGGATCGCATG-3′) containing the target DNA sequence into the large Bam HI–Sph I fragment of pBR322. The 269-bp Nhe I/Hinc II fragment used in cleavage reactions was labeled with ^{32}P by filling in the Nhe I 5′ overhang with Sequenase version 2.0 (USB).
29. F. Michel *et al.*, *Nature* **347**, 578 (1990).

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Oligoclonal Expansion and CD1 Recognition by Human Intestinal Intraepithelial Lymphocytes

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A human intestinal intraepithelial lymphocyte (IEL) T cell line was established from jejunum to characterize the structure and function of the αβ T cell antigen receptors (TCRs) expressed by this population. Single-sided polymerase chain reaction (PCR) amplification cloning and quantitative PCR amplification of the TCR chains from the cell line and from fresh IELs demonstrated that IELs were oligoclonal. The IEL T cell line exhibited CD1-specific cytotoxicity and a dominant IEL T cell clone was CD1c-specific. Thus, human jejunal intraepithelial lymphocytes are oligoclonal and recognize members of the CD1 gene family.

THE EPITHELIAL SURFACE OF THE intestine contains a distinct population of IELs, the function of which is unknown (*1*). Human and murine intestinal IELs are enriched for T lymphocytes that express the γδ TCR (*1*). However, the majority of human intestinal IELs express the αβ TCR, and most of these human αβ IELs express the CD8 accessory molecule (*2*). The predominant expression of CD8 by human intestinal αβ IELs distinguishes them from T cells in the lamina propria and peripheral blood and suggests that their TCRs are directed at major histocompatibility complex (MHC) class I or class I–like molecules. The TCRs expressed by murine γδ IELs have been shown to utilize a small number of variable region genes but to have extensive junctional diversity (*3*). No comparable analysis, however, has been

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performed on human or murine αβ IELs.

To characterize the αβ TCRs expressed by human intestinal IELs and to identify ligands recognized by this population of lymphocytes, we established a human IEL T cell line from normal jejunal IELs (*4*). After 3 weeks in culture, the cell line was analyzed by flow cytometry and was 91% TCRαβ$^+$, 7% TCRγδ$^+$, 90% CD8$^+$, and 11% CD4$^+$, which was consistent with the phenotype of freshly isolated IELs (*4, 5*). Concurrently, RNA was extracted and analyzed for TCR expression by PCR amplification.

The TCRα chains expressed by the cells in the line were amplified and cloned by a modification of the single-sided PCR amplification method (*6*) (Fig. 1). Independent isolates were sequenced and 25 functionally rearranged TCRα chains were identified. Only four TCRα chains, with one chain predominating, were detected. The dominant α chain utilized V_α13.1-J_αN and represented 19 of the 25 chains isolated (Fig. 1). The next most frequent isolates were V_α14.1-J_αW (three isolates) and V_α1.2-J_αT (two isolates) (Fig. 1). A single V_α8.2 cDNA was also identified. Similarly, single-sided PCR amplification and cloning of the TCRβ chains from the line only detected two transcripts, V_β3.1-J_β2.1 (4/7 isolates) and V_β7.2-J_β2.1 (3/7 isolates) (Fig. 1).

The restricted expression of TCRα variable regions by the line was confirmed by quantitative PCR amplification (*7*). The cDNA was amplified by PCR with a panel of specific V_α oligonucleotide primers and a constant region antisense primer, and the PCR products were analyzed by hybridization with an internal C_α probe. V_α13 was the major product after 24 cycles of amplification, and smaller amounts of V_α14, V_α15 (*8*), and V_α1 PCR products could also be identified (Fig. 2A). An additional transcript, V_αBK6, was identified after 30 cycles (Fig. 2B). V_αBK6, which is related to the V_α2 family, was cloned from a CD1a reactive T cell clone (*9*). After 38 cycles, the majority of the oligonucleotides used in this panel yielded detectable transcripts (Fig. 2C). Therefore, both PCR analyses indicated the presence of a dominant clone that expressed V_α13 and the expansion of several other clones.

The distribution of TCRs was also analyzed by determination of the TCR usage of three CD8$^+$, TCRαβ$^+$ T cell clones that were isolated from the cell line by limiting dilution (*10*). These clones, JRS1 to JRS3, expressed V_α13, V_α14, and V_α19, respectively, as determined by quantitative PCR amplification (*11*). The JRS1 TCRα chain was also cloned by single-sided PCR amplification and was identical to the dominant V_α13.1-J_αN receptor cloned from the cell line (Fig. 1). The JRS3 TCRα chain was similarly cloned and found to be V_α19 combined with a new J region (Fig. 1).

The TCRβ chains expressed by JRS1, JRS2, and the IEL T cell line were also analyzed by quantitative PCR amplification (*11*). JRS1 and JRS2 expressed V_β17 and V_β7, respectively. The dominant TCRs expressed by the cell line were V_β3, V_β7, and V_β17, each representing approximately one-third of the amplified products. The JRS1 TCRβ chain was cloned by single-sided PCR amplification and was a V_β17.1-J_β2.7 combination (Fig. 1). A Bgl II site was identified in the 5′ untranslated region of the JRS1 V_β17 chain that may have precluded the isolation of this chain during the random V_β cloning from the IEL cell line (Fig. 1). The V_β17 PCR product from the cell line was also subcloned and all of the isolates (7/7) were identical to the JRS1 TCRβ chain (*12*). These results provided further confirmation that the IEL cell line was oligoclonal and contained a dominant clone, JRS1, that expressed a V_α13.1-V_β17.1 TCR. Taken together, the TCRβ chain quantitative PCR analysis and limiting dilution cloning suggested that the JRS1 clone represented approximately one-third of the IELs in the line. The higher estimates for JRS1 yielded by the PCR analyses of TCRα chains (Figs. 1 and 2) may have been due to higher expression by JRS1 of the message from the TCRα chain versus that from the TCRβ chain.

To confirm that this oligoclonal expansion was present in vivo, we analyzed fresh jejunal IELs from a second individual by single-sided PCR amplification and cloning. Twenty-seven functionally rearranged TCRβ chains were identified by DNA sequencing. The distribution of these receptors (Fig. 3) indicated that these freshly isolated cells were also oligoclonal. The dominant receptor was a V_β12.3-J_β1.6 combination that was present in 13 of 27 isolates (*13*). The V_β12.3 gene was also rearranged to J_β2.2 in one isolate. The remaining receptors used predominantly the V_β3 gene family and four different J regions (J_β1.2, J_β2.1, J_β1.6, and J_β2.7) (Fig. 3). Single isolates of a V_β7.2 and a V_β6.5 chain were also identified. The TCRα chains from the fresh preparation of IELs were also cloned by single-sided PCR amplification. Six of the nine functionally rearranged isolates were a V_α3.1 combined with a J_α not previously described (Fig. 3). The remaining isolates were a V_α20-J_αO combination (2/9) (Fig. 3) (*14*) and a V_α7 (1/9).

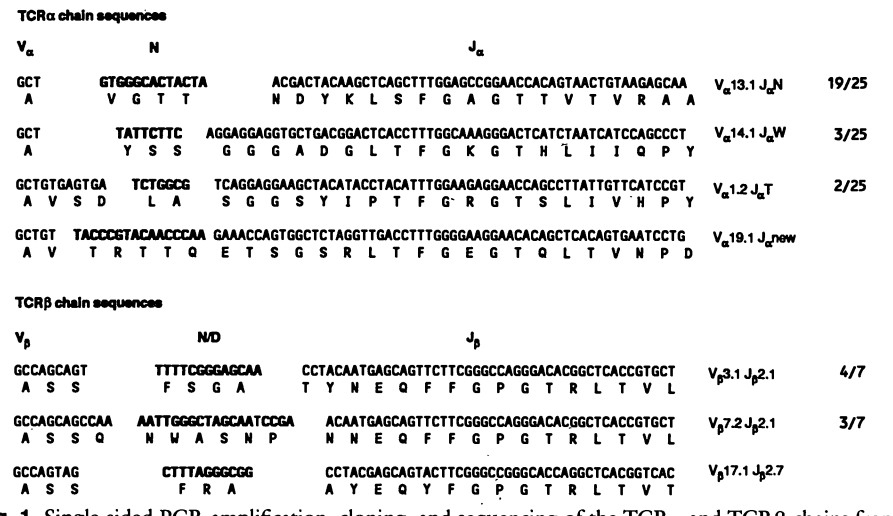

Fig. 1. Single-sided PCR amplification, cloning, and sequencing of the TCRα and TCRβ chains from a jejunal IEL T cell line. The nucleotide and deduced amino acid sequences of the 3′ end of each variable (V) region and the complete joining (J) region sequences are shown for the TCRα and TCRβ chains cloned from an IEL T cell line. The 3′ and 5′ ends of the V and J regions, respectively, were determined from genomic clones or approximated on the basis of previous cDNA isolates. Unless otherwise indicated, the nomenclature of the TCRα and TCRβ chains is that of Toyonaga and Mak (*13*). The sequences between the V and J regions [N region diversity for the α chains; N plus diversity (D) regions for the β chains] are shown in boldface, and the frequency of each isolate is indicated. Each isolate differed in the length of the 5′ end, which indicated that the isolates were derived from distinct transcripts. The V_α19.1 chain was isolated directly from the JRS3 clone. It contains a new J_α, and therefore the junction between N and J is uncertain. The V_β17-J_β2.7 chain was isolated directly from the JRS1 clone. Single-sided PCR amplification was carried out by a modification of published methods using a deoxycytidine homopolymer tail and an oligo(dG) primer (GATAGTCGACGGGGGGGGGG) (*6*). Transformed colonies were screened with an internal ^{32}P-labeled oligonucleotide probe and independent isolates sequenced. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

Fresh jejunal IELs from three additional patients were examined by quantitative PCR amplification of their TCRβ chains. The first patient expressed predominantly $V_\beta 3$ and lower levels of $V_\beta 6$ and $V_\beta 7$. Subcloning and sequencing of $V_\beta 3$ PCR products showed that all of the isolates (21/21) were identical (*12*). Subcloning and sequencing of the $V_\beta 7$ PCR products from this patient also showed a single clone (6/6 isolates). Clonally expanded populations expressing $V_\beta 3$ and $V_\beta 6$ were similarly detected in the second patient, and clonal populations expressing $V_\beta 2$, $V_\beta 3$, and $V_\beta 8$ were detected in the third patient. These results indicated, therefore, the oligoclonal expansion of human intestinal IELs in vivo.

The expression of CD8 and the utilization of a limited number of TCRα and TCRβ chains suggested that an intestinal epithelium–specific MHC class I–like molecule might be a ligand for intestinal IELs. Human intestinal epithelial cells express a nonpolymorphic MHC class I–like molecule, CD1d (*15*). Similar molecules [CD1 and the thymus leukemia antigen (TL)] have been detected on murine intestinal epithelial cells (*16*). In addition, CD1- and TL-specific cytolytic T cell clones have been identified (*9, 17*). The human CD1 molecules were, therefore, tested as target ligands for human intestinal IELs. The human leukocyte antigen–A,B (HLA-A,B)–negative cell line, C1R (*18*), stably transfected with either the pSR-α Neo expression vector (*19*) (mock) or the pSR-α Neo vector containing the CD1a, CD1b, CD1c, or CD1d cDNA (*15, 20*), was used as a target cell in ^{51}Cr release assays.

The IEL T cell line had significant cytolytic activity against CD1a to CD1d, and CD1c was the most effective target (Fig. 4A). Most of the cytolytic activity against CD1c could be accounted for by the JRS1 clone, which demonstrated potent CD1c-specific cytolytic activity (Fig. 4B). The ability of JRS1 to discriminate between CD1a to CD1d and its inhibition with CD3-specific (SPV-T3b) and TCRαβ-specific (WT31) monoclonal antibodies (*21*) indicated that CD1c was recognized by the TCR-CD3 complex. Finally, the lysis of the CD1a, CD1b, and CD1d transfectants by the line, but not by the JRS1 clone, indicated that additional clones from the IEL T cell line were likely to be cytolytic for CD1a, CD1b, and CD1d.

The clonal expansion of TCRαβ, $CD8^+$ intestinal IELs indicates that human intestinal IELs respond to a restricted group of intestinal epithelial antigens, with the CD1 molecules implicated as candidate ligands. The human CD1 locus encodes four nonpolymorphic, β_2-microglobulin–associated, MHC class I–like molecules, CD1a to CD1d (*15, 20, 22*). Although CD1d is expressed by the majority of intestinal epi-

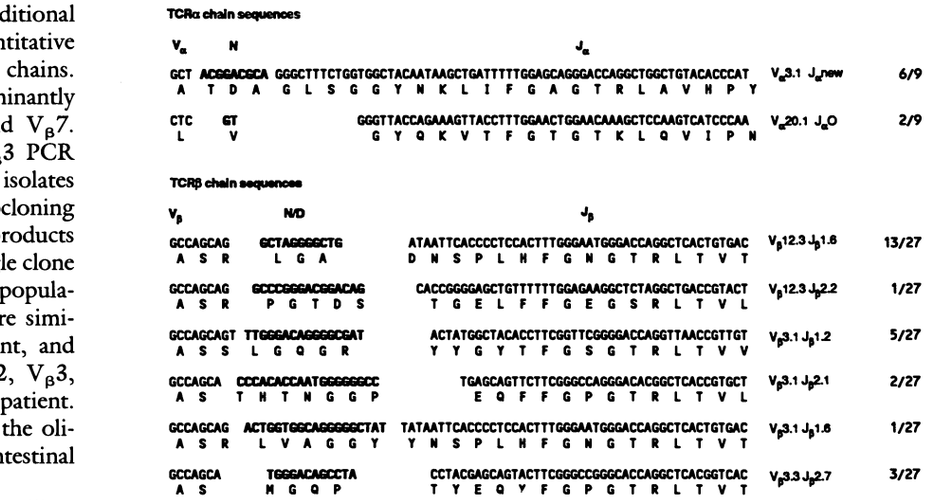

Fig. 3. Single-sided PCR amplification, cloning, and sequencing of the TCRα and TCRβ chains from fresh human jejunal IELs, performed as described in Fig. 1. The frequency of each isolate is indicated.

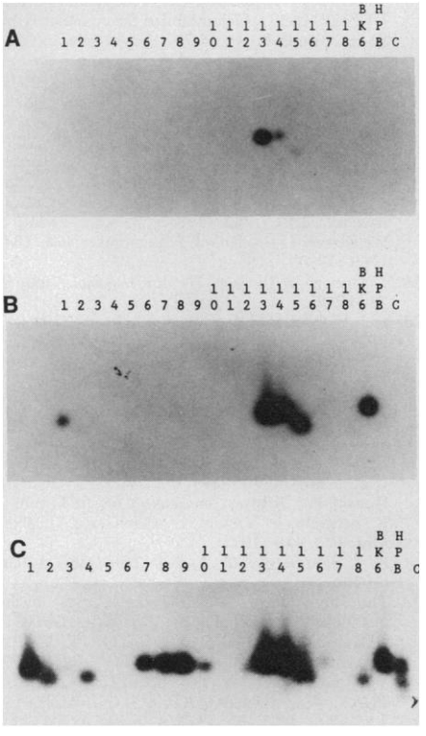

Fig. 2. Quantitative PCR amplification of the TCRα chains from an IEL T cell line. Reaction mixtures (0.04 ml) contained approximately 100 cell equivalents of cDNA, 80 ng of a V_α primer (approximately 10 pmol) (*29*), 80 ng of a C_α antisense primer (GGCAGACAGACTTGTCACTGGAT), 1 U of Taq 1 polymerase, 0.2 mM of each dNTP, 10 mM tris (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, and 0.001% gelatin. PCR cycles were 94°C for 20 s, 55°C for 30 s, and 72°C for 60 s. Aliquots (10 μl) from the amplification were withdrawn after 24 (**A**), 30 (**B**), and 38 (**C**) cycles. Lane C represents a negative control without any added V_α oligonucleotide in the PCR reaction. The PCR products were then separated on a 1.0% agarose gel, transferred to a nitrocellulose filter, and hybridized with a ^{32}P-labeled internal C_α (CGGCAGGGTCAGGGTTCT) probe.

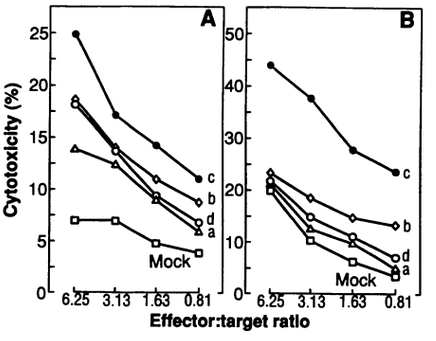

Fig. 4. Cytotoxicity of an IEL T cell line and IEL T cell clone. An IEL T cell line (**A**) and the IEL T cell clone (JRS-1) (**B**) were exposed to ^{51}Cr-labeled C1R cells transfected with either the pSR-α Neo vector (mock) or the pSR-α Neo vector containing the CD1a, CD1b, CD1c, or CD1d cDNAs (*30*) at effector-to-target ratios of 6.25:1, 3.125:1, 1.625:1, and 0.8125:1 in triplicate. Specific cytotoxicity in a 4-hour release assay was determined. Spontaneous and maximal releases were measured by incubation of target cells with medium alone or with medium containing 1% Nonidet P-40, respectively. The percent cytotoxicity was calculated by the formula: [experimental release (cpm) − spontaneous release (cpm)]/[maximal release (cpm) − spontaneous release (cpm)] × 100. The figure shows the average of three separate experiments. Sample standard deviations were less than 5% of reported values in all cases except in one of three experiments in (A) at effector-to-target ratios of 6.25:1 and 0.8125:1 with the CD1c and CD1b transfectants, respectively.

thelial cells (*15*), CD1a, CD1b, and CD1c are expressed primarily by immature thymocytes and not by intestinal epithelial cells (*23*). We propose, however, that CD1a to CD1c are expressed by intestinal epithelial cells during inflammation, infection, or neoplastic transformation (*24*). In support of this hypothesis, CD1a expression has been detected on gingival epithelial cells after exposure to interleukin-1 (IL-1) and on keratinocytes from several dermatoses (*25*). The expression of CD1a to CD1c by abnormal intestinal epithelial cells would likely provide a stimulus for expansion of CD1-reactive IELs that have migrated to the epithelium and that could, in turn, be important in immunosurveillance of the intestinal epithelium. Within this context, CD1d normally expressed on the surface of intestinal epithelial cells might not be a target for cytolytic IEL in vivo. Instead, CD1d may play a role in the selection or extrathymic education, or both, of CD1a- to CD1c-responsive T cells recruited to the epithelium. It remains possible, however, that the CD1a to CD1c recognition observed here represents cross-reactivity with another intestinal ligand. This cross-reactive ligand could either be unrelated to CD1 or, more likely, CD1d that is associated with an intestinal antigen such as a stress protein induced in injured epithelial cells (*26*). Further studies will be required to determine the mechanisms that stimulate the expansion of these clones, identify their precise target ligands, and determine whether similar populations are present in other epithelial surfaces (*27*).

REFERENCES AND NOTES

1. P. B. Ernst, A. D. Befus, J. Bienenstock, *Immunol. Today* **6**, 50 (1985); M. Bonneville *et al.*, *Nature* **336**, 479 (1988); T. Goodman and L. LeFrancois, *ibid.* **333**, 855 (1988); K. Deusch *et al.*, *Eur. J. Immunol.* **21**, 1053 (1991).
2. N. Cerf-Bensussan, E. E. Schneeberger, A. K. Bhan, *J. Immunol.* **130**, 2615 (1983); P. Brandtzaeg *et al.*, *Scand. J. Immunol.* **30**, 123 (1989); R. P. Bucy, C. H. Chen, M. D. Cooper, *J. Immunol.* **142**, 3045 (1989); V. Groh *et al.*, *J. Exp. Med.* **169**, 1277 (1989); L. K. Trejdosiewicz *et al.*, *Immunology* **68**, 7 (1989).
3. S. Kyes, E. Carew, S. R. Carding, C. A. Janeway, A. Hayday, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 5527 (1989); D. M. Asarnow, T. Goodman, L. LeFrancois, J. P. Allison, *Nature* **341**, 60 (1989); Y. Takagaki, A. DeCloux, M. Bonneville, S. Tonegawa, *ibid.* **339**, 712 (1989); S. Itohara *et al.*, *ibid.* **343**, 754 (1990).
4. IELs were isolated from jejunal mucosa obtained from healthy individuals undergoing gastric bypass surgery for morbid obesity, as described [E. C. Ebert, *Gastroenterology* **97**, 1372 (1990)]. A T cell line was established by culturing of the IELs in a 24-well plate in Dulbecco's modified Eagle's medium containing 5% fetal bovine serum, 2% pooled human AB^+ serum, penicillin-streptomycin (100 units per milliliter), gentamicin (50 μg/ml), Fungizone (1 μg/ml), 2 mM L-glutamine, recombinant IL-2 (rIL-2) (5 units per milliliter; Boehringer Mannheim), rIL-4 (4 units per milliliter; Genzyme), and phytohemagglutinin (PHA) (1.0 μg/ml; Difco, Detroit, MI) at a concentration of 2×10^6 cells per milliliter. The cells were fed at day 3 or 4 without PHA and at day 7 with PHA (1.0 μg/ml) and irradiated allogeneic peripheral blood mononuclear cells (PBMNC) (7.5×10^5 cells per well). The stimulation was repeated on day 14 with PHA and irradiated PBMNC and every 7 to 10 days thereafter.
5. Immunofluorescence of the cell line and clones was performed as described (*28*) with the WT31, δ1, OKT4, and OKT8 monoclonal antibodies (MAb). WT31 is a mouse immunoglobulin G1 (IgG1) MAb to human TCRαβ framework [W. Tax, H. Willems, P. Reekers, P. Capel, R. Koene, *Nature* **304**, 445 (1983)]. δ1 is a mouse IgG1 MAb to human TCRδ framework [H. Band *et al.*, *Science* **238**, 682 (1987)]. The MAbs to CD8 and CD4, OKT8 and OKT4, are murine IgG2b and IgG2a, respectively [E. Reinherz, P. Kung, G. Goldstein, R. Levey, S. Schlossman, *Proc. Natl. Acad. Sci. U.S.A.* 77, 1588 (1980)].
6. Whole cellular RNA from approximately 10^6 cells was extracted in a solution of 3 M LiCl and 6 M urea [C. Auffray and F. Rougeon, *Eur. J. Biochem.* **107**, 303 (1980)], and cDNA was synthesized with M-MLV reverse transcriptase [Bethesda Research Laboratories, Bethesda, MD (BRL)] and oligo$(dT)_{12\text{-}18}$ (Pharmacia). Single-sided PCR amplification was performed by a modification of published methods [M. A. Frohman, M. L. Dush, G. R. Martin, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 8998 (1988); E. Y. Loh, J. F. Elliott, S. Cwirla, L. L. Lanier, M. M. Davis, *Science* **243**, 217 (1989)]. Homopolymer tailing of the cDNA was performed in a 0.025-ml reaction mixture containing 0.4 mM deoxycytidine triphosphate and 30 U of terminal deoxytransferase in the buffer supplied by the manufacturer (BRL) for 10 min at 37°C, which adds a deoxycytidine tail of approximately 10 to 15 bases. The RNA was then removed by alkaline hydrolysis. Second-strand synthesis was performed with the Klenow fragment of DNA polymerase I and an oligo(dG) primer with a Sal I site (GATAGTCGACGGGGGGGGGG; Sal-dG_{10}) in a 0.025-ml reaction mixture containing 50 ng of primer (about 7.5 pmol), 0.1 mM of each deoxynucleotide triphosphate (dNTP), and 15 U of enzyme. Each 0.1-ml PCR reaction contained cDNA, 100 ng of Sal-dG_{10} primer, 100 ng of an antisense C_α (GAGGGAGCACAGGCTGTCTT) primer (located at the 3′ end of the constant region), 2.5 U of Taq 1 polymerase, 0.2 mM of each dNTP, and buffer, as described by the manufacturer (Perkin-Elmer Cetus). Each cycle of amplification consisted of 94°C for 20 s, 55°C for 30 s, and 72°C for 2 min for 30 cycles. One microliter of the product was then reamplified as above with an internal antisense C_α (GGCAGACAGACTTGTCACTGGAT) primer. The PCR products were digested with Sal I and an enzyme cutting in C_α (Pvu II) and cloned into pBluescript (Stratagene). A more 3′ nested primer and an internal Hind III site were also used in parallel for V_α cloning. TCRβ chains were amplified and cloned similarly with a 3′ C_β primer (CCTTTCTCTTGACCATGGCCATCA) and a nested C_β primer (GCCTTTTCCCTGTGGGAGAT) which both recognize $C_\beta 1$ and $C_\beta 2$. A Bgl II site in C_β was used for cloning into pBluescript.
7. Y. Choi *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 8941 (1989).
8. The $V_\alpha 15$ primer used in the PCR amplification was derived from clone AC24 [M. H. Klein *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 6884 (1987)] and has been redesignated as $V_\alpha 19$ [K. Wilson, E. Lai, P. Concannon, R. K. Barth, L. E. Hood, *Immunol. Rev.* **101**, 149 (1988)].
9. S. Porcelli *et al.*, *Nature* **341**, 447 (1989); S. P. Balk, S. Porcelli, M. Brenner, unpublished data.
10. The IEL T cell line was cloned by limiting dilution at less than one cell per well with 10^5 allogeneic, irradiated (5000 rads) PBMNC in Dulbecco's modified Eagle's medium containing PHA (1.0 μg/ml), rIL-2 (5 U/ml), and rIL-4 (4 U/ml) in 96-well V-bottom plates, as described [S. A. Brod, M. Purvee, D. Benjamin, D. A. Hafler, *Cell. Immunol.* **125**, 426 (1990)].
11. Quantitative PCR amplification of DNA from the IEL T cell clones, cell line and fresh IELs was performed as described (Fig. 2), using a panel of V_α or V_β oligonucleotide primers (*29*).
12. As described in the legend to Fig. 2, cDNA from the cell line or from fresh IELs was amplified by PCR for 35 cycles with a specific oligonucleotide. The PCR-amplified product was then subcloned into pBluescript and multiple isolates were sequenced.
13. The $V_\beta 12.3$ chain was originally isolated from clone HBP 34 and was designated as $V_\beta 13.2$ [N. Kimura *et al.*, *J. Exp. Med.* **164**, 739 (1986)]. We have, however, followed the convention of merging the $V_\beta 13$ family into $V_\beta 12$ [B. Toyonaga and T. W. Mak, *Annu. Rev. Immunol.* **5**, 585 (1987)].
14. The $V_\alpha 20$ chain was originally designated $V_\alpha 16.1$ (*8*).
15. S. P. Balk, P. A. Bleicher, C. Terhorst, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 252 (1989); R. S. Blumberg *et al.*, *J. Immunol.*, in press.
16. P. A. Bleicher *et al.*, *Science* **250**, 679 (1990); R. Hershberg *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 9727 (1990).
17. J. Bluestone, R. Cron, M. Cotterman, B. Houlden, L. Matis, *J. Exp. Med.* **168**, 1899 (1988); M. Bonneville *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 5928 (1989); K. Ito *et al.*, *Cell* **62**, 549 (1990); F. Faure, S. Jitsukawa, C. Miossec, T. Hercend, *Eur. J. Immunol.* **20**, 703 (1990).
18. J. Alexander, J. Payne, R. Murray, J. Frelinger, P. Cresswell, *Immunogenetics* **29**, 380 (1989).
19. Y. Takebe *et al.*, *Mol. Cell. Biol.* **8**, 466 (1988).
20. A. Aruffo and B. Seed, *J. Immunol.* **143**, 1723 (1989).
21. The cytolytic activity of the JRS1 clone was blocked by preincubation with the CD3 (SPV-T3b) [H. Spits *et al.*, *Hybridoma* **2**, 423 (1983)] and TCRαβ (WT31) MAbs, which inhibited the cytotoxicity by 81 ± 3% and 40.3 ± 6.7%, respectively, in three experiments.
22. L. H. Martin, F. Calabi, F. A. Lefebvre, C. A. G. Bilsland, C. Milstein, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 9189 (1987).
23. A. J. McMichael *et al.*, *Eur. J. Immunol.* **9**, 205 (1979); E. L. Reinherz, P. C. Kung, G. Goldstein, R. H. Levey, S. F. Schlossman, *Proc. Natl. Acad. Sci. U.S.A.* 77, 1588 (1980); M. A. Amiot *et al.*, *J. Immunol.* **136**, 1752 (1986); B. Kahn-Perles, J. Wietzerbin, D. H. Caillol, F. Lemonnier, *ibid.* **134**, 1759 (1985).
24. C. Janeway, B. Jones, A. Hayday, *Immunol. Today* **9**, 73 (1988).
25. L. J. Walsh and G. J. Seymour, *J. Invest. Dermatol.* **90**, 13 (1988); B. Dreno, B. Milpied, J. F. Stadler, G. Guillet, P. Litoux, *Acta Derm. Venereol.* **76**, 406 (1987); M. Drijkoningen, C. D. Wolk-Peeters, V. Desmet, *Br. J. Dermatol.* **115**, 511 (1986).
26. D. M. Asarnow *et al.*, *Cell* **55**, 837 (1988).
27. V. Groh, M. Fabbi, F. Hochstenbach, R. T. Maziarz, J. L. Strominger, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 5059 (1989); J. L. Viney, T. T. MacDonald, P. J. Kilshaw, *Immunology* **66**, 583 (1989); R. Benedita, P. Vassalli, D. Guy-Grand, *J. Exp. Med.* **173**, 483 (1991).
28. R. S. Blumberg *et al.*, *J. Biol. Chem.* **265**, 14036 (1990).
29. The family of V_α oligonucleotides used in the quantitative PCR amplification were $V_\alpha 1$, CTGCTGCTCGTCCCAGTGCTCGAGGTGATT; $V_\alpha 2$, ACAGTGACCGAGTTTCCCAGTCCTTCTTCT; $V_\alpha 3$, GGAGTGTCTTTGGTGATTCTATGGCTTCAA; $V_\alpha 4$, CTCCACAATCAGTGGAACTGATTACATACA; $V_\alpha 5$, AACTATACAAACTATTCTCCAGCATACTT; $V_\alpha 6$, CTGAAGGTGGTCACAGCTTCACTGTGGCTA; $V_\alpha 7$, TGGGGAGTTTTCCTTCTTTATGTTTCCATG; $V_\alpha 8$, CGAGCTTTATTTATGTACTTGTGGCTGCAG; $V_\alpha 9$, CCCACCCTCATCTCAGTGCTTGTGATAATA; $V_\alpha 10$, ACCCAGCTGCTGGAGCAGAGCCCTCAGTTT; $V_\alpha 11$, GCTTTGCAGAGCACTCTGGGGGCGGTGTGG; $V_\alpha 12$, TTGAGGGCAGTCATAGCCTCCATCTGTGTT; $V_\alpha 13$, GCTCTGCTGGGGCTCTTGAGTGCCCAGGTT; $V_\alpha 14$, GCATGCCCTGGCTTCCTGTGGGCACTTGTG; $V_\alpha 15$, TGTTTCCTTGTTTATGCTGAGCTCAGGGAA; $V_\alpha 16$, ATCTCGATGCTTGCGATGCTCTTCACATTG; $V_\alpha 17$, ATATCTATAAGTTCCATTAAGGATAAAAAT; $V_\alpha 18$, TGTCAGGCAATGACAAGGGAAGCAACAAAG; $V_\alpha BK6$, GAATCCTCAGTGAACCAGGGCAGA; and $V_\alpha HPB$, CGGAGCACGGAACATTTCACTCAGGG. The $V_\alpha BK6$ oligonucleotide was derived from the CD1a reactive T cell clone, BK6 (*9*), and the $V_\alpha HPB$ oligonucle-

otide from the HPB-ALL T cell line. The V_β family of oligonucleotides used were as described [K. Wucherpfennig *et al.*, *Science* **248**, 1016 (1990)].

30. The HLA-A,B-negative B cell line, C1R, was transfected with the pSR-α Neo expression vector (mock) and pSR-α Neo expression vector containing the CD1a, CD1b, CD1c, or CD1d cDNA by electroporation, as described (*28*). The transfectants were selected in geneticin sulfate (G418; 2.0 mg/ml; specific activity 450 μg/mg, Gibco) and CD1-expressing cells were identified by indirect immunofluorescence. The transfected C1R cells were cloned by limiting dilution and maintained in complete medium containing geneticin sulfate (2.0 mg/ml).

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A Mechanosensitive Channel in Whole Cells and in Membrane Patches of the Fungus *Uromyces*

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Bean leaf stomata provide a topographical signal that induces germlings of the phytopathogen *Uromyces appendiculatus* to develop specialized infection structures. Protoplasts from germ tubes of this fungus, when examined with patch-clamp electrodes, displayed the activities of a 600-picosiemen mechanosensitive ion channel. This channel passes a variety of cations, including Ca^{2+}, and is blocked by Gd^{3+} at 50 micromolar. This channel could transduce the membrane stress induced by the leaf topography into an influx of ions, including Ca^{2+}, that may trigger differentiation.

MOST PHYTOPATHOGENIC FUNGI must locate and recognize an appropriate site for entry into their hosts in order to establish a successful parasitic interaction. The rust fungi are the causal agents of the plant diseases wheat rust, coffee rust, and bean rust. Urediospore germlings of the bean rust fungus *Uromyces appendiculatus* are guided by the topography of the leaf surface to stomata, where they cease polarized growth and differentiate appressoria from the germling apices. Such appressoria are the first in a series of specialized infection structures needed for the pathogen to enter the plant host (*1*). The topographical signal that triggers formation of the appressorium is provided by a feature of the stomatal architecture that can be simulated on chemically inert plastic replicas of the epidermis (*2*) and on polystyrene membranes bearing microfabricated ridges 0.45 to 0.7 μm in height (*3*) (Fig. 1A).

The various postulated mechanisms by which a fungal germling can sense minute surface features include an ionic or electric change mediated by mechanosensitive (MS) channels. MS channels that respond to physiological stretch forces in the plasma membrane have been implicated in the regulation of cell volume, morphogenesis, osmotic pressure, guard cell responses, and neuronal growth-cone functions (*4, 5*). This type of ion channel has been identified in animal and plant cells and microbes, including the budding yeast, a free-living fungus (*5*). Contact of the bean rust germling with an inductive topography could produce localized stretching of the plasma membrane and cause MS channels to open. The resulting changes in membrane potential or cytoplasmic concentrations of ions, such as Ca^{2+}, may induce appressorium formation. We therefore examined the plasma membrane of *U. appendiculatus* germ tube protoplasts for MS channels with the patch-clamp method.

Collected urediospores were germinated and converted into protoplasts 4 to 7 μm in diameter (*6*). Standard patch-clamp electrodes were used to form gigaohm seals with the plasma membrane of selected protoplasts (Fig. 1B) (*7*). We report here the activities of a large conductance MS channel. Other smaller conductance channels were also detected that were voltage-sensitive but not MS. We detected the MS channel activities in every one of more than 50 protoplasts examined in the whole-cell mode or in excised inside-out or outside-out patches. In contrast to *Lymnaea* neurons (*8*), *Uromyces* protoplasts readily displayed whole-cell MS currents. Small pressures applied through the patch pipette tended to open these channels, yielding discrete, stepwise changes in the conductance of the membrane channels (Fig. 2). Opening appears not to be cooperative among these channels (Fig. 2B).

The probability that channels will open in response to a given applied pressure (open probability) fits a Boltzmann distribution in which the mechanical energy partitions the channel molecule between its open and closed conformations (Fig. 3). In three cells analyzed systematically, this curve inflects at pressures between 10 and 20 mmHg, which is similar to the sensitivities of MS channels of other species (*4, 5*). Because we have approached saturation of the open probability without breaking the protoplasts, we can estimate the number of MS channels in a protoplast. In one example (Fig. 3), there were 111 such MS channels in the whole membrane of this protoplast. The average density was about two channels per square micrometer in the ten protoplasts analyzed. The open probability of this channel under a small pressure increases strongly upon depolarization and slightly upon hyperpolarization below −50 mV (Fig. 3C). However, we could not activate the channel with voltage alone.

The conductance of the channel was 601 ± 10 pS (mean ± SD, $n = 9$) in symmetric 290 mM KCl solutions (Fig. 4A). This conductance is large for ion channels among eukaryotic species. We investigated the ion selectivity in the whole-cell mode by examining the changes in the current-voltage (*I-V*) plots of the unitary current after changing the ionic species and concentrations of the bath. Diluting the bath KCl concentration from 290 to 58 mM caused a shift of the *I-V* curve such that the current reversed at −35 mV, close to the calculated equilibrium potential of K^+ (Fig. 4A). Similar results were obtained in three other five-

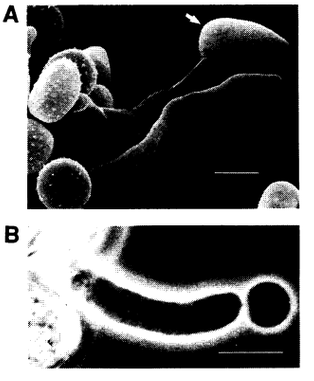

Fig. 1. Germlings of *U. appendiculatus*. (**A**) Scanning electron micrograph of two germlings grown on a polystyrene substrate bearing ridges 0.5 μm high and 2.0 μm wide, prepared as in (*3*). One of the germlings encountered an inductive ridge and developed an appressorium (arrow). (**B**) Protoplasts derived from young germlings (*6*). Bars represent 10 μm.

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