pairings. Each class had a minimum of five pairs from the same reef and five pairs from different reefs (one 1-3 interreef pair was later lost). Pairs were distributed in ten groups at a 10-m depth along Aguadargana Reef, with different types of pairings evenly distributed among the groups to avoid confounding location with treatment. Initially healthy areas of paired corals were separated by 1 to 2 mm, and monitored daily. Corals were subsequently collected within a 3-day interval for laboratory analyses.
12. J. C. Lang and E. A. Chornesky, in *Coral Reefs*, Z. Dubinsky, Ed. (Elsevier, Amsterdam, 1990), pp. 209–252.
13. After 6 days, only 35% of 60 intramorph pairs showed aggressive reactions; median area damaged for aggressive pairs was 27 mm^2 with a median delay of 5 days prior to first observation of mortality. In contrast, all 34 intermorph pairings were aggressive, with a median area damaged of 202 mm^2 and a median delay of 1 day. Differences between intra- and intermorph aggressive pairs are highly significant ($P \leq 0.0002$, Mann Whitney U tests). Data from intra- and interreef pairings were combined for these analyses as there were no statistically significant differences between them ($P > 0.05$, Mann Whitney U tests). For statistical analyses, SPSS-X [*SPSS-X User's Guide* (SPSS, Chicago, ed. 3, 1987)] was used, except where noted below.
14. F. J. Ayala, in *Protein Polymorphism: Adaptive and Taxonomic Significance*, G. S. Oxford and D. Rollinson, Eds. (Academic Press, London, 1983), pp. 3–26.
15. D. M. Hillis and C. Moritz, *Molecular Systematics* (Sinauer, Sunderland, MA, 1990).
16. Standard methods (*15*) were employed, with minor modifications for corals: grinding buffer [J. A. Stoddart, *Mar. Biol.* **76**, 279 (1983)]; homogenate passed through Miracloth (Calbiochem, La Jolla, CA) to reduce mucus on wicks. Loci scored were TPI-1, TPI-2, GPI-1, ME-1, GDH-1, GDH-2, PGM-1, PEP A-1, PEP D-1 [H. Harris and D. A. Hopkinson, *Handbook of Enzyme Electrophoresis in Human Genetics* (American Elsevier, New York, 1976)]. Minimum sample sizes per locus were 43, 44, 45, and 26, for *M. annularis* morphotypes 1, 2, 3, and *M. cavernosa*, respectively. Portions of colonies completely bleached by maintaining them in darkness for 40 days gave identical results as clonemate controls maintained in daylight, indicating that symbiotic zooxanthellae did not influence results (J. A. Stoddart, *ibid.*). Color variants within morphotypes showed no pattern with respect to allele frequencies. Samples of *M. cavernosa* included several growth forms. Electrophoretic data were analyzed by BIOSYS-1 [D. L. Swofford and R. B. Selander, *J. Hered.* **72**, 281 (1981)].
17. For a review of analyses of *Montastraea*, see A. F. Budd, *Syst. Bot.* **15**, 150 (1990).
18. J. P. Thorpe, in *Protein Polymorphism: Adaptive and Taxonomic Significance*, G. S. Oxford and D. Rollinson, Eds. (Academic Press, London, 1983), pp. 131–152.
19. Colony values for corallite measures were the average of ten randomly chosen, mature calices (five each at 2 cm from the edge and 2 cm from the center of colony). Colonies (a subsample of those previously assayed by aggression and electrophoresis) came from a single reef at depths of 2.5 to 8 m (morphotype 1), 2.5 to 10 m (morphotype 2), and 7.5 to 18.5 m (morphotype 3); 50% of each morphotype came from 7.5 to 12 m.
20. A. F. Budd, personal communication.
21. Height to diameter [A. B. Foster, *Coral Reefs* **2**, 19 (1983)].
22. Morphotype 1 is the type species, based on the original illustration [J. Ellis and D. Solander, *The Natural History of Many Curious and Uncommon Zoophytes* (Benjamin White, London, 1786), plate 53, figure 1] and (*20*). Formal nomenclature and descriptions for morphotypes 2 and 3 and more detailed comparisons of all three species will be presented elsewhere (E. Weil and N. Knowlton, in preparation).
23. A. B. Foster, *J. Exp. Mar. Biol. Ecol.* **39**, 25 (1979); R. R. Graus and I. G. Macintyre, *Science* **193**, 895 (1976).
24. _____, *Smithson. Contr. Mar. Sci.* **12**, 441 (1982).
25. P. Dustan, *Bull. Mar. Sci.* **29**, 79 (1979).
26. Vertical, linear growth-rate measurements were made on contact prints from x-radiographs of sectioned colonies in areas of clear banding and active growth; sections were made down the center of the specimen parallel to the main axis of growth. Skeletal material representing the last 2 years of growth from the section was sent to Beta Analytic (University Branch, Coral Gables, FL; Beta-43731-43760) for isotopic analysis. For each morphotype, corals came from several reefs (a subsample of those previously assayed by aggression and electrophoresis) to obtain adequate sample sizes of colonies large enough to section. Depths ranged from 2.5 to 14 m; 37 of 40 sectioned colonies and all but one analyzed for isotopes came from 6- to 12.5-m depth.
27. T. McConnaughey, *Geochim. Cosmochim. Acta* **53**, 151 (1989).
28. Bleaching of Jamaican *M. annularis* occurs in two forms, and that resembling morphotype 3 is temporally stable and unrelated to sea temperature [R. D. Gates, *Coral Reefs* **8**, 193 (1990)]; see also A. M. Szmant and N. J. Gassman, *ibid.*, p. 217. If morphotypes differ in propensity to bleach, a morphotype difference in $\delta^{18}O$ could be mistaken for a temperature signal, because the differences are comparable in magnitude [J. W. Porter, W. K. Fitt, H. J. Shapiro, C. S. Rogers, M. W. White, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 9342 (1989)].
29. J. P. Grassle and J. F. Grassle, *Science* **192**, 567 (1976).
30. We thank E. Brunetti, A. Budd, E. Gomez, C. Hansen, J. Jackson, J. Lang, A. Szmant, D. West, and others for their help and the Smithsonian Institution for financial support. The Government of Panama (Recursos Marinos) and the Kuna Nation granted permission for fieldwork and collections. N.K. was a Visiting Scholar at Wolfson College and the Department of Zoology, University of Oxford, while preparing the manuscript.

30 May 1991; accepted 2 October 1991

Induction of Broadly Cross-Reactive Cytotoxic T Cells Recognizing an HIV-1 Envelope Determinant

HIDEMI TAKAHASHI,* YOHKO NAKAGAWA, CHARLES D. PENDLETON, RICHARD A. HOUGHTEN, KOZO YOKOMURO, RONALD N. GERMAIN, JAY A. BERZOFSKY*

An immunodominant determinant for cytotoxic T lymphocytes (CTLs) exists in the hypervariable portion of human immunodeficiency virus-1 (HIV-1) gp160. Three mouse CTL lines (specific for isolates MN, RF, and IIIB) were examined for recognition of homologous determinants from distinct isolates. Only MN-elicited CTLs showed extensive interisolate cross-reactivity. Residue 325 played a critical role in specificity, with MN-elicited CTLs responding to peptides with an aromatic or cyclic residue and IIIB-induced cells recognizing peptides with an aliphatic residue at this position. CTL populations with broad specificities were generated by restimulation of IIIB-gp160 primed cells with MN-type peptides that have an aliphatic substitution at 325. This represents an approach to synthetic vaccines that can generate broadly cross-reactive CTLs capable of effector function against a wide range of HIV isolates.

THE ENVELOPE GLYCOPROTEIN GP160 has been used in numerous prototype vaccine preparations designed for prophylaxis against or immunotherapy of infection by HIV-1 or its close simian lentivirus relatives (*1–4*). Studies in humans and mice have revealed a small region of this protein, called the V3 loop, between cysteine residues 303 and 338, that evokes the major neutralizing antibodies to the virus (*5–7*) and stimulates both helper and cytotoxic T cell responses in both species (*8–11*). This same region is one of the most variable in sequence among different clonal isolates (*12, 13*); this variation may arise by selection of mutant virus as a result of the intense immune pressure directed against this region of the molecule (*14–17*). Thus, this segment of gp160 is both an attractive candidate for a major component of an acquired immunodeficiency syndrome (AIDS) vaccine because of its known antigenic properties, and a problem for the design of useful vaccines because of the extensive diversity in its structure. Because an effective anti-HIV vaccine strategy must anticipate to the greatest extent possible such potential changes in viral antigenicity, we have examined in detail the specificity of CTL recognition of diverse HIV-1 isolates and describe a method for immunization that generates broadly reactive CTLs with an enhanced capacity to respond to variant sequences at this critical immunodominant site.

We could elicit from BALB/c (H-2^d) mice CTL specific for the peptide SITKGPGRVIYATGQ (18RF), the segment of the HIV-1 RF isolate corresponding to the 315 through 329 region of gp160 IIIB previous-

H. Takahashi, Y. Nakagawa, K. Yokomuro, Department of Microbiology and Immunology, Nippon Medical School, 1-1-5 Sendagi, Bunkyo-Ku, Tokyo 113, Japan.
C. D. Pendleton and J. A. Berzofsky, Molecular Immunogenetics and Vaccine Research Section, Metabolism Branch, National Cancer Institute, National Institutes of Health (NIH), Bethesda, MD 20892.
R. A. Houghten, Torrey Pines Institute for Molecular Studies, San Diego, CA 92121.
R. N. Germain, Lymphocyte Biology Section, Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, NIH, Bethesda, MD 20892.

*To whom correspondence should be addressed.

ly shown to be important for CTL recognition (*8, 16*). These RF-specific CTLs did not cross-reactively kill targets treated with homologous peptides from HIV-1 strains IIIB or MN (18IIIB or 18MN, respectively). Because we had already obtained HIV IIIB and MN envelope-specific CTL lines from BALB/c mice, both restricted by the same D^d class I molecule (*8, 16*), we had three noncross-reactive, type-specific CTL lines that could kill targets infected with the appropriate gp160-expressing recombinant vaccinia virus and targets treated with the appropriate peptide. Taking advantage of these CTL lines and a series of synthetic peptides corresponding to the homologous portion of 14 different HIV isolates (Table 1), we analyzed the cross-reactivity of each CTL line for each peptide presented by $H\text{-}2^d$ cells (Table 1). Neither IIIB-specific CTLs nor RF-specific CTLs cross-reactively lysed targets incubated with peptides derived from other HIV isolates. However, MN-specific CTLs did cross-reactively kill targets incubated with the SC-, Z321-, SF2-, and, weakly, NY5-derived peptides.

In previous studies, we have demonstrated that the amino acid at position 325 plays a critical role in the specificity of CTL responses to 18IIIB and 18MN (*16, 17*). The peptides recognized by MN-specific CTLs share a common structure of -(I)-GPGRAF-X-(T)-, where X is a variable amino acid at position 325 whose identity appeared to determine target sensitivity to lysis by a given CTL population. To examine more systematically the effect of changes at this site, we synthesized a series of substituted peptides, each with a single amino acid substitution at position 325 in 18MN (*18*). The IIIB-specific CTL line strongly lysed targets sensitized with peptide 18MN(Y-V) consisting of the 18MN sequence with 325 Y replaced by V (Table 2) (*16*). These CTLs also recognized the substituted peptides 18MN(Y-I) and 18MN(Y-L) in which 325(Y) was replaced by either I or L, respectively. The MN-specific CTL line strongly lysed targets sensitized with 18MN(Y-F), 18MN(Y-H), or 18MN(Y-P) and moderately lysed targets with 18MN(Y-W) but failed to lyse cells treated with 18MN(Y-R). These results are compatible with the results obtained with naturally occurring variants (Table 1). Our findings suggest a chemical basis for the observed cross-reactive CTL function, in that IIIB-specific CTLs tend to see an aliphatic amino acid at 325 like V, I, or L, whereas MN-specific CTLs tend to see an aromatic amino acid like Y, F, H, or W or a ring structure such as P (*19*). This can explain why such CTLs did not cross-react with IIIB [325(V)] or WMJ-2 [325(R)]–derived peptides. However, the failure of MN-specific CTLs to lyse cells incubated in RF peptide, which has Y at 325, indicates that other residues play important roles in the recognition of this determinant.

To compare the potency of the cross-reactive peptides for the IIIB-specific CTL line, we titrated either peptide concentration at the same effector to target ratio (Fig. 1) or effector to target ratio at a constant concentration of peptide (*20*). The potency of 18MN(Y-V) was within 10-fold of that of the original peptide 18IIIB, whereas both 18MN(Y-I) and 18MN(Y-L) were 10 to 100 times less active (Fig. 1A). For the MN-specific CTL line (Fig. 1B), the 18MN(Y-F) substitution reduced the ability to sensitize targets approximately 10-fold compared to 18MN, the 18MN(Y-H) and 18MN(Y-P) substitutions reduced the potency nearly 30-fold, and the 18MN(Y-W) substitution reduced the potency 100-fold. For both CTL lines, it is the bulkiest substitution in the relevant chemical category of

Table 1. Cross-reactive CTL activity for the homologous portion of the gp160 immunodominant site from different HIV isolates. Mice were immunized intravenously with 10^7 plaque-forming units (PFUs) of recombinant vaccinia viruses. The recombinant vSC8 containing the bacterial *lacZ* gene as well as vSC-25, vMN, and vRF expressing the HIV env glycoprotein gp160 of the HIV IIIB, MN, RF isolates, respectively, without other HIV structural or regulatory proteins, have been described (*16, 23*). Four to eight weeks later, immune spleen cells [5×10^6 per milliliter in 24-well culture plates in complete T cell medium (*17*)] were restimulated for 6 days in vitro with either 0.3 μM peptide 18IIIB (RIQRGPGRAFVTIGK), 1 μM 18MN variant peptide (RIHIGPGRAFYTTKN), or 1 μM 18RF variant peptide (SITKGPGRVIYATGQ) plus 10% Rat Con-A supernatant-containing medium [Rat T cell monoclone (Collaborative Research, Bedford, Massachusetts)]. After culture for 6 days, cytolytic activity of the restimulated cells was measured as described (*8*) in a 6-hour assay with various ^{51}Cr-labeled targets. For testing the peptide specificity of CTLs, effectors and ^{51}Cr-labeled targets were mixed with various concentrations of peptide at the beginning of the assay (*8*). The variant peptides were synthesized as described (*16, 24*). The percent-specific ^{51}Cr release was calculated as 100 × [(experimental release − spontaneous release)/(maximum release − spontaneous release)]. Maximum release was determined from supernatants of cells that were lysed by addition of 5% Triton-X 100. Spontaneous release was determined from target cells incubated without added effector cells. Standard errors of the means of triplicate cultures were always less than 5% of the mean. In each sequence, the amino acid residue 325 is underlined.

HIV isolates	Sequence			Specific lysis (%)* at various peptide concentrations					
				IIIB-specific CTLs		MN-specific CTLs		RF-specific CTLs	
	↓315		329↓	10 μM	1 μM	10 μM	1 μM	10 μM	1 μM
IIIB	RIQRGPGRAF	V	TIGK	42.3	53.9	−0.5	−1.1	1.8	3.0
MN	RIHIGPGRAF	Y	TTKN	0.3	−2.3	43.3	50.3	1.5	1.7
RF	SITKGPGRVI	Y	ATGQ	0.3	−1.2	−0.9	−1.2	38.5	40.4
SC	SIHIGPGRAF	Y	ATGD	0.2	−1.6	46.2	42.2	3.0	−0.6
WMJ-2	SLSIGPGRAF	R	TREI	0.7	−1.2	4.2	−1.0	1.8	−0.1
Z321	SISIGPGRAF	F	ATTD	−0.3	−1.2	30.3	18.0	2.5	0.6
SF2	SIYIGPGRAF	H	TTGR	0.3	−0.4	25.9	13.8	1.6	0.4
NY5	GIAIGPGRTL	Y	AREK	−0.4	−0.7	10.2	1.0	1.4	−0.2
CDC4	RVTLGPGRVW	Y	TTGE	0.0	−2.0	1.0	−1.5	2.6	−0.2
Z3	SIRIGPGKVF	T	AKGG	0.5	−2.3	−1.7	−1.2	1.5	4.0
MAL	GIHFGPGQAL	Y	TTGI	0.2	−2.3	−1.4	−2.4	2.9	1.9
Z6	STPIGLGQAL	Y	TTRG	−0.6	−1.7	−2.1	−2.7	−0.7	−0.9
JY1	STPIGLGQAL	Y	TTRI	0.3	−2.3	1.2	−1.6	0.1	1.7
ELI	RTPTGLGQSL	Y	TTRS	0.4	−0.6	−0.5	−2.5	2.1	0.9

*Effector to target ratio is 10:1.

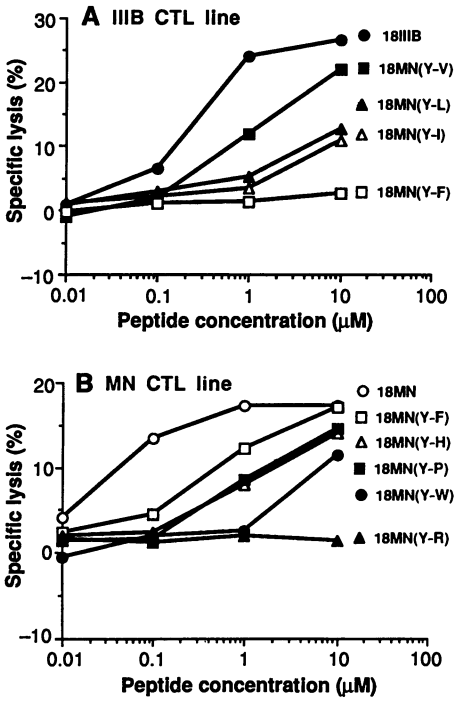

Fig. 1. Relative sensitization potencies of substituted MN peptides. CTL lines specific for IIIB (**A**) or MN (**B**) were cocultured with ^{51}Cr-labeled BALB/c 3T3 fibroblast targets in the presence of the indicated concentrations of peptides at a 5 to 1 effector to target ratio.

amino acid that had the least activity. This observation suggests that the T cell receptors (TCRs) of these CTLs have relatively hydrophobic pockets that can distinguish aliphatic from aromatic residues but are too constrained to accept easily the bulkiest of each category of side chain.

Highly isolate-specific CTL immunity on vaccination is unlikely to provide appropriate protection against the range of HIV-1 variants present in the population. To examine whether a broader range of effector CTL specificities could be induced by varying the epitopic structure of the antigen during priming and boosting, we restimulated immune spleen cells from mice primed with recombinant vaccinia virus expressing IIIB gp160 with either 18IIIB or several different 18MN-like peptides substituted at position 325. The pattern of CTL cross-reactivity induced by restimulation with the original 18IIIB appears the same as that of the IIIB-specific CTL line (Table 2). However, we could generate CTL populations with significantly broader specificity when the IIIB-gp160–primed spleen cells were restimulated with 18MN-based peptides containing aliphatic substitutions such as 18MN(Y-V), 18MN(Y-I), or 18MN(Y-L) (Table 2). Such CTLs cross-reactively lysed targets sensitized not only with the aliphatic substituted peptides themselves, but also targets exposed to 18MN(Y-F), 18MN(Y-R), 18MN(Y-K), and, more weakly, 18MN, 18MN(Y-W), and 18MN(Y-Q). Despite the cross-reactive killing by such effectors of targets sensitized with 18MN and 18MN-related peptides with aromatic or basic residues at 325, restimulation of IIIB-gp160–primed spleen cells with 18MN itself [325(Y)] or 18MN-substituted peptides containing such aromatic or basic substitutions did not induce any cross-reactivity or indeed much specific CTL activity (Table 2). The increased breadth of cross-reactivity elicited by the procedure of priming with gp160 IIIB-expressing vaccinia virus and boosting with 18MN(Y-V) peptide was also apparent when the CTLs were tested on natural HIV variant sequences. The CTLs elicited by this procedure now lysed cells incubated with peptides corresponding to isolates RF, MN, SF2, and WMJ-2 (26, 28, 12, and 7% specific lysis, respectively), whereas CTLs raised only against the IIIB isolate did not (<1% lysis) (Table 1). They also lysed targets infected with recombinant vaccinia viruses vIIIB (vSC25) and vMN expressing the HIV-1 IIIB and MN gp160 proteins endogenously, whereas CTLs elicited by restimulation with the IIIB peptide only lysed targets infected with vIIIB (*20*).

We also tried the analogous experiment with MN; spleen cells immunized with recombinant vaccinia virus expressing the HIV-MN gp160 gene were restimulated either with several aromatic substituted 18MN peptides or with 18IIIB [325 (V-Y)]. However, we could not generate any significant amount of specific CTL activity by restimulation with the former peptides, despite the fact that we could elicit specific CTLs from such immune cells when they were restimulated with 18MN itself, and restimulation with 18IIIB[325 (V-Y)] resulted in CTLs with specificity no different from that of CTLs stimulated with 18MN (*20*). A similar discordance between the capacity of a peptide to stimulate a response and to sensitize a target for lysis was observed when we tried to restimulate IIIB-primed T cells with MN peptides lacking aliphatic residues at 325. Such restimulation did not yield active CTLs, yet CTLs from the same primed cells restimulated with the aliphatic substituted MN peptides were able to kill targets sensitized with the aromatic variants.

These latter findings are consistent with recent data indicating that priming or restimulation of T cells is a much more stringent process than elicitation of effector function, especially cytotoxicity, from already stimulated CTLs (*21*). At present, it remains unclear if these differences reflect simple quantitative changes in thresholds for intracellular signaling or more complex events such as receptor-dependent elicitation of costimulatory molecules on the antigen-presenting cell (*22*). Such costimulation is needed for synergizing with TCR signals in driving a T cell out of the resting state but not for evoking effector functions independent of new gene activation.

Whatever the mechanism, these observations suggest that vaccine design should take into account the nonreciprocal nature of determinant cross-reactivity in the various stages of T cell priming and effector function. Our data suggest a method of immunization (IIIB priming followed by aliphatic MN substitution antigen boosts) that may be able to elicit CTL populations or individual clones with broad specificity. Although the immunization experiments presented here could be carried out only in an animal model, because the same epitope is also recognized by human CTLs with more than one histocompatibility antigen (HLA) class I molecule (*11*), the same approach, if not the identical substituted peptides, should be applicable to human immunization. These cross-reactivity experiments thus provide an approach for eliciting broadly cross-reactive CTLs that may suffice to protect against at least low-level exposure to a variety of HIV isolates.

Note added in proof: Evidence recently reported suggests the selection of HIV escape mutants by $CD8^+$ CTLs in infected humans (*25*). One way to deal with such a problem is to induce broadly cross-reactive CTLs by the approach we have indicated to prevent the outgrowth of escape mutants rather than require the immune system to deal with them after they arise.

Table 2. Effect of position 325 substitutions on CTL effector function and induction of CTL with broader specificities. CTL lines shown on the left, specific for either the IIIB or MN HIV gp160, were cocultured during the CTL assay with ^{51}Cr-labeled BALB/c 3T3 fibroblast targets in the presence of a series of substituted MN peptides at 10 μM. Effector to target ratio was 5 to 1. On the right, immune spleen cells from mice immunized with recombinant vaccinia virus vSC25 expressing the IIIB-gp160 gene (vIIIB) were restimulated for 6 days with either 18IIIB or 18MN peptides (1 μM) substituted at position 325, plus interleukin-2. The resulting CTLs were assayed on targets incubated with the indicated peptides. Effector to target ratio was 10 to 1.

Substituted peptides used to sensitize targets (10 μM)	Specific lysis (%)									
	CTL line		vIIIB-immunized spleen cells restimulated with peptide (1 μM):							
	IIIB line	MN line	18IIIB	18MN	18MN (Y-V)	18MN (Y-I)	18MN (Y-L)	18MN (Y-F)	18MN (Y-R)	18MN (Y-K)
18IIIB	40.5	−1.2	45.8	3.8	71.3	65.8	35.8	9.7	12.0	13.0
18MN	5.7	62.3	6.9	4.1	34.6	39.4	18.0	1.1	8.6	7.3
18MN(Y-V)	35.9	−2.9	27.3	5.0	61.0	62.7	33.1	8.5	20.6	13.8
18MN(Y-I)	34.4	−0.9	33.3	4.2	65.0	50.1	38.3	6.9	20.0	10.9
18MN(Y-P)	1.7	39.2	2.2	3.4	3.7	2.9	6.8	0.8	−1.4	0.8
18MN(Y-L)	22.6	−0.7	18.5	3.8	74.7	62.3	37.0	7.3	16.5	9.1
18MN(Y-W)	5.8	28.1	4.2	3.1	22.9	15.9	13.5	7.4	18.4	11.7
18MN(Y-F)	11.3	50.9	8.4	5.2	43.2	46.5	23.3	10.6	10.6	13.0
18MN(Y-S)	4.9	2.5	5.4	4.0	15.1	12.2	9.8	4.7	3.5	11.0
18MN(Y-E)	3.8	0.6	2.3	2.9	15.3	11.3	9.9	3.4	15.9	13.5
18MN(Y-R)	6.6	1.0	7.6	5.0	49.7	41.1	24.3	7.2	14.5	8.8
18MN(Y-H)	2.5	48.3	1.3	4.5	16.9	17.6	11.5	9.1	6.8	11.7
18MN(Y-K)	7.3	−0.8	5.5	4.6	57.1	51.9	23.9	11.1	19.9	15.0
18MN(Y-Q)	7.3	−0.3	4.9	4.2	37.0	36.4	19.1	7.7	14.0	12.8
No peptide	−0.5	0.1	−0.1	2.5	2.7	3.5	6.3	−0.1	−1.4	1.0

REFERENCES AND NOTES

1. P. W. Berman *et al.*, *Nature* **345**, 622 (1990).
2. D. Zagury *et al.*, *ibid.* **332**, 728 (1988).
3. M. Clerici *et al.*, *Eur. J. Immunol.* **21**, 1345 (1991).
4. R. R. Redfield *et al.*, *N. Engl. J. Med.* **324**, 1677 (1991).
5. T. J. Palker *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 1932 (1988).
6. J. R. Rusche *et al.*, *ibid.*, p. 3198.
7. J. Goudsmit *et al.*, *ibid.*, p. 4478.
8. H. Takahashi *et al.*, *ibid.*, p. 3105. 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.
9. H. Takahashi, R. N. Germain, B. Moss, J. A. Berzofsky, *J. Exp. Med.* **171**, 571 (1990).
10. M. Clerici *et al.*, *Nature* **339**, 383 (1989).
11. M. Clerici *et al.*, *J. Immunol.* **146**, 2214 (1991).
12. G. Myers *et al.*, *Human Retroviruses and AIDS 1989* (Los Alamos National Laboratory, Los Alamos, NM, 1989).
13. G. J. LaRosa *et al.*, *Science* **249**, 932 (1990).
14. J. Albert *et al.*, *AIDS* **4**, 107 (1990).
15. P. L. Nara *et al.*, *J. Virol.* **64**, 3779 (1990).
16. H. Takahashi *et al.*, *Science* **246**, 118 (1989).
17. H. Takahashi *et al.*, *J. Exp. Med.* **170**, 2023 (1989).
18. Peptide analogs of 18MN (*16*) were synthesized by solid-phase peptide synthesis [J. M. Stewart and J. D. Young, *Solid Phase Peptide Synthesis* (Pierce Chemical, Rockford, IL, 1984)] and purified by gel filtration and high-performance liquid chromatography (HPLC). We purified the peptides to single peaks on reverse-phase HPLC using C-18 columns and a buffer system of 0.1% trifluoroacetic acid–water and 0.1% trifluoroacetic acid–acetonitrile. Each peptide had the expected amino acid analysis.
19. Although precedent exists for a response to be due to a shorter peptide contaminant in a synthetic preparation [T. N. M. Schumacher, M. L. H. De Bruijn, L. N. Vernie, W. M. Kast, C. J. M. Melief *et al.*, *Nature* **350**, 703 (1991)] or for possible trimming by proteases once a peptide is bound to a class I MHC molecule [G. M. Van Bleek and S. G. Nathenson, *ibid.* **348**, 213 (1990); O. Rötzschke, K. Falk, K. Deres, H. Schild, M. Norda *et al.*, *ibid.*, p. 252; K. Falk, O. Rötzschke, S. Stevanovic, G. Jung, H.-G. Rammensee, *ibid.* **351**, 290 (1991)], the reciprocal nature of the recognition by different T cells, correlating with the chemical character of a single core residue, when the rest of the peptide, the presenting class I major histocompatibility complex (MHC) molecule, D^d, and the antigen-presenting cells and their processing machinery are held constant, could not simply be explained by arbitrary levels of peptide contaminants or differences in antigen processing.
20. H. Takahashi *et al.*, unpublished observations.
21. M. A. Alexander, C. A. Damico, K. M. Wieties, T. H. Hansen, J. M. Connolly, *J. Exp. Med.* **173**, 849 (1991).
22. B. D. Evavold and P. M. Allen, *Science* **252**, 1308 (1991).
23. S. Chakrabarti, M. Robert-Guroff, F. Wong-Staal, R. C. Gallo, B. Moss, *Nature* **320**, 535 (1986).
24. R. A. Houghten, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 5131 (1985).
25. R. E. Phillips *et al.*, *Nature* **354**, 453 (1991).
26. We thank B. Moss and S. Merli for the generous gifts of recombinant vaccinia viruses and W. Biddison and J. Yewdell for critical reading of the manuscript and helpful suggestions. This work was supported in part by grants from the Ministry of Education, Culture, and Science and from the Ministry of Health, Japan.

23 August 1991; accepted 18 November 1991

Evidence of a Pre-Angiosperm Origin of Endosperm: Implications for the Evolution of Flowering Plants

WILLIAM E. FRIEDMAN

The formation of a polyploid endosperm tissue has long been considered a unique and defining feature (autapomorphy) of angiosperms. Contemporaneous with the fertilization of an egg nucleus by a sperm nucleus in *Ephedra trifurca* (a nonflowering seed plant closely related to angiosperms), a second fertilization event has previously been shown to occur between a second sperm nucleus and the sister nucleus of the egg nucleus. Development of the second fertilization product is now shown to be fundamentally similar to that of endosperm in primitive flowering plants: both are characterized by an initial period of free nuclear proliferation followed by a process of cellularization. In *Ephedra*, however, the second fertilization product ultimately yields additional embryos. If double fertilization in *Ephedra* and angiosperms is evolutionarily homologous, it is likely that endosperm evolved from a supernumerary fertilization event that originally produced embryos into one that produced a specialized nonembryo tissue dedicated to the nourishment of the zygotic embryo.

"The unravelling of the history of the phylogenetic evolution of the process of endosperm formation should prove one of the most interesting developments in botany, and if accomplished will go far to solve the problem of the origin of Angiosperms." These words, written in 1907 by the botanist E. N. Thomas (*1*) are still true today. Despite considerable research into developmental, physiological, and ecological aspects of endosperm (*2*), relatively little is known of the sequence of evolutionary events that led to the origin and establishment of this distinctive feature of angiosperm reproduction.

Double fertilization and the associated formation of polyploid endosperm have long been considered unique and defining features of angiosperms (*3*). Recent studies, however, have established that a process of double fertilization also occurs in members of the genus *Ephedra* (*4–6*). These findings are significant in view of the critical phylogenetic position of *Ephedra*, which is a basal member of the most closely related extant group of seed plants (Gnetales) to angiosperms (*7*). Thus, the presence of double fertilization in *Ephedra* and angiosperms suggests that this important feature of reproduction may be evolutionarily homologous in both groups of seed plants, having been inherited from a common pre-angiosperm ancestor (*4, 5, 8*).

Although a pattern of double fertilization has been established in *Ephedra nevadensis* (*4, 5*) and *Ephedra trifurca* (*6*), the fate of the second fertilization product has remained uncertain. In this report, evidence is advanced that supernumerary embryos are formed from the fusion product of the second fertilization event in *E. trifurca*. These findings have profound implications for our interpretation of the evolutionary history of endosperm and the origin of angiosperms.

Ovules of *E. trifurca* were collected from naturally occurring populations near Tucson, Arizona, from 1987 through 1990.

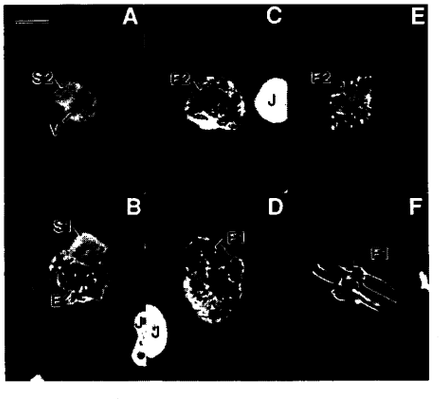

Fig. 1. Fluorescence views (stained with DAPI) of double fertilization and early post-fertilization free nuclear development in three individual egg cells of *E. trifurca*. Early stage of double fertilization: (**A**) second sperm nucleus (S2) and ventral canal nucleus (V) and (**B**) first sperm nucleus (S1) and egg nucleus (E). Fusion products of double fertilization: (**C**) second fertilization product (F2) and (**D**) first fertilization product (F1). Mitosis of fusion products of double fertilization: (**E**) second fertilization product and (**F**) first fertilization product. Jacket cell nuclei (J) in jacket cells adjacent to the egg cell are visible in several frames. Scale bar, 25 μm.

Department of Botany, University of Georgia, Athens, GA 30602.