reduce the Tc1 copy number and also make the MJ562 Tc1 profile in the genome similar to that for N2. The standard genetic markers used for outcrossing were the *tpa-1*–linked genes *dpy-9(e12)*IV and *dpy-9(e12)unc-17(e113)*IV.
9. B. Rosenzweig, L. W. Liao, D. Hirsh, *Nucleic Acids Res.* **11**, 4201 (1983).
10. The cDNA library was constructed in λgt10 from mRNAs of the worms at various developmental stages.
11. Template DNA for sequencing was prepared as described [J. Vieira and J. Messing, *Methods Enzymol.* **153**, 3 (1987)]. Deletions were constructed by the exonuclease III method [S. Henikoff, *Gene* **28**, 351 (1984)] using mung bean nuclease. Nucleotide sequences were determined by the dideoxy chain termination method [F. Sanger *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463 (1977)].
12. The DNA sequence common to both the 2.4-kb fragment and cDNA-1 is internally contained in the sequence of the latter.
13. P. J. Parker *et al.*, *Science* **233**, 853 (1986).
14. L. Coussens *et al.*, *ibid.*, p. 859.
15. S. Ohno *et al.*, *Nature* **325**, 161 (1987).
16. A. Rosenthal *et al.*, *EMBO J.* **6**, 433 (1987).
17. J. L. Knopf *et al.*, *Cell* **46**, 491 (1986).
18. Abbreviations for the amino acid residues are: 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. X represents any amino acid.
19. G. L. Greene *et al.*, *Science* **231**, 1150 (1986); J. L. Arriza *et al.*, *ibid.* **237**, 268 (1987); S. Green *et al.*, *Nature* **320**, 134 (1986).
20. R. M. Tufty and R. H. Kretsinger, *Science* **187**, 167 (1975).
21. M. J. Geisow *et al.*, *Nature* **320**, 636 (1986).
22. Y. Ono *et al.*, *J. Biol. Chem.* **263**, 6927 (1988).
23. S. Ohno, *et al.*, *Cell* **53**, 731 (1988).
24. S. K. Hanks *et al.*, *Science* **241**, 42 (1988).
25. Y. Nishizuka, *ibid.* **233**, 305 (1986).
26. J. Karn, S. Brenner, L. Barnett, *Methods Enzymol.* **101**, 3 (1983).
27. E. M. Southern, *J. Mol. Biol.* **98**, 503 (1975).
28. We thank A. Otsuka for introducing us to nematode gene cloning and S. Siddiqui for critically reading the manuscript. We also thank E. Hedgecock for NJ82, R. Waterston and D. Moerman for RW7097, and J. Kimble and J. Ahringer for the *C. elegans* cDNA library. The Tc1-containing plasmid pCe2003 was constructed by S. Emmons and provided to us through A. Otsuka.

19 August 1988; accepted 19 December 1988

In Vivo Modulation of Cytolytic Activity and Thy-1 Expression in TCR-γδ$^+$ Intraepithelial Lymphocytes

LEO LEFRANCOIS AND THOMAS GOODMAN

Although the functional aspects of the αβ T cell antigen receptor (TCR) found on most peripheral T cells are well described, the function of the γδ TCR remains unclear. Murine intraepithelial lymphocytes (IEL) of the small intestine are CD8$^+$, express the γδ TCR, and are constitutively lytic. Fresh IEL from germ-free mice had no lytic activity. Moreover, whereas IEL from normal mice are 30 to 50 percent Thy-1$^+$, IEL from germ-free did not express Thy-1. Acclimation of germ-free mice to nonsterile conditions resulted in the generation of Thy-1$^+$ IEL and induction of lytic activity. Thus CD8$^+$ TCR-γδ IEL were regulated by externally derived stimuli via a specific functional interaction between IEL and gut-associated antigens.

THE MUCOSAL IMMUNE SYSTEM IS one of the first lines of defense against bacterial and viral invasion. In the small intestine at least three anatomically distinct lymphoid areas are evident: (i) Peyer's patches, (ii) lamina propria of the villi, and (iii) intraepithelial lymphocytes (IEL) residing between the columnar epithelial cells of the villi. In the mouse, IEL are made up of both Thy-1$^+$ and Thy-1$^-$ CD8$^+$ CD3$^+$ T cells (*1, 2*). This latter subset of T cells has been detected only in this location. The relation of the Thy-1$^-$ IEL to Thy-1$^+$ IEL and to other peripheral T cells is unknown. Recently, we reported that murine CD8$^+$ IEL express heterodimeric T cell receptors (TCRs) composed of γ and δ chains (*3*). Most other murine TCR-γδ–expressing cells described are of the CD4$^-$8$^-$ phenotype and are found in very small numbers in the thymus (*4, 5*), spleen (*6*), or peripheral blood (*5*), or in the skin as dendritic epidermal cells (DEC) (*7, 8*). Most of the CD8$^+$ T cell populations express TCRs composed of α and β chain heterodimers (*9, 10*). In addition, freshly isolated IEL are constitutively lytic, unlike other CD8$^+$ peripheral T cells, implying that in situ activation of IEL occurs under normal circumstances (*3*). However, the origin of the antigens involved in the in vivo activation of IEL has not been determined. Thus, although it is clear that TCR-γδ expressing CD8$^+$ IEL constitute a T cell lineage distinct from other CD8$^+$ T cells, their role in vivo remains unclear.

To determine the nature of the antigens involved in IEL activation, we analyzed IEL from germ-free mice for lytic activity. We used a redirected lysis assay in which a monoclonal antibody (MAb) to CD3 is bound to an Fc receptor–positive target cell to detect all lytic activity regardless of receptor specificity (*11, 12*). The IEL in all experiments were ~95% CD3$^+$8$^+$. In five of five experiments with IEL isolated from a total of 50 Swiss (NIH) germ-free mice virtually no lytic activity was detectable, although the same MAb efficiently directed the lytic activity of a cytolytic T cell clone. Results from a representative experiment are depicted in (Fig. 1). In contrast, IEL from Swiss (NIH) mice reared under standard conditions or IEL from C57BL/6 or BALB/c mice, had cytolytic activity (Table 1) (*13*). The absence of lytic activity in IEL from germ-free mice indicated that environmental antigens were responsible for the in vivo activation of IEL.

We also phenotypically characterized IEL from germ-free mice. Although all CD8$^+$ IEL from germ-free mice expressed CD3, there were few cells with detectable Thy-1 antigen (Fig. 2). However, 35% of IEL from Swiss (NIH) mice were Thy-1$^+$, a percentage similar to our results with IEL from C57BL/6 mice (*3*). Therefore, IEL in the absence of antigenic stimuli in vivo were not cytolytic and did not express Thy-1. If there were a relation between lytic activity and Thy-1 expression in IEL, then removal of Thy-1$^+$ cells from IEL from normal mice should result in the loss of lytic activity. We isolated IEL from C57BL/6 or Swiss (NIH) mice and removed Thy-1$^+$ cells with MAb to Thy-1 and complement. The remaining populations, which were routinely <5% Thy-1$^+$, were assayed for cytolytic activity (Table 1). In all cases, the lytic activity of IEL was reduced to background levels after removal of Thy-1$^+$ cells; thus the lytic activity of IEL resided primarily in the Thy-1$^+$ subset.

To further substantiate the involvement of antigenic stimulation in the generation of Thy-1$^+$ IEL and cytolytic activity, we introduced germ-free mice into our standard mouse colony. After four weeks, IEL from such "acclimated" mice were assayed for cytolytic activity and for Thy-1 expression

Table 1. Lytic activity of IEL after removal of Thy-1$^+$ cells. IEL were treated with antibody to Thy-1, followed by two successive treatments with complement (C). The resulting populations were <5% Thy-1$^+$. Starting populations were 37%, 34%, and 42% Thy-1$^+$ for experiments 1 to 3, respectively. IEL were isolated from C57B1/6J mice (experiments 1 and 2) or from Swiss (NIH) mice (experiment 3). One lytic unit is the number of effector cells required to achieve 20% specific lysis of 2.5×10^3 target cells. Target cells were ^{51}Cr-labeled P815 (DBA/2 mastocytoma) with the addition of 1 μg of anti-CD3 per milliliter. Lytic activity in all cases without the addition of anti-CD3 was negligible.

Experiment	Lytic activity (lytic units per 10^6 cells)	
	C alone	anti–Thy-1 + C
1	9.3	<0.1
2	8.0	<0.1
3	9.2	<0.1

Department of Cell Biology, The Upjohn Company, Kalamazoo, MI 49001.

(Fig. 3). IEL from germ-free acclimated mice were 31% Thy-1$^+$ and had cytolytic activity comparable to that of IEL from normal mice. The increase in Thy-1 expression and lytic activity increased in proportion to the length of time the germ-free mice were housed under standard conditions. These results indicated that in vivo stimulation of IEL was required for Thy-1 expression and for induction of cytolytic activity.

Because Thy-1 antigen expression was determined by the presence of externally derived stimuli, it was also possible that TCR-γδ expression was affected. To determine whether in vivo antigenic stimulation was required for the presence of TCR-γδ$^+$ IEL, we immunoprecipitated the TCR complex of IEL from germ-free mice using antibody to CD3 (Fig. 4). The precipitate was analyzed under reducing conditions and contained proteins of 34, 35, 40, and 46 kD, as well as smaller proteins of the CD3 complex. These results were consistent with our results on IEL from normal mice and with the results of others, in that the TCR protein profile resembled γδ heterodimers (*14–16*). To prove the presence of TCR-γδ in IEL from germ-free mice and to analyze in more detail the constant regions present in these TCR, we used an antisera specific for the $C_{\gamma 1}$, $C_{\gamma 2}$, and $C_{\gamma 3}$ families (*4*). By first precipitating with anti-CD3 and reducing and alkylating the precipitated proteins, we determined which species were reactive with this sera. Immunoprecipitation of reduced and alkylated material yielded proteins of 34 and 35 kD; thus, these proteins were γ chains that contained either $C_{\gamma 1}$ and $C_{\gamma 2}$ constant regions since $C_{\gamma 3}$ is encoded by a pseudogene (*17*). Two-dimensional gel electrophoresis showed that the 34- and 35-kD proteins were disulfide-linked either to the 40- or 46-kD proteins, indicating that the latter were δ chains (*18*). This pattern of γδ proteins was similar to that obtained with IEL from normal C57BL/6 mice (*3*) or Swiss (NIH) mice. Thus, although Thy-1 antigen expression was regulated by antigenic stimulation, the expression of TCR-γδ, at least at the biochemical level, was not. It remains to be seen whether in vivo antigenic stimulation affects TCR usage in terms of variable region usage and combinatorial diversity.

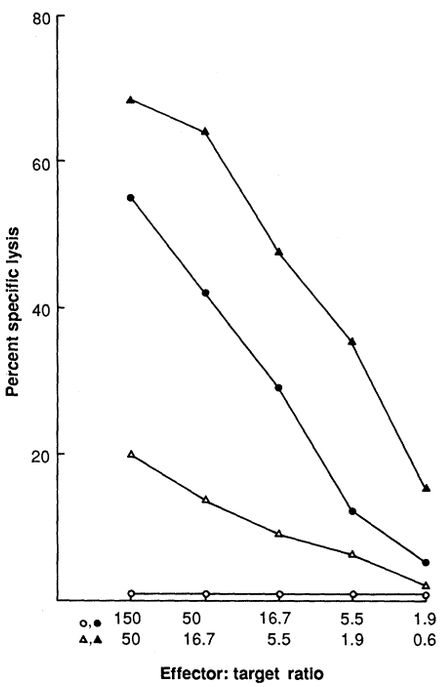

Fig. 1. Cytolytic activity of IEL from normal and germ-free mice. IEL were isolated as previously described (*3*) from groups of four to ten normal Swiss (NIH) mice (●) or Swiss (NIH) germ-free mice (○). The cells were assayed for lytic activity against ^{51}Cr-labeled P815 cells in the presence of a MAb specific for the ε chain of the CD3 complex (1 μg/ml) (*11*). As a control, T cell clone Bc.3 cytolytic for H-2^b was tested in the presence (▲) or absence of anti-CD3 (△). Serial dilutions of effector cells were incubated in 96-well round bottom microtiter plates with 2.5 × 10^3 target cells and incubated at 37°C for 4 hours. Percent-specific lysis was calculated as 100 × [(counts per minute released with effectors) − (counts per minute released alone)]/[(counts per minute released by detergent) − (counts per minute released alone)]. Spontaneous release was <10%.

The function of Thy-1, a phosphatidylinositol-linked membrane protein (*19, 20*) in vivo is unknown, but it is clear that it can transduce activation signals (*21, 22*). Transgenic mice with inappropriate expression of Thy-1 exhibit disorders associated with unregulated proliferation, suggesting that Thy-1 can affect cell growth in vivo (*23, 24*). Our results describe the only in vivo example of Thy-1 expression that is regulable during antigen encounter by T cells. Moreover, antigenic regulation of Thy-1 occurred in the only major murine CD8$^+$ T cell population described to date that expresses TCRs composed of γδ chain heterodimers. It is formally possible that the presence of antigen affects the migration of Thy-1$^+$ cells into the small intestine. However, if this were the case, the T cells entering the epithelium from the periphery would necessarily be of similar phenotype and would express similar TCRs as the Thy-1$^+$ IEL. It is perhaps more plausible that Thy-1$^-$ IEL, once activated, become cytolytic and begin to express Thy-1. Indeed, our preliminary results suggest that activation of Thy-1$^-$ IEL via the TCR-γδ can result in Thy-1 expression (*25*). In either case, the appearance of Thy-1$^+$ IEL was dependent on the presence of external stimuli.

The dichotomy in the expression of TCR-αβ versus TCR-γδ is not yet understood. Our data provide evidence that the explanation may lie in the nature of the antigens that confront the two types of receptors. Whereas CD8$^+$ TCR-αβ bearing T cells would be expected to encounter predominantly viral antigens within the body, CD8$^+$ TCR-γδ$^+$ IEL are likely to encounter large amounts of bacterial antigens in the small intestine and, to a lesser extent under normal circumstances, viral antigens. Furthermore, it is unlikely that food-derived antigens were responsible for induction of lytic activity in IEL, since normal and germ-free mice were fed similar diets, albeit sterile in the latter

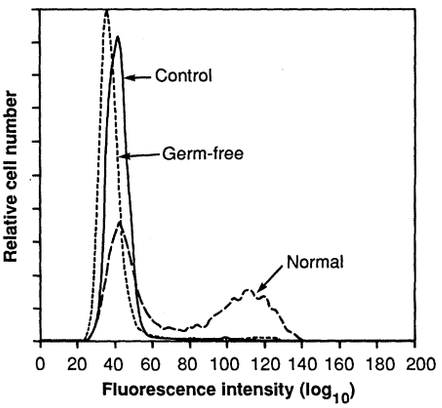

Fig. 2. Thy-1 expression of IEL from normal and germ-free mice. Purified IEL were reacted with a fluorescein isothiocyanate (FITC)–conjugated MAb to Thy-1 [T24, (*29*), germ-free, normal] or with an irrelevant FITC-conjugated antibody (control). Relative fluorescence intensities of individual cells were measured with an Ortho Cytofluorograph model 50H. The results are presented as fluorescence histograms with the relative number of cells on a linear scale plotted versus the relative fluorescence intensity on a four-decade logarithmic scale, both in arbitrary units.

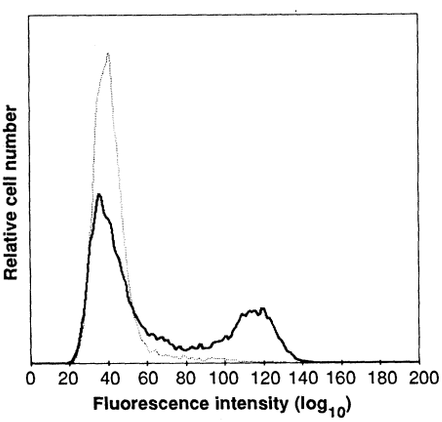

Fig. 3. Induction of Thy-1 expression and cytolytic activity by environmental antigens. Germ-free mice were housed under standard conditions for 4 weeks and IEL were then isolated and tested for Thy-1 expression (solid line) and for lytic activity (26.8 lytic units per 10^6 cells) as described in Figs. 1 and 2. Control, stippled line. One lytic unit is the number of effector cells required to achieve 20% specific lysis of 2.5 × 10^3 target cells. Similar results were obtained in several experiments.

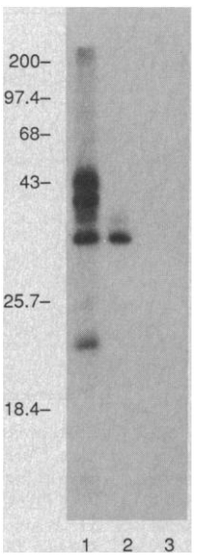

Fig. 4. Immunoprecipitation of T cell receptors of IEL from germ-free mice. Cell surface proteins of IEL isolated from germ-free mice were ^{125}I-labeled by the iodogen method (*30*). Precipitation by anti-CD3 was carried out essentially as described (*31*) with the use of anti-CD3 coupled to Sepharose 4B. For antibody to γ chain precipitation, the anti-CD3 precipitates were disrupted by boiling in the presence of 1% SDS and 10 m*M* dithiothreitol as the reducing agent. The reduced proteins were then alkylated by incubation with 30 m*M* iodoacetamide. The samples were diluted with buffer containing 10 m*M* triethanolamine, 0.15*M* NaCl (*p*H 7.8), 0.5% NP-40, 1 m*M* phenylmethylsulfonyl fluoride, 1 m*M* EDTA, and 1 μg of leupeptin per milliliter. Rabbit antiserum against the γ chain (*9*) was added with protein A coupled to Sepharose 4B, and the mixture was incubated for 2 hours at 4°C with mixing. The precipitates were washed six times with the above buffer and analyzed by SDS–12% polyacrylamide gel electrophoresis. Lane 1, anti-CD3; lane 2, antiserum to γ chain; and lane 3, control antibody.

case. The normal mice used in these studies were free of antibody to virus but we cannot rule out low levels of inapparent viral infection. This is not to imply that IEL are not capable of responding to viral antigens, only that their repertoire may be skewed toward recognition of bacterially derived determinants. The primordial immune system would require protection against invasion of a primitive digestive tract prior to developing a surveillance system of the internal milieu, and thus TCR-γδ may have been the earliest TCRs, as has been previously proposed (*26, 27*). CD8$^+$ T cells have been implicated in the immune response to pathogenic intracellular bacteria but the TCR status of these cells has not been analyzed (*28*). Although the TCRs of CD8$^+$ IEL could be specific for bacterially derived antigens, the nature of these antigens and the antigen-processing mechanisms required for recognition by IEL will require further study. The phenotypic and functional characteristics of IEL coupled with their highly restricted anatomical distribution should allow a detailed analysis of the in vivo function of T cells expressing TCR-γδ.

REFERENCES AND NOTES

1. D. M. V. Parrot *et al.*, *Ann. N.Y. Acad. Sci.* **409**, 307 (1983).
2. A. Petit *et al.*, *Eur. J. Immunol.* **15**, 211 (1985).
3. T. Goodman and L. Lefrancois, *Nature* **333**, 855 (1988).
4. A. M. Lew *et al.*, *Science* **234**, 1401 (1986).
5. L. L. Lanier and A. Weiss, *Nature* **322**, 179 (1986).
6. R. Q. Cron *et al.*, *J. Immunol.* **141**, 1074 (1988).
7. F. Koning *et al.*, *Science* **236**, 834 (1987).
8. G. Stingl *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 4586 (1987).
9. S. Hedrick *et al.*, *ibid.* **82**, 531 (1985).
10. M. Kronenberg *et al.*, *Nature* **313**, 647 (1985).
11. O. Leo, M. Foo, D. H. Sachs, L. E. Samelson, J. A. Bluestone, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 1374 (1987).
12. O. Leo *et al.*, *J. Immunol.* **137**, 3874 (1986).
13. Lefrancois and Goodman, unpublished results.
14. M. B. Brenner *et al.*, *Nature* **322**, 145 (1986).
15. A. Weiss, M. Newton, D. Crommie, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 6998 (1986).
16. D. M. Pardoll *et al.*, *Nature* **326**, 79 (1987).
17. A. C. Hayday *et al.*, *Cell* **40**, 259 (1985).
18. L. Lefrancois and T. Goodman, unpublished results.
19. M. G. Low and P. W. Kincade, *Nature* **318**, 62 (1985).
20. A. G. D. Tse, A. N. Barclay, A. Watts, A. F. Williams, *Science* **230**, 1003 (1985).
21. K. C. Gunter, T. R. Malek, E. M. Shevach, *J. Exp. Med.* **159**, 716 (1984).
22. R. A. Kroczek, K. C. Gunter, R. C. Germain, E. M. Shevach, *Nature* **322**, 181 (1986).
23. S. Chen, F. Botteri, H. van der Putten, C. P. Landel, G. A. Evans, *Cell* **51**, 7 (1987).
24. G. Kollias *et al.*, *ibid.*, p. 21.
25. L. Lefrancois and T. Goodman, unpublished results.
26. D. M. Pardoll, A. M. Kruisbeek, B. J. Fowlkes, J. E. Coligan, R. H. Schwartz, *FASEB J.* **1**, 103 (1987).
27. C. A. Janeway, B. Jones, A. Hayday, *Immunol. Today* **9**, 73 (1988).
28. S. H. E. Kaufmann, *ibid.*, p. 9.
29. G. Dennert, R. Hyman, J. Lesley, I. S. Trowbridge, *Cell. Immunol.* **53**, 350 (1980).
30. M. A. K. Markwell and C. F. Fox, *Biochemistry* **17**, 4807 (1978).
31. H. C. Oettgen, C. L. Pettey, W. L. Maloy, C. Terhorst, *Nature* **320**, 272 (1986).
32. We thank J. Coligan for the gift of the antiserum to γ chain and L. Puddington and A. Berger for reviewing the manuscript and for many helpful discussions.

21 October 1988; accepted 25 January 1989

Defensive Behaviors in Infant Rhesus Monkeys: Environmental Cues and Neurochemical Regulation

NED H. KALIN AND STEVEN E. SHELTON

To survive, primates must detect danger in time to activate appropriate defensive behaviors. In this study, the defensive behaviors of infant rhesus monkeys exposed to humans were characterized. It was observed that the direction of the human's gaze is a potent cue for the infant. Infants separated from their mothers were active and emitted frequent distress vocalizations. When a human entered the room but did not look at the infant, it became silent and froze in one position. If the human stared at the infant, it responded with aggressive barking. Alterations of the opiate system affected the frequency of the infant's distress calls without affecting barking and freezing, whereas benzodiazepine administration selectively reduced barking and freezing. This suggests that opiate and benzodiazepine systems regulate specific defensive behaviors in primates and that these systems work together to mediate behavioral responses important for survival.

TO SURVIVE, PRIMATES MUST DETECT potentially dangerous situations and then activate appropriate defensive behaviors. These behaviors appear to originate from genetic programs (*1, 2*) and may be similar in rhesus monkeys and human infants (*3, 4*). For example, rhesus infants begin to respond to fearful visual stimuli at 2 to 4 months of age (*1*). Human infants experience a similar period of fearfulness toward strangers beginning between 7 and 9 months of age (*5*). Through experience (*6*) and maturation, infants acquire a more refined understanding of what is dangerous. Consequently, the circumstances that elicit fear-related behaviors become more specific.

Eyes receive and communicate information important for survival. Staring is frequently associated with aggression and may predict or prevent an attack (*7*). The implications of staring have been exploited by various species through the evolution of protective "eyespot" markings (*8*). Initially, we observed that infant rhesus monkeys (*Macaca mulatta*) briefly separated from their mothers dramatically alter their behavior when they detect a human intruder and that their behavioral responses differ greatly, depending on whether the intruder stares at the infant or averts his gaze. We then found selective regulation of these different behavioral patterns by the infant's opiate and benzodiazepine systems.

To characterize these behaviors, we tested 11 infant monkeys (8 females and 3 males, 6 to 11 months old) twice. During the first test, the infant was separated from its mother and placed in a cage in a different room. It remained alone (A_1) for 10 min, while its behavior was recorded on videotape. A human then entered the room and remained motionless 2.5 m from the cage, gazing at the wall and presenting his profile to the infant. At no time did the human engage the

Department of Psychiatry, University of Wisconsin–Madison, and William S. Middleton Veterans Hospital, Madison, WI 53705.