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Cell Cycle Dependence of Laminar Determination in Developing Neocortex

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The neocortex is patterned in layers of neurons that are generated in an orderly sequence during development. This correlation between cell birthday and laminar fate prompted an examination of how neuronal phenotypes are determined in the developing cortex. At various times after labeling with [^{3}H]thymidine, embryonic progenitor cells were transplanted into older host brains. The laminar fate of transplanted neurons correlates with the position of their progenitors in the cell cycle at the time of transplantation. Daughters of cells transplanted in S-phase migrate to layer 2/3, as do host neurons. Progenitors transplanted later in the cell cycle, however, produce daughters that are committed to their normal, deep-layer fates. Thus, environmental factors are important determinants of laminar fate, but embryonic progenitors undergo cyclical changes in their ability to respond to such cues.

SEVERAL BRAIN REGIONS, INCLUDING the neocortex, segregate neurons with characteristic dendritic and axonal morphologies into distinct cellular layers. [^{3}H]thymidine "birthdating" studies of the developing cerebral cortex have shown that its six layers are generated in an orderly sequence. Neurons destined for the deepest layers leave the mitotic cycle first and migrate to form the cortical plate. Subsequently neurons of the more superficial layers are added, following an inside-out gradient of development (*1, 2*). This correlation between a neuron's birthday and its ultimate laminar fate suggests that the time at which a cell is generated may determine its identity in the cortex. Lineage studies using retroviral vectors indicate that early cortical progenitors can produce neurons in several layers (*3, 4*), presumably through asymmetric cell divisions (*4*). A sequential production of different neuronal phenotypes could result from progenitors that are intrinsically programmed first to produce deep-layer neurons and later upper-layer cells. Alternatively, multipotential progenitors or their daughters may respond to environmental cues that trigger the production of appropriate neuronal phenotypes. Transplantation studies can directly test the commitment of cells to their normal fates, thus distinguishing between cell-autonomous and environmental determinants of neuronal identity. Previous isochronic (*5, 6*) and heterochronic (*6*) transplants have shown that transplanted cortical neurons can migrate, differentiate, and form long-distance axonal connections, and have suggested that the layer to which a neuron migrates depends on the environment in which it goes through its final mitotic division (*6–8*). We examined the developmental potential of newly generated cortical neurons and their progenitors and the timing of laminar commitment by transplanting cells into host ferret brains at different times in the cell cycle.

[^{3}H]thymidine birthdating studies in the ferret (*2, 6*) show that neurons generated on embryonic day (E) 29 are normally destined for layer 6 and the subplate (*9*). To test the commitment of E29 cells to a deep-layer fate, we transplanted embryonic cells into postnatal host brains in which layer 2/3 neurons were being generated (*2, 6*). E29 cells were labeled in utero with [^{3}H]thymidine; cells developed undisturbed for 0 to 24 hours so that [^{3}H]thymidine-labeled cells could be caught in various stages of the cell cycle at the time of transplantation (Fig. 1A). Dissociated cells from the posterior (presumptive visual) cerebral wall were then injected into or near the occipital ventricular zone of a neonatal host ferret (*10*); hosts survived for at least 6 weeks, which allowed transplanted neurons to migrate and settle into a cortical layer. We first examined the developmental potential of newly generated postmitotic neurons, before migration. Cells were transplanted 24 hours after [^{3}H]thymidine labeling, an interval that allowed labeled progenitors to complete their current round of cell division before transplantation. Neurons committed to their normal fates should behave autonomously and migrate to the deep layers of the cortex, but if environmental cues determine laminar fate, transplanted neurons should adopt the host fate by migrating to layer 2/3 (Fig. 1B). As in previous heterochronic transplants (*6*), only a fraction of the cells labeled with [^{3}H]thymidine migrated into the host cortex (Fig. 1C); the remainder formed a small island of cells buried in the white matter at the injection site. However, those neurons that did migrate into the cortex were found in layer 6, appropriately for their birthday. In sum, over 85% of neurons transplanted after a 24-hour interval migrated to the infragranular layers (below layer 4), with the

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majority in layer 6 and the subplate, positions typical of their birthday (Fig. 2D). At least some transplanted neurons in layer 6 formed long-distance axonal projections to the thalamus, a target normal for their birthday (*6*). Thus, in contradiction to the notion that the postmigratory position and local environment of young neurons determine their phenotypes, neurons born on E29 are committed to a deep-layer fate before they initiate migration toward the cortical plate. Layer-specific adhesion molecules may mediate the cell's recognition of its correct laminar position (*11*).

Are cortical progenitors preprogrammed to produce deep-layer neurons exclusively on E29? Such a developmental clock has been proposed to regulate the production of photoreceptors from multipotential precursors in the retina (*12*). Alternatively, cortical progenitors may be multipotent and rely on instructive cues from the environment to generate neurons with appropriate phenotypes. To distinguish between these possibilities, we labeled progenitors with [^{3}H]thymidine in vitro (*13*) and transplanted them during the S-phase of the cell cycle, thereby allowing cells to complete that cell cycle in a new environment. The majority (about 85%) of resulting [^{3}H]thymidine-labeled neurons switched their normal laminar fates and migrated into layer 2/3, the position typical of newly generated host neurons (Fig. 2A). Thus, E29 progenitors are multipotent; the laminar phenotype of their daughters can be altered by local environmental cues (*14*). These results raise the question of which cell makes the decision to become a layer 6 cell, the progenitor or its daughter? To ascertain the time of commitment, E29 fetuses were injected with [^{3}H]thymidine in vivo; then cells were removed and transplanted at various times after the injection. When the donor cerebral mantle was removed from the host brain 8 hours after labeling, heavily labeled neurons adopted laminar positions typical of their birthdays, with roughly 88% in the infragranular layers (Fig. 2C). After only a 4-hour wait between [^{3}H]thymidine injection and removal of the donor brain, transplanted cells migrated specifically to layer 6 (Fig. 2B). Thus, commitment occurs rapidly after labeling in S-phase, at times that seem short relative to typical mammalian cell cycle lengths (*15*), and it is likely that the decision to generate a deep-layer neuron is made by the progenitor before mitosis. This could be tested by ascertaining the DNA content of committed cells removed 4 hours after [^{3}H]thymidine labeling. Should labeled cells at 4 hours have diploid DNA contents (Fig. 1A), commitment could occur postmitotically, after DNA contents have been halved. Should labeled cells have doubled DNA contents, commitment must occur before mitosis.

We followed the progression of S-phase progenitors through the cell cycle by labeling cells with the thymidine analog 5-bromo-2′-deoxyuridine (BrdU) and performing flow cytometry to ascertain their DNA contents. Cell suspensions were prepared from E29 fetuses at different times after injection with BrdU and were stained immunocytochemically with a monoclonal antibody to BrdU and a fluorescein-conjugated secondary antibody (*16*), enabling the fluorescence-activated cell sorter to distinguish BrdU-labeled and unlabeled cells. Staining with propidium iodide (PI), a DNA-intercalating dye, revealed cellular DNA contents. At least 10^4 cells per suspension were analyzed by flow cytometry for both BrdU fluorescence and DNA content, and two-dimensional contour plots of these parameters were generated (Fig. 3). The progression of labeled cells through the cell cycle was quantified by measurement of the fraction of BrdU-labeled cells in S-phase, G2/M, and G1 at different times after labeling (*17*) (Fig. 4). The total length of the cell cycle at E29 appears to be roughly 24 hours. From these graphs, we could determine the distribution of DNA contents of BrdU-labeled cells that were removed and dissociated 4 hours after BrdU injection. Few, if any, labeled cells had completed mitosis at this time; the vast majority were in late S-phase or in G2/M at 4 hours (Fig. 4). Thus, the decision to generate a deep-layer neuron must be made before mitosis, conceivably as early as late S-phase or near the transition into G2 of the cell cycle (*18*).

These experiments suggest that laminar determination in the cortex is a dynamic process correlated with the progression of precursor cells through the cell cycle. So far we have considered the fates of neurons heavily labeled with [^{3}H]thymidine, which have completed a single mitotic division before migrating into the cortex. What is the

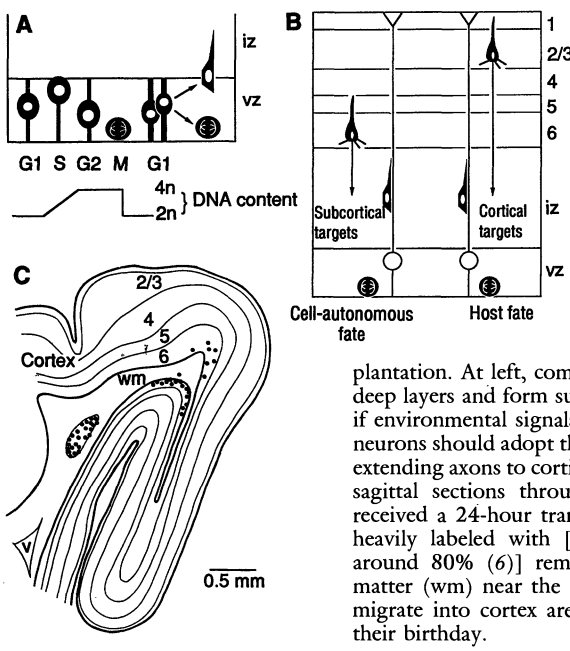

Fig. 1. (**A**) Summary of cell cycle progression in the cortical ventricular zone (vz). Cells in G1, with diploid DNA contents, translocate their nuclei upward on entering S-phase and initiating DNA replication. Nuclei of G2 cells (doubled DNA content) move down toward the ventricular surface, where mitotis (M) takes place. With division, DNA contents are halved; postmitotic daughters then migrate toward the cortical plate (intermediate zone, iz). (**B**) Two possible outcomes of heterochronic transplantation. At left, committed neurons should migrate to the deep layers and form subcortical axonal projections. At right, if environmental signals determine laminar fate, transplanted neurons should adopt the host fate, migrating to layer 2/3 and extending axons to cortical targets. (**C**) Reconstruction of four sagittal sections through a heterochronic host brain that received a 24-hour transplant as a neonate. Dots show cells heavily labeled with [^{3}H]thymidine. Many cells [typically around 80% (*6*)] remain clustered deep within the white matter (wm) near the injection site. Nearly all neurons that migrate into cortex are in layer 6, which is appropriate for their birthday.

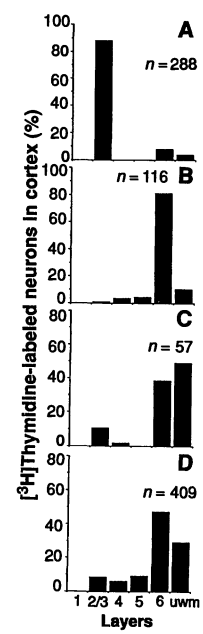

Fig. 2. Histograms of the laminar positions of heavily labeled neurons removed and transplanted at four different times after labeling with [^{3}H]thymidine. Only those cells that migrated into the cortex are included. (**A**) Zero hours (n is the number of cells from six hemispheres of four animals); (**B**) 4 hours (five hemispheres, three animals); (**C**) 8 hours (four hemispheres, three animals); (**D**) 24 hours (fifteen hemispheres, eight animals). [Underlying white matter (adult remnant of the embryonic subplate), uwm.] Cells transplanted immediately after [^{3}H]thymidine labeling are multipotent, but at a range of later times their progeny are committed to a deep-layer fate. The fraction of neurons found in layer 6 or the subplate (uwm) in (B) through (D) is variable, but both of these layers constitute normal destinations of E29 neurons. The variability between experiments might be explained by variability in the gestational age of donors (±1 day), which could influence the number of subplate neurons labeled. Additionally, because most subplate neurons die in the first months of postnatal life (*9*), differences in the age at which the animals were killed could alter the fraction of subplate neurons recovered.

fate of progenitors that cycle again in a foreign cortical environment? We predict that cells reentering S-phase in an older host should produce upper-layer daughters, regardless of their ancestors' decisions in previous cell cycles. To test this, we reexamined transplant brains for lightly labeled neurons that had undergone additional cell divisions in the new environment, thus diluting the [^{3}H]thymidine label so that it was below the criterion for heavy labeling (*19*). Lightly labeled cells migrated specifically to layer 2/3, regardless of the time between [^{3}H]thymidine labeling and transplantation (of 3543 cells from all time intervals, 98.3% were in layer 2/3; range, 95.5 to 99.8%). Thus, cells that reentered the cell cycle after transplantation generated daughters that switched their normal fates (*20*) and adopted laminar positions typical of the host environment, whereas the migration of heavily labeled cells reflected their fate as determined by their position in the cell cycle in the donor at the time of transplantation. One interpretation of this finding is that each progenitor progresses through a cycle of plasticity in the ventricular zone: multipotent cells are sensitive to environmental instructions early in the cell cycle but then decide to generate neurons with specific phenotypes late in the cell cycle. Daughters that reenter S-phase lose the cellular memory of this decision and regain the plasticity typical of S-phase progenitors. Alternatively, the decision to generate a layer 6 cell may be inherited asymmetrically: only terminally postmitotic daughters inherit a laminar commitment, whereas daughters that retain a proliferative potential also retain a multipotency of laminar fate (*21*).

The information required for the layered patterning of the cerebral cortex does not use a mechanism based strictly on cell lineage. Instead, cell types are specified progressively by environmental cues that must change over developmental time. Although their nature is unknown, possible cues include temporally regulated growth factors or hormones, neuropeptides, neurotransmitters from ingrowing afferents, or factors regulated by differentiating cortical neurons (*22*). There is, however, only a narrow window of time during the cell cycle in which the environment can exert this determinative effect. Neurons generated early in cortical development become committed to a laminar position before their final mitotic division; these early decisions ultimately result in the segregation of functionally similar neurons into distinct layers.

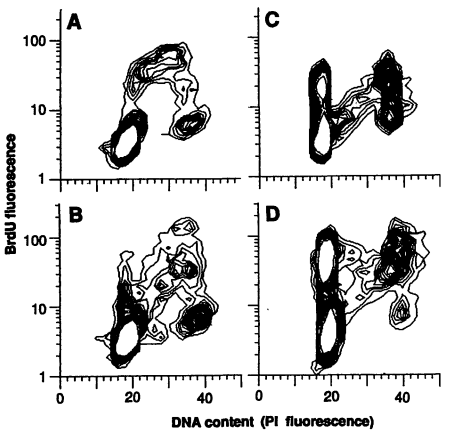

Fig. 3. Contour plots of bivariate BrdU-DNA distributions from flow cytometry of E29 cells, obtained at four times after BrdU labeling. (**A**) At 0 hours, BrdU-labeled cells (top half of plot) are in S-phase with intermediate DNA contents, as BrdU is only incorporated during S-phase. Unlabeled cells (bottom half) have predominantly diploid and doubled DNA contents. With increasing time after the BrdU pulse, the shifting DNA contents of labeled cells in the top half of each plot reflect their progress through the cell cycle. (**B**) At 6 hours, many BrdU-labeled cells have completed S-phase; their DNA contents have shifted right, to the G2/M level. (**C**) At 12 hours, after mitosis, labeled cells with diploid DNA contents appear on the left side of the plot, above unlabeled G1 cells. (**D**) At 24 hours, small numbers of BrdU-labeled cells have reentered S-phase, showing intermediate DNA contents.

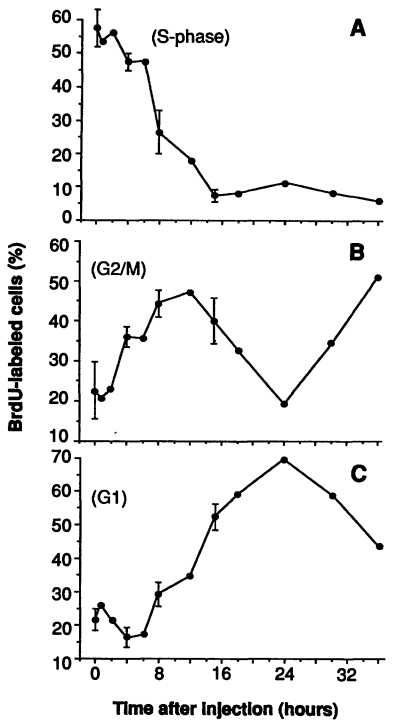

Fig. 4. The percentages of BrdU-labeled cells found in three stages of the cell cycle (S-phase, G2/M, and G1), as determined by their DNA contents measured at various times after labeling with BrdU on E29. Percentages were determined from histograms like those in Fig. 3 (*17*). Each point represents the average from two samples of cells obtained from a litter of E29 ferret fetuses; points with error bars represent the averages of two samples obtained from each of two litters (error bars, ±SD). (**A**) The number of labeled cells in S-phase decreases with time as cells complete DNA replication and move into G2. (**B**) The fraction of labeled cells in G2/M increases as cells leave S-phase. (**C**) As labeled cells in G2 complete mitosis, their DNA contents return to the diploid level and the fraction of labeled cells in G1 increases. Few if any cells have completed mitosis at 4 hours.

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5. Isochronic controls in which presumptive layer 2/3 neurons were transplanted into hosts of the same age have shown that transplanted neurons migrate specifically to layer 2/3 [S. K. McConnell, *Science* **229**, 1268 (1985)] and form corticocortical connections (*6*).
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7. In previous heterochronic transplants (*6*), transplanted neurons were found in both deep and superficial layers; a mixture of intervals between labeling and transplantation from 1 to 4 hours was used. One hypothesis that could account for this heterogeneity in laminar positions is that cells were in different stages of the cell cycle at the time of transplantation.
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10. At the appropriate time after [^{3}H]thymidine injection, the occipital cerebral wall was dissected from donor fetuses and dissociated enzymatically with papain [J. E. Huettner and R. W. Baughman, *J. Neurosci.* **6**, 3044 (1986)]. Control donor brains were fixed for autoradiography; labeled cells remained in the ventricular zone up to 24 hours after labeling (and thus had not initiated migration at the time of dissociation). Following methods described in (*6*), dissociated cells were pressure-injected through a micropipette into the ventricular region of neonatal host ferrets, in which upper-layer neurons were being generated (*2, 6*). Cortical neurogenesis continues for 2 weeks after birth (E41) in the ferret (*2*). Host brains were sectioned and analyzed by autoradiography for the positions of neurons heavily labeled with [^{3}H]thymidine (*6*).
11. Migration to the deepest cortical layers appeared to be specific, and not to be a result of cells' inability to penetrate the cortical plate. In heterochronic transplants of E31-32 cells (including presumptive layer 5 neurons), a proportionately larger number migrate to layer 5 (*6, 8*); this contrasts with the present experiments, in which few cells were in layer 5 (an inappropriate position for E29 neurons). This correlation between the transplanted neurons' birthdays and their specific laminar positions suggests that migrating neurons actively recognize the layers appropriate for their birthday.
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14. Definitive proof that these cells have switched fates awaits characterization of their axonal projections; normal upper-layer neurons form corticocortical connections. In the *reeler* mutant mouse, defective cell migration places neurons in incorrect laminar positions, yet cells develop axonal connections typical of their birthdays [U. C. Dräger, *J. Comp. Neurol.* **201**, 555 (1981); V. Lemmon and A. L. Pearlman, *J. Neurosci.* **1**, 83 (1981); M. C. Pinto-

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16. Pregnant ferrets were injected with BrdU (5 mg per fetus, intrauterine) on E29. Fetal brains were removed at various times after injection and were dissociated (*10*). For the 0-hour time point, cells were labeled in vitro with BrdU (20 mM) during dissociation. Cells were fixed in ice-cold 70% ethanol, immunoreacted with a monoclonal antibody against BrdU (IU-4, Caltag, 1:150), then with fluorescein-conjugated antibody to a mouse IgG (Sigma), and stained with PI (10 μg/ml, Sigma) [C. A. Hoy, G. C. Rice, M. Kovacs, R. T. Schimke, *J. Biol. Chem.* **262**, 11927 (1987)]. Cells were analyzed by 488-nm laser excitation on an EPICS flow cytometer (Coulter), using a 514-nm band-pass filter for fluorescein and a 600-nm long-pass filter for PI fluorescence.
17 The total number of BrdU-labeled cells in each two-dimensional plot (Fig. 3) was determined with a Quadstat data analysis program. A horizontal line was positioned in the valley between the upper (BrdU-labeled) and lower (unlabeled) halves of each plot. A vertical line then delineated cells on the far right with G2/M DNA contents from those with intermediate (S-phase) DNA contents. Another vertical line was drawn to delineate G1 from S-phase cells, and the percentage of BrdU-labeled cells in each phase was calculated. The placement of vertical lines was based on the positions of unlabeled cells with G1 and G2/M DNA contents; although the exact placement of each line was somewhat arbitrary, identical positions were used for all suspensions in an experiment. Thus, any inaccuracies in this simple measurement of the fraction of cells in G1, S, and G2/M are consistent among all time points. Theoretically, 100% of labeled cells should be in S-phase at time 0 (Fig. 4A); however, our measurements underestimate this fraction because DNA contents of early S-phase cells overlap with G1, and those of late S-phase cells with G2/M. This underestimate should not affect the kinetics of cell movement between phases.
18. Times in Fig. 4 and text refer to the times at which cells were removed from donors; the dissociation procedure took an additional 1.5 to 2 hours. Thus, the real time intervals between labeling and transplantation were about 2 hours longer than those indicated in graphs. Because the total length of S-phase is about 8 hours (roughly a third of the cell cycle), even cells in early S-phase at the time of labeling would have had about 6 hours to reach late S-phase if removed from donors at 4 hours.
19. Lightly labeled cells were defined as cells labeled above background but not meeting the criterion for number of grains for a heavily labeled cell. Typical lightly labeled cells had 4 to 15 silver grains over their nuclei, compared to 20 or more for heavily labeled cells.
20. The normal destinations of lightly labeled cells after an E29 [^{3}H]thymidine injection are layers 5 and 6 (*2, 6*). The presence of lightly labeled cells exclusively in layer 2/3 suggests that the laminar distributions of heavily labeled cells do not result from the differential selection or survival of precommitted cells. According to a selection hypothesis, committed deep-layer cells would be incapable of migrating when transplanted in S-phase, whereas committed upper-layer cells would only be able to migrate when transplanted in S-phase. Because lightly labeled cells migrate to layer 2/3 at every time interval, the S-phase environment may determine laminar fate.
21 It is not known whether, as development proceeds, cortical progenitors retain the full developmental potential displayed by early precursors, or whether the competence of cells to respond to early environmental cues is lost over time.
22. A "feedback control" mechanism has been proposed to regulate neurogenesis in layered neural structures [T. A. Reh and T. Tully, *Dev. Biol.* **114**, 463 (1986); T. A. Reh and I. J. Kljavin, *J. Neurosci.* **9**, 4179 (1989); D. J. Anderson, *Neuron* **3**, 1 (1989); S. K. McConnell, *Annu. Rev. Neurosci.* **14**, 269 (1991)]. The production of layer 6 neurons could conceivably feed information back to progenitors to trigger the production of the next cortical layer. Interestingly, growth cones of recently generated cortical neurons extend near or along the upper surface of the ventricular zone (G. Y. Kim, C. J. Shatz, S. K. McConnell, *J. Neurobiol.*, in press), providing a possible source of communication between postmitotic neurons and progenitors.

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Long-Term High-Titer Neutralizing Activity Induced by Octameric Synthetic HIV-1 Antigen

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A titer for homologous viral neutralization activity (>1:19,683) was observed after a 3.5-year immunization period with an octameric, branching peptide representing the principal neutralizing determinant (PND) of the human immunodeficiency virus–1_{IIIB} envelope protein. Booster immunizations elicited persistent and potent antibodies in guinea pigs, exceeding responses produced by a conventional bovine serum albumin conjugate by 100-fold. Peptide length, central presentation of a conserved sequence, and inclusion of an upstream sequence contributed to immunogenicity. Titers (>1:1,000) of heterotypic neutralizing antibodies also developed. Octameric PND peptides are a promising approach for an acquired immunodeficiency syndrome (AIDS) vaccine.

THE THIRD HYPERVARIABLE REGION (V_3) of the human immunodeficiency virus–1 (HIV-1) external envelope glycoprotein (gp120) contains a cysteine loop that constitutes the PND (*1–6*). A relatively conserved GPGR (*7*) sequence lies at the tip of the loop, flanked by type-specific sequences (*1, 2*). The V_3 loop is also a target for cytotoxic T lymphocytes and for antibody-dependent, cell-mediated cytotoxicity; and the loop is involved in syncytium formation, replication, and cell tropism (*2, 8*). Neutralizing antibodies (NAs) to the V_3 loop, though low-titer, short-lived, and type-specific, can protect chimpanzees from viral challenge (*9, 10*) and correlate with a lower incidence of maternal transmission of HIV-1 in humans (*11, 12*). Investigators have shown that antibodies to a specific V_3 peptide neutralize a significant proportion of field HIV isolates (*6, 13*), suggesting that a limited repertoire of synthetic peptides based on varied PND epitopes may meet the requirements for a broadly protective vaccine (*14*).

In an effort to enhance the immunogenicity of PND peptides, we investigated the presentation of V_3-derived peptides as radial octamers (*5*) using the multiple-antigen peptide method (*15*). A heptalysyl core bearing eight reactive NH_2-termini served as a carrier onto which eight V_3 peptides were attached by solid-phase synthesis (*16*). This antigenic PND matrix, with an eightfold molar excess of peptide over the core, forms greater than 95% of the mass of the octamer, as opposed to 6 to 28% of the mass in a comparable V_3 peptide–bovine serum albumin (BSA) conjugate (range for molar ratio of peptide to carrier 1:1 to 6:1) and <3% of the mass of the envelope glycoprotein in the viral lysate.

The immunogenicity and neutralizing responses elicited by two forms of synthetic peptide (p127) [QSVEINCTRPNNNTR-KSIRIQRGPGRAFVTIGK (residues 297 to 329 of HIV-1_{IIIB} gp120) (*7*)], which overlaps the PND-V_3 region, were evaluated. The immunogenicity of octameric p127 was compared to that of monomeric p127 (*16*) coupled to BSA by conventional glutaraldehyde cross-linking (p127-BSA) and to that of the whole, inactivated HIV. Both p127-BSA and octameric p127 induced specific and high-titer (>1:1000 to 1:10,000) antibodies to their respective peptides after one boost (3 weeks after initial immunization). Reactivity of both antisera to native HIV-1_{IIIB} gp120 protein was detected by protein immunoblot after 5 to 6 weeks

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