Chemotropic guidance of developing axons in the mammalian central nervous system

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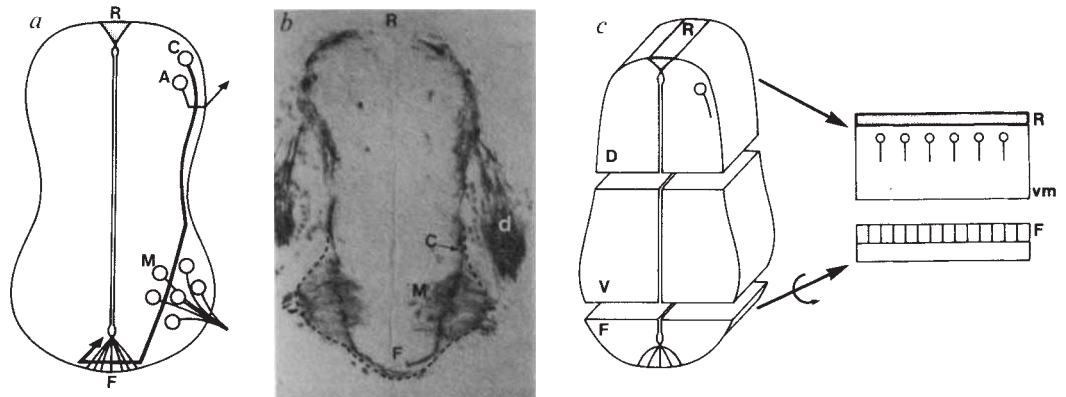Guy's Hospital Medical School, London, UK

In the developing nervous system, axons project considerable distances along stereotyped pathways to reach their targets. Axon guidance depends partly on the recognition of cell-surface and extracellular matrix cues derived from cells along the pathways[1]. It has also been proposed that neuronal growth cones are guided by gradients of chemoattractant molecules emanating from their intermediate or final cellular targets[2]. Although there is evidence that the axons of some peripheral neurons in vertebrates are guided by chemotropism[3,4] and the directed growth of some central axons to their targets is consistent with such a mechanism[5-7], it remains to be determined whether chemotropism operates in the central nervous system. During development of the spinal cord, commissural axons are deflected towards a specialized set of midline neural epithelial cells, termed the floor plate[8-10], which could reflect guidance by substrate cues or by diffusible chemoattractant molecules. Here we provide evidence in support of chemotropic guidance by demonstrating that the rat floor-plate cells secrete a diffusible factor(s) that influences the pattern and orientation of commissural axon growth in vitro without affecting other embryonic spinal cord axons. These findings support the hypothesis[2] that chemotropic mechanisms guide developing axons to their intermediate targets in the vertebrate CNS.

The existence of a floor plate-derived chemotropic factor was established by examining the effect of floor-plate explants and defined medium conditioned in vitro by the floor plate on axon outgrowth from explants of dorsal spinal cord. Dorsal explants were taken from rat embryos at embryonic day (E) 11, an age at which commissural neurons are beginning to differentiate and extend processes[8] (Fig. 1a and b). Explants were cultured in a collagen gel matrix (Fig. 1c) capable of stabilizing gradients of diffusible molecules[11]. In control experiments, there was no outgrowth from 75% of dorsal explants cultured alone for 39-44 h, and only sparse outgrowth from the remainder (Fig. 2a and Table 1, n = 107). In contrast, dorsal explants cultured with an E11 floor-plate explant placed 100-400 μm from their ventral-most edge (Fig. 1c) showed clear axon outgrowth within 20-24 h (not shown). After 39-44 h, a characteristic pattern of outgrowth was observed from all of these explants, with most axons projecting to the floor plate from their ventral-most edge in thick fascicles (Fig. 2b and Table 1, n = 116 co-cultures). The axons that projected into the collagen gel were not preceded or accompanied by migrating cells, as assessed by staining with the nuclear dye, Hoechst 33258 (n = 8). Marked outgrowth was also observed from dorsal explants cultured alone but exposed to medium conditioned by E11 floor plate (Table 1), but in this case axons emerged from all edges of the explant (M.P. et al, manuscript in preparation). These experiments show that the floor plate secretes a diffusible factor that promotes axon outgrowth from dorsal explants.

The axons that projected from dorsal explants in the presence

Fig. 1 a and b, Trajectory or commissural axons in the embryonic rat spinal cord. a, Schematic diagram of a transverse section of an E12 rat spinal cord (E0, day of vaginal plug) showing the location of the first three classes of differentiated neurons and their prospective axonal trajectories. Motoneurons (M) differentiate in the ventral region of the spinal cord from E10.5 (refs 8, 20) and extend axons to their target muscles. Commissural (C) and association (A) neurons differentiate from E11 (refs 8, 20) in the dorsal region of the spinal cord, adjacent to the roof plate[10] (R). Association axons project laterally to join the ipsilateral lateral funiculus[8,20] (arrow). Commissural axons grow ventrally along the lateral margin of the spinal cord to the motor column, then alter their trajectory and course directly through the nascent motor column to the floor plate (F). Because these axons appear to home in on the floor plate through a cellular environment that does not contain a preformed pathway[6,9], it seems possible that they are guided by chemoattractant molecules secreted by the floor plate. After crossing the midline of the spinal cord at the floor plate, commissural axons turn by 90° to form longitudinal projections in the contralateral ventrolateral funiculus[20] (arrow). b, Transverse section of an E12 rat spinal cord at the cervical level, stained by the immunoperoxidase method with a monoclonal antibody (4D7) (ref. 21) to the TAG-1 antigen (see ref. 10 for methods). Motor and commissural axons, but not association axons, express TAG-1 (ref. 10). Commissural axons project towards the floor plate (arrow). TAG-1⁺ neurons in the dorsal root ganglia[10,21] (d) can also be seen. Note that the spinal cord shown here is at a more advanced stage of development than those used for explant cultures (see below). Calibration, 100 μm. c, Schematic diagram of the main experimental protocol. Each section of E11 spinal cord (see below) was dissected into three portions: a 'dorsal explant' (D) comprising the dorsal third or half, a 'floor-plate explant' (F) comprising the ventral-most fifth, and the remaining 'ventral explant without floor plate' (V) which contained most of the motoneurons. Explants were then embedded in three-dimensional collagen matrices in appropriate orientations. For instance, dorsal and floor-plate explants were co-cultured as depicted on the right: the dorsal explant was placed with its ventral-most edge (vm) facing the ventral-most edge of the floor-plate explant.

Methods. Spinal cord explants were taken from rat embryos at the 24-27 somite stage (E11-E11.5). Embryos were placed in L15 medium (Gibco), and tungsten needles were used to remove a two-segment portion of the vertebral column 14 segments from the most recently segmented somites. These pieces of tissue were incubated with Dispase (Boehringer, 1 mg ml⁻¹ in L15) for 30 min at room temperature, which facilitated excision of the spinal cord. After further dissection, the explants were embedded in a three-dimensional collagen gel matrix as described[3], and cultured in supplemented Ham's F12 medium[22] with 5% horse serum (Gibco) at 37 °C in a 5% CO_2 environment.
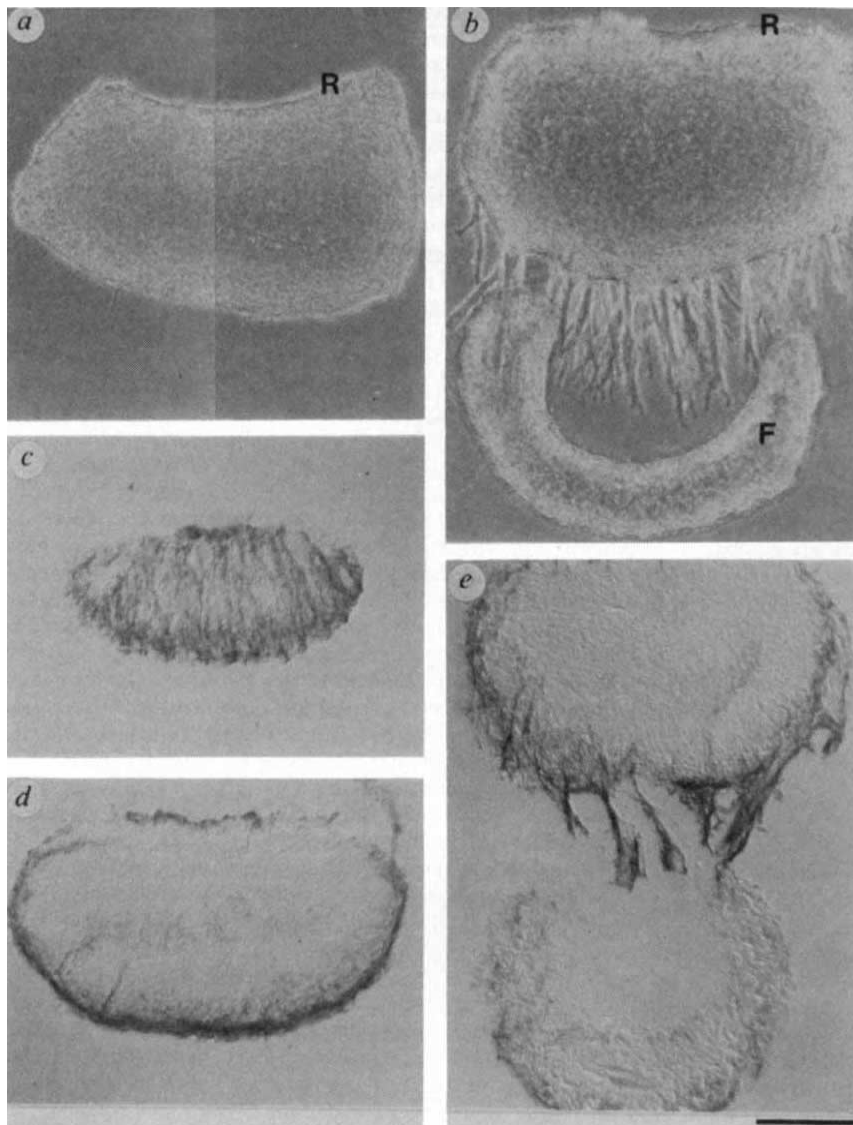
Fig. 2 The floor plate promotes commissural axon outgrowth from dorsal spinal cord explants. All explants were taken from E11 rat embryos and cultured in collagen gels, as described in Fig. 1c. In a-e, the dorso-ventral axis of dorsal explants is oriented vertically, ventral downwards. a, Little axon outgrowth is observed from a dorsal spinal cord explant cultured alone for 44 h. Abbreviations as in Fig. 1. b, Extensive axon outgrowth is apparent from a dorsal explant cultured for 44 h with a floor-plate explant. c and d, TAG-1 expression observed by immunoperoxidase labelling in cryostat sections through two different levels of a dorsal explant cultured without floor plate for 44 h. Extensive growth of TAG-1^{+} axons, presumably from commissural neurons[10,12], is observed along a dorso-ventral trajectory (c). These axons then extend along the inside perimeter of the explant (d), and do not project into the collagen gel. e, TAG-1 expression observed in a cryostat section of a dorsal explant cultured with floor plate for 40 h. TAG-1^{+} axons are oriented along a dorso-ventral trajectory (not shown), and project into the collagen gel. Note that the floor-plate explant contains a few TAG-1^{+} motor axons. Calibrations: a, b, 150 μm; c, d, 135 μm; e, 130 μm. R and F as in Fig. 1.

Methods. For immunostaining, explants were fixed after 39-44 h in culture by immersing the collagen gels in 0.12 M phosphate buffer (pH 7.4) containing 4% paraformaldehyde for 3 h at 4 °C, then transferred to 30% sucrose in 0.1 M phosphate buffer for 48-72 h at 4 °C. A piece (~0.6 cm × 0.6 cm) of each gel containing the explants was then cut out and frozen in OCT (TissueTek) compound, and 15-20 μm cryostat sections were collected on to gelatin-subbed slides. Staining was as described[10].

of floor plate appear to derive from commissural neurons as they express the TAG-1 glycoprotein (Fig. 2e, n = 51 explants sectioned). Studies both in vivo and in vitro indicate that TAG-1 is selectively expressed by commissural neurons in the dorsal spinal cord (refs 10 and 12; Fig. 1b). Many TAG-1^{+} axons were also observed within control explants (Fig. 2c and d, n = 16 explants sectioned) and were oriented along the original dorso-ventral axis of the explant (Fig. 2c). Instead of projecting into the collagen gel, however, these axons remained confined to the explant, extending along its inside perimeter (Fig. 2d). The floor plate-derived factor therefore appears to promote commissural axon outgrowth from the neural epithelium into the collagen gel without detectable effect on the differentiation or survival of commissural neurons. By 60 h in culture, many axons had projected from control explants (15.5 ± 1.8 (s.e.) axon bundles per explant, n = 11), which could suggest that the effect of the floor plate is simply to increase the rate of commissural axon growth. Several lines of evidence argue against this interpretation, however. First, many of the axons that emerged by 60 h were TAG-1^{−} (not shown), and may therefore have derived from association neurons (see below). Second, most of these axons emerged from the sides and not the ventral-most edge of dorsal explants (not shown), unlike the projection pattern observed in the presence of floor plate. Finally, the density and length of TAG-1^{+} axons in dorsal explants cultured for 20 h (that is, a time at which axons are beginning to project from

dorsal explants cultured with floor plate) appeared to be similar in the presence (n = 8) and absence (n = 10) of floor plate, showing that the floor plate does not markedly enhance the initial rate of commissural axon growth.

The ability to evoke commissural axon outgrowth from E11 dorsal explants was confined largely to the floor plate over the period in which commissural axons project to the floor plate in vivo (that is E11-14)[8]. Explants of E13-14 floor plate had the same effect as E11 floor plate in evoking profuse outgrowth of TAG-1^{+} axons from dorsal explants (Table 1). This effect was not mimicked by explants of E11-14 dorsal spinal cord (n = 27) or E11 ventral spinal cord (n = 16), but E13-14 ventral spinal cord did evoke the outgrowth of a small number of TAG-1^{+} axons above background (Table 1). It is possible that ventral spinal cord cells at this age secrete low amounts of a factor that affects commissural axon outgrowth. Alternatively, the factor secreted by the floor plate may bind to the ventral spinal cord in vivo and be released slowly by ventral explants in vitro.

The effect of the floor plate on axon outgrowth appears to be selective for commissural neurons. Neurofilament-reactive axons which did not express TAG-1 and presumably derived from association neurons (ref. 10 and Fig. 1), were also present within dorsal explants cultured for 39-44 h with floor plate (not shown), but they were not observed projecting from the explants towards the floor plate (n = 10). Also, motor axons extended into the collagen gel from ventral explants grown alone (not
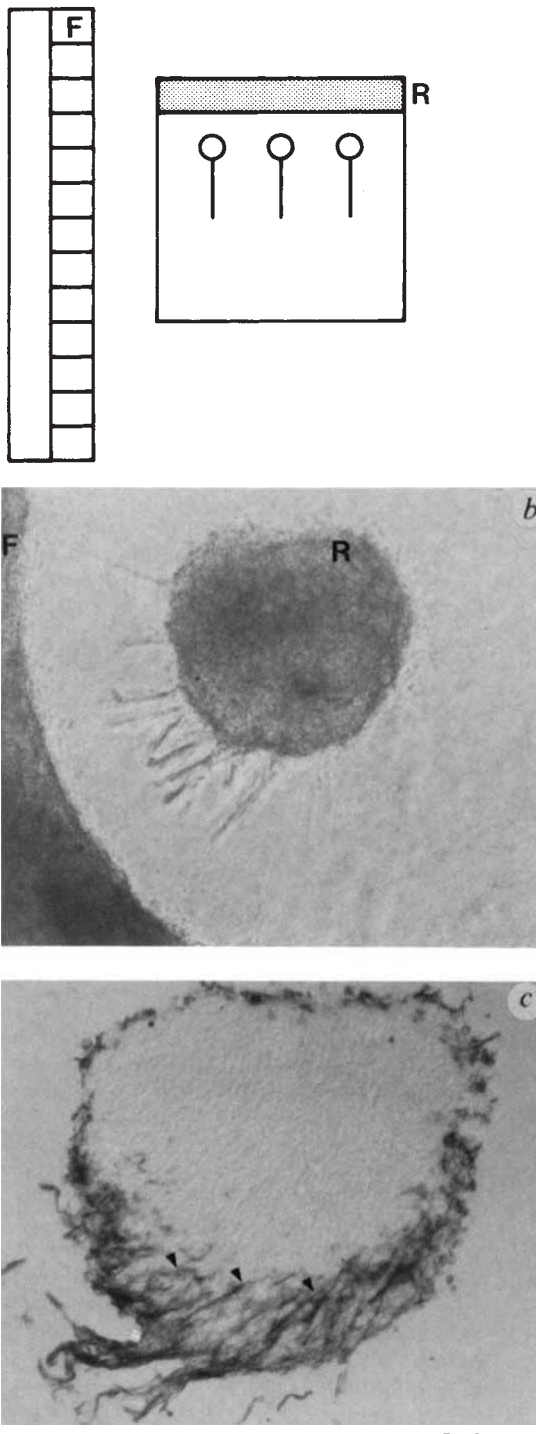
a

b

c

Fig. 3. The floor plate orients the growth of commissural axons *in vitro. a,* Schematic diagram of the experimental protocol. An E11 dorsal explant was embedded in a collagen gel with its roof plate placed at right angles to a long strip of E11 floor plate. To prevent the floor plate from curling up extensively (see Fig. 2*b* and *e*), the ventral half of the spinal cord was left attached to the floor plate. *b,* After 42 h, most of the axons projecting from a dorsal explant emerged near to, and were oriented towards, the floor plate. In 30 similar co-cultures, the majority of the emerging axons were oriented towards the floor plate. This orienting effect was observed whether or not the floor-plate explant curled. *c,* TAG-1 immunostaining of a cryostat section from a similar explant to that shown in (*b*). TAG-1⁺ axons are oriented towards the floor plate within the dorsal explant itself (arrowheads). This behaviour was observed in the 14 explants cultured with floor plate that were sectioned, but not in explants cultured with E11 dorsal (*n* = 4) or E11 ventral (*n* = 6) spinal cord. Calibrations: *b,* 120 μm; *c,* 50 μm.

Table 1 Characteristics of axon bundles emerging from E11 dorsal spinal cord explants

Condition	Number of explants	Number of bundles per explant Mean ($\pm$ s.e)	Bundle length (μm) Mean ($\pm$ s.e)
Dorsal explant (D)	107	0.4 ± 0.1	63 ± 3.4
D + E11 floor plate	64	17 ± 1.1	129 ± 5.4
D + E11 floor plate-conditioned medium	13	25 ± 2.5	126 ± 11
D + E13–14 floor plate	13	29 ± 3.5	154 ± 12
D + E13–14 ventral spinal cord	27	3.2 ± 0.6	88 ± 8.3

Dorsal explants were cultured for 39–44 h under the following conditions: alone (control), 100–400 μm from different explants as indicated, or with E11 floor plate-conditioned medium (see below). Measurements were made from camera-lucida tracings or photographs of the explants. The number and length of bundles emerging from dorsal explants cultured with E11 or E13–14 floor plate explants, or with E11 floor plate-conditioned medium were significantly greater than those in control explants ($P < 0.0005$, Student's *t* test). E13–14 ventral spinal cord evoked a small but statistically significant amount of axon outgrowth above background ($P < 0.0005$), but this effect was significantly smaller than that observed with floor plate or floor plate-conditioned medium ($P < 0.0005$). Moreover, as shown here, the bundles that emerged in these cases were considerably shorter than those observed with floor plate ($P < 0.0005$), and were thinner (not shown). The number and length of bundles emerging from dorsal explants cultured with E11–14 dorsal spinal cord, E11 ventral spinal cord, or medium conditioned by the remainder of the spinal cord were not significantly different from those observed in control explants ($P > 0.1$). For conditioned-medium experiments, culture medium (Fig. 1 legend) was exposed for 48 h to E11 floor plate tissue or to the remainder of the E11 spinal cord (M.P. *et al.,* manuscript in preparation). In both cases, tissue from 100 E11 embryos was pooled to condition 400 μl medium.

shown), but the extent and pattern of motor axon outgrowth was not altered when they were cultured with floor plate (*n* = 7).

These findings show that the floor plate promotes commissural axon outgrowth, but not whether it can orient the growth of these axons. To test for a chemotropic action, dorsal explants were cultured adjacent to an E11 floor-plate explant placed parallel to the original dorso-ventral axis of the spinal cord (Fig. 3*a*). Most axons that emerged from dorsal explants projected towards the floor plate (Fig. 3*b, n* = 30). Moreover, TAG-1⁺ axons within the dorsal explants themselves were clearly oriented towards the floor plate (Fig. 3*c, n* = 14 explants sectioned), and were not oriented in this way when E11 dorsal (*n* = 4) or ventral (*n* = 6) explants were substituted for floor plate. The floor plate thus causes commissural axons to deviate from their expected trajectory, consistent with a chemotropic action of the floor plate-derived factor. In particular, these observations show that the floor plate can orient the growth of commissural axons within a piece of explanted neural epithelium, which presumably approximates the *in vivo* environment of the axons more closely than a collagen gel.

The identity of the factor(s) secreted by the floor plate is unknown. The effect of the floor plate on commissural axon outgrowth was not mimicked by 7.5S nerve growth factor (100 ng ml⁻¹, *n* = 16), which can exert a chemotropic action on peripheral neurites *in vitro*[13,14], or by EHS-laminin (100 μg ml⁻¹, added to the collagen gel and the culture medium, *n* = 17), which promotes neurite outgrowth from many central and peripheral neurons[15-17].

The idea that axons are guided by a series of tropic factors released by their successive intermediate targets was first proposed in 1892 (ref. 2), but it has not been generally accepted[18]. Our findings have established that a defined intermediate target, the floor plate, secretes a diffusible factor(s) that functions as a selective chemoattractant for embryonic commissural axons

in vitro. Chemotropism may be only one of several guidance mechanisms that operate successively or coordinately to define the trajectory of commissural axons *in vivo*. Their initial ventral trajectory through the dorsal spinal cord (see Figs 1 and 2c) may be determined by extracellular matrix cues, in particular laminin (ref. 19; J.D. *et al.*, manuscript in preparation). Moreover, once commissural growth cones arrive at the midline, their subsequent guidance may be regulated by contact-dependent interactions with floor-plate cells[10]. Identification of the floor plate-derived chemotropic factor should permit experimental analysis of the contribution of chemotropism to the guidance of commissural axons *in vivo*. More generally, our observations raise the possibility that chemotropism represents a major mechanism of axon guidance in both the central and peripheral nervous systems.

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A malaria T-cell epitope recognized in association with most mouse and human MHC class II molecules

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An ideal vaccine should elicit a long lasting immune response against the natural parasite, both at the T- and B-cell level. The immune response should occur in all individuals and be directed against determinants that do not vary in the natural parasite population. A major problem in designing synthetic peptide vaccines is that T cells generally recognize peptide antigens only in association with one or a few of the many variants of major histocompatibility complex (MHC) antigens[1,2]. During the characterization of epitopes of the malaria parasite *Plasmodium falciparum* that are recognized by human T cells, we analysed a sequence of the circumsporozoite protein, and found that synthetic peptides corresponding to this sequence are recognized by T cells

in association with many different MHC class II molecules, both in mouse and in man. This region of the circumsporozoite protein is invariant in different parasite isolates[3,4]. Peptides derived from this region should be capable of inducing T-cell responses in individuals of most HLA-DR types, and may represent good candidates for inclusion in an effective anti-malaria peptide vaccine.

We had observed that peripheral blood mononuclear cells (PBMC) from the majority of immune and non-immune donors responded *in vitro* to a peptide, CS.T3, corresponding, with the exception of two cysteine-alanine substitutions, to residues 378-398 of the circumsporozoite (CS) protein[5]. This suggested that the peptide could associate with many different MHC class II molecules. To test this hypothesis, cells from 20 non-immune donors (all with titres of anti-sporozoite antibodies <1:80) were challenged *in vitro* with CS.T3 at 10 μM. We derived a total of 298 CS.T3-specific T-cell clones from eight different donors. None of the clones proliferated in the presence of a control peptide (CS protein 325-341, data not shown).

The MHC restriction of CS.T3-specific T-cell clones was assessed using anti-MHC-class II monoclonal antibodies (mAbs). The proliferation of all 187 clones tested was inhibited by the mAb E.31 (ref. 6), which recognizes a monomorphic HLA-DR determinant. Neither anti-DP[7] nor anti-DQ[8] mAbs inhibited T-cell proliferation, and fibroblasts transfected with the DRα and DRβ genes presented antigen to selected clones, confirming (Table 1) that the DR molecule is the restriction element for CS.T3-specific T-cell clones. The DR restriction specificity of each T-cell clone was then determined using a panel of HLA-DR-homozygous antigen-presenting cells. As shown in Table 1, T-cell clones responded equally well to CS.T3 when presented on autologous Epstein-Barr virus-transformed B (EBV-B) cells or on an EBV-B line homozygous for one of the donor's DR specificities. The peptide CS.T3 was recognized in association with at least seven different DR molecules (DR1, 2, 4, 5, w6, 7 and 9).

To determine whether T-cell clones restricted by different DR molecules recognize different determinants on CS.T3, we assayed proliferative responses to a series of peptides truncated at either the N or C terminus of the CS.T3 sequence (Table 2). Peptides 380-398, 378-395 and larger peptides were stimulatory for all T-cell clones examined. However, when shorter peptides were tested, distinct recognition patterns emerged. At the two extremes stand the DR2- and DR5-restricted clones. The minimal regions that are stimulatory for DR2- and DR5-restricted clones correspond to residues 385-395 and 381-390 respectively. The minimal stimulatory region for DR4-restricted clones lies between residues 383 and 394, the regions for DRw6 and DR7 correspond to residues 381-392 and 381-394 respectively, and for DR1 to residues 382-395 (data not shown) or 381-392. DR9-restricted T-cell clones (four out of four tested) did not recognize peptides 382-398 and 381-398 over a wide range of concentrations. Further removal of Lys 382 and Ile 383, however led to reappearance of recognition. The still shorter peptide 385-398 was not stimulatory at all (Table 2). In conclusion, closely overlapping epitopes between residues 381-395 are recognized in slightly different ways in association with different DR antigens, consistent with earlier studies which showed a correlation between the MHC class II restriction element and the fine specificity of antigen recognition by mouse T cells[9-11].

To see whether CS.T3 could induce helper T-cell function *in vivo*, we coupled the peptide to $(NANP)_3$; the repetitive NANP (Asn-Ala-Asn-Pro) sequence in the central domain of the CS protein being the major epitope recognized by anti-sporozoite antibodies[12-14] The $(NANP)_3$-CS.T3 peptide was administered to seven different mouse strains, and anti-$(NANP)_n$ and anti-sporozoite antibody responses were measured (Table 3). All the strains mounted an antibody response against both $(NANP)_n$ and sporozoites. Although C57BL/6 T cells can recognize $(NANP)_n$, T cells from the other strains do not respond to the