Major Histocompatibility Complex–expressing Nonhematopoietic Astroglial Cells Prime Only CD8+ T Lymphocytes: Astroglial Cells as Perpetuators but not Initiators of CD4+ T Cell Responses in the Central Nervous System

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Summary

The potential of cells within the central nervous system (CNS) to initiate T lymphocyte responses is not known and was the subject of this study. Using the ability of virgin T lymphocytes to proliferate in a primary response to allogeneic determinants on antigen-presenting cells (APC), we have examined the capacity of major histocompatibility complex (MHC)-expressing astroglial cells to act as stimulators of primary and secondary T cell responses. Neither freshly isolated astrocytes nor primary astrocyte cultures pretreated with interferon γ (IFN-γ) to upregulate MHC class I and II expression stimulated unfractionated lymph node (LN) cell populations in the primary mixed lymphocyte reaction. In mixing experiments, astrocytes did not inhibit the T cell response to allogeneic LN stimulators. Purified responder CD4+ T cells also were not stimulated to proliferate or secrete interleukin 2 (IL-2) by MHC class I– and II–expressing astrocytes. In contrast to their inability to stimulate virgin, alloreactive CD4+ T cells, astrocytes were able to specifically stimulate an alloreactive CD4+ T cell line. Unprimed CD8+ T cells, however, exhibited some weak autonomous proliferation to astrocyte stimulators but this response was only substantial in the presence of exogenous IL-2, the latter predominantly being a CD4+ T cell product. Those CD8+ T cells responding in the presence of IL-2 were mainly T cell receptor α/β^+ IL-2 receptor $(\alpha \text{ chain})^+$, and a majority had shifted from high to low CD45R expression. Given the virtual dependence of CD8+ T cells in these studies, on CD4+ T cell help, and the complete absence of activation of this latter subset by astrocytes, it is clear that in the context of this resident CNS cell, further activation of either T cell subset by astrocytes within the CNS can only follow priming by another type of APC. The implications of these results for the induction of T cell responses in the CNS are discussed.

The site of initiation of central nervous system (CNS)[1] auto-immune inflammatory diseases is generally unknown, except of course under those laboratory conditions such as in experimental autoimmune encephalomyelitis (EAE), where CD4+ T cells with specificity for CNS autoantigens are generated by peripheral immunization (1). However, spontaneously arising CNS inflammatory diseases with a suspected autoimmune base such as multiple sclerosis (MS) could conceivably be initiated in the CNS itself or alternatively could be a consequence of peripheral autoreactive T cell stimulation. Evidence for these two opposing views has recently been reviewed (2, 3).

To begin to understand the induction of these types of CNS inflammatory diseases one must first consider the set of unique anatomical and immunological circumstances present (reviewed in reference 4). Foremost among these is the virtual absence of cells, either resident or of bone marrow origin, expressing MHC class I or class II molecules which are the prerequisite elements for presentation of antigenic peptides to CD8+ and CD4+ T lymphocytes, respectively (reviewed in reference 5). Under these conditions it is difficult to envisage how an inflammatory reaction can be initiated. It is known, however, that products of activated T lymphocytes such as IFN-γ can in-

[1] *Abbreviations used in this paper:* AST, astrocytes; CNS, central nervous system; DC, dendritic cells; EAE, experimental autoimmune encephalomyelitis; GalC, galactocerebroside; GFAP, glial fibrillary acidic protein; LEW, Lewis; MS, multiple sclerosis.

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duce the expression of MHC class II on parenchymal elements like astrocytes (AST; reference 6) and microglia (7) but one could then argue that the MHC induction observed on these cells in diseases like MS (8, 9) is an event secondary to the activation of the IFN-γ–secreting T cell so the resident CNS elements could not have initiated the response. Within this framework, however, it is nevertheless feasible that once glial cells are induced to express MHC antigens, essentially as bystander cells of, for example, CNS inflammation after a viral or bacterial infection, these glial cells would have the capacity to play an active role in presentation of self antigens to T cells that are then present within the CNS. Indeed it has been previously demonstrated (10–12) that the bulk of T cells present in a CNS inflammatory infiltrate accumulate nonspecifically subsequent to damage mediated by a small number of specific effector T cells and it is possible that at least some of these inflammatory T lymphocytes may be (auto)reactive for CNS antigens. Another related way in which CNS autoimmune inflammatory disease may be precipitated is after direct MHC induction on glial cells mediated by viral particles via an IFN-γ–independent mechanism (13).

An important consideration, however, is whether glial cells, once induced to express MHC antigens (by whatever mechanism), can then initiate T cell responses or if they merely perpetuate a response that has been initiated elsewhere, that is, outside the CNS, and by another cell type. Primarily using the MLR as a test system, it has been established that certain cell types of bone marrow derivation, most notably dendritic cells (DC) and activated B cells (14, 15, reviewed in reference 16), are effective stimulators of primary $CD4^+$ T lymphocyte responses while other cell types such as macrophages and resting B cells efficiently only stimulate $CD4^+$ T cells once they have been activated (16, 17). Similarly, unprimed $CD8^+$ T cells are stimulated by DC in the primary MLR (18) but, unlike $CD4^+$ T cells, are also reportedly activated by macrophages and possibly a range of other non-DC APC (19, 20). There is no similar definition of the ability of MHC-expressing, CNS-resident cells, to stimulate primary and/or secondary $CD4^+$ and $CD8^+$ T cell responses. The ability of glial cells to directly participate in the induction of immune responses is obviously relevant to potential mechanisms of autoreactive T lymphocyte activation.

In this report we examine the potential of a nonhematopoietic glial cell, the AST, to stimulate unprimed $CD4^+$ and $CD8^+$ T lymphocytes in the primary allogeneic MLR. Collectively our results do not provide evidence for a role of CNS astroglial cells as initiators of $CD4^+$ T lymphocyte responses, while their capacity to stimulate unprimed $CD8^+$ T cells rests largely on the availability of exogenous IL-2. However, the data indicate that AST are potentially important as perpetuators of $CD4^+$ T cell responses that have been initiated after interaction with APC present in peripheral lymphoid organs.

Materials and Methods

Animals. Full-term pregnant rats, mothers with 7–10-d-old litters, and 8–10-wk-old female rats were obtained from Zentralinstitut für Versuchstierzucht (Hannover, Germany or Moellegard, Ltd., Skensved, Denmark). All animals were Specific Pathogen Free (SPF) and from the Lewis (LEW, $RT1^l$), PVG ($RT1^c$), and Brown Norway (BN, $RT1^n$) strains.

Monoclonal and Polyclonal Antibodies. Mouse mAbs were: MRC OX6 (anti-rat monomorphic MHC class II, RT1B (I-A)), MRC OX8 (anti-rat CD8), MRC OX12 (anti-rat κ chain), MRC OX18 (anti-rat monomorphic MHC class I, RT1A), MRC OX21 (anti-human C3bi and not rat cells), MRC OX22 (anti-rat CD45R), W3/25 and MRC OX35 (noncompeting anti-rat CD4), MRC OX39 (anti-rat IL-2R, α chain), MRC OX30 (anti-rat CD45; L-CA), MRC OX42 (anti-rat CD11b, C3bi), R73 (anti-rat αβ TCR), 1A29 (anti-rat CD54, ICAM-1), 4B6 (anti-RAN-2) and anti-galactocerebroside (GalC). See (11, 21–27) for cross reference details of mAb. Supernatants of MRC OX21 and MRC OX39 were provided by Don Mason and Mike Puklavec (MRC Cellular Immunology Unit, Oxford, U.K.), 1A29 ascites was kindly provided by M. Miyasaka (Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan) and 4B6 supernatant by M. Raff (University College, London, UK). All other mAb were prepared from hybridomas obtained from Alan Williams (MRC Cellular Immunology Unit, Oxford, U.K.), Thomas Hünig (Institute for Virology and Immunobiology, Würzburg, Germany), (R73 mAb) and B. Ranscht (Max-Planck Society Laboratories, Tübingen, Germany) (anti-GalC mAb). A rat mAb, R2/15S (28), which binds to a determinant on the polymorphic classical MHC Class I molecule (RT1.A) and is positive on LEW ($RTI.A^l$) but not on BN ($RT1.A^n$), was purified from tissue culture supernatants by anti-rat IgG affinity chromatography, and biotinylated.

Affinity-purified rabbit anti-mouse Ig for coating of SRBC for rosetting was prepared by standard immunization and solid-phase affinity adsorbent procedures. FITC-conjugated donkey or goat anti-mouse Ig and PE-streptavidin were from Dianova (Hamburg, Germany). Rabbit anti-glial fibrillary acidic protein (GFAP) recognizing AST but not other CNS parenchymal cells was from DAKOPATTS (Hamburg, Germany).

Astrocyte Preparation

Direct Isolation of AST. A method was devised for the isolation of AST direct from the CNS of 13–16-d-old rats. At this age, both glial development and GFAP expression in situ are maximal (29). We did not succeed in isolating AST directly from newborn or adult brain. Cerebral lobes were removed from BN or LEW rats, dissociated through a fine metal sieve, and the mixture subjected to collagenase and DNAse digestion and fractionated over a multistep Percoll gradient as described for microglial cell isolation (J. Sedgwick, S. Schwender, H. Imrich, R. Dörries, V. ter Meulen, and G. Butcher, manuscript submitted for publication). Cells recovered at a density of 1.065 g/ml consisted of ~70% microglia defined by the mAb MRC OX42 (CD11b/C3bi, reference 30), 3–5% $GalC^+$ cells (oligodendrocytes) and the remainder a heterogeneous population of larger cells, many with a granular appearance. Microglia, $GalC^+$ cells and leukocytes were removed by labeling with mAb against CD11b, CD45, αβ TCR, rat Ig and GalC and depleting with magnetic beads (see below) to give a population of cells enriched for AST (see Results). Recoveries from the cerebral lobes of four rats was ~4 × 10^6 cells and after Percoll fractionation and mAb depletion, between 10 and 15% of this number of cells remained.

Preparation of Cultured AST and MHC Class II Induction. Primary AST cultures were prepared from the meninges-stripped cerebral lobes of 1- to 2-d-old rats as described (31) and grown to confluence in 270-ml flasks (Falcon, Becton Dickinson, U.S.A.). AST were used within a maximum of 21 d of culture establishment but mostly by 14 d when confluence was generally first

reached. To induce MHC Class II, 20 U/ml rat IFN-γ (from Dr. P. H. van der Meide, TNO Primate Center, Rijswijk, The Netherlands) was added fresh to some of the AST on each of 4 d before harvesting of the cells. Routinely, around 95% of cells were positive for the AST marker, GFAP. Less than 2% of cells were positive for either L-CA (CD45, present on cells of hematopoietic origin including microglia, reference 32), MRC OX42 (recognizing rat microglia and some macrophages, reference 30), or GalC. Note that AST recovered from primary culture were also immediately irradiated and used as stimulator cells so the above data are indicative of AST populations that were present in the MLR studies.

Preparation of Lymphocyte Subpopulations. Lymphoid cells used as both stimulators and responders were exclusively derived from pooled cervical and mesenteric LN. LN cells in the rat generally respond better in the MLR than do splenocytes, probably due to the presence of suppressive macrophages in the latter (33). Single cell suspensions of LN were prepared in cold PBS-0.2% BSA and used unfractionated (see below), or depleted of various populations by incubating the cells with a cocktail of mAb tissue culture supernatants followed by a rosetting procedure (34). LEW rat $CD4^+$ T cells were prepared by depletion of all cells expressing CD8 (MRC OX8), MHC class II (MRC OX6) and surface Ig (MRC OX12) in a single rosetting step. LEW $CD8^+$ T cells were prepared by depletion of cells expressing CD4 (W3/25 and MRC OX35), MHC class II and surface Ig by rosetting followed by a second purification using sheep anti-mouse Ig-coated magnetic beads (Dynal A. S., Oslo, Norway) to remove residual contaminating cells.

Primary MLR. Responder leukocytes were unfractionated LN cells, $CD4^+$ or $CD8^+$ T lymphocytes. These were titrated onto a constant number of 2,000 rad (γ) irradiated syngeneic or allogeneic stimulator cells consisting of unfractionated LN cells at 5×10^5 per well, fresh AST at 5×10^4 per well, or cultured AST at 2×10^4 per well. This number of cultured AST rapidly adhere to the well floor and within 24 h form a monolayer. Addition of higher numbers results in cell clumping and retraction of the AST monolayer. Culture vessels were flat-bottomed 96-well plates (A/S Nunc, Kamstrup, Denmark). Medium was RPMI 1640 supplemented with β-ME, penicillin, streptomycin, glutamine, sodium pyruvate, and either 5% heat inactivated FCS or 5% normal rat serum (obtained from 5–6-wk-old male LEW rats). Results were similar with either serum source. Human rIL-2 (Cellular Products Inc., Buffalo, NY) at 2–50 U/ml and W3/25 (anti-rat CD4) IgG at 5 μg/ml were added to some of the wells. Responder cell proliferation was assessed by incorporation of [^{3}H]thymidine (0.5 μCi/well; Amersham, Braunschweig, Germany) at varying times after culture establishment (see Results).

Alloreactive T Cell Line. A bulk primary MLR was established between LEW unfractionated LN responder and irradiated BN LN stimulator cells at a ratio of 1:2, respectively, in 270-ml flasks. Between 4 and 6 d later, blast cells were isolated over a BSA or metrizoate/Ficoll gradient and a T cell line produced by sequential rounds of resting in IL-2 containing media and allogeneic stimulus with irradiated BN LN cells as described (24). The phenotype of this line is detailed in Results. Line cells were recovered after the fourth and fifth 10-d IL-2 resting phase and proliferation against LN or AST stimulator cells assessed.

Cytofluorographic Analyses. Aliquots of 10^5 to 10^6 leukocytes or AST were labeled for 1 h at 4°C with mAb diluted in PBS-0.2%, BSA-10 mM sodium azide, washed, then incubated at 4°C for 1 h with FITC-conjugated antibody at 10 μg/ml diluted in PBS-1%, BSA-10% rat serum-10 mM sodium azide. 10^4 live gated events were analyzed on a FACScan® (Becton Dickinson & Co., Heidelberg, Germany).

IL-2 Determinations. A continuous IL-2-dependent mouse CTL line (designated clone 3, prepared by Thomas Hünig, Würzburg) was used to measure the IL-2 content of MLR supernatants. 5×10^3 clone 3 cells were mixed with twofold dilutions of supernatants in flat-bottomed 96-well plates and incubated for 20 h then pulsed for 4 h with 0.5 μCi [^{3}H]thymidine and proliferation assessed. Supernatant IL-2 content was quantified by relating proliferation values to a standard curve of human rIL-2 to give a units per milliliter value.

Statistics. Student's *t* test was used to compare mean cpm scores of responder lymphocytes on syngeneic or allogeneic stimulator cells.

Results

Direct Isolation of AST and Stimulator Capacity. Responder lymphocytes in these studies were predominantly from the LEW strain, which is not only highly susceptible to the induction of inflammatory CNS reactions (35), but whose T cells exhibit a substantially greater response that those from a number of other rat strains after stimulation by alloantigen (36) or CNS autoantigens (35) in vitro. Strictly speaking, the mixture of AST and T cells is not a mixed "leukocyte" reaction, but for ease, this term will be used to cover such cell mixtures as well as those that are between two different populations of leukocytes.

Our first aim was to assess whether freshly isolated and minimally cultured AST were able to stimulate unprimed T cells in the MLR and, to this end, a method was devised to separate AST from the bulk of other CNS cells (see Materials and Methods). Cells isolated from the brain of 13–16-d-old rats after Percoll density fractionation and depletion of microglia, oligodendrocytes, and leukocytes were virtually all large granular cells with high forward and side scatter and routinely contained <2% of the above cell types as contaminants. Around 60% of these cells were weakly $GFAP^+$ as determined by immunofluorescence on cells applied to adherence slides (Superior, Paul Marienfeld KG, Bad Mergentheim, Germany) and stained immediately. 25–30% of the total cells formed a distinct positive population by FACScan analysis after labeling with the anti-RAN-2 mAb, 4B6, and a further 20–25% were very weakly positive for this marker. The expression of RAN-2 is heterogeneous even on cultured AST (26). Collectively, the results indicated that at least 60% of the cells were AST.

As expected, there was no MHC expression by these cells (not shown) but to preinduce these molecules would involve at least 1–2 d of IFN-γ treatment. To avoid any extension of culture time, irradiated syngeneic or allogeneic (BN) AST were mixed directly with unfractionated LEW LN cells in the presence of IFN-γ and responder cell proliferation assessed. Fig. 1 indicates that freshly isolated BN AST are not stimulatory for LEW responder LN cells. The AST nevertheless appear to induce some degree of nonspecific responder cell proliferation. Neither the AST in mixing experiments, nor the presence of IFN-γ, inhibit the LN cell response to allogeneic LN stimulators (Fig. 1).

At the end of the 90-h culture in the presence of IFN-γ, all irradiated stimulator AST are weakly MHC class I^+ and

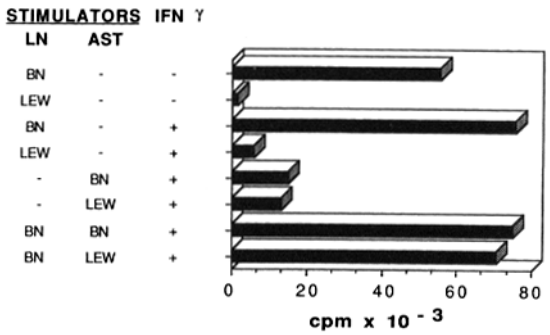

Figure 1. Inability of freshly isolated astrocytes to stimulate proliferation of allogeneic lymphocytes. 2.5 × 10^5 unfractionated responder LN cells from LEW rats were mixed with combinations of irradiated LN (5 × 10^5) or freshly isolated AST (5 × 10^4) stimulator cells in the presence or absence of 50 U/ml IFN-γ. Responder cell proliferation was assessed after a 90-h incubation time inclusive of an 18-h pulse with [^{3}H]thymidine. Data shown are means of triplicate determinations.

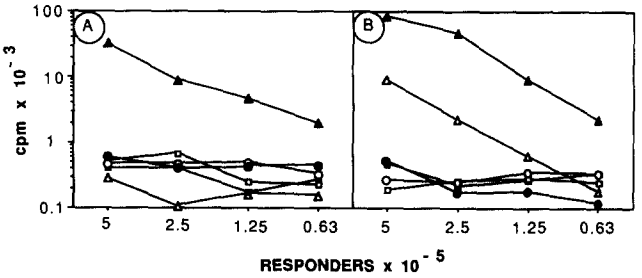

Figure 3. MHC class I- and II-expressing astrocytes do not stimulate unprimed allogeneic lymphocytes. Unfractionated responder LN cells from LEW (*A*) or PVG (*B*) rats were titrated onto syngeneic (*open symbols*) or allogeneic (*closed symbols*) irradiated stimulator LN cells (△, ▲), AST (□, ■), or IFN-γ treated AST (○, ●). Allogeneic stimulators were from BN rats in A and LEW rats in B. Responder cell proliferation was assessed after a 90-h incubation time inclusive of an 18-h pulse with [^{3}H]thymidine. Data shown are means of triplicate determinations and are representative of three separate experiments.

there is a low level expression of MHC class II (not shown). Despite this, no stimulation of responder cells was observed. The inability of these AST to act as stimulators could, at least in part, be related to the low level MHC expression which presumably can only be enhanced by extending the time of in vitro exposure to IFN-γ. We therefore turned to primary AST cultures which are not only of higher purity but whose MHC expression can be readily manipulated.

AST MHC Expression. AST in situ do not express MHC class I and II, but upregulate MHC class I during growth in vitro remaining MHC class II negative (Fig. 2, left hand panels, no IFN-γ). In all three rat strains, the majority of cells expressed MHC class II after IFN-γ treatment as evidenced by a shift of the whole population to the right. However, substantial expression was seen in only ~55% of AST from the LEW strain and 35–40% of cells from the BN and PVG strains. These differences (at least between LEW and BN) have been reported previously (31). As a comparison, ~25–30% of the total LEW AST population and 15–20% of BN and PVG AST express levels of MHC class II equivalent to that of normal resting B lymphocytes with a further 5–8% in LEW and BN rats expressing MHC class II levels similar in intensity to that seen with DC (16) or B blasts (our own observations). MHC class I expression was further enhanced after IFN-γ treatment but no strain differences were observed.

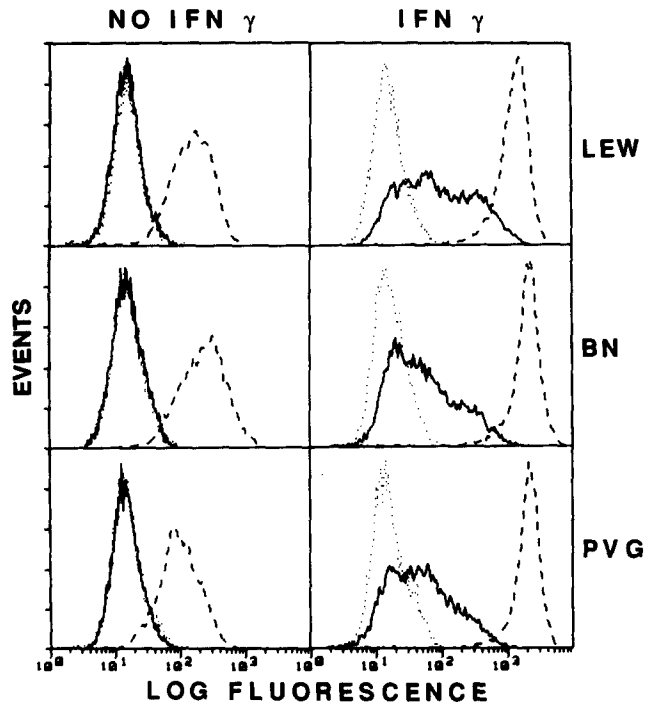

Figure 2. MHC expression of cultured primary astrocytes. Auto fluorescence (MRC OX21 mAb, *dotted line*), MHC Class II (*solid line*), and MHC Class I (*dashed line*). IFN-γ treatment was for 4 d before staining, as described in Materials and Methods.

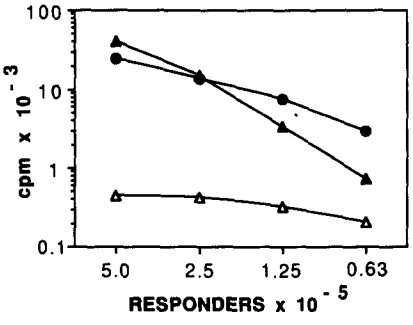

Figure 4. AST enhance but do not inhibit the T cell response to allogeneic LN stimulators. Unfractionated responder LN cells from LEW rats were titrated onto 5 × 10^5 LEW syngeneic (*open symbols*) or BN allogeneic (*closed symbols*) irradiated stimulator LN cells in the presence (●) or absence (△, ▲) of 2 × 10^4 IFN-γ pretreated BN AST. Addition of LEW rather than BN AST gave similar results. Responder cell proliferation was assessed after a 90-h incubation time inclusive of an 18-h pulse with [^{3}H]thymidine. Data shown are means of triplicate determinations.

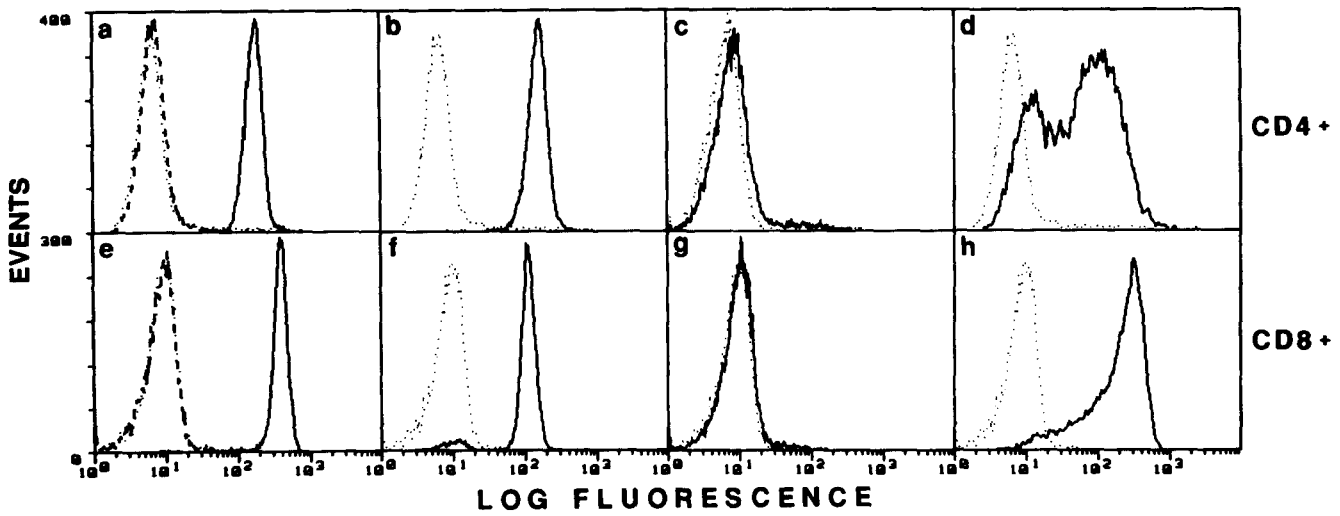

Figure 5. Purity and phenotype of T cell subsets. CD4+ (top row) and CD8+ (bottom row) T cells were isolated from LEW rat LN and immediately labeled with a variety of mAb for cytofluorographic analysis. Auto fluorescence in all cases is indicated by the dotted lines using cells stained with anti-human C3bi mAb, MRC OX21. (*a*) dashed line (which overlays the dotted autofluorescence plot) is CD4+ T cells labeled with anti-CD8 mAb and solid line is cells stained with anti-CD4 mAb. (*e*) dashed line (which overlays the dotted autofluorescence plot) is CD8+ T cells labeled with anti-CD4 mAb and solid line is cells stained with anti-CD8 mAb. (*b*, *f*) αβ TCR. (*c*, *g*) IL-2R (α chain). (*d*, *h*) CD45R (MRC OX22 mAb).

Unfractionated LN Cells Do Not Respond to Allogeneic AST Stimulators. Like freshly isolated cells (Fig. 1), allogeneic BN AST preinduced to express MHC class II also do not stimulate the unprimed T lymphocytes within a mixture of responder LEW LN cells (Fig. 3 *A*). BN LN stimulator cells, in contrast, induce a substantial and responder cell dose-dependent response which is reduced by ~90% in the presence of anti-CD4 mAb (data not shown). It should be noted (Fig. 2) that the percentage of BN AST expressing MHC class II as well as the level of expression after IFN-γ treatment is generally lower than, for example, the LEW strain. Possibly the LEW strain is unusual in this respect given that a third strain (the PVG) was comparable to BN. However, we also examined the response of PVG LN cell responders to syngeneic as well as allogeneic (LEW) LN and AST stimulator cells and again, no response to AST was observed (Fig. 3 *B*). Thus, the lower levels of MHC class II expression on BN AST is probably not the sole reason for the inability of these cells to stimulate unprimed T lymphocytes.

Fresh AST were not inhibitory in mixing experiments (Fig. 1). Mixing of cultured AST with LN stimulators (Fig. 4) resulted in a slight inhibition of the response only at the highest responder cell number (probably a crowding effect) but appeared to have an enhancing effect at the lower responder cell numbers. Additionally, addition of indomethacin did not reverse the inability of AST to prime T lymphocytes (data not shown).

Weak Autonomous CD8+ But Not CD4+ T Cell Response to AST Stimulators. Given the heterogenous mixture of responder cells present in the Fig. 3 experiments, it was feasible that a positive response from a minority T cell population may have been overlooked. To examine this possibility, highly purified LEW responder CD4+ and CD8+ T cells were prepared and tested in the primary MLR against AST stimulator cells.

As shown in Fig. 5, the purity of the two T cell subpopulations was high (~99%) with negligible numbers of CD8+ T cells in the CD4+ T cell population (Fig. 5 *a*) or CD4+ T cells in the CD8+ T cell population (Fig. 5 *e*). Virtually all the CD4+ cells were TCR-α/β+ (Fig. 5 *b*) while 10% of the CD8+ T cells were TCR-α/β- (Fig. 5 *f*). γδ TCR+ cells in the rat have recently been shown to predominantly lie within this CD8+ TCR-α/β- population (37). Between 5% and 8% of CD4+ (Fig. 5 *c*) and <2% of CD8+ T cells (Fig. 5 *g*) were IL-2R, α chain positive. MRC OX22 (CD45R) was highly expressed on around two-thirds of CD4+ T cells (Fig. 5 *d*) and most (90%) CD8+ T cells (5 h).

Both in mice and rats (20, 38), CD8+ T cell responses appear to peak early and then decline. The optimal time to assess proliferative responses of both CD4+ and CD8+ T

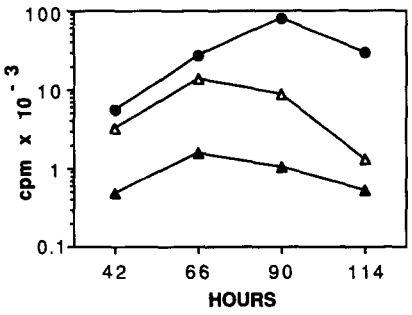

Figure 6. Early decline of the CD8+ T cell response to allogeneic LN stimulators. 5 × 10^5 responder CD4+ (●) or CD8+ (△, ▲) T cells from LEW rats were added to the same number of BN allogeneic irradiated stimulator LN cells, in the presence (△) or absence (●, ▲) of 50 U/ml rIL-2. Responder cell proliferation was assessed at the times shown which are inclusive of an 18-h pulse with [^{3}H]thymidine. Data are means of triplicate determinations and are representative of two comparable experiments.

cells was determined in a time course experiment (Fig. 6). In response to allogeneic LN stimulator cells, CD8+ T cells in the presence of IL-2 and CD4+ T cells, responded similarly up to 66 h. After this time, the CD8+ T cell response leveled off and had declined sharply by 114 h. CD8+ T cells without IL-2 revealed a low level response with kinetics comparable to CD8+ T cells in the presence of IL-2. The reduction in the CD4+ T cell response at 114 h probably reflects cell death or exhaustion of the culture medium as the medium at this time was extremely acid. Addition of IL-2 to the CD4+ T cells did not substantially alter their response. A compromise culture period of 78 h was chosen reflecting a time of peak CD8+ T cell proliferation but also high CD4+ T cell responsiveness.

As with the unfractionated LN responder cells in Fig. 3, CD4+ T lymphocytes showed no response to AST stimulators (Fig. 7, top three panels). By microscopic examination there was, furthermore, no evidence of CD4+ T cell blast transformation which would, if it occurred, be an indication that there had been some positive interaction. Anti-CD4 mAb (W3/25) substantially reduced the response of the CD4+T cells to allogeneic LN stimulator cells as expected and also blocked the background CD4+ T cell proliferation seen in the syngeneic MLR mixture. Comparable data have been generated in four other similar experiments.

CD8+ T cells did show some, albeit a low level, response to AST stimulators. In Fig. 7, we have produced results from three completely separate experiments (*A*, *B*, *C*) which essentially illustrates the difficulty, at least in our hands, of detecting autonomous primary CD8+ T lymphocyte responses. Nevertheless, the responses are real and significant and could no be blocked by anti-CD4 mAb. Even with LN stimulators cells (left hand panels), allogeneic CD8+ T cell responses were at best (experiment *B*), 7–10 times higher than background and usually only two to five times greater but with a magnitude some 20–40 times less than the equivalent number of CD4+ T cells. After IFN-γ treatment, allogeneic (BN) AST were more consistent stimulators for CD8+ T cells but the response was of low magnitude and the titration curves rather flat. Addition of higher numbers of CD8+ responder cells (10^6) usually resulted in a lower response than with 5×10^5 responder cells and extension of the incubation time was also detrimental to the magnitude of the response (not shown).

IL-2 Production by CD4+ and CD8+ T Cells. LEW CD4+ or CD8+ T cells were cultured in the presence of ir-

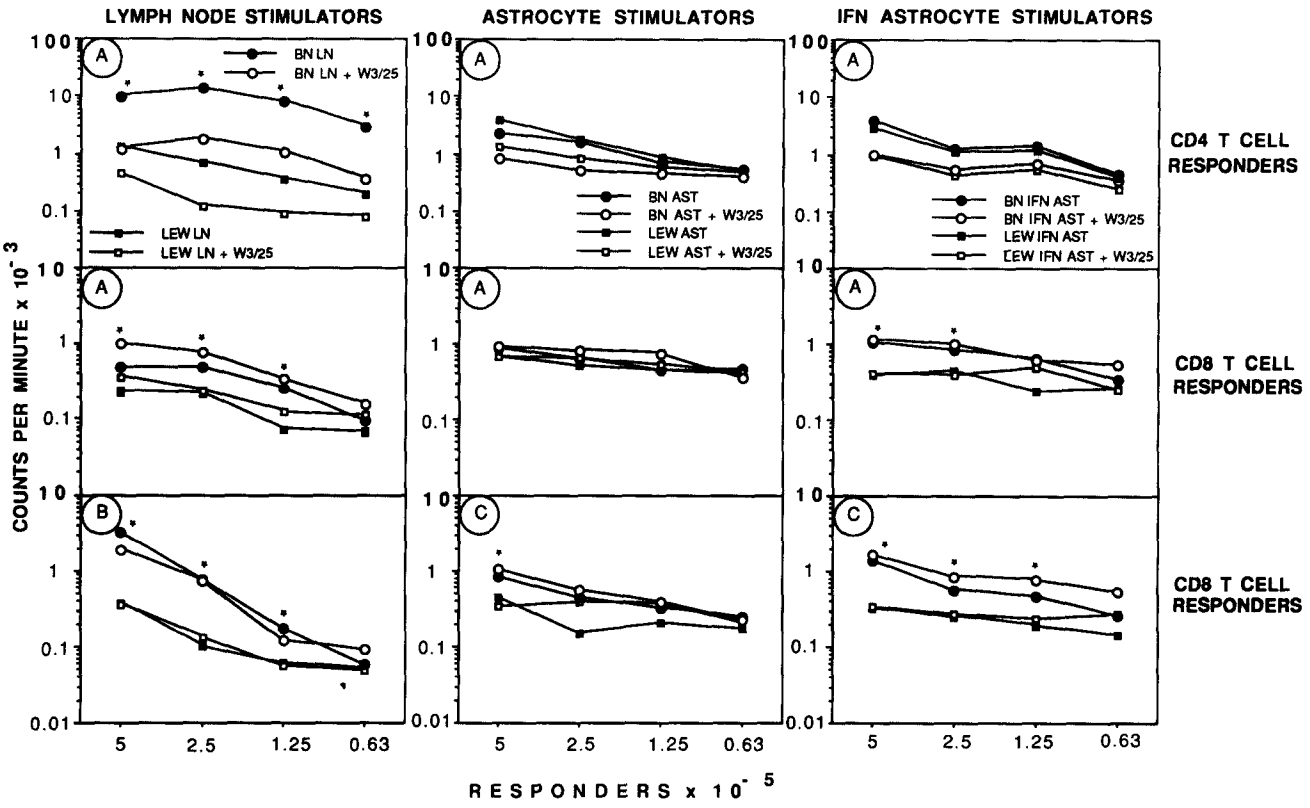

Figure 7. Weak autonomous CD8+, but not CD4+ T cell proliferation in response to allogeneic astrocyte stimulator cells. Responder LEW rat CD4+ and CD8+ T cells were titrated onto constant numbers of irradiated BN or LEW stimulator cells as described in Fig. 3. Purified W3/25 IgG (anti-rat CD4 mAb) was added to some of the wells at a final concentration of 5 μg/ml. The incubation time was 60-h + 18-h [^{3}H]thymidine pulse. Data shown are means of triplicate determinations. Three separate experiments (*A*, *B*, and *C*) are illustrated. The key in the top boxes refers to the stimulator cells and is also consistent for the two underlying graphs. Note the logarithmic scale difference between CD4+ T cell responders (10^5 cpm) and CD8+ T cell responders (10^4 cpm). *Proliferation of T cells with allogeneic stimulators is significantly greater ($p < 0.05$) than when mixed with syngeneic stimulator cells. For CD8+ T cells, data from wells both with and without anti-CD4 mAb are combined for statistical analysis.

Table 1. *MHC Class II-expressing AST Do Not Stimulate IL-2 Production by CD4+ T Cells*

	IL-2 in culture supernatants						T lymphocyte proliferation	
	24 h		48 h		72 h			
Stimulator cells	CD4+	CD8+	CD4+	CD8+	CD4+	CD8+	CD4+	CD8+
BN LN	–	–	35 ± 10*	–‡	750 ± 300	–	43,170‖	2,835‖
LEW LN	–	–	–	–	–	–	302	234
BN IFN γAST	–	–	–	–	–	–	520	588¶
LEW IFN γAST	–	–	–	–	–	–	612	458

5×10^5 LEW CD4+ or CD8+ T cells were cultured together with irradiated stimulator cells, and supernatants were collected at the times shown and tested for IL-2 content. Proliferation of T cells in parallel cultures was determined at 78 h inclusive of an 18-h [^{3}H]thymidine pulse (mean cpm of triplicate wells).

* Means ± 1 SD of supernatants from triplicate wells (U/ml).

‡ No proliferation of indicator cells above background.

‖ Proliferation of T cells is significantly different to the syngeneic control ($p<0.001$).

¶ In this experiment, no significant CD8+ T cell proliferation against AST stimulators was observed ($p>0.1$).

radiated syngeneic or allogeneic LN and IFN-γ–treated AST stimulator cells and supernatants collected and tested for T cell growth factor (IL-2) content (Table 1). IL-2 was detected only in wells containing LEW CD4+ T cells and allogeneic (BN) LN stimulator cells. CD8+ T cells proliferated in response to BN LN stimulators but no IL-2 was detectable. In this experiment, no CD8+ T cell proliferation above background was detected when cultured together with BN-IFN-γ–treated AST.

AST as Stimulators of Primary CD8+ T Cell Responses in the Presence of IL-2. Table 2 illustrates the response by CD8+ T cells following addition of rIL-2 which resulted in levels of proliferation many times greater than that seen in the absence of IL-2. A number of features of this response are noteworthy. First, in every experiment we have conducted, the CD8+ T cell response in the presence of AST stimulators is at least four to five times greater than that seen with LN stimulators. Second, there is no requirement for further AST MHC Class I upregulation (by treatment with IFN-γ) to elicit a strong CD8+ T cell proliferation although such treatment of the AST is clearly beneficial to the magnitude of the CD8+ T cell response. Third, the specificity, that is the relative responsiveness to syngeneic vs. allogeneic stimulators, of the CD8+ T cell response in the presence of IL-2

Table 2. *No CD4+ but Strong Primary CD8+ T Cell Response to AST Stimulators in the Presence of IL-2*

		Stimulator cells					
		No IL-2			50 U/ml IL-2		
Responder cells	Stimulator cells	BN	LEW	*p*	BN	LEW	*p*
CD4+	LN	19,799	131	<0.001	25,527	1,672	<0.001
	AST	653	492	NS	2,164	1,946	NS
	IFN-γAST	534	500	NS	4,670	3,938	NS
CD8+	LN	774	63	<0.001	3,460	1,941*	0.05<*p*<0.1
	AST	369	227	NS	16,742	4,414	<0.001
	IFN-γ AST	247	356	NS	26,373	7,931	<0.001

1.25×10^5 LEW CD4+ or CD8+ cells were cultured together with irradiated stimulator cells in the presence or absence of rIL-2 for 78 h inclusive of an 18-h [^{3}H]thymidine pulse. Data shown are mean cpm of triplicate determinations. NS; no statistically significant difference in the T cell response between syngeneic (LEW) and allogeneic (BN) stimulator cells.

* CD8+ T cells in the presence of IL-2 but in the absence of any stimulator cells exhibited proliferation indices similar to that seen when syngeneic LN cells were added.

is not as clearly defined as is seen in the absence of IL-2 (compare CD8+ T cell response to LN stimulators with and without IL-2), or indeed in the response of CD4+ T cells + IL-2 which proliferate strongly and specifically to LN stimulators but still show no response to AST. Reducing the amount of IL-2, for example to 2 U/ml, resulted in an improvement in the specificity of the CD8+ T cell response but the level of proliferation was only around 2 times greater than that of CD8+ T cells in the absence of exogenous IL-2. Comparable results to these have been obtained with medium supplemented with either FCS or normal rat serum and when employing a 10% supernatant of Con A–activated splenocytes as a source of IL-2.

The phenotype of the responding LEW CD8+ T cells in the presence of IL-2 and BN AST stimulators is shown in Fig. 8. A clearly defined minority blast population could be distinguished (high forward scatter) representing 20–25% of the total CD8+ T cells at the end of culture. As expected from the purity of the input cells (Fig. 5), the cells recovered from culture were exclusively CD8+ and CD4−. Blasts appeared to express higher amounts of CD8 than the small cells. The input cells were around 90% αβ TCR+ and the blast population essentially retained this phenotype. Only the blast cells expressed the α chain of the IL-2R and, significantly, around two-thirds of the blast cells were CD45R^{low} while most small cells retained the CD45R^{high} phenotype of the input CD8+ T cells (Fig. 5).

AST and Secondary Alloreactive CD4+ T Cell Responses. The MHC class II–expressing AST used in these experiments are clearly unable to prime CD4+ T cells, but can they restimulate such cells once they have been primed by another cell type? Preliminary studies to determine if AST have some allostimulatory capacity for CD4+ T cells employed a LEW anti-BN T cell line. The line (designated LEBN-1) was typical in being CD4+ CD8− TCR-α/β+. The important phenotypic changes that distinguish these CD4+ T cells from those used in the primary MLR studies (Fig. 5) are illustrated in Fig. 9. All cells were now completely CD45R (MRC OX22) negative and IL-2R (α chain) positive despite having been rested in IL-2-containing medium in the absence of BN stimulator cells, before phenotypic analysis. IL-2R ex-

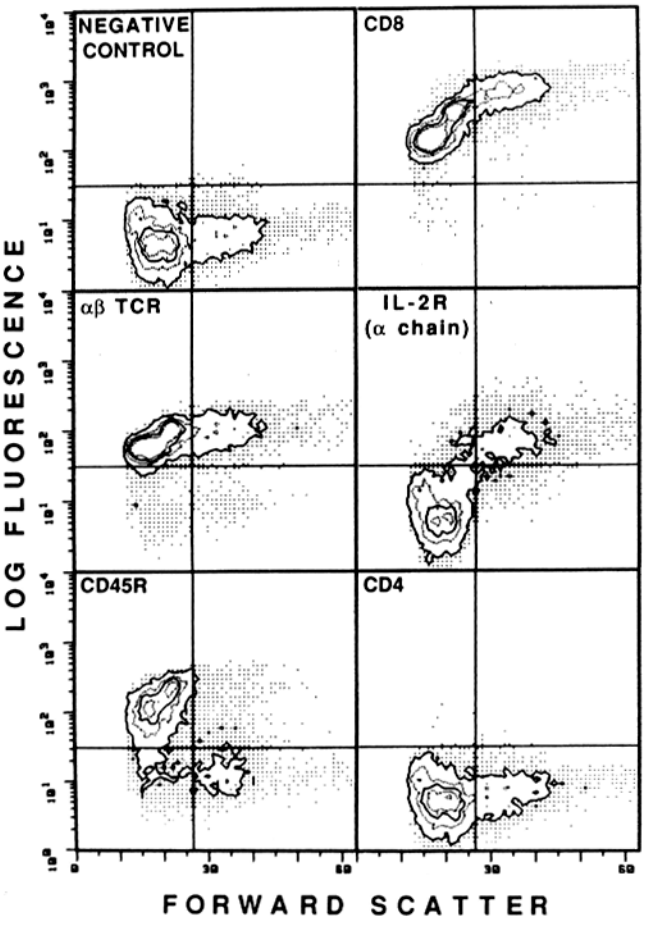

Figure 8. Phenotype of responding CD8+ cells in the MLR. LEW CD8+ T cell/BN AST cultures with added rIL-2 comparable to those described in Table 2 were established and cells recovered after 78-h incubation. Cells were double labeled with the biotinylated rat antibody, R2/15S which binds LEW but not BN MHC class I and mouse mAb against either rat CD8, αβ TCR, IL-2R, CD45R, CD4 or human C3bi (control). R2/15S was detected with 10 μg/ml streptavidin-PE and the mouse mAb with anti-mouse FITC (see Materials and Methods). Shown is the green (FITC) channel vs. forward scatter of the cells gated to exclude both dead cells and R2/15S− cells, the latter being the BN stimulator population.

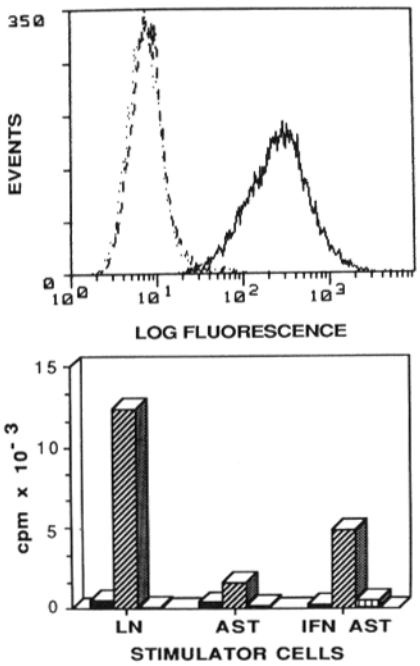

Figure 9. Astrocytes stimulate an alloantigen-specific CD4+ T cell line. (*Top panel*) Cells from the LEBN-1 (LEW anti-BN) CD4+ T cell line were rested in IL-2–containing medium then stained for FACScan® analysis. Dashed line (which overlays the dotted MRC OX21 autofluorescence plot) is CD45R (MRC OX22 mAb) and the solid line is IL-2R (α chain). (*Bottom panel*) 10^4 resting LEBN-1 cells were added to 5×10^5 LN or 2×10^4 AST stimulator cells from LEW (■), BN (▨) or PVG (□) rats. Incubation time was 48 h + a 12-h pulse with [^{3}H]thymidine. Data are means of triplicate determinations and are representative of two separate experiments.

pression immediately after stimulation on irradiated BN LN cells was further enhanced above that shown here, as expected.

Fig. 9 shows that BN AST are indeed capable of specifically stimulating the LEBN-1 line to proliferate. Reactivity to self (LEW) or a third party (PVG) is minimal. There was some response to AST not pretreated with IFN-γ as reported previously employing CD4$^+$ T cell lines specific for CNS autoantigens (39). The response to BN AST is ~40% of that against BN LN cells, the latter being the stimulator population that was used to raise the LEBN-1 line. It is not known why the difference exists but the presence of this positive response against AST is nevertheless significant in view of the previous data with unprimed CD4$^+$ T cells.

Discussion

There has been a significant shift in opinion away from the concept that MHC class II expression on nonhematopoietic cells may be an important factor in the induction of some tissue-specific autoimmune diseases. In particular, the evidence accumulated from transgenic mouse studies involving targeted MHC expression on elements such as pancreatic β cells have indicated that such expression alone is not sufficient to induce an autoimmune inflammation (reviewed in reference 40). It has been proposed, but to our knowledge not yet directly tested, that one reason MHC expression on such cells does not result in autoimmune inflammation, is because the cells lack the ability to prime T lymphocyte responses.

The studies reported here are consistent with and further extend these views in as much as they provide evidence for the first time that MHC class II expression on astrocytes (AST) which are one of the more thoroughly studied nonhematopoietic cells exhibiting APC activity, is unlikely to lead to the induction of an autoreactive T lymphocyte response because these MHC-expressing cells are unable to act as APC for primary T cell responses.

One should qualify this statement to the extent that CD8$^+$ T cells do appear to be stimulated by AST (Fig. 7) which is in keeping with previous studies (20, 41) showing that mouse CD8$^+$ T cells can be triggered by a wider range of non-DC accessory cells than can CD4$^+$ T cells. However, in the absence of IL-2 (which is predominantly a CD4$^+$ T cell product, Table 1), CD8$^+$ T cells did not respond to any substantial degree (Fig. 7 and Table 2). This virtual dependence of rat CD8$^+$ T cells on CD4$^+$ T cell help confirms studies from other groups (38, 42) but differs from a number of reports (18, 20, 43) in which mouse mouse CD8$^+$ T cells have been shown to proliferate substantially, to secrete IL-2 and to differentiate into CTL, all in the absence of CD4$^+$ T cells. However, at least in the mouse, this effect appears to be strain dependent (20) and it is thus unclear to what extent CD8$^+$ T cell autonomy can be considered a general phenomenon, particularly in response to nonhematopoietic stimulator cells. Indeed, recent in vivo studies in mice involving allogeneic MHC class I expression on pancreatic β cells (44) and tumour cell rejection in the eye (45), generally do not support the concept that CD8$^+$ T cells can respond effectively to antigen on nonhematopoietic cells in the absence of CD4$^+$ T cell help.

The response of CD8$^+$ T cells in the present study also demonstrated two other unusual features after addition of exogenous IL-2. First, CD8$^+$ T cells, regardless of the stimulator population (LN or AST) responded to the added IL-2, at least to some extent, without an apparent requirement for specific receptor interaction as evidenced by a high background proliferation in the presence of syngeneic stimulators (see Table 2). These results are remarkable considering the essentially IL-2R (α chain) negative condition of the CD8$^+$ T cells at the initiation of culture (Fig. 5). NK and CD8$^+$ but not CD4$^+$ T cells in the human appear to constitutively express the IL-2R (p75) β chain (46) and we are currently assessing whether this differential expression is also present in rat T cell subpopulations. Notably, the responding CD8$^+$ blast cells in our experiments (Fig. 8) were also predominantly TCR-α/β^+ and not, therefore, NK cells which are also CD8$^+$ in the rat (37). The change from high to low CD45R expression in the blast cell population (Fig. 8) is consistent with previous studies on the CD45R phenotype of virgin and memory CD4$^+$ T lymphocytes (47) and has also been observed on human CD8$^+$ T cells stimulated by allogeneic DC (48). The continuing CD45R^{high} phenotype of some of the blast cells (Fig. 8) in these relatively short-term cultures (78 h) may be due to the slow turnover rate of this surface molecule (49).

The second unusual feature of the IL-2-dependent CD8$^+$ T cell response in the present studies was the substantial levels of proliferation observed when AST were used as stimulator cells. Such proliferation indices were never observed using LN cells as allogeneic stimulators. This may be partly related to the levels of MHC class I expressed by the AST (Fig. 2) but a number of potential stimulators of CD8$^+$ T cells present in LN (such as DC) are almost certain to express equivalent or higher levels of this molecule. Alternatively, the AST may secrete cytokines such as IL-6 (50) to which the CD8$^+$ T cells respond optimally. It is noteworthy that even background proliferation in the presence of syngeneic AST stimulators and IL-2 (Table 2) was higher than when syngeneic LN stimulators were present (see also Fig. 1) suggesting that AST provided something promoting T cell growth that LN stimulator cells did not.

Why do unprimed CD4$^+$ T cells not respond to MHC class II positive AST stimulator cells? A simple solution would be that AST are unable to interact with CD4$^+$ T cells but this is patently not the case given their capacity to restimulate already primed alloreactive CD4$^+$ T cells as illustrated in Fig. 9. Inadequate levels of MHC class II may be one explanation, however a low percentage of AST expressed quite high levels of MHC class II and our system was probably sensitive enough to detect even a low level of CD4$^+$ T cell proliferation that resulted from interaction with these AST. Moreover, in one experiment (not shown) we pretreated AST with a combination of human rTNF-α and rat rIFN-γ. In this case, more than 60% of BN-strain AST were MHC class II positive and the level of expression was also increased. Again, no primary allogeneic CD4$^+$ T cell response was observed.

We did not examine this further as TNF-α is known to have a variety of effects on target cells such as inducing cytokine release (50) which makes the system somewhat more difficult to interpret. Possibly, certain cytokines which are required for priming of CD4$^+$ T cells may have been absent but addition of supernatant from Con A-activated splenocytes which presumably contained a variety of cytokines in addition to IL-2, did not result in CD4$^+$ T cell priming by MHC class II positive AST (not shown). Finally, it is conceivable that important adhesion molecules were absent either on the AST or the CD4$^+$ T cells. The AST used here constitutively express high levels of ICAM-1 which is further enhanced in the presence of IFN-γ (E. Kraus, S. Schneider-Schaulies, M. Miyasaka, and J. Sedgwick, manuscript in preparation), but other adhesion molecules may be of greater significance.

What do these results mean in terms of the induction and enhancement of an autoimmune response against tissue-specific antigens and more specifically, those in the CNS? First, as there is no evidence from our studies that MHC Class II expressing AST can prime CD4$^+$ T cells then it is doubtful that these cells will initiate an autoreactive T cell response. However, once the response is initiated by another cell type(s), not only can the autoreactive T cell induce MHC Class II expression on the AST (or presumably a range of other nonhematopoietic cells) via secretion of factors like IFN-γ, but the AST may then restimulate the CD4$^+$ T cell and perpetuate and enhance the response further. This latter property of AST is certainly not a feature of all MHC class II–expressing nonhematopoietic cells as, for example, pancreatic β cells (40) and keratinocytes (51) appear to switch off rather than reactivate already primed T cells. Second, even if an unprimed autoreactive CD8$^+$ T cell is activated after interaction with AST, there is also little evidence from our studies to suggest that any substantial proliferative event would result unless (CD4$^+$ T cell) help was provided for this cell. In the case of the CNS (and for arguments sake, taking the AST in isolation), this means that development of CNS inflammation is unlikely to happen unless prior peripheral sensitization has occurred.

There are of course other cells in the CNS to consider in this context and foremost among those which may potentially prime T cell responses, are macrophage-like microglia which, in contrast to AST, are probably of bone marrow origin (32). However, in preliminary studies using mixed glial cultures as stimulators containing 10–15% macrophage/microglia as well as AST, we have not seen any evidence that these cells have a priming capability. As expected, MHC-expressing microglia have been shown to stimulate secondary T cell responses in vitro (7). We are currently fully assessing the capacity of these cells, in isolation, to stimulate unprimed CD4$^+$ and CD8$^+$ T cells.

The results of tissue engraftment studies in the rat CNS (52) are particularly relevant to the concepts addressed here. This study demonstrated that without prior peripheral sensitization (that is, by careful placement of allografts only into the CNS), the allografts were very slowly rejected. After concomitant engraftment of tissue into the periphery (under the kidney capsule), the CNS grafts were rapidly eliminated. Overall therefore, the data are consistent with the concept that diseases like MS, while manifest in the CNS, are nevertheless peripherally initiated as there is little evidence, direct or circumstantial, that suggests there is any cell normally resident in the CNS that is capable of priming an (autoreactive) T cell response.

The question still remains as to how T cells (in the circulation) come to recognize and interact with antigen in the CNS. The role of T blast cell traffic (4) and/or interactions at the level of the brain vascular endothelium (53) are currently being considered.

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