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## Recognition of Major Histocompatibility Complex Antigenes on Murine Glial Cells

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### Summary

Recognition of autologous major histocompatibility complex (MHC) antigens by T cells is an essential step in the induction of an immunologic reaction to either endogenous or exogenous antigens. We investigated the ability of murine glial cells of different ages to stimulate clones of allospecific T lymphocytes. We also investigated the effects of supernatants from cultures of activated T cells on the immunologic recognition of MHC antigens on murine glial cells.

Lymphocyte clones specific for Class I, Class II and non-MHC, background antigens were obtained from C57Bl/6J-anti-DBA/2 mixed lymphocyte cultures. Glial cell cultures were prepared from newborn syngeneic (C57Bl/6J) and allogeneic (DBA/2) mouse brains. Glial cultures 1–4 weeks of age were able to stimulate  $\alpha$ -Class I-specific clones. No stimulation of  $\alpha$ -Class II or  $\alpha$ -background clones was noted. Incubation of glial cells with supernatants from cultures of alloantigen-activated spleen cells (C57Bl/6J-anti-DBA/2) resulted in a decreased ability of glial cells to stimulate  $\alpha$ -Class I responses. In contrast supernatant-treated cultures acquired the capacity to stimulate  $\alpha$ -Class II-specific clones. No responses were noted in clones responsive to non-MHC antigens. The ability to stimulate  $\alpha$ -Class II-specific clones was most prominent with one-week-old glial cultures and was lost by four weeks of culture. The increased susceptibility of younger glial

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cultures to the modulatory effects of lymphokines from activated T cells may be a factor in the increased susceptibility of the immature central nervous system to persistent viral infections and the development of autoimmune phenomena.

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Key words: *Murine glial cells – Major histocompatibility complex antigens*

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## Introduction

It has recently been demonstrated that central nervous system cells, especially glia, have immunologic activity. For example, astrocytes can produce an IL-1-like substance (Fontana et al. 1978), present antigen to sensitized lymphocytes (Fontana et al. 1984), and stimulate lymphocytes in mixed lymphocyte culture (Wong et al. 1985). Since antigens of the major histocompatibility complex (MHC) play a critical role in the recognition of 'foreign' and self-antigens by the immune system (Snell 1978) their presence on brain cells may be important in autoimmune reactions to brain. In the present report we addressed the following questions: (a) Do monolayer cultures of developing mouse glial cells stimulate alloantigen-specific murine T cell clones? (b) Can soluble products of activated lymphocytes affect the ability of glial cells to stimulate these clones? (c) Do glial cell cultures change with age in their ability to stimulate lymphocytes? (d) Do glial cell responses to lymphocyte culture supernatants change with glial cell age? The latter two questions were posed because of the particular susceptibility of the immature nervous system to both autoimmune phenomena and persistent viral infection (Miller et al. 1956; Stone and Lerner 1965; Kennedy 1968; Currier et al. 1974; Wolinsky et al. 1976; Lublin et al. 1981).

## Materials and Methods

### *Glial cultures*

Cerebral cortices from newborn C57Bl/6J (B6) and DBA/2 (DBA) mice were prepared as single cell suspensions by trypsinization and trituration (Swaiman et al. 1982). Cells were plated at varying concentrations ( $8 \times 10^5$ /ml to  $2.4 \times 10^6$ /ml) in 16 mm wells of plastic, 24 well plates (Nunc Interned) in medium consisting of Eagle's MEM supplemented with  $\text{NaHCO}_3$ , 3.7 g/l, D-glucose, 6 g/l, penicillin and streptomycin, 100 units/ml and 10% heat-inactivated fetal calf serum (Gibco Labs, Chagrin, OH). Higher cell concentrations were used in cultures to be tested after one week to ensure full monolayer formation. Medium was changed at 24 and 48 h after explantation and then twice weekly. Medium at that time consisted of Eagle's MEM with no antibiotics and 10% heat-inactivated horse serum (HyClone, Logan, UT). Cultures were grown at 37°C in an atmosphere of 10%  $\text{CO}_2$ .

### *Characterization of glial cultures*

Cultures prepared as above were grown for one week or three weeks and then fixed in cold ( $-20^\circ\text{C}$ ) ethyl alcohol. Cultures were kept in fixative for one to three

weeks at  $-20^{\circ}\text{C}$ . Cultures were stained with mouse monoclonal antibody to (1) glial fibrillary acidic protein (GFAP) (Boehringer Mannheim Biochemica, Indianapolis, IN), (2) myelin basic protein (MBP) (Hybridtech, San Diego, CA), (3) murine IA<sup>b</sup> (American Type Tissue Culture Collection, Rockville, MD; ATCC No. HB-37) and (4) mouse Ia<sup>d</sup> (ATCC No. HB-3). Binding of monoclonal antibodies to brain cells was determined using an avidin-biotin immunoperoxidase technique (Vector Laboratories, Burlingame, CA). At both one and three weeks of culture age 90–95% of the flat, polygonal cells forming the bottom layer of cells in the cultures were GFAP positive. A slightly lesser percentage of these cells was also MBP positive. In addition, bipolar cells, lying on top of the flattened polygonal cells were almost all MBP positive. No cells were stained with the anti-IA<sup>b</sup> or anti-IA<sup>d</sup> antibodies.

The results indicate that almost all of the cells in our cultures were of glial origin. In addition, since macrophages and B cells constitutively express Ia antigens, the absence of staining with anti-IA antibodies would suggest little contamination of our cultures with macrophages.

#### *Anti-MHC antigen-specific clones*

Clones specific for Class I, Class II and non-MHC-linked antigens were prepared as described by MacDonald et al. (1980). Briefly, alloantigen-specific T cell clones were derived from bulk B6 anti-DBA mixed lymphocyte cultures (MLC) by micro-manipulation or deposition of single cells into microwell plates using a FACS IV Flow Cytometer (Becton Dickinson, Mountain View, CA) (MacDonald et al. 1980). Clones were grown in Dulbecco's MEM supplemented with 5% fetal calf serum, amino acid supplements, irradiated, fresh DBA spleen cells and an optimal concentration of 2° mixed lymphocyte culture supernatant (2° SN) (Ryser et al. 1978). Specificity of the clones was determined by measuring their proliferative responses to allogeneic, T cell-depleted spleen cells and cytolytic activity against P815 tumor cells or concanavalin A or lipopolysaccharide-stimulated spleen cells from a panel of H-2 recombinant mice (MacDonald et al. 1980).

Two clones, BD6-13 and C2-11, were cytolytic toward P815 tumor target cells and were both lytic and proliferated when confronted with cells from strains DBA/2, B10.D2, B10.A and B10.A(5R), but not B10.HTG. These clones were thus categorized as reactive with H-2D/L<sup>d</sup> (Class I) antigen. Clones BD6-13 and C2-11 were Thy-1<sup>+</sup>, Lyt-1<sup>-</sup>, Lyt-2<sup>+</sup> and L3T4<sup>-</sup>. Clones BD6-6 and C2-15 were not directly cytolytic toward P815 cells nor lymphoblasts from the recombinant panel but exhibited proliferative activity against cells from DBA, B10.D2 and B10.HTG but not B10.A nor B10.A(5R). These cells presumably react with H-2I<sup>d</sup> antigens (Class II). Clone C2-16 was not directly lytic but proliferated in response to DBA/2 but not B10.D2 and is therefore specific for non-H-2 (background) genes. This clone was Thy-1<sup>+</sup>, Lyt-1<sup>+</sup>, Lyt-2<sup>-</sup>, L3T4<sup>+</sup>.

Cell line CTLL-20 was also used (a gift of Dr. Frank Fitch). This line is extremely sensitive to Il-2 and was used to determine whether residual Il-2 could be detected on the surfaces of 2° SN-treated glial cells.

The  $\alpha$ -Class I clones used in our experiments (C2-11 and BD6-13) were helper cell-independent, cytolytic clones (Widmer and Bach 1981). These clones both

proliferated and exhibited cytolytic activity when stimulated with appropriate antigens. Clones C2-15 and BD6-6 were non-cytolytic, IL-2-secreting clones as was clone C2-16.

#### *Preparation of supernatant from secondary MLC (2° SN)*

The procedure of Ryser et al. was used (1978). Briefly B6 $\alpha$ DBA cultures were prepared and incubated for 12–14 days. Cells were washed and restimulated with fresh DBA cells. After 24–48 h of additional culture, cells were removed by centrifugation and the culture supernatants collected and stored at  $-20^{\circ}\text{C}$ .

#### *Treatment of glial cells with 2° SN*

Confluent glial cell cultures of different ages were exposed for three days to medium containing 30% 2° SN. Control cultures were incubated with clone medium alone. After three days, cultures were washed four times with medium and allowed to incubate at  $37^{\circ}\text{C}$  for 1–2 h with fresh medium prior to addition of clone cells. All cultures were also irradiated with 3000 R prior to addition of clone cells to reduce background radionucleotide incorporation.

Supernates from cultures of activated lymphocytes contain factors that increase the proliferation rate of glial cells (Merrill et al. 1984). To determine the effects of 2° SN on our glial cultures we counted cell nuclei in 100 random microscopic fields of view in both 2° SN-treated and untreated glial cultures. Numbers of cells in both treated and untreated cultures were equivalent. Using a microscope micrometer we calculated the number of glial cells/well to be about  $4 \times 10^5$  cells. Our ratio of T cells to glial cells in our experiments was 1 : 4.

#### *Assay for the stimulatory capacity of glial cells*

Clones were added to glial cultures eight days after their last feeding with fresh medium and stimulator cells.  $1 \times 10^5$  clone cells were added to each well of glial cells. Additional controls consisted of clone cells added to wells containing  $5 \times 10^6$  irradiated, fresh DNA or B6 spleen cells or medium containing 30% 2° SN. All cultures were incubated for an additional 40 h. At the end of that time medium in the wells was vigorously pipetted to dislodge clone and glial cells and triplicate 0.1 ml aliquots from each 16 mm well were prepared and placed into round-bottom, 96 micro-well plates (Flow Labs, McClean, VA). An additional 0.1 ml fresh clone medium was then added to each well along with 2  $\mu\text{Ci}$  of [ $^3\text{H}$ ]thymidine, spec. act. 20 Ci/mM (New England Nuclear, Boston, MA). Cells were cultured for an additional 8 h at  $37^{\circ}\text{C}$ , harvested onto glass fiber filter paper and counted in a scintillation counter. Results are expressed as the counts per minute of [ $^3\text{H}$ ]thymidine incorporated  $\pm$  SE. In all experiments clones in control wells proliferated only in the presence of allogeneic (DBA) spleen cells or in the presence of medium containing 2° SN.

Proliferation was used as a measure of immunologic response even with the  $\alpha$ -Class I helper-independent cytotoxic clones. Since such clones proliferate only when stimulated with the appropriate antigen (Widmer and Bach 1981), we felt these assays were accurate measures of antigen-specific immune responses.

## Results

Four separate experiments were performed and results of each were similar. A representative experiment, utilizing the  $\alpha$ -Class I clone C2-11 is shown in Fig. 1. This clone proliferated when stimulated with one-, two-, three- and four-week-old cultures of DNA glial cells but not when cultured with syngeneic B6 glial cells. Proliferative responses of equivalent numbers of C2-11 cells to  $5 \times 10^6$  allogeneic DBA spleen cells were usually 3- to 5-fold less than those observed with DBA glial cells. Therefore, it is unlikely that the proliferation noted with glial cells was the result of contamination of these cultures by large numbers of hematopoietic cells. Class I MHC antigens, as defined by induction of clone proliferation, thus could be detected very early in culture and remained detectable thereafter.

Clones C2-15 and BD6-6, reactive with Class II (presumably Ia<sup>d</sup>) antigens, did not proliferate when cultured with untreated DBA glial cells of any age. A representative experiment with Clone BD6-6 is shown in Fig. 2. This clone did respond to allogeneic spleen cells (data not shown). Clone C2-16, responsive to background, non-H2-linked antigens also did not proliferate when stimulated with DBA glial cells (data not shown).

When glial cells were cultured for three days with medium containing 30% 2° SN, marked changes in the patterns of T cell clone proliferation were observed (Figs. 1

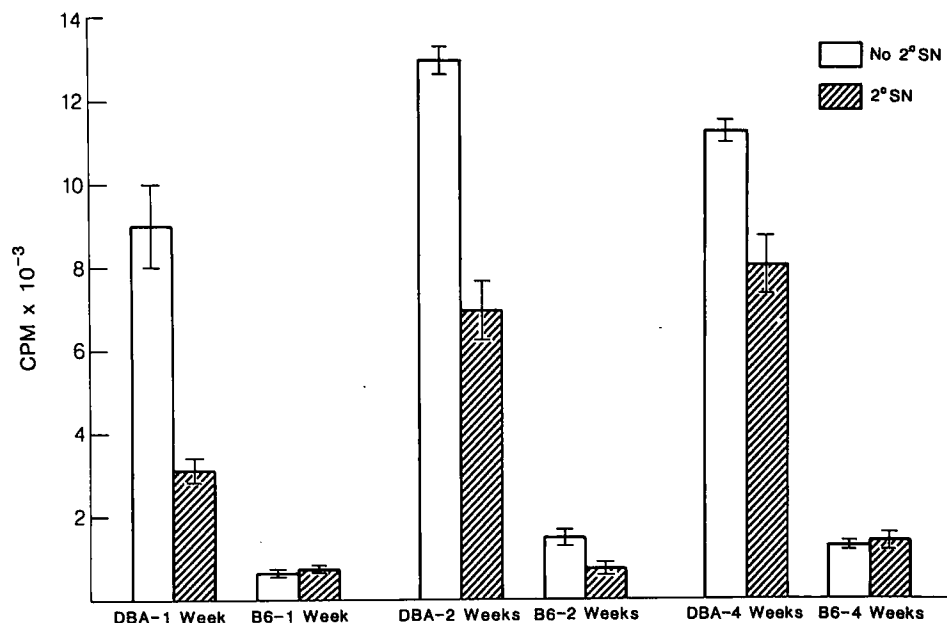


Fig. 1. The  $\alpha$ -Class I clone C2-11 was cultured with syngeneic and allogeneic mouse glial cells with and without 2° SN treatment. The proliferative responses of the clone were measured using a [<sup>3</sup>H]thymidine incorporation assay. Results are expressed as counts per minute (cpm) of [<sup>3</sup>H]thymidine incorporated. Background cpm of glial cells cultured alone were always <1000 cpm and usually < 500 cpm.

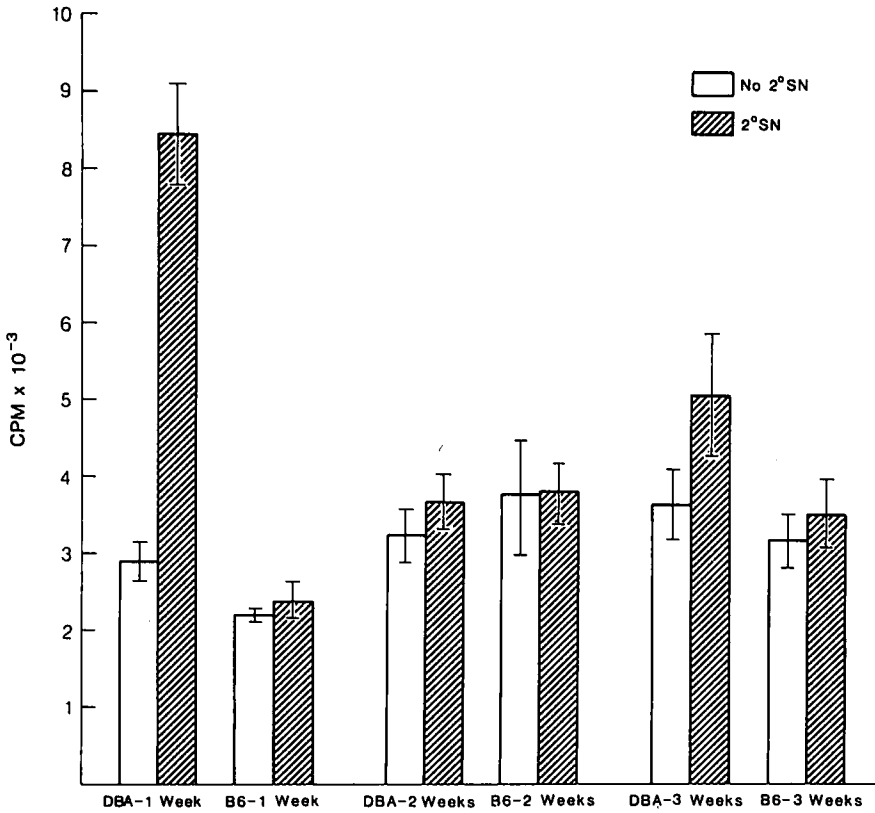


Fig. 2. The proliferative responses of the  $\alpha$ -Class II clone BD6-6 are shown. Details are as described in Fig. 1.

and 2). Proliferation of  $\alpha$ -Class I clone C2-11 was markedly decreased in one-, two- and four-week cultures. In contrast an  $\alpha$ -Class II-specific clone (BD6-6) response could now be detected but only with treated glial cultures that were one week post-explantation (Fig. 2). Proliferative responses of BD6-6 cells to  $5 \times 10^6$  allogeneic mouse spleen cells were equivalent to those noted with 2° SN-treated, allogeneic glia. After two weeks of in vitro growth the ability of treated DBA glia to stimulate BD6-6 cell proliferation was lost (Fig. 2). Supernatant-treated glia of any age did not induce proliferation of the  $\alpha$ -non-H2, background clone. Thus, based on the limited numbers of clones tested, the effects of 2° SN on clonal proliferation were specific for clones responsive to MHC antigens.

To determine whether the  $\alpha$ -Class II-proliferative response resulted from 2° SN passively bound to glia, cells from an Il-2 indicator line (CTLL-20) were added to glial cultures that had been exposed to 2° SN for three days and then washed. No proliferation was observed. Thus, passive binding of functionally detectable amounts of Il-2 to cultures of 2° SN-treated glia cells was not noted.

## Discussion

We have demonstrated that cultured mouse glial cells have the capacity to stimulate  $\alpha$ -Class I H-2 antigen-specific clones but not  $\alpha$ -Class II antigen-specific clones. We have also demonstrated that exposure of cultured mouse glial cells to the various lymphokines present in the supernatants from activated lymphocyte cultures results in an interesting reciprocal effect on the ability of these cultures to stimulate  $\alpha$ -Class I and  $\alpha$ -Class II antigen-specific clones. Treatment of glial cells with 2° SN reduced their ability to induce  $\alpha$ -Class I clonal proliferative responses but resulted in their ability to induce  $\alpha$ -Class II responses. In addition, the ability to induce  $\alpha$ -Class II responses was culture age related, disappearing by two weeks after explantation.

Our results corroborate and extend the observations of other investigators. Hirsch et al. (1983) and Wong et al. (1984) were able to induce Class II antigens on glial cells with  $\gamma$ -interferon. Serologic techniques were used and functional studies of T cell activation were not described. Since antibodies and T cells respond to different epitopes on MHC antigens (Cairns 1985), these differences in technique could explain the inability of Hirsch et al. (1983) and Wong et al. (1984) to detect MHC antigens on untreated glial cells. Antigen presentation by astrocytes to lines of sensitized lymphocytes was noted by Fontana et al. (1984) suggesting that functional Class II antigens were present on glial cells even though they could not be serologically detected. Of additional interest is their observation that glial cells exposed to activated lymphocytes became Class II antigen positive. More recently, using both glioma cultures (Takiguchi et al. 1985) and normal brain cultures (Wong et al. 1985) investigators have shown that such cells can activate bulk cultures of allogeneic spleen cells to proliferate in MLC. These effects were increased by treating cells with  $\gamma$ -interferon. To our knowledge our observations of the reciprocal effects of 2° SN on Class I and Class II antigen-specific lymphocyte proliferation have not been described.

Cell proliferation is a complex function and many variables determine whether such proliferation takes place. Therefore, the decreased proliferative responses of  $\alpha$ -Class I clones to 2° SN-treated glial cells may be explained in several ways: (1) decreased expression of Class I antigen, (2) decreased production of lymphocyte growth factors necessary for  $\alpha$ -Class I clone proliferation, (3) a selective decrease of glial cells expressing Class I antigens, or (4) an enhanced cytotoxic response of  $\alpha$ -Class I clones to treated vs. untreated glial cells, resulting in fewer stimulator cells being present. To evaluate the latter possibility we microscopically inspected 2° SN-treated and untreated glial cultures incubated with the  $\alpha$ -Class I clones for loss or disruption of the glial cell monolayers. None could be detected. Therefore the lower proliferative responses of  $\alpha$ -Class I clones to supernatant-treated glial cells did not appear to be the result of fewer glial cells being present in treated cultures. Experiments to investigate the other possibilities are in progress.

Our results may have important implications for our understanding of both persistent viral infections of the central nervous system and the development of autoimmune responses to this organ. Recognition of viral antigens by anti-viral



cytotoxic cells occurs in conjunction with recognition of Class I MHC antigens (Snell 1978). If products of lymphocytes activated during a viral infection can interfere with the recognition of this class of MHC antigens *in vivo*, less efficient killing of virus-infected cells may occur, resulting in incomplete elimination of the virus and possible persistence. Similarly, if products of activated lymphocytes allow recognition of Class II MHC antigens on brain cells not normally expressing them, helper T cells, responsive to antigens in the context of Class II MHC antigens, may be stimulated. Such helper cells are necessary for the induction of antibody responses to thymic-dependent antigens (Snell 1978), the generation of cytotoxic T cells (Snell 1978) and have been implicated in the development of various autoimmune diseases (Theofilopoulos et al. 1979; Krakauer et al. 1981; Miller and Salem 1982). Thus functional recognition of Class II antigens occurring as a result of lymphokine release during a CNS viral infection, could trigger an autoimmune response to brain components. The work of Watanabe and colleagues (1983), demonstrating acute EAE in recipients of cells from donors having viral encephalitis is indirect evidence in support of this hypothesis.

The effects of 2° SN were most marked in young glial cultures and decreased or disappeared with prolonged culture of glia *in vitro*. It has been demonstrated that ontogenic changes in cultures of newborn brain parallel those seen *in vivo* (Nelson 1975; Block 1982). If our observations can be extrapolated to the *in vivo* condition, they may partially explain the increased susceptibility of the immature brain to autoimmune phenomena (Miller et al. 1956; Stone and Lerner 1965; Currier et al. 1974; Lublin et al. 1981) and persistent viral infections (Kennedy 1968; Wolinsky et al. 1976).

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