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Modulation of immune-associated surface markers and cytokine production by murine retinal glial cells

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Abstract

Murine retinal glia are normally negative for major histocompatibility complex (MHC) Class II antigens and express low levels of MHC Class I and intercellular adhesion molecule-1 (ICAM-1) as detected by avidin–biotin–peroxidase immunohistochemistry. These surface molecules associated with immune function were either induced (Class II) or upregulated (Class I and ICAM-1) on cultured retinal glial cells in a dose- and time-dependent manner following exposure to recombinant interferon-γ (rIFN-γ). MHC Class I and II expression by passaged and primary cells was maximal (> 90% positive) after incubation with 100 U/ml of rIFN-γ for 48 h. ICAM-1 expression by primary and passaged cells tripled between 48 and 72 h after exposure to 25 or 50 U/ml of rIFN-γ. By 72 h after exposure to 100 U/ml of rIFN-γ, 62% of the retinal glia were positive for ICAM-1, whereas under normal culture conditions these molecules were detected on < 3% of the retinal glia. Bacterial lipopolysaccharide (LPS), a known stimulator of central nervous system (CNS) astrocytes, increased ICAM-1 expression only 3-fold to 9% of cells staining positively, but neither MHC Class I nor Class II expression was altered from baseline levels. Surface expression of ICAM-1, MHC Class I, and MHC Class II was unaffected by exposure to either rTNF-α (1000 U/ml) or rIL-6 (100 U/ml) for 24 h. Under normal culture conditions, intracellular interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) were detected immunohistochemically. Exposure to either rIFN-γ or LPS induced more intense staining which correlated with increased secreted levels of both cytokines in culture supernatants. Levels of secreted TNF-α increased 6-fold after stimulation with LPS for 24 h, while secreted IL-6 increased over 9-fold. These results support the hypothesis that retinal glia may participate in intraretinal immune processes following stimulation during inflammatory and infectious processes via either cell surface- or soluble mediator-dependent mechanisms or a combination of both.

Keywords: Müller cells; Retina; Glia; Intercellular adhesion molecule-1; Interferon-γ; Major histocompatibility complex antigens; Cytokines; Mouse; Interleukin-6; Tumor necrosis factor-α

1. Introduction

Glial cells of Müller influence development and function of neuronal cells in the retina (Ripps and Witkovsky, 1985). The role of Müller cells in intraocular immune and antiviral responses, however, is unknown. While Müller cells are considered to be the functional equivalent of CNS astrocytes, the two cell types do not share a common precursor. In addition to Müller cells, the retina also contains two other types of glial cells, retinal astrocytes and microglia. Müller cells are derived from a multipotential precursor cell common to both Müller cells and retinal neurons (Cepko, 1995). Retinal astrocytes (type 1 astrocytes) are found in the nerve fiber layer and migrate into the retina from the optic nerve (Shaw and Weber, 1983; Ling and Stone, 1988; Huxlin et al., 1992); microglia are thought to be derived from a monocyte precursor cell (Linden et al., 1986; Hickey and Kimura, 1988). In the normal murine retina, Müller cells are negative by immunohistochemistry for glial fibrillary acidic protein (GFAP), a unique component of cells of the astrocyte lineage and Müller cells (Eng, 1985). GFAP expression is observed only in astrocytes in the nerve fiber layer of normal retina. However, both retinal Müller cells and CNS astrocytes from several species respond in vivo to injury or inflammation by proliferation and upregulated GFAP expression (Bignami and Dahl, 1979; Erickson et al., 1987;

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Ekstrom et al., 1988; Sarthy and Fu, 1989; Whittum-Hudson, 1992; Geiger et al., 1994). In the human CNS, various disease states including multiple sclerosis (MS) lead to altered surface marker expression or cytokine production by astrocytes (Traugott and Raine, 1985; Mucke and Eddleston, 1993; Merrill and Jonakait, 1995). Production of TNF-α and IL-6 is known to be modulated by IFN-γ in many systems, though little is known regarding the ability of retinal glia to produce soluble mediators. Identification of immunologically relevant surface markers on retinal glia and the ability of these cells to produce soluble mediators may aid in the further elucidation of the potential role(s) of these cells in intraretinal immune or inflammatory responses after ocular infection or other injury such as retinal degeneration.

Surface molecules required for a cell to act as an antigen-presenting cell (APC) include MHC Class I or II, ICAM-1, as well as other co-modulatory elements. Generally, intracellular antigens such as viral peptides are presented to cytotoxic $CD8^+$ T cells in an MHC Class I-restricted manner, while presentation of exogenous antigens to $CD4^+$ T-helper cells is MHC Class II-restricted (Berzofsky and Berkower, 1989). ICAM-1 is the ligand for the lymphocyte function-associated antigen (LFA-1) and is important in antigen presentation. In addition, ICAM-1 plays a vital role in inflammatory cell migration and extravasation to sites of inflammation as well as in subsequent cell–cell interactions (Springer, 1990). Upregulated ICAM-1 expression on Müller cells during intraretinal inflammatory responses may influence infiltration of the retina by inflammatory cells due to the proximity of Müller cells to retinal vessels.

The ability of glia to modulate their expression of surface molecules following exposure to products of activated T lymphocytes has important implications for localized immune and inflammatory responses. In vitro studies have clearly demonstrated that CNS astrocytes, in response to exposure to IFN-γ, modulate their expression of MHC Class I (Wong et al., 1984; Cannella and Raine, 1989), Class II (Hirsch et al., 1983; Wong et al., 1984; Fierz et al., 1985; Cannella and Raine, 1989; Benveniste et al., 1993), and ICAM-1 (Satoh et al., 1991; Benveniste et al., 1993). Exposure to other proinflammatory soluble mediators including TNF-α, IFN α/β and interleukin-1 (IL-1) can alter the expression of these surface molecules on a variety of cell types (Lavi et al., 1988; Cannella and Raine, 1989; Springer, 1990; Vidovic et al., 1990; Satoh et al., 1991; Benveniste et al., 1993; Smith et al., 1993b). While immune-associated surface marker expression may be a protective response for the host in many tissues, inappropriate expression could contribute to irreversible pathology in those immunologically privileged sites which normally are isolated from systemic immune responses.

In addition to surface marker changes, production of soluble mediators such as TNF-α and IL-6 is known to be an important means of initiating or controlling a local inflammatory or immune response. TNF-α and IL-6 production have been localized to CNS astrocytes under a variety of pathological processes (Selmaj et al., 1991; Genis et al., 1992). However, the functional significance of these cytokines in the infected or inflamed retina or CNS has not been determined.

In this study we investigated changes in surface marker expression and cytokine production by cultured murine retinal glial cells after exposure to rIFN-γ or LPS. Immunohistochemical methods were used to examine the modulation of MHC Class I and II and ICAM-1 molecules on retinal glial cells derived from newborn BALB/c and C57Bl/6 (B6) mice. Immunostaining and capture ELISA were used to detect altered cytokine production and secretion after exposure to viral and other immunologically relevant stimuli. The results reported here demonstrate that expression of MHC antigens and ICAM-1 is altered on retinal glial cells upon exposure to IFN-γ. Production of TNF-α and IL-6 is markedly upregulated after exposure to either LPS or IFN-γ, thus supporting the potential of these retinal cells to actively participate in intraretinal immune or inflammatory responses.

2. Materials and methods

2.1. Mice

Breeding pairs or pregnant BALB/c or C57Bl/6 (B6) mice were obtained from Harlan Sprague Dawley (Indianapolis, IN), Charles River Breeders (Wilmington, MA), or Jackson Labs (Bar Harbor, ME). Mice were maintained in the animal facilities of the Wilmer Institute in The Johns Hopkins Medical Institution. All studies comply with the NIH Guidelines for the Use of Animals in Research.

2.2. Retinal glial cell cultures

Primary retinal glial cell cultures were established using our published techniques (Merges and Whittum-Hudson, 1990) which are a modification of the method of Politi et al. (1988). Briefly, newborn BALB/c or B6 mice (24–48 h old) were sacrificed by decapitation and their eyes removed. The retinas were removed and dissociated by treatment with 0.25% trypsin (Sigma, St. Louis, MO) and 75 μg of DNase from bovine pancreas (Sigma). Enzymatic digestion was stopped using soybean trypsin inhibitor (0.25%; Sigma), after which cells were resuspended in L-EBM (consisting of Eagle's Basal Media (Gibco, Gaithersburg, MD), α-D(+)glucose (27.7 mM; Sigma) and sodium bicarbonate (1 mM; J.T. Baker; Phillipsburg, NJ)). Single cell suspensions were obtained by trituration through siliconized pipettes and filtration through nylon mesh, after which cells were suspended in complete Dulbecco's minimal essential medium (D-MEM, Gibco, Long Island, NY) containing 10% fetal calf serum (Gibco),

penicillin (100 U/ml (Gibco)), streptomycin sulfate (100 μg/ml (Gibco)), 2 mM L-glutamine (Gibco), and Fungizone (0.5 mg/ml (Gibco)). Cells were plated at high density (1×10^6 cells/ml) and maintained at 37°C in 5% CO_2 until they became semi-confluent. The primary cultured glia exhibit flat-cell morphology and 100% are positive for GFAP by ABC immunoperoxidase staining (Merges and Whittum-Hudson, 1990).

Retinal glial cell lines from both B6 and BALB/c mice were maintained in complete D-MEM. Cells from passage numbers of less than 20 were compared to those from over 100 passages. The passaged cells replicated rapidly, were fibrous in appearance when confluent, and over 99% of the cells continued to express GFAP. GFAP expression was periodically reconfirmed by ABC immunoperoxidase staining.

2.3. In vitro stimulation

Primary or passaged retinal glia were cultured with 0, 25, 50 or 100 U/ml of murine rIFN-γ (Gibco) in duplicate wells of 8-well glass chamber slides for 24–72 h, washed with PBS, and fixed in chilled acetone. For some studies, cells were co-cultured with supernatants obtained from concanavalin A-stimulated (ConA (Sigma); 10 μg/ml) murine splenocytes following 48 h of bulk-culture in RPMI supplemented with 5% FCS, penicillin (100 U/ml), streptomycin sulfate (100 μg/ml), 2 mM L-glutamine, and 5×10^{-5} M 2-mercaptoethanol. In other experiments, glial cells were bulk-cultured in T-25 flasks in a total volume of 10 ml for 24 h in the presence of 10 μg/ml of LPS (*Escherichia coli* O26:B6; Sigma), 1000 U/ml murine rTNF-α (Gibco) or 100 U/ml murine rIL-6 (PharMingen, San Diego, CA). Cells were trypsinized, washed, and resuspended in phosphate-buffered saline containing 0.1% bovine serum albumin (PBS-0.1% BSA; Sigma). Cells were cytocentrifuged onto ethanol-cleaned glass slides, air-dried, and fixed for 10 s in chilled acetone for storage at −20°C until the time of staining.

2.4. Immunohistochemical staining

Staining was performed by the ABC immunoperoxidase method as previously described (Whittum-Hudson and Pepose, 1987; Merges and Whittum-Hudson, 1990). Slides were thawed and fixed in chilled acetone for an additional 5 min and then rehydrated in PBS prior to blocking with the appropriate normal serum. Primary antibodies, or normal IgG as negative controls, were applied and slides incubated at room temperature for 60–90 min. After PBS washes, biotinylated secondary antibodies were applied for 60 min. Table 1 summarizes the antibodies used in this study. Following PBS washes, the ABC Elite reagent (Vector, Burlingame, CA) was applied according to the manufacturer's instructions. The reaction product was visualized with 3-amino-9-ethylcarbazole (AEC; Sigma) in a 0.02 M sodium acetate buffer (pH 5.0–5.2) containing 1.5 M dimethyl sulfoxide (DMSO) and 0.01% hydrogen peroxide. Cells were lightly counterstained with Harris' hematoxylin (Harleco, Gibbstown, NJ) and coverslips applied with glycerin/gelatin mounting media. ConA-stimulated splenocytes or LPS-stimulated macrophages served as a known positive control in each assay. Residual ConA was neutralized with 0.1 M methyl α-D mannoside (Sigma) prior to cytocentrifugation. At least 1000 cells were counted per well or cytospin. Two experiments were performed per antibody for both primary and passaged glia. Each treatment in an experiment was performed in duplicate.

Table 1
Antibodies used in immunohistochemistry

Specificity	Host species	Source	Dilution [a]
Primary antibodies			
ICAM-1	Hamster	PharMingen	1:200
Class II ($I\text{-}A^{b,d}$)	Rat	ATCC	1:50 (ascites)
Class I ($H\text{-}2^{d}$)	Mouse	PharMingen	1:100
Class I ($H\text{-}2^{b}$)	Mouse	ATCC	Neat (cult sup)
IL-6	Rat	PharMingen	1:50
TNF-α	Rabbit	Genzyme	1:50
Secondary antibodies			
Hamster IgG	Goat	Caltag	1:100
Rat IgG	Rabbit	Vector	1:200
Mouse IgG	Horse	Vector	1:200
Rabbit IgG	Goat	Vector	1:200

[a] All dilutions were made with PBS (pH = 7.2). Regardless of primary and secondary antibodies, all slides received the ABC Elite reagent for 45 min and were subsequently developed with AEC/DMSO in 0.02 M sodium acetate buffer (pH 5.0–5.2).

2.5. Capture ELISA for cytokines

The IL-6 capture ELISA was performed using monoclonal antibody pairs (PharMingen) against two different IL-6 epitopes, with some modification of the manufacturer's protocol. Plates were coated with 50 μl of a monoclonal rat anti-mouse IL-6 antibody (clone MP5-20F3) at a final concentration of 2 μg/ml in 0.1 M sodium bicarbonate buffer (pH 8.2) and incubated overnight at 4°C. All subsequent steps were performed in a total volume of 100 μl. Following PBS washes, wells were blocked with PBS/10% FCS for 2 h at room temperature after which serial dilutions of the IL-6 standard (PharMingen) or samples (diluted in PBS/10% FCS) were added and incubated overnight at 4°C. The plates were washed with 0.5% PBS/Tween, and the detecting antibody (biotinylated monoclonal rat anti-mouse IL-6 (clone MP5-32C11)) was added at a final concentration of 1 μg/ml and incubated at room temperature for 45 min. Avidin-alkaline phosphatase (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) was added (1:1000 dilution) and incubated at room temperature for 30 min. The

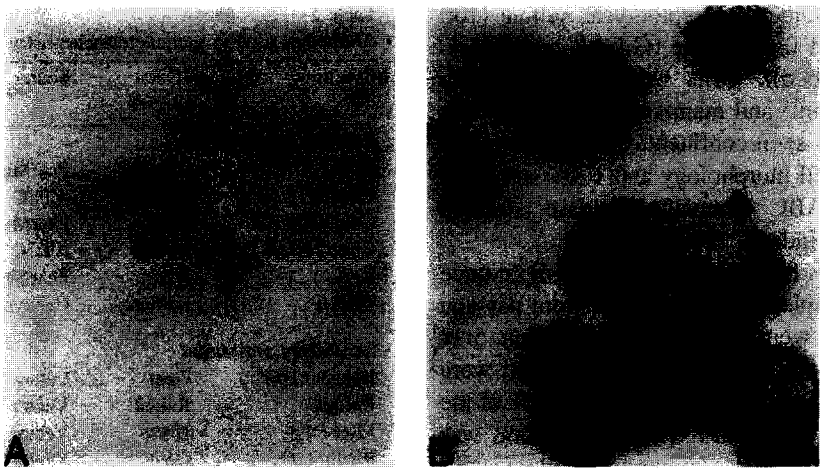

Fig. 1. Expression of MHC Class I antigens is upregulated on cultured BALB/c murine retinal glia by rIFN-γ. ABC immunoperoxidase staining for Class I antigens was performed on cytocentrifuged cells. (**A**) Less than 2% of unstimulated retinal glial cells (passage 22) stained positively for MHC Class I (H-2^d) antigens (400 ×). (**B**) Following exposure to 50 U/ml of rIFN-γ for 48 h, over 90% of the cells expressed MHC Class I antigens (400 ×). Red reaction product is seen as black (arrows).

reaction was developed in the dark with 1 mg/ml para-nitrophenyl phosphate (p-NPP; Sigma, St. Louis, MO) in buffer consisting of 9.7% diethanolamine, 3 mM sodium azide, and 0.4 mM magnesium chloride (pH 9.8). The plate was read on a Bio-Tek 312E plate reader (Burlington, VT) at 450 nm.

Similar methods were used to assay secreted TNF-α using a TNF-α capture ELISA kit obtained from Endogen (Cambridge, MA) and performed per the manufacturer's instructions using their pre-coated wells and reagents.

3. Results

3.1. Induction of MHC antigens on retinal glial cells

Prior to exposure to rIFN-γ, less than 2% of passaged BALB/c retinal glial cells expressed MHC Class I antigens (H-2^d) (Fig. 1A). The expression of Class I antigens was increased in a dose- and time-dependent fashion by rIFN-γ. Maximal expression (> 90% positive) of Class I was detected after co-culture with either 50 or 100 U/ml of rIFN-γ (Fig. 1B). No staining for Class I was observed on BALB/c cells when a primary antibody to an irrelevant MHC haplotype (anti-H-2^b) was used, regardless of treatment (not shown). Results of a typical experiment with passage 30 cells are shown in Fig. 2. Responses of primary retinal glial cells, which were also negative for MHC Class I antigens under standard culture conditions, were maximal between 48 and 72 h of exposure to rIFN-γ (50 or 100 U/ml), with Class I antigens detected on > 97% of the primary glial cells. These findings are summarized in Table 2. Class I expression was unaltered by exposure to LPS (10 μg/ml), rTNF-α (1000 U/ml), or rIL-6 (100 U/ml) for 24 h (not shown).

Both primary and passaged unstimulated retinal astrocytes are normally negative for MHC Class II antigens (Fig. 3A). As observed for other cell types (Fierz et al., 1985; Vidovic et al., 1990; Sun, 1991), rIFN-γ induced the expression of Class II antigens (Fig. 3B). Upregulation occurred in a dose- and time-dependent manner (Fig. 4). A maximum number of passaged BALB/c glial cells were Class II antigen-positive (> 85%) after 48 h exposure to 50 or 100 U/ml of rIFN-γ. Glial cells from both BALB/c and B6 mice responded similarly to rIFN-γ (not shown). Incubation with conditioned media from ConA-stimulated lymphocytes (20% v/v) for 48 h also induced MHC Class II antigens on over 90% of the passaged BALB/c or B6 glia. In contrast, incubation with LPS, rTNF-α, or rIL-6

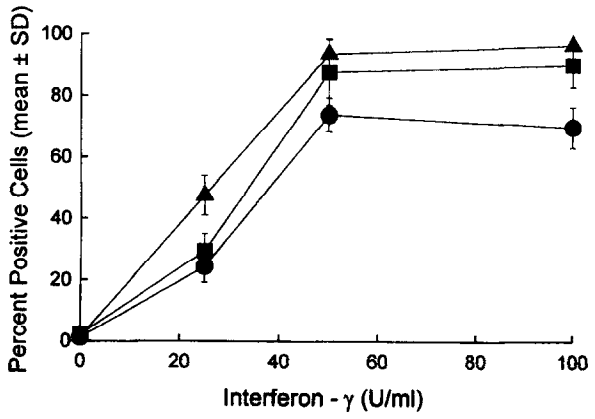

Fig. 2. rIFN-γ-induced Class I expression on BALB/c retinal glia is dose- and time-dependent. Passaged retinal glia (passage 30) were exposed to varying doses of rIFN-γ for 24 (circles), 48 (squares) or 72 (triangles) h prior to immunohistochemical staining. Maximal expression of MHC Class I antigens was induced by 48 h exposure to 50 U/ml of rIFN-γ. Similar results were obtained with primary glia. Results are expressed as the mean ± S.D. of duplicate wells.

Table 2
rIFN-γ upregulates surface antigens on primary BALB/c retinal glial cells [a]

Antibody specificity	Time of in vitro exposure (h)	Percent positive cells at different concentrations of rIFN-γ (U/ml)			
		0	25	50	100
Class I (H-2^d)	0	0.2 ± 0.1			
	24		1.9 ± 0.7	4.7 ± 1.6	5.2 ± 3.4
	48		74.1 ± 3.9	85.6 ± 2.3	90.1 ± 5.6
	72		88.3 ± 4.2	97.8 ± 0.1	97.5 ± 1.1
Class II (I-$A^{b,d}$)	0	0.1 ± 0.1			
	24		11.0 ± 3.6	26.0 ± 2.9	54.8 ± 6.3
	48		60.7 ± 5.5	91.1 ± 5.7	93.9 ± 2.5
	72		80.8 ± 8.1	97.2 ± 0.3	98.0 ± 0.4
ICAM-1	0	4.9 ± 1.4			
	24		11.1 ± 3.7	15.5 ± 4.8	16.3 ± 1.2
	48		12.4 ± 2.8	18.1 ± 2.3	31.9 ± 7.2
	72		36.1 ± 5.9	61.0 ± 7.7	64.9 ± 6.1
	96		35.4 ± 6.4	69.0 ± 8.4	72.4 ± 4.7

[a] Retinal glia primary cultures grown in chamber slides to semi-confluence were exposed to varying amounts of rIFN-γ for the times shown. Percentages were calculated by dividing the number of positive cells by the total cells counted and multiplying by 100. A minimum of 1000 cells/well were counted in duplicate for each treatment group; results are expressed as the mean percent positive cells ± S.E.M. Similar results were obtained for primary cultures of cells from the C57Bl/6 mouse strain, and in duplicate experiments with each cell type.

for 24 h had no effect on the expression of MHC Class II by retinal glia from either mouse strain. Primary BALB/c retinal glial cells appeared to be slightly more responsive to rIFN-γ than passaged glia, with increased percentages of cells positive for Class II antigens at 48 (> 90% positive) and 72 h (> 97% positive) following exposure to either 50 or 100 U/ml of rIFN-γ (Table 2) compared to 85% by the passaged cells. These differences may simply reflect the states of activation or differentiation of the unpassaged and passaged cells.

3.2. Upregulation of ICAM-1 expression on retinal glial cells

Less than 3% of the passaged retinal glia expressed ICAM-1 under baseline culture conditions (Fig. 5A). Exposure to 100 U/ml rIFN-γ increased ICAM-1 expression so that 62% of the passaged glial cells were positive by 72 h (Fig. 5B). Prior to 72 h, less than one-third of the primary and passaged retinal glia expressed ICAM-1 even at the highest dosage of rIFN-γ (100 U/ml). The greatest increases in ICAM-1 expression were detected on both primary and passaged cells 48–72 h after exposure to 50 or 100 U/ml rIFN-γ, when the percentage of positively staining cells more than tripled (Fig. 6, Table 2). Minimal increases in ICAM-1 expression were induced on primary cultures by longer exposure, and induction of ICAM-1 at the 25 U/ml concentration remained 50% less than observed for higher rIFN-γ concentrations. LPS had only a modest effect on ICAM-1 expression by retinal glia; less than 10% of the cells were positive after 24 h of exposure to LPS, although this represented a 3-fold increase over

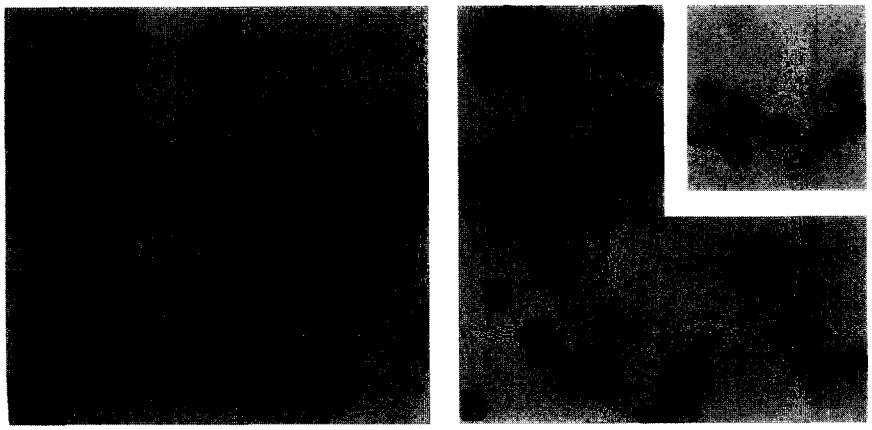

Fig. 3. Modulation of Class II antigen expression on BALB/c retinal glial cells. Retinal glia (passage 22) were stained by the ABC immunoperoxidase method for MHC Class II antigens after co-culture with rIFN-γ or ConA-conditioned medium by the ABC immunoperoxidase method. (**A**) Unstimulated cultured glial cells were negative for Class II MHC antigens (250 ×). (**B**) Exposure to 50 U/ml of rIFN-γ for 48 h induced expression of MHC Class II antigens by > 90% of cells (250 ×). Red reaction product is seen as black. Inset: BALB/c retinal glia exposed to rIFN-γ as in (**B**), but normal rat IgG was substituted for the anti-Class II antibody and no staining was observed.

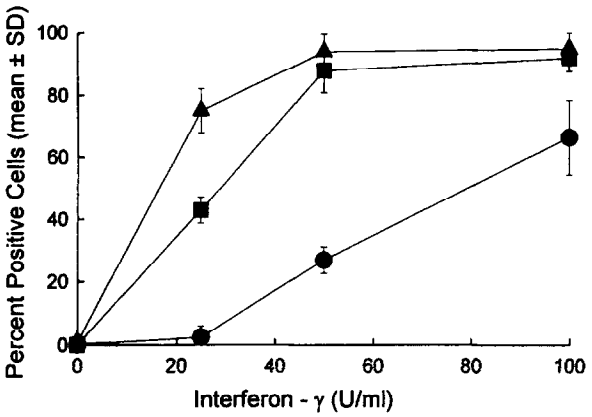

Fig. 4. rIFN-γ induces Class II antigen expression on BALB/c retinal glial cells. Passaged Müller cells (passage 19) were exposed to varying doses of rIFN-γ for 24 (circles), 48 (squares) or 72 (triangles) h prior to immunohistochemical staining. The percentage of positive cells was maximal after exposure to 50 U/ml of rIFN-γ for 48 h. The results shown are the mean ± S.D. of duplicate wells.

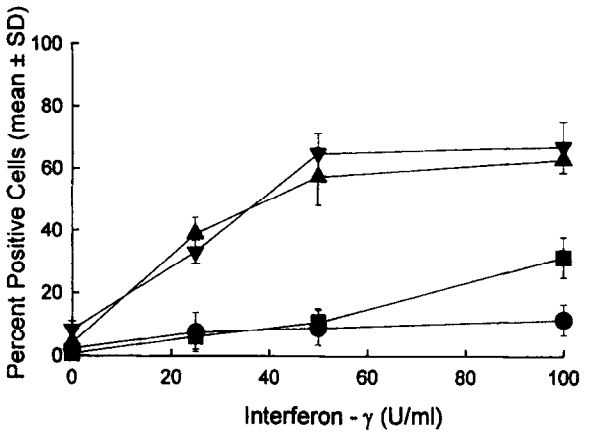

Fig. 6. rIFN-γ upregulates ICAM-1 on BALB/c retinal glial cells. Passaged retinal glia (passage 23) were exposed to rIFN-γ for 24 (circles), 48 (squares), 72 (triangles) or 96 (inverted triangles) h prior to immunohistochemical staining for ICAM-1. Maximal staining was induced by exposure to 50 U/ml of rIFN-γ for 72 h. Results are expressed as the mean ± S.D. of duplicate wells.

unstimulated cells. No alteration in ICAM-1 expression was observed following exposure of the cells to either rTNF-α (1000 U/ml) or rIL-6 (100 U/ml) for 24 h (not shown).

3.3. Immunohistochemical evidence of IL-6 and TNF-α production by cultured retinal glia

Unstimulated BALB/c retinal glia were weakly positive for IL-6 by immunohistochemistry (Fig. 7A). In contrast, cell-associated IL-6 was markedly upregulated by exposure to HSV (m.o.i. = 10; Fig. 7B) or LPS (10 μg/ml; not shown) for 24 h. Following exposure to rIFN-γ, IL-6 positive cells increased in a dose- and time-dependent manner (Fig. 8). While both passaged and primary cultured glia responded to rIFN-γ in a dose- and time-dependent manner, the maximal IL-6 responses were observed in unpassaged glia. At 72 h after exposure to 100 U/ml of IFN-γ, over 80% of the unpassaged cells were IL-6 positive (Table 3) compared to only 60% of the passaged cells (Fig. 8). Differences in response levels may relate to the states of cell activation at the time of exposure to rIFN-γ.

Approximately 60% of unstimulated glial cells expressed TNF-α as detected by immunohistochemistry. rIFN-γ (50 U/ml for 48 h) increased the percentage of cells staining positively to over 95%. However, two populations of TNF-α positive cells were apparent, one of

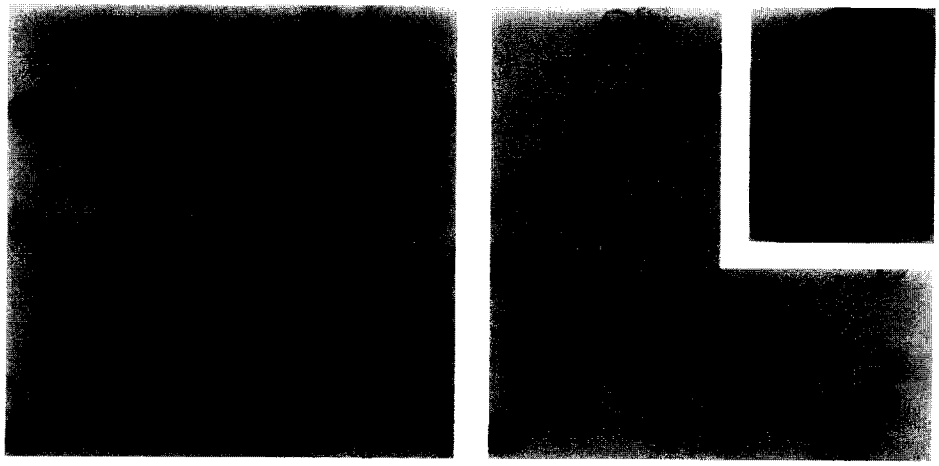

Fig. 5. ICAM-1 expression on passaged BALB/c retinal glial cells (passage 22). ABC immunoperoxidase staining was performed using cytocentrifuged cells. (**A**) Unstimulated glial cells expressed low levels of ICAM-1, with less than 5% of the cells staining positively (250 ×). (**B**) After exposure to 50 U/ml of rIFN-γ for 72 h, 60% of the cells expressed detectable ICAM-1 (250 ×). Inset: Higher power view of ICAM-1 ring-staining. Red reaction product is seen as black in these photographs. The Hematoxylin counterstain appears gray.

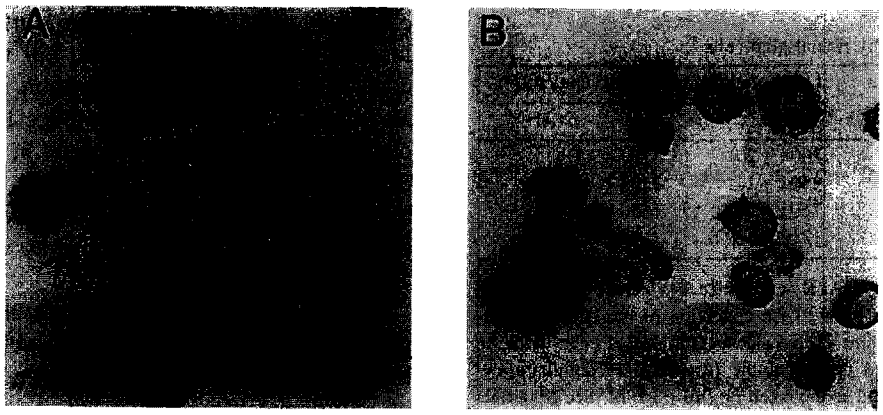

Fig. 7. HSV upregulates cell-associated IL-6 expression in cultured murine retinal glia. ABC immunoperoxidase staining for IL-6 was performed on cytocentrifuged cells. (A) Under standard culture conditions, retinal glia express low levels of IL-6 as demonstrated by immunohistochemistry. (B) After exposure to HSV for 24 h, over 90% of the retinal glia stain positively for IL-6.

which was highly positive and exhibited intense staining, and another which exhibited less intense staining (Table 4). Stimulation with LPS for 24 h induced similar staining for TNF-α.

3.4. HSV-1 and other stimulants upregulate IL-6 and TNF-α secretion by retinal glia

Capture ELISAs were performed to determine if TNF-α and IL-6 were secreted by the cultured retinal glia or if in vitro stimulation merely upregulated synthesis of the cell-associated form of these cytokines. Retinal glial cell culture supernatants were tested for altered secretion of TNF-α by capture ELISA after exposure to the stimulant for 24 h. Unstimulated retinal glia secreted low levels of TNF-α (200 pg/ml). LPS was a potent inducer of TNF-α secretion, and secretion increased by 6-fold over levels produced by unstimulated cells to 1210 pg/ml (Table 5). Co-culture with 50 U/ml of rIFN-γ for 24 h increased secretion of TNF-α by 3.6-fold to 710 pg/ml, whereas exposure to HSV-1 for the same period had a minimal effect on levels of TNF-α secreted.

IL-6 secretion was increased more markedly than TNF-α after exposure to the same stimuli. Basal levels of IL-6 secretion by the glial cells were 325 pg/ml. LPS was the most effective stimulator of cytokine secretion, with > 3000 pg/ml of IL-6 detected in the supernatants of the

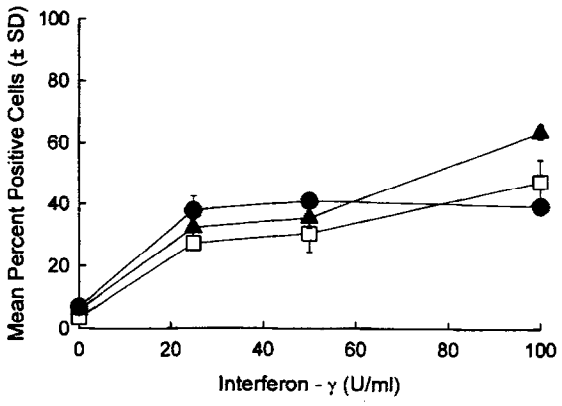

Fig. 8. Time- and dose-dependence of rIFN-γ-induced upregulation of IL-6 production by retinal glia. Passaged retinal glia (passage 28) were exposed to varying doses of rIFN-γ for 24 (circles), 48 (squares), or 72 (triangles) h prior to immunohistochemical staining for IL-6. Results are expressed as the mean ± S.D. of duplicate wells.

Table 3
rIFN-γ upregulates intracellular interleukin-6 production by primary BALB/c retinal glia [a]

Time of in vitro exposure (h)	Percent IL-6-positive cells at different concentrations of rIFN-γ (U/ml)			
	0	25	50	100
0	8.8 ± 0.3			
24		10.0 ± 0.9	15.1 ± 2.1	27.3 ± 1.4
48		36.5 ± 1.3	40.8 ± 1.5	60.0 ± 3.9
72		48.3 ± 4.4	44.2 ± 2.6	81.9 ± 4.8

[a] Semi-confluent retinal glia (flat cells) grown in chamber slides were exposed to varying amounts of rIFN-γ for the time shown. Percentages were calculated by dividing the mean number of positive cells by the total cells counted times 100. A minimum of 2000 cells were counted per treatment group (1000 cells/well) and results are expressed as the mean percent positive cells ± S.E.M.

Table 4
TNF-α responses of passaged BALB/c retinal glia to rIFN-γ and LPS [a]

Stimulus	Percent TNF-α-positive cells	
	High [b]	Low [b]
None	45.4 ± 2.7	14.8 ± 1.1
LPS	64.2 ± 2.1	31.3 ± 2.4
IFN-γ	72.9 ± 1.0	24.1 ± 1.5

[a] Semi-confluent retinal glia grown in T25 flasks were exposed to 10 μg/ml of LPS or 50 U/ml of rIFN-γ for 24 h. Percentages were calculated by dividing the mean number of positive cells by the total cells counted multiplied by 100. A minimum of 1000 cells were counted per treatment group; results are expressed as mean percent positive cells ± S.E.M.

[b] High and low represent intensity of staining.

Table 5
In vitro cytokine secretion by retinal glial cells [a]

Stimulant	Concentration	Level of cytokine detected (pg/ml)	
		TNF-α	IL-6
None	–	200 (±20)	325 (±45)
HSV	m.o.i. = 10	350 (±14)	750 (±46)
IFN-γ	50 U/ml	710 (±49)	1050 (±10)
LPS	10 μg/ml	1210 (±202)	> 3000 (±186)

[a] Cultured retinal glial cells (BALB/c; passage 14) were exposed to the stimulus indicated for 24 h prior to the collection of the culture supernatants for analysis by capture ELISA. Concentrations of each cytokine were derived from standard curves of the respective recombinant cytokines. Results are expressed as the mean pg/ml ± S.E.M. See Materials and methods for assay details.

glial cell cultures (a greater than 9.2-fold increase). rIFN-γ upregulated IL-6 secretion by more than 3-fold to 1050 pg/ml. IL-6 secretion levels increased more than 2-fold to 750 pg/ml following exposure to HSV. Although dose-response effects of these stimuli on secretion of IL-6 and TNF-α were not tested, the immunocytochemical results suggest that dose and duration of exposure would correlate with increased protein secretion.

4. Discussion

In the present study, murine retinal glia were shown to rapidly modulate their expression of surface molecules and production of selected cytokines associated with immune function in response to rIFN-γ, LPS, or HSV-1. Immunohistochemically detectable MHC class I and II antigens and the adhesion molecule, ICAM-1, were induced or upregulated by rIFN-γ or ConA-conditioned splenocyte medium in a dose- and time-dependent manner. In some species, surface marker expression varies in a strain-specific manner (Linke and Male, 1994). However, we found no difference between BALB/c and B6-derived retinal glia in their ability to upregulate surface molecule expression despite differences in their in vivo susceptibility to many pathogens including HSV (Lopez, 1975; Pepose and Whittum-Hudson, 1987). LPS, TNF-α, and IL-6, all shown to modulate surface marker expression by several cell types including cultured human CNS astrocytes (Springer, 1990; Satoh et al., 1991; Chang et al., 1994), had no effect on the expression of these surface markers by cultured murine retinal glia. Our results for IFN-γ-mediated induction of MHC antigens on murine retinal glia differ somewhat from those of Mano et al. (1991) who investigated the effects of IFN-γ on expression of MHC antigens by human retinal Müller (glia) cells using flow cytometry techniques. Those authors observed lower percentages of cells positive for MHC antigens after exposure to higher doses of recombinant human IFN-γ for longer times. Alterations in ICAM-1 expression were not tested for in that study. Our studies of retinal glia did not exclusively distinguish between surface and intracellular localization of MHC antigens, but the ring staining was consistent with surface localization. MHC antigens could also be seen in the Golgi complex after stimulation, indicating new synthesis. Differences in our results and those of Mano et al. (1991) may reflect differences between human and inbred mouse retinal cells, the ages of cell donors (adult vs. newborn mice), assay conditions, or other factors (Barres, 1991).

ICAM-1 expression influences the extravasation and migration of inflammatory cell as well as antigen presentation (Simmons et al., 1988; Springer, 1990). In the absence of an inflammatory response, most cells express low levels of adhesion molecules which are upregulated after exposure to inflammatory mediators including IFN-γ, TNF-α and IL-1 (Springer, 1990). The blood–brain barrier (BBB) and the blood–retinal barrier (BRB), once thought to be impermeable to cells and molecules, can be penetrated by activated T cells in non-disease states (Hickey et al., 1991; Prendergast et al., 1994). The end foot processes of the Müller cells are a component of the BRB, and increased adhesion molecule expression on these cells may promote lymphocyte/monocyte extravasation from the blood into the inflamed retina. The mechanism(s) for an altered BRB that allows lymphocytes to enter the neuroretina in experimental autoimmune uveoretinitis (EAU) has not been well defined, though ICAM-1 expression correlates with retinal pathology in EAU (Greenwood, 1992). Inappropriate ICAM-1 expression in the eye has been associated with other disease states in both mice and man. For example, ICAM-1 has been shown to be induced on cells of the iris and ciliary body in a murine model of endotoxin-induced uveitis, and disease is inhibited by in vivo treatment with anti-CD11b/CD18 antibody (Whitcup et al., 1993). In eyes of humans with sickle cell disease or diabetes, aberrant ICAM-1 localization in retina has been described at a site consistent with Müller cells (McLeod et al., 1995). MHC antigen and adhesion molecule expression on normally negative CNS astrocytes has been described in vivo during several pathological processes in humans (Traugott and Raine, 1985; Frohman et al., 1989a, b) and in experimental animals. Studies of the CNS have also shown altered surface marker expression during disease (Traugott and Raine, 1985; Haegel et al., 1993; Smith et al., 1993a). T lymphocytes are often present in these lesions and may provide the stimulus for such MHC antigen upregulation. Alternatively, pathology in some disease states is due to the overproduction of soluble mediators in the host, and adhesion molecules may provide a means of regulating cytokine production. In vitro studies have demonstrated that anti-ICAM-1 antibody profoundly inhibits the production of TNF-α, IFN-γ, and IL-1 by human mitogen-activated mononuclear cells (Geissler et al., 1990). This finding suggests that retinal glial cell cytokine production could be regulated through receptor–ligand interactions with infiltrating T cells. We have demonstrated that cul-

tured murine retinal glial cells produce the cytokines TNF-α and IL-6. The ability of the cells to express ICAM-1 may provide one means to regulate production of these cytokines.

Virus-mediated upregulation of surface molecules provides another possible mechanism by which Müller cells or other retinal glia could participate in local immune responses. ICAM-1 was upregulated on rat CNS astrocytes by in vitro exposure to measles virus (Massa et al., 1987). Both Class I and Class II antigens were induced on murine CNS astrocytes in vitro by West Nile virus infection (Liu et al., 1989). In vivo, CNS astrocytes of coronavirus-infected rats expressed Class II, even if animals were first IFN-γ-depleted, and support direct virus-mediated effects on MHC expression (Massa et al., 1987). Alternatively, virus-infected cells are susceptible to lysis by cytotoxic T lymphocytes if viral peptides are presented in the context of MHC Class I (Brodsky and Guagliardi, 1991). In vitro studies by others have shown that flavivirus-infected murine astrocytes are lysed by specific CTL (Liu et al., 1989). We demonstrated previously that cultured retinal glia and Müller cells in intact retina are susceptible to infection with HSV (Whittum-Hudson and Pepose, 1987; Merges and Whittum-Hudson, 1990), which combined with our demonstration of inducible MHC antigens support the potential of Müller cells to serve as CTL targets during infection. Direct anti-viral effects of Müller cells have not yet been demonstrated though both TNF-α and IL-6 are known to have anti-viral capacities in other systems (Wong et al., 1986; Wietzerbin et al., 1990). CNS astrocytes are known to produce these cytokines during HIV-1 infection (Genis et al., 1992), but it has not been determined if these mediators prevent or contribute to pathology.

The similarity of results with passaged and primary cultures of newborn retinal glial cells and the reproducibility with cells obtained from many independently established cultures suggest that the responses by these cells reflect the in vivo capacity of Müller cells to express and upregulate these surface markers and cytokines. Due to its relative immune privilege and limited access to systemic mediators, it is logical to suggest that resident retinal cell populations would contribute to control of infection and inflammation via the production and release of soluble mediators. However, the retinal microenvironment may negatively influence the in vivo functions of Müller cells by local production of downregulatory molecules such as transforming growth factors. Studies by Caspi et al. (1987) demonstrated that rat Müller cell lines continuously exposed to ConA-conditioned splenocyte media in vitro were immunosuppressive for T cell proliferation, apparently because of IL-1 secreted by the Müller cells (Roberge et al., 1988); other cytokines were not tested for in the latter study. Whether the observed suppressive effect of cultured rat Müller cells is operative in vivo for inflammatory or resident retinal cells is currently unknown. Many cytokines, including TNFα and IL-6, have been identified within the eye during inflammatory responses, and intraocular injection of these and other cytokines has been shown to induce uveitis (reviewed by De Vos et al., 1992). In order to better understand control mechanisms which could be invoked to counter inflammatory reactions within the eye, it will be important to distinguish between resident and infiltrating cells as the source(s) of immunoregulatory cytokines and to determine the temporal relationships of resident cell-derived mediators and those produced by inflammatory cells reaching the retina to altered surface molecule expression on these populations. These issues have been addressed in studies of experimental and clinical uveitis (e.g. Whitcup et al., 1992), but not during HSV-induced ocular inflammation.

Both IL-6 and TNF-α have known roles in the rescue of photoreceptor cells during retinal degeneration in the Royal College of Surgeons rat (LaVail et al., 1992). These cytokines also have opposing roles in induction of apoptosis (e.g. Smith et al., 1994). Thus, retinal Müller cells may play a role in maintenance of normal retinal integrity via their secretion of TNFα, IL-6, and other soluble mediators commonly associated with immunological function. The dynamics of surface marker modulation and altered cytokine secretion by Müller cells in these normal and pathological retinal processes remain to be elucidated. Studies are in progress to further define the key functions of these cells in intraocular immune responses.

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