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Differential effects of Sendai virus infection on mediator synthesis by mesangial cells from two mouse strains

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Differential effects of Sendai virus infection on mediator synthesis by mesangial cells from two mouse strains.

Background. Recently, we observed that the severity of glomerulonephritis in an experimental model of immunoglobulin A nephropathy (IgAN) induced by Sendai virus differs between C57BL/6 and BALB/c mouse strains. The determinants of differing renal insufficiency are not understood. In the present study, we examine the capacity for mesangial cells to support Sendai viral replication and assess the direct effects of Sendai virus on the production of selected cytokines, chemokines, and eicosanoids by mesangial cells, comparing C57BL/6 to BALB/c mouse strains.

Methods. Sendai virus replication was measured by viral plaque assay using LLCMK2 cells. Production of cytokines [interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α)], chemokines (JE and KC), and eicosanoids [prostaglandin E_2 (PGE$_2$) and thromboxane B_2 (TxB$_2$)] in culture medium was evaluated by sandwich enzyme-linked immunosorbent assay (ELISA) or competitive enzyme immunoassay (EIA) after 48 hours' incubation with infectious or inactivated Sendai virus.

Results. Sendai virus replicates equally well in mesangial cells from both strains, and infection evokes increased IL-6, JE, KC, and PGE$_2$ production in relation to viral dose. BALB/c mesangial cells produce significantly more IL-6 and JE than those from C57BL/6, and the dose response for KC is steeper in BALB/c mesangial cells than those from C57BL/6. Synthesis of PGE$_2$ in BALB/c mesangial cells is higher than that of C57BL/6 mesangial cells, both under basal conditions and in response to infectious Sendai virus, again in a dose-dependent manner. There is no TNF-α or thromboxane response to viral stimulation.

Conclusion. We conclude that different mesangial cell responses to this common mucosal viral pathogen might influence the severity of IgAN in our model system.

Immunoglobulin A nephropathy (IgAN), the most common form of glomerulonephritis in the world, is a mesangial proliferative glomerulonephritis characterized by granular deposition of predominantly IgA and C3 in the glomerular mesangium [1–5]. Although IgAN is now recognized as a major cause of end-stage renal disease (ESRD), its pathogenetic mechanisms remain obscure [1–5]. To date, the characteristics of the deposited IgA as antibody and the functional responses of mesangial cells to this IgA, other immunoglobulin co-deposits, complement, and antigens have been extensively investigated [1–5].

Most patients with IgAN initially manifest disease and develop exacerbation in association with acute illnesses, apparently infections, involving the respiratory and/or gastrointestinal tract. Several lines of evidence suggest that IgAN represents an immune complex disease instigated by specific IgA antibody bound to antigens derived from mucosal pathogens [1–4, 6]. However, only a small minority of individuals with mucosal infections develops IgAN. Therefore, some research on the pathogenesis of IgAN has focused on regulation of the mucosal immune response to viral or bacterial pathogens [1, 2, 7–9]. Recently, the principle that defective regulation of mucosal immunity underlies the genesis of IgAN [1, 2, 4] has gained wider acceptance and clinical corroboration [10–12]. We recently developed a murine model of IgAN induced by Sendai virus, a parainfluenza virus similar to human respiratory viruses [13], to probe infection-related IgAN and identify mechanisms potentially responsible for IgAN [1, 2, 6, 9]. In this experimental system, the frequency of nephritis is similar among several mouse strains, but BALB/c mice develop more severe nephritis with acute renal insufficiency than C3HeB and C57BL/6 mice, which rarely develop renal insufficiency [9]. Since glomerular mesangial immune deposits predominate both in the murine model induced by Sendai virus and in naturally occurring IgAN in patients [1, 2], interaction of mesangial cells with virus could be a critical determinant of disease. Accordingly, we postulated that geneti-

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cally determined differences in the response of mesangial cells to virus or viral antigens underlie the different severity of nephritis that develops in different mouse strains upon Sendai virus infection. Therefore, we compared the capacity for Sendai virus to generate a productive infection of mesangial cells and/or elicit release of inflammatory mediators from C57BL/6 and BALB/c mesangial cells.

In response to diverse stimuli, mesangial cells produce a broad variety of inflammatory mediators [14–26], many of which are increased in renal tissue and/or urine in glomerulonephritis. Moreover, there is evidence that some of these factors play functional roles. Notably, there is increased interleukin-6 (IL-6) expression by renal tissues in IgAN; production of IL-6 appears to both induce and serve as a marker of mesangial cell proliferation [19, 21, 27–29]. Furthermore, the production of chemokines such as monocyte chemoattractant protein-1 (MCP-1), IL-8, and interferon-induced protein 10 (IP-10) by mesangial cells in IgAN and other glomerulonephritides might contribute to infiltration of macrophages or neutrophils into glomeruli, and thereby modulate glomerular damage [14, 22, 25, 26, 30–33]. Therefore, we focused on the potential for mesangial cells to produce inflammatory mediators implicated in pathogenesis in response to stimulation with Sendai virus, comparing C57BL/6 to BALB/c mesangial cells.

METHODS

Cell cultures

Mesangial cells were cultured from isolated mouse glomeruli, essentially as previously described [14, 34]. Renal cortices (10 mice per primary culture) from female C57BL/6 or BALB/c mice (The Jackson Laboratory, Bar Harbor, ME, USA), 6 weeks of age, were dissected from the medulla and capsule, minced to a paste, gently pressed through a 75 μm steel sieve and then suspended in Hanks' balanced salt solution (HBSS) (Life Technologies, Rockville, MD, USA) without calcium or magnesium. The suspension was passed repetitively through a 26-gauge needle and through a 106 μm steel sieve to remove large tissue fragments. Glomeruli, retained on a 70 μm nylon sieve, were incubated with 750 U/mL collagenase (type IA) (Sigma Chemical Co., St. Louis, MO, USA) in HBSS without calcium or magnesium at 37°C for 25 minutes. After centrifugation (200 $\times$ g) (SORVALL®, RT 6000B) (Kendro Laboratory Products, Newtown, CT, USA) at 4°C for 3 minutes, glomeruli were resuspended in 6 mL of RPMI 1640 (Life Technologies) supplemented with ITS Pre-mix® (0.55 μg/mL transferrin, 1 μg/mL insulin, and 0.67 ng/mL selenium-A) and with 100 U/mL penicillin, 100 μg/mL streptomycin, and 17% fetal bovine serum (FBS) (all supplements from Life Technologies). Equal volumes (1 mL) of this suspen-

sion were plated into individual wells in a 6-well culture plate. Cultures were incubated at 37°C in a 5% CO_2 conditioned humidified atmosphere, and subcultured in 75 cm^2 flasks using the same medium with 10% FBS.

Sendai virus

Sendai virus strain 52 (ATCC catalog No. VR-105, American Type Culture Collection, Rockville, MD, USA) was propagated in 11-day-old embryonated eggs as described [35, 36]. Virus was purified from allantoic fluid after ultracentrifugation and stored at -70°C. For inactivation, virus was treated for 18 hours at 4°C with 0.05% β-propriolactone, and then incubated for 1 hour at 37°C to detoxify β-propriolactone. Infectious virus was quantified by plaque assay, and the protein concentrations in both infectious and inactivated virus suspensions were determined as described [35].

Viral titers

LLCMK2 monkey kidney cells were propagated in medium 199 (M199) supplemented with 5% FBS, 2 mmol/L glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. Plaque assay for virus was performed as previously detailed [35, 36]. Mesangial cell supernatants were incubated with trypsin [four parts supernatant and one part 12.5 μg/mL trypsin] for 30 minutes at 37°C and added to LLCMK2 cells for 90 minutes at 37°C. The inoculum was removed, and the cells were overlaid with 2 mL of M199 containing no serum, 0.5% Bacto Agar (Difco Laboratories, Detroit, MI, USA), and 2.5 μg/mL of trypsin (Life Technologies). Plaques were visualized after 3 days by hemadsorption with a 0.001% suspension of freshly washed and pelleted guinea pig red blood cells. The virus titer for each sample (suspension, culture medium, or cell lysate) was calculated as the common logarithm of the number of plaques [35, 36].

Limulus amebocyte lysate test (LAL test)

Endotoxin was measured using an end point method with diazo-coupling (Pyrochrome®) (Associates of Cape Cod, Inc., Falmouth, MA, USA). Briefly, 50 μL of infectious or inactivated virus suspension or endotoxin standard at varying concentrations were incubated with 50 μL of Pyrochrome [a proprietary mixture of limulus polyphemus extract, dextran, ethylenediaminetetraacetic acid (EDTA), $CaCl_2$ and Boc-Leu-Gly-Arg-p-nitroanilide with 0.2 mol/L Tris (hydroxymethyl)-aminomethane HCl, pH 8] for 47 minutes at 37°C in 96-well microplates (Associates of Cape Cod, Inc.). After incubation, 50 μL of 0.4 mg/mL sodium nitrite in 0.48 N HCl, 3 mg/mL ammonium sulfamate, and 0.7 mg/mL N-(naphthyl) ethylenediamine dihydrochloride were added to each well. The optical density at 550 nm was determined in a microplate reader (Bio-Kinetics Reader Model EL312) (Bio-Tek Instruments, Inc., Winooski,

VT, USA). Endotoxin concentrations in unknown duplicate samples were determined by interpolation of the respective optical density into the standard curve.

Experimental protocols

Mesangial cells from C57BL/6 or BALB/c mice were cultured to near confluence in 6-well culture plates; cell growth was arrested in RPMI 1640 containing 0.5% FBS for 24 hours. The cells were incubated in 0.45 mL medium with various numbers of infectious (1.25, 2.5, 5, and 10 pfu/cell) or inactivated (5 virions/cell) Sendai virus, or with no virus added. After 2 hours' incubation, the supernatants were collected for analysis of prostaglandin E_2 (PGE_2) and thromboxane B_2 (TxB_2) content. The cells were washed once with culture medium, and 1 mL fresh culture medium was applied. In several independent pilot experiments comparing the response to 5 pfu/cell Sendai virus to medium alone (data not shown), all mediators reached peak levels after an additional 48 hours in culture. Accordingly, for the definitive experiments presented herein, supernatants and cells were harvested for various assays at this time point. Calcium ionophore A23187 (1 μg/mL) or recombinant mouse IL-1β (500 pg/mL) was added to some cultures as positive controls for eicosanoid, cytokine or chemokine production.

Eicosanoid synthesis

PGE_2 and TxB_2 content in supernatant from each culture well were measured in duplicate using a competitive enzyme immunoassay (EIA) system (Amersham Pharmacia Biotech Inc, Piscataway, NJ, USA) according to the manufacturer's instructions. Briefly, 50 μL of supernatant or standard was incubated for 1 hour at room temperature on a plate shaker with a fixed mixture of either mouse anti-PGE_2 antibody and PGE_2 conjugated to horseradish peroxidase (HRP) in wells precoated with a goat antimouse IgG or with rabbit anti-TxB_2 antibody and TxB_2- HRP in wells precoated with a donkey antirabbit IgG. After washing the plate, 150 μL of substrate solution [3,3′,5,5′ tetramethylbenzidine (TMB)/hydrogen peroxide in 20% (vol/vol) dimethylformamide, preequilibrated at room temperature] was added for 30 (PGE_2) or 15 (TxB_2) minutes. Following the addition of 100 μL 2 N sulfuric acid to all wells, the optical density at 450 nm was determined in a microplate reader (Bio-Tek Instruments, Inc., Winooski, VT, USA). PGE_2 and TxB_2 concentrations in unknown duplicate samples were determined by interpolation of the respective optical density into the appropriate standard curve.

Cytokine production

IL-6 and tumor necrosis factor-α (TNF-α) production were determined by sandwich enzyme-linked immunosorbent assay (ELISA) (DuoSet®) (R&D Systems, Min-

neapolis, MN, USA) according to the manufacturer's instructions. All incubations were performed at room temperature. Briefly, 96-well microplates were coated with 2 μg/mL rat antimouse IL-6 or 0.8 μg/mL goat antimouse TNF-α antibody in phosphate-buffered saline (PBS) overnight. After three washes (0.05% Tween 20 in PBS, pH 7.4), the plates were blocked by adding 1% bovine serum albumin (BSA), 5% sucrose in PBS with 0.05% NaN_3 to the wells for 1 hour. Plates were washed three times, and 100 μL of sample or standard was incubated for 2 hours. After three washes, biotinylated goat antimouse IL-6 antibody (200 ng/mL) or goat antimouse TNF-α antibody (300 ng/mL) in 1% BSA in PBS was incubated for 2 hours. Plates were washed three times and developed with an HRP-conjugated streptavidin, followed by substrate solution (1:1 mixture of TMB and hydrogen peroxide) (R&D Systems). Interferon-α (IFN-α) content in supernatant from each culture well was measured using a sandwich ELISA Kit with proprietary formulations, according to the manufacturer's instructions (R&D Systems). Based on pilot experiments, samples from incubations with infectious virus were diluted 1:2; all others were assayed undiluted. Cytokine concentrations in unknown duplicate samples were determined by interpolation of the respective optical density into the appropriate standard curve.

Chemokine production

The concentrations of JE and KC, the murine homologs of human MCP-1 and IL-8, respectively, were measured by sandwich ELISA according to the manufacturer's instructions (DuoSet®) (R&D Systems). Capture and detection antibodies for JE (0.2 μg/mL goat antimouse JE and 50 ng/mL biotinylated goat antimouse JE) or KC (2 μg/mL rat antimouse KC and 200 ng/mL biotinylated goat antimouse KC) were utilized for assays otherwise the same as those for cytokine measurement. Murine interferon-inducible protein-10 (IP-10) was determined using a previously published protocol [37].

Assessment of mesangial cell proliferation

Mesangial cell proliferation was determined at 48 or 72 hours of incubation by direct counting of trypan blue–excluding cells in a hemocytometer and, in parallel cultures, by bioreduction of a tetrazolium salt (CellTiter® 96 AQ$_{ueous}$ One Solution Cell Proliferation Assay) (Promega, Madison, WI, USA). Cellular protein was quantified by dye binding (BCA Protein Assay Kit) (Pierce, Rockford, IL, USA). To assess the effect of endogenous IL-6 on proliferation, parallel wells were incubated in the presence of 15 ng/mL monoclonal rat antimouse IL-6 neutralizing antibody (R&D Systems). As a positive control for IL-6–induced cell proliferation, 200 pg/mL of recombinant mouse IL-6 (R&D Systems) was added to additional culture wells.
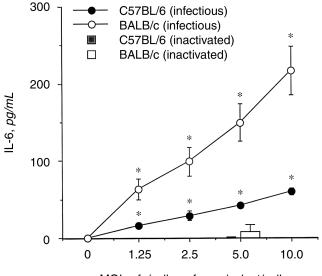
Statistics and data analysis

Two independent cell cultures were established from each of the two strains of mice at separate times from distinct groups of animals (four separate primary explant cultures in all). In each of the four experiments, triplicate culture wells for each of six incubation conditions (four levels of infectious virus, a single level of inactivated virus or medium control) were established. Each individual supernatant sample was assayed in duplicate for each analyte, and the arithmetic mean of the duplicates was taken as the result for the individual well. Three-way analysis of variance (ANOVA) (stratified for strain, incubation condition, and experiment) and two-way ANOVA (stratified for the combination of strain and incubation condition and for experiment) indicated that there was no significant difference between the two replicate experiments performed with each of the two strains, except for PGE$_2$ production by BALB/c cells. Accordingly, the data from the two experiments within each strain were pooled for all but PGE$_2$ synthesis by BALB/c, and the data plotted in the figures are the mean ($\pm$ SD) for the six replicate wells (three in each of two experiments) for each condition in each strain. Three-way ANOVA (stratified for strain, incubation condition, and experiment) and two-way ANOVA (stratified for strain and incubation condition, with the two experiments for each strain combined) indicate a statistically significant effect for strain and incubation condition. All t statistics quoted are Dunnett's post-hoc ts from one-way ANOVA (Statview II, Abacus Concepts, Inc., Berkeley, CA, USA); each stratification group represents a particular combination of strain and incubation condition. Unless otherwise specified (e.g., PGE$_2$ results), effects are considered statistically significant only if the comparison is significant by one-way ANOVA for each independent experiment separately (three replicates per incubation condition in each of the two experiments) and for both experiments pooled (i.e., six replicates per incubation condition).

RESULTS

Viral titers in culture medium of mouse mesangial cells

No virus is recovered from mesangial cells inoculated with inactivated Sendai virus (5 pfu equivalents/cell) or medium controls. In contrast, copious virus is recovered from mesangial cells inoculated with infectious (5 pfu/cell) Sendai virus ($t > 44$, $P < 0.001$ versus medium control or cells incubated with inactivated virus), with no difference between C57BL/6 and BALB/c mesangial cells [3.66 $\pm$ 0.08 and 3.69 $\pm$ 0.04 log (pfu/mL), respectively, data not shown].

Endotoxin is undetectable (less than 0.005 endotoxin units (EU) per mL) in suspensions of either infectious or inactivated Sendai virus (data not shown).

Fig. 1. Interleukin-6 (IL-6) production by cultured mesangial cells stimulated with Sendai virus. IL-6 production by C57BL/6 or BALB/c mesangial cells stimulated with infectious Sendai virus is significantly higher than with medium alone and increases in a dose (MOI)-dependent manner (*$t \geq 5.7$ versus the value produced by cells of the same strain cocultured in medium only, MOI = 0 and $t \geq 3.3$ versus the values of the immediately lower MOI within the same strain). Mesangial cells from BALB/c mice produce significantly more IL-6 than those from C57BL/6 after infectious Sendai virus stimulation ($t \geq 6.0$ between strains at the same MOI). Inactivated virus added at an MOI of 5 has no effect (P = NS versus medium control for both mouse strains). Data are expressed as mean $\pm$ SD of two independent experiments for each strain (i.e., for each plotted point $N = 6$, three for each independent experiment), harvested at 48 hours after 2 hours' incubation with virus (most errors for C57BL/6 are too small to be visualized). Statistical significances shown are also attained within each individual experiment.

Cytokine responses to Sendai virus

Both C57BL/6 and BALB/c mesangial cells produce increasing IL-6 in relation to the dose, or multiplicity of infection (MOI), of infectious Sendai virus added (Fig. 1). At all MOI, IL-6 significantly exceeds the level produced by cells in medium only. Mesangial cells from BALB/c mice produce significantly more IL-6 in response to infectious virus than those from C57BL/6, even though C57BL/6 and BALB/c cells produce identical amounts of IL-6 in response to IL-1β (~111 pg/mL, data not shown). Inactivated Sendai virus (at 5 pfu equivalents/cell) elicits no increase in IL-6 production by either C57BL/6 or BALB/c mesangial cells relative to the basal IL-6 level.

No TNF-α production is detectable in cultures of mesangial cells of either mouse strain under any conditions tested, even if stimulated by IL-1β (data not shown).

As anticipated, infectious, but not inactivated, virus evokes heavy production of (type I) IFN-α. Supernatants from C57BL/6 or BALB/c mesangial cells incubated for 48 hours after infection with 5 MOI of Sendai virus contain 57.5 $\pm$ 4.7 pg/mL or 915 $\pm$ 54 pg/mL IFN-α,
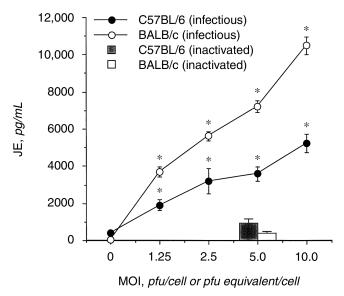
Fig. 2. JE production by cultured mesangial cells stimulated with Sendai virus. JE production by C57BL/6 or BALB/c mesangial cells stimulated with infectious Sendai virus is significantly higher than in medium alone, and increases as a function of the MOI (*$t > 6.3$ versus the value for the same strain in medium only, MOI = 0 and $t > 5.5$ versus the values of the immediately lower MOI within the same strain), except for 5 MOI applied to C57BL/6 cells. Mesangial cells from BALB/c mice produce significantly more JE than those from C57BL/6 after infectious virus exposure (all $t > 8.9$ between strains at the same MOI). Inactivated virus added at an MOI of 5 has no effect (P = NS versus medium control for both mouse strains). Data are expressed as mean ± SD of two independent experiments for each strain, harvested at 48 hours after 2 hours' incubation with virus. Statistical significances shown are also attained within each individual experiment.

Fig. 3. KC production by cultured mouse mesangial cells exposed to Sendai virus. KC production by C57BL/6 or BALB/c mesangial cells stimulated with higher doses of infectious Sendai virus is significantly higher than in medium control, and increases at higher MOI (*$t > 4.1$ versus the value for the same strain in medium only, MOI = 0 and $t > 2.7$ versus the values of the immediately lower MOI within the same strain). No significant difference in KC production by C57BL/6 mesangial cell between infectious and inactivated virus stimulation at 5 virions/cell. Mesangial cells from C57BL/6 mice produce significantly more KC than those from BALB/c with 1.25, 2.5, and 5 virions/cell, but BALB/c cells produce more KC at 10 virions/cell ($t \geq 2.3$ between strains within the same MOI stimulation). Data are expressed as mean ± SD of two independent experiments for each strain, harvested at 48 hours after 2 hours' incubation with virus. Statistical significances shown are also attained within each individual experiment. However, addition of 1.25 pfu/cell to BALB/c mesangial cells elicits a statistically borderline KC response; specifically, although significance is not achieved in either of the two experiments individually, KC is significantly increased when the two experiments are pooled.

respectively ($t = 62$, $P < 0.001$ for C57BL/6 versus BALB/c), but all supernatants from cells incubated with 5 MOI equivalents of inactivated virus or maintained in culture medium alone are below the detection threshold of 12.5 pg/mL ($t > 50$, $P < 0.001$ for both BALB/c controls and $t > 2.6$, $P < 0.05$ for C57BL/6 controls versus cells infected at 5 MOI, data not shown).

Chemokine responses to Sendai virus

Infectious Sendai virus stimulates JE production by either C57BL/6 or BALB/c mesangial cells, in relation to the dose (MOI); all stimulated cultures are significantly higher than medium controls (Fig. 2). Basal production of JE by C57BL/6 mesangial cells (421.3 ± 57.1 pg/mL) is tenfold that produced by BALB/c cells (44.0 ± 8.4 pg/mL), and IL-1β (data not shown) stimulates C57BL/6 mesangial cells to produce fourfold more JE (7460.5 ± 2122.3 pg/mL) than BALB/c cells (1832.1 ± 484.7 pg/mL). Yet, at all MOI tested, BALB/c cells produce significantly more JE in response to virus than C57BL/6 cells. When JE production is normalized to total cellular protein content, the difference between the larger (and more protein-rich) BALB/c cells and the smal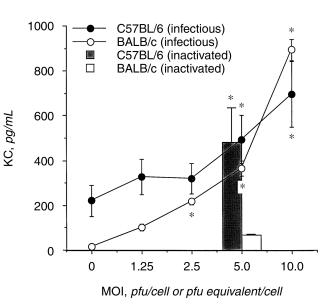ler C57BL/6 cells is eliminated (data not shown). No significant increase in JE production above control is elicited from either C57BL/6 or BALB/c mesangial cells by inactivated Sendai virus.

Production of KC by both C57BL/6 and BALB/c mesangial cells is significantly higher than basal upon stimulation with higher levels (MOI = 5 or 10 pfu/cell) of infectious Sendai virus (Fig. 3). Basal production of KC by C57BL/6 mesangial cells (220.1 ± 69 pg/mL) is 17-fold higher than that by BALB/c mesangial cells (13.3 ± 3.4 pg/mL), and IL-1β elicits threefold higher KC from C57BL/6 mesangial cells (9980.8 ± 936.3 pg/mL) than from BALB/c cells (3513.8 ± 802.2 pg/mL, data not shown). Nonetheless, only BALB/c cells produce significantly increased KC in response to lower MOI (2.5 and perhaps 1.25 pfu/cell), and the effect of dose upon KC response is significantly steeper with BALB/c mesangial cells than with C57BL/6 cells at the higher MOI. Inactivated Sendai virus (5 pfu equivalents/cell) elicits heightened KC production by C57BL/6, similar to (P = NS) an equal amount (5 pfu/cell) of infectious virus, but
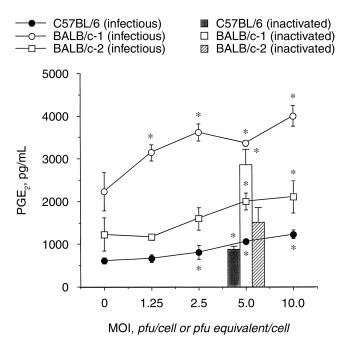
Fig. 4. Prostaglandin E₂ (PGE₂) synthesis by cultured mouse mesangial cells stimulated with Sendai virus. At all but the lowest dose of virus (MOI =1.25), PGE₂ synthesis by C57BL/6 mesangial cells (data are mean ± SD from two independent experiments which did not differ from each other) stimulated with infectious Sendai virus is significantly higher than control, and increases in relation to the MOI (*t > 3.3 versus the value for the same strain in medium only, MOI = 0 and t ≥ 2.4 versus the values of the immediately lower MOI within the same strain). PGE₂ synthesis by BALB/c mesangial cells (data are mean ± SD from each of two independent experiments) exposed to infectious Sendai virus is significantly higher than in medium alone (*t > 3.3 versus the value for the same strain in medium only, MOI = 0), except for 1.25 and 2.5 MOI in experiment 2. Although PGE₂ synthesis increases in relation to the MOI, significant dose effect is recognized only at 10 MOI in experiment 1 (t ≥ 2.4 versus the values of the immediately lower MOI within the same strain). Mesangial cells from BALB/c mice synthesize significantly more PGE₂ than C57BL/6 cells under basal conditions and in response to infectious and inactivated virus (all t ≥ 3.2 versus between strains within the same MOI stimulation).

does not significantly influence BALB/c mesangial cells (Fig. 3).

Infectious Sendai virus (5 pfu/cell) evokes synthesis of IP-10 from both C57BL/6 (4050 ± 1115 pg/mL) and BALB/c (9044 ± 1885 pg/mL) mesangial cells (both $t > 6.9$, $P < 0.001$ versus syngeneic medium controls). Inactivated virus stimulates trivial (< 700 pg/mL) levels that do not differ significantly from the lower limit of detection for the assay (50 pg/mL) or from medium controls. Again, BALB/c cells respond to virus infection significantly ($t = 9.3$, $P < 0.001$) more vigorously than C57BL/6 mesangial cells.

Eicosanoid responses to Sendai virus

PGE₂ synthesis by C57BL/6 mesangial cells upon stimulation with 2.5 pfu Sendai virus/cell increases significantly (by 30%), and increases further (by 75% and 108%) at higher MOI (5 and 10 pfu/cell), relative to medium control (Fig. 4). PGE₂ synthesis by C57BL/6

cells in response to inactivated virus (5 pfu equivalents/cell) is also significantly higher (by 40%) than control, albeit significantly less than the response (75% increase relative to basal) to the same number of infectious virions. Synthesis of PGE₂ by BALB/c mesangial cells varies widely among experiments; we show the results of two independent representative experiments with BALB/c mesangial cells individually (Fig. 4). At 5 and 10 pfu/cell, respectively, infectious virus elicits ~55% and ~75% increases in PGE₂ synthesis compared to basal levels in multiple experiments, numerically less than but similar in character to the response by C57BL/6 mesangial cells. In contrast to C57BL/6 cells, lower doses (1.25 and 2.5 pfu/cell) of infectious virus and inactivated virus at an MOI of 5 exert inconsistent effects on PGE₂ synthesis by BALB/c cells. Basal synthesis of PGE₂ by BALB/c mesangial cells is consistently two to three-fold higher than by C57BL/6 mesangial cells, but the relative response to calcium ionophore A23187 is higher in C57BL/6 (4912.2 ± 110.2 pg/mL, eightfold basal) than in BALB/c cells (4930.3 ± 1411.3 pg/mL, threefold basal). Total stimulated production is approximately equal in the two strains.

No change in TxB₂ synthesis is observed in response to any of the stimuli by either strain of mice (data not shown).

Mesangial cell proliferative responses to Sendai virus

By direct enumeration, BALB/c mesangial cells incubated with infectious virus (5 pfu/cell) exhibit 22% or 24% greater proliferation at 48 and 72 hours, respectively, compared to control cells cultured in medium for the same period. Viability (by trypan blue exclusion) remains high and does not differ across incubation conditions or over time. Exogenous IL-6, added at a concentration (200 pg/mL) somewhat higher than that achieved in supernatants from cells incubated with infectious virus, elicits proliferation (20% greater than medium control) similar to that instigated by infectious virus itself at 72 hours (Table 1). Collateral assessments of cell growth produce qualitatively similar results (data not shown). Specifically, over 48 hours, infectious virus elicits a 15% increase in the reduction of a proprietary tetrazolium dye relative to cells in medium alone ($t = 4.63$, $P < 0.001$). The dye reduction closely correlates with but is not identical to cell replication assessed by counting or DNA content. By 72 hours, however, this signal wanes, and cells incubated in medium or with infectious virus exhibit similar degrees of dye reduction ($P = NS$). Infectious virus (MOI = 5) also stimulates 42% to 63% higher cellular protein content compared to medium controls in BALB/c mesangial cells at 48 and 72 hours, respectively. Protein content in C57BL/6 mesangial cell lysates is 40% higher at 48 hours after incubation with infectious virus compared to medium controls, but no further evaluation

Table 1. Mesangial cell proliferation exposed to Sendai virus

	48 hours		72 hours	
Incubation	Cell number ×10⁴/well	Inhibition %	Cell number ×10⁴/well	Inhibition %
Medium	26.6	NA[a]	26.0	NA
Infectious virus	32.5[b]	NA	32.1[b]	NA
Infectious virus + anti-IL-6 antibody	30.0	42	25.5[c]	100
IL-6 (200 pg/mL)	27.5	NA	31.1	NA

IL-6 is interleukin-6. Data are expressed as mean ± SD.
[a] NA: not applicable
[b] $t > 2.1$ vs. medium
[c] $t = 2.7$ vs. infectious virus alone, P = NS vs. medium

of C57BL/6 response is undertaken. The protein content of the viral suspensions for these experiments is much less than 1% of the total protein recovered in the samples, and does not contribute significantly to the measured increase in protein content. Exogenous IL-6 increases dye reduction at levels similar to infectious virus, but has no significant effect on cellular protein content. Inactivated virus has no or inconstant effects on cell number, dye reduction or cellular protein content (data not shown). Although addition of exogenous blocking antibody specific for IL-6 inhibits the proliferative response to infectious virus at 72 hours, anti-IL-6 antibody inhibits the proliferative response at 48 hours by only 42% (Table 1).

DISCUSSION

In response to Sendai virus infection of the respiratory tract, immunized BALB/c and C57BL/6 mice experience a similar incidence of IgAN, yet the former develop more severe nephritis [9]. The purpose of the present study was to investigate potential differences between BALB/c and C57BL/6 mesangial cells in susceptibility to Sendai virus infection and consequent production of inflammatory mediators. Our data indicate that Sendai virus can infect mesangial cells, and in so doing up-regulates the synthesis and release of selected mediators believed to play a role in nephritis. Despite copious deposition of Sendai virus antigen in the mesangium, we cannot distinguish infection of mesangial cells from deposition of immune complexes in the experimental model [9]. However, if applicable in vivo, infection of mesangial cells by Sendai or other viruses may elicit mediator release, and thereby contribute to virally associated IgAN in both mice and humans. Interestingly, both mouse strains are equally susceptible to Sendai viral infection and fatal bronchopneumonitis in vivo [13], and viral replication does not differ between C57BL/6 and BALB/c mesangial cells in vitro. Nonetheless, BALB/c mesangial cells consistently produce more inflammatory mediators than C57BL/6 mesangial cells in response to viral infection. Therefore, the higher IL-6, JE, KC, and IP-10 responses by BALB/c cells are not explicable by the level of viral

replication. Somehow, the genetic background might control the metabolic response of mesangial cells [38]. Type I interferons are potent stimulators of a variety of cytokines and chemokines, with IL-6, MCP-1/JE, and IL-8/KC notable among them. In addition to IFN-α, IFN-β has very similar effects and is generally recognized as being regulated via the same pathways as IFN-α [39, 40]. Both IFN-α and IFN-β are prominent components of the response to infection of most mammalian cells by a wide array of viruses. Unfortunately, murine IFN-β is not accessible to assay at present. Although the expression of type I interferon we observed may well account for all of the other responses by mesangial cells we report in response to Sendai virus infection, it is important to note that many of these responses can ensue independently of interferon synthesis as well [41, 42]. Indeed, at the present time, reliable distinction between interferon-dependent and interferon-independent responses requires highly specialized transfected cell lines, and is impractical in essentially all primary cultures. Additional experiments beyond our present scope will be needed to clarify these issues.

Among a variety of cytokines implicated, IL-6 and TNF-α seem most likely to participate in the pathogenesis of glomerular injury in IgAN [1, 3, 4, 16–21, 24, 27–29]. IL-6, a multifunctional cytokine, promotes a wide variety of biologic responses, including cell proliferation. IL-6 may be an autocrine cofactor in the pathogenesis of mesangioproliferative glomerulonephritis such as IgAN, and there is correlation among IL-6 expression in renal tissues, the level of urinary IL-6, and disease activity [1, 3, 4, 17, 27–29]. Mesangial cells of both mouse strains produced IL-6 in response to stimulation by infectious but not inactivated virus. However, the higher IL-6 production by BALB/c mesangial cells compared to C57BL/6 cells might account for the different risk for renal insufficiency between the two strains. We observed herein a mitogenic effect of exogenous IL-6 similar to that reported previously by others [16, 17]. Moreover, at a dose that elicits IL-6 levels similar to those employed by others, Sendai virus promoted proliferation of mesangial cells. Although antibody that blocks IL-6 inhibited the

proliferative response to Sendai virus, the blockade was incomplete at earlier time points. This suggests that exogenous antibody is relatively ineffective in blocking autocrine, as compared to paracrine, growth stimulation. Alternately, infectious virus may elicit an early response that is independent of IL-6 and a later phase that requires IL-6 stimulation. This latter view is supported by the observation that IL-6 itself has little effect on cell number or dye reduction at 48 hours, and requires 72 hours for full effect. Although TNF-α exerts many effects on mesangial cells [1, 3, 4, 19, 21, 43], the potential for mouse mesangial cells to produce TNF-α is controversial [44, 45]. Under our experimental conditions, TNF-α production by mesangial cells was undetected, even after stimulation by IL-1β.

In IgAN, several studies suggest that the infiltration of monocytes/macrophages, in turn governed by adhesion molecules and chemoattractants, could contribute to the severity of glomerular injury [1, 3, 4, 25, 26, 30–33]. One member of the CC-chemokine family, MCP-1, is a specific and powerful chemoattractant for monocytes, and is produced by various cell types, including endothelial cells, vascular smooth muscle cells, mesangial cells, and renal tubular epithelial cells, as well as monocytes [25, 26, 30–33]. MCP-1 expression appears to promote monocyte migration into the kidney, and renal MCP-1 expression correlates well with the degree of the histologic changes, and particularly with the severity of tubulointerstitial lesions [25, 30–33]. Furthermore, urinary MCP-1 excretion, mesangial proliferation, and interstitial infiltration are well correlated in IgAN [31, 32]. Expression of JE, the murine homolog of human MCP-1, is increased by mesangial cells of both strains in response to infectious but not inactivated virus. Furthermore, BALB/c mesangial cells produced significantly more JE than C57BL/6 cells (on a per cell basis), even though C57BL/6 mesangial cells produce much more JE than BALB/c mesangial cells when stimulated with IL-1β. If the effects of Sendai virus on JE production by BALB/c compared to C57BL/6 mesangial cells apply in vivo, the greater production of JE might underlie the propensity for renal insufficiency in BALB/c mice.

Although basal production of KC by C57BL/6 mesangial cells is significantly higher than that of BALB/c, the responsiveness of BALB/c mesangial cells to infectious Sendai virus exceeds that of C57BL/6 cells. KC is one of the CXC chemokines and generally acts on neutrophils, but not monocytes, whereas the CC chemokines act primarily on monocytes. Several reports indicate that KC is expressed by mesangial cells in response to proinflammatory mediators and contributes to neutrophil activation and renal infiltration [46, 47]. In patients with IgAN, the CXC chemokine IL-8, a human homolog of mouse KC, is mainly observed in glomeruli, whereas MCP-1 is expressed principally in vascular endothelial cells, tubular epithelial cells, and infiltrating interstitial mononuclear cells [32]. Urinary IL-8 excretion is elevated only during the acute phase of IgAN and is correlated with glomerular endocapillary proliferation and the degree of hematuria [32]. Therefore, KC and JE might be involved in the pathogenesis of IgAN at distinct phases or in association with particular pathologic lesions. As for JE, the greater production of KC by BALB/c cells may also contribute to the higher risk for renal insufficiency of BALB/c compared to C57BL/6 mice [9].

Eicosanoids may also play a role in mediating glomerular hemodynamic and proinflammatory events in glomerulonephritis [23, 24, 48–50]. For example, increased PGE_2 may antagonize thromboxane- and/or vasoconstrictor peptide-induced reductions in glomerular filtration rate and renal blood flow, thereby preserving kidney function in glomerulonephritis [51]. Moreover, PGE_2 can regulate local cell growth and influence the synthesis and actions of cytokines produced by infiltrating and resident glomerular cells [23, 24, 52, 53]. Infection of mesangial cells by Sendai virus evokes enhanced synthesis of PGE_2, without affecting thromboxane. This may indicate a selective increase in PGE_2 isomerase activity or a plateau of thromboxane synthase because calcium ionophore A23187 did not significantly increase thromboxane synthesis by these mouse mesangial cells [48]. Of note, BALB/c mesangial cells produce more PGE_2 than C57BL/6 cells, both in response to Sendai virus and under basal conditions. Although increased PGE_2 synthesis has multiple effects upon kidney function and we could not draw any conclusion regarding the role of glomerular eicosanoids in this study, Sendai virus clearly alters arachidonate metabolism, which in turn might contribute to glomerular pathophysiology in vivo.

CONCLUSION

Cultured mesangial cells of either mouse strain produce eicosanoids, cytokines, and chemokines in response to infectious Sendai virus. Although several factors may contribute to renal insufficiency, the tendency of the BALB/c cells to produce higher levels of inflammatory mediators might underlie the proclivity of that strain toward renal insufficiency relative to C57BL/6 mice in the Sendai virus-induced model of IgAN. The critical determinants of the difference in renal insufficiency between strains might be related to this result, but the role of other factors in the pathogenesis of human IgAN such as the aberrant glycosylation of IgA molecules, immune complexes containing virus or viral proteins, and/or antigen-specific T cells, must also be explored.

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