Original Article

Molecular epidemiology and virulence characteristics of prevalent group A streptococci recovered from patients in northern India

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Abstract
Introduction: In this study, the prevalence of M types of Group A *Streptococcus* (GAS) in North India, invasive behavior of prevalent M types, and inflammatory immune response by host cells were studied.
Methodology: A total of 1,047 clinical samples were collected between 2004 and 2010. Confirmation of GAS was determined by serotyping and M types were identified by *emm* gene sequencing. The most prevalent serotypes were selected to study their invasive behavior and inflammatory immune response under different temperatures and salt concentrations in A549 and HEp-2 cells.
Results: Ninety-two isolates were identified as GAS of which 17 were M types with 18.5% heterogeneity. The most prevalent M types were M1 (21.73%) and M49 (8.7%), respectively. M1 and M49 were used to study virulence potential and inflammatory immune responses. The efficiency of cell infection decreased with increased temperature for both M types, increasing with lowering temperatures compared to the uninfected control (37°C). As salt concentration was increased, cell infection efficiency was lowered with some exceptions; the infection efficiency of M1 strain in A549 cells with 0.6 M NaCl was 50 fold higher ($p \leq 0.03$). Significantly increased production of IL-6 and IL-8 was observed in both cell lines infected with GAS and when grown under different environmental conditions compared to uninfected cell lines.
Conclusions: This study determined the prevalence of different M types in North India and showed that environmental conditions can regulate cell infection by GAS . This information may influence the selection of GAS serotypes used in vaccine development.

Key words: group A streptococcus; *emm* typing; invasiveness; cytokine; environmental regulation

Introduction

Group A streptococcus (GAS), or *Streptococcus pyogenes*, is a Gram-positive human pathogen. It causes a number of infections, such as pharyngitis, scarlet fever, impetigo, streptococcal toxic shock syndrome (STSS), rheumatic fever (RF), rheumatic heart disease (RHD), and acute glomerulonephritis, leading to high morbidity and mortality [1]. Several methods have been used to classify GAS into different serotypes, but M typing is the most accurate method known so far [2-7]. The *emm* gene of GAS encodes M protein, which is a major virulence factor [8]. The surface-exposed amino termini of M proteins are heterogeneous and provide the basis for identifying more than 150 different serologic M types [9].

Distribution of M types of GAS from several countries has been explored successfully [7,9-12] but few reports are available from India regarding the M type distribution of GAS [13-19]. The differences in serotype distribution among various populations may also reflect differences in pathogenesis among the serotypes. Ongoing monitoring of the distribution of GAS serotypes is important for charting changes in serotype prevalence and determining the components of an effective GAS vaccine. Furthermore, information regarding universal M type distribution does not only provide an insight into epidemiological data of GAS isolates, but is also useful for designing and developing vaccine strategies. Based on such epidemiological studies, the most prevalent M types from a particular region may be included in the development of a multivalent vaccine to prevent and control disease [20].

Innate proinflammatory responses to GAS infection include the synthesis and release of cytokines, prostaglandins, and nitric oxide. Cytokines

are also important mediators in host defenses against bacterial and viral infections [21]. In general, proinflammatory cytokines, such as TNF-α, IL-1β, IL-10, IL-6, IL-8, and IL-12p70, function as part of signaling cascades that orchestrate the recruitment and activation of inflammatory cells and the induction of effectors [21,22]. Invasion of bacteria into host tissue is mediated through a complex series of events that involves changes in surface proteome [23]. Apart from genotypic and phenotypic characteristics, environmental stimuli are also deciding factors for variable invasive behavior of GAS [24].

The present study was conducted to explore the epidemiology of GAS to determine the prevalent serotypes in the North Indian population from the clinical samples collected between 2004 and 2010 so that future vaccine preparations can be checked against the prevalent North Indian serotypes [7,20]. We also identified GAS types that cause throat and skin infections within this community. Furthermore, we studied the virulence potential of GAS grown under different environmental conditions using prevalent GAS M types by measuring the *in vitro* proinflammatory immune response by host cells against selected GAS strains. Infection efficiency was checked using a conventional method [25] under different temperature and salt stress conditions. We observed environmentally regulated infectious behavior of GAS and variable proinflammatory immune responses by host cells.

Methodology

Bacterial strains

An epidemiological survey of GAS infection in North India during 2004-2010 was performed using samples from four hospitals and five pathology laboratories in New Delhi and Jaipur city (Rajasthan). These pathology laboratories have sample collection centers in major as well as in minor (in terms of population) towns of North India. The study was approved by the human research ethics committee of the hospitals involved in the study. A total 1,047 samples were collected from patients with clinical manifestations similar to GAS infection, *i.e.*, sore throat, tonsillar and/or pharyngeal exudates, and/or pharyngeal erythema, skin eruptions/lesions. Clinical samples were subjected to preliminary examination for β-hemolysis on blood agar plates (BAP) incubated overnight at 37°C in 5% CO_2. Of the 1,047 specimens, 160 β-hemolytic streptococci were identified and these were further checked for the presence of GAS by serotyping using a commercially available antisera kit

(Streptex; Murex Biotech Ltd., Dartford, UK). All identified GAS isolates (n = 92) were used for M typing. All GAS isolates were grown in Todd Hewitt Broth (THB) (HiMedia Labs, Mumbai, India) at 37°C and 5% CO_2. Isolates were preserved with 10% glycerol and kept in a deep freezer at –80°C. Colony-forming unit (CFU) counts were estimated for all M1 and M49 serotypes grown at different temperatures (25°C, 30°C, 37°C and 41°C) and salt concentrations (0.3 and 0.6 M NaCl) when an OD_{650} of 0.5 was obtained in THB.

M typing

After genomic DNA was extracted manually using the cetyl trimethyl ammonium bromide method (CTAB) [26], the strains were typed by *emm* gene amplification using universal primers (*emm* forward 5' TATTCGCTTAGAAAATTAA 3' and *emm* reverse 5' GCAAGTTCTTCAGCTTGTT 3') as provided on the CDC website (http://www.cdc.gov/ncidod/biotech/strep/protocol_emm-type.htm). PCR products and sequencing primer *emm* seq2 (tattcgcttagaaaattaaaaacagg) were used exactly as described (Applied Biosystems, Foster City, USA). The amplified PCR product was sequenced by Microsynth (Microsynth, Balgach, Switzerland), and the homology of the DNA sequences were determined using the CDC *emm* database and by BLAST search analysis (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi). Pair-wise comparison of the nucleotide homology for the first 150 bases of the hypervariable region of matured M protein was conducted. Strains with ≥ 95% homology with the reference strain were designated the particular parental M type. Furthermore, subtypes were designated according to the information available on the CDC website (http://www.cdc.gov/ncidod/biotech/strep/assigning.htm). In brief, subtypes were assigned based on the exact match of 150 bases of matured M protein to already catalogued M types and subtypes in the CDC database.

Cell lines

The HEp-2 cell line (larynx carcinoma cell line; ATCC CCL-23) was obtained from the National Center for Cell Sciences, Pune, India, and A549 (human lung alveolar carcinoma epithelial cell line; ATCC CCL-185) was kindly provided by Prof. R. K. Saxena, School of Life Sciences, Jawaharlal Nehru University, New Delhi, India. HEp-2 [18] and A549 [27] cell lines have been previously used for invasion

experiments. These cell lines were maintained as described [27]. In brief, both cell lines were maintained in RPMI 1640 culture medium (GIBCO, Invitrogen, Carlsbad, USA) with 1% L-glutamine and 10% fetal bovine serum (FBS) (GIBCO) without antibiotics at 37°C with 5% CO_2 (CO_2 Incubator, Shel Lab, Sheldon Manufacturing Inc., Cornelius, USA). These were incubated for four days (A549) and three days (HEp-2) in 60 mm cell culture dish to reach the confluency (~90%). The medium was replaced one day before the experiment. By the beginning of the assays, the cell number had increased to approximately 3.5×10^6 cells per dish for A549 and 2.3×10^6 cells per dish for the HEp-2 cell line. Cell viability was determined by 0.4% trypan blue exclusion, and total cells were counted using a hemocytometer.

Cell culture Infection assay

Infection efficiency of all M1 (n = 20) and M49 (n = 8) strains grown at normal conditions (0.1 M NaCl, 37°C) were checked with A549 cells. One highly infectious strain belonging to each of M1.27 and M49.4 serotypes was selected to check its infectivity with A549 and HEp-2 cells and corresponding proinflammatory immune response by host cells under different environmental conditions.

The cell culture infection assay was performed using a conventional method with some modifications described elsewhere [25,27]. In brief, GAS strains (M1.27 and M49.4 strains) were grown at three different temperatures (25°C, 30°C, and 41°C) and two salt concentrations (0.3 and 0.6 M NaCl) to an OD_{650} of 0.5 in THB. GAS strains grown at the normal condition of 0.1 M NaCl and 37°C were used as controls in all infection and cytokine assays. Only GAS strains (M1.27 and M49.4) and not A549 and HEp-2 cells were grown under different environmental conditions for the experiments because the goal was to check the comparative change in infection behavior of GAS strains when grown under different environmental conditions, and not the host cells themselves. The bacteria were centrifuged (7000 *g* for five minutes at 4°C), and the pellet was suspended in RPMI 1640 (without FBS). The A549 and HEp-2 cells were cultured to reach to confluency (~90%) in 60 mm culture dishes and were infected at MOI (ratios of bacterial cell and mammalian cells) of 1:1 and placed in a 5% CO_2 incubator at 37°C. After two hours of incubation, infected monolayers were washed three times with phosphate-buffered saline (PBS, pH 7.4). Next, three mL of fresh RPMI 1640 medium with 10% FBS containing gentamicin (100 μg mL^{-1}) and

penicillin G (5 μg mL^{-1}) (HiMedia Labs, Mumbai, India) was added and plates were incubated in a 5% CO_2 incubator for two hours to kill extracellular bacteria. Monolayers were rinsed five times with PBS (pH 7.4) to remove antibiotics completely [27], and cells were detached by the addition of 0.2 mL of 0.25% trypsin-EDTA (GIBCO); the mixture was incubated for five minutes in a CO_2 incubator. Monolayers were lysed by the addition of Triton X-100 (Sisco Research Laboratories, Mumbai, India) (0.8 mL of 0.025% solution), transferred quantitatively to microtubes, and mixed on a vortex mixer for one minute. Appropriately diluted aliquots in 0.25% Triton X-100 were plated on tryptic soy 5% sheep BAPs. The plates were incubated at 37°C overnight. The number of intracellular GAS was determined by viable plate counts. The percentage of infecting GAS was calculated with the formula (CFU of intracellular GAS/CFU of original inoculum) x 100 [27]. Each test was done in quadruplicate.

Induction of cytokines from A549 and HEp-2 cells

Selected M1.27 and M49.1 strains were used in the experiments involving cytokine production by A549 and HEp-2 cells. Induction of cytokines was studied by growing M1 and M49 serotypes to an OD_{650} of 0.5 in THB media under the previously mentioned environmental conditions, infecting them at an MOI of 1:1 onto A549 and HEp-2 monolayers in duplicate, and allowing them to interact. The supernatant fluid was removed after two hours of incubation in a 5% CO_2 incubator at 37°C. Supernatant fluid from the dish containing no bacteria was taken as the negative control. The supernatant fluids were centrifuged and filtered through a 0.22 μm pore-size filter (Millipore, Billerica, USA) and were stored at –70°C for further use [28].

Cytokine assay

Cytokine production (IL-1β, IL-6, IL-8, IL-10, IL-12p70, and TNFα) was checked in culture supernatant collected after a two-hour interaction of M1 and M49 serotypes with A549 and HEp-2 cells. A Cytometric Bead Array (CBA) Human Inflammation Kit (BD Biosciences, Franklin Lakes, USA) was used to assay all six proinflammatory cytokines simultaneously. CBA technique is based on microparticles matched with antibodies, in which the particles are dyed to six different fluorescence intensities [29]. The dye has a maximal emission wavelength of approximately 650 nm (FL-3). The particles were matched by a covalent linkage with an antibody against one of the six

cytokines (IL-1β, IL-6, IL-8, IL-10, IL-12p70, or TNFα). When the particles are used as a mixture, six separate cytokines can simultaneously be detected. The cytokines are directly detected in the immunoassay using six different antibodies matched with a phycoerythrin (PE), which emits at 585 nm (FL-2). The PE-conjugated detector antibody is used to complete the sandwich and the medium intensity in FL-2 is proportional to the concentration of the cytokine in the sample, which is quantified from a calibration curve. An important characteristic of the assay system is that the calibrators, the primary reagent and the second antibody reagent, are each made of the mixture of all six cytokines. Six standard curves (standard ranging from 0 to 5000 pg mL^{-1}) were obtained from one set of calibrators and six results were obtained on one test sample. All samples were prepared in duplicate as per the manufacturer's instructions before data acquisition with flow cytometry.

Cytometric analysis

To detect the fluorescent dye labeled cytokine particles in the samples, cytometric analysis was conducted. Two-color cytometric analysis was carried out using FACS calibur flow cytometry (BD Biosciences, Franklin Lakes, USA). Data were obtained and analyzed by CBA software. The dot-plot forward *vs.* side scatter was used to distinguish between particles and to detect the cytokine particles. Data were analyzed in dot-plot FL-2 *vs.* FL-3; the six FL-3 particles dyed to different intensities were distributed along the BDy-axis. The concentration of the cytokine calibrators was expressed (y-axis) *vs.* medium fluorescence intensity (FL-2) (MFI) in the standard curves. The concentrations of cytokines that were below the limit of detection of the assay were given a zero value of concentration. Each assay tube and sample was analyzed on FACS calibur (BD Biosciences, Franklin Lakes, USA) and documented with the help of BD CBA software provided by BD Bioscience, USA.

Statistical analysis

The significance of GAS infection for two different variables was determined by the unpaired *t*-test with Welch correction. P values of < 0.05 were considered significant. These analyses were performed with Sigma Stat version 2.0 (USA).

Results

M typing

A total of 1,047 clinical samples were collected from different hospitals and pathology laboratories in North India between 2004 and 2010. A total 160 specimens showed β-hemolysis (15.28%) and, out of these, 92 isolates (57.50%) were identified as GAS. Clinical samples from patients with positive throat culture for GAS were diagnosed as pharyngitis and those with skin cultures positive for GAS were diagnosed as impetigo. A total 45 cases of pharyngitis and 47 cases of impetigo were recorded. A total 17 M types were identified, revealing 18.5% heterogeneity among all GAS isolates. The most prevalent M type found was M1 (21.73%), followed by M49 (8.7%), M42 (6.52%), and M48 (6.52%). Other reported GAS serotypes were M8, M15, M66, M74, M77, M78, M86, M100, M104, M112, M114, M116, and St1389. Moreover M15, M66, M74, M78, M104, M114, and St1384 were isolated exclusively from skin-infected patients and M1.13tss, M8, M49, M100, and M112 were isolated exclusively from throat-infected patients. All isolates were categorized into M types and subtypes and these results are summarized in Table 1.

Growth pattern and CFU counts

Infection efficiency of all M1 (n = 20) and M49 (n = 8) GAS strains grown at normal conditions (0.1 M NaCl, 37°C) were checked with A549 cells (data not shown here). Out of these strains, the most invasive strains belonging to each of M1.27 and M49.4 serotypes (both from pharyngitis patients) were selected to check infection efficiency with A549 and HEp-2 cells and corresponding proinflammatory immune response by host cells under different environmental conditions.

For further studies, growth pattern and CFU counts of selected M1 and M49 strains were checked under different environmental conditions such as salt stress (0.3, 0.6 and 0.9 M NaCl) and temperature stress (25°C, 30°C, and 41°C) and were compared with the control conditions of growth (37°C and 0.1 M NaCl) (Figures 1 and 2, Table 2). The growth rate decreased as the salt concentration was increased for other strains (Figures 1A and B). M1 serotype of GAS did not show any growth at the 0.9 M NaCl condition (Figure 1B). The growth rate decreased when serotypes M1 and M49 were grown at temperatures of 25°C and 30°C, but the rate increased when grown at 41°C compared to normal conditions (Figure 2).

Table 1. Distribution pattern of M serotypes of GAS isolates from North India

M type	Subtypes[Δ]	Symptoms[Δ]	No. of isolates
M1	M1.27 (4)	Pharyngitis (2), Impetigo (2)	20
	M1.tss (8)	Pharyngitis (5), Impetigo (3)	
	M1.13tss (8)	Pharyngitis (8)	
M8	M 8.0 (4)	Pharyngitis (3), Impetigo (1)	4
M15	M15.1 (4)	Impetigo (4)	4
M42	M42.1 (6)	Pharyngitis (2), Impetigo (4)	6
M48	M48.1 (6)	Pharyngitis (3), Impetigo (3)	6
M49	M49.4 (8)	Pharyngitis (6), Impetigo (2)	8
M66	M66.0 (4)	Impetigo (4)	4
M74	M74 (4)	Pharyngitis (2), Impetigo (2)	4
M77	M77.0 (4)	Pharyngitis (1), Impetigo (3)	4
M78	M78.0 (4)	Pharyngitis (1), Impetigo (3)	4
M86	M86.2 (4)	Pharyngitis (1), Impetigo (3)	4
M100	M100.3 (4)	Pharyngitis (4)	4
M104	M104.0 (4)	Pharyngitis (1), Impetigo (3)	4
M112	M112.2 (4)	Pharyngitis (4)	4
M114	M114.2 (4)	Impetigo (4)	4
M116	M116.1 (4)	Pharyngitis (2), Impetigo (2)	4
St1389	St1389.1 (4)	Impetigo (4)	4
Total	-	Pharyngitis (45), Impetigo (47)	**92**

[Δ]Number of isolates for each subtypes and symptoms are mentioned in parentheses

Table 2. CFU counts of GAS serotypes at 0.5 OD_{650nm} under normal and varied environmental conditions

Growth conditions	Average CFU ± SD [mL^{-1} (x 10^7)]	
	Serotype M1	Serotype M49
Normal[Δ]	14.6 ± 1.2	2.725 ± 0.14
0.3 M NaCl	38 ± 1.6	1.3 ± 0.18
0.6 M NaCl	2.3 ± 0.18	0.506 ± 0.023
25°C	3.76 ± 0.32	9.0 ± 0.72
30°C	7.0 ± 0.53	32.4 ± 4.6
41°C	0.28 ± 0.014	3.42 ± 0.37

[Δ]Normal conditions: 37°C and 0.1 M NaCl

Table 3. Infection into A549 and HEp-2 cells by M49 and M1 serotypes grown under varied environmental conditions

| GAS serotype	Environmental conditions	Cell culture infection assays			
		A549		HEp-2	
		Mean CFU±SD (x10^5) (p value)	%	Mean CFU±SD (x10^5) (p value)	%
M49	Normal[Δ]	1.30316 ± 0.156	3.72	1.00693 ± 0.058	4.38
	25°C	4.26579 ± 0.188 (< 0.05)	12.2	0.67920 ± 0.056 (< 0.05)	2.95
	30°C	1.67494 ± 0.275 (> 0.05)	4.78	1.99986 ± 0.199 (< 0.05)	8.69
	41°C	1.1994 ± 0.04 (< 0.05)	0.34	0.01199 ± 0.004 (< 0.05)	0.052
	0.3M NaCl	0.84918 ± 0.042 (< 0.05)	2.42	0.01069 ± 0.002 (< 0.05)	0.046
	0.6M NaCl	0.62086 ± 0.07 (<0.05)	1.77	0.53456 ± 0.049 (< 0.05)	2.32
M1	Normal[Δ]	0.02312 ± 0.0057	0.066	0.04677 ± 0.0016	0.203
	25°C	0 (< 0.05)	0	0 (< 0.05)	0
	30°C	0.00251 ± 0.0002 (< 0.05)	0.0072	0.04742 ± 0.007 (> 0.05)	0.206
	41°C	0.00105 ± 0.0002 (< 0.05)	0.003	0 (< 0.05)	0
	0.3M NaCl	0.00155 ± 0.0002 (< 0.05)	0.0044	0.00809 ± 0.001 (< 0.05)	0.035
	0.6M NaCl	1.26473 ± 0.108 (< 0.05)	3.61	0.03758 ± 0.007 (< 0.05)	0.163

[Δ]Normal conditions: 37°C and 0.1 M NaCl; *P values were calculated by unpaired *t*-test between control sets (infection efficiency of GAS strains grown under normal conditions) and respective experimental sets (infection efficiency of GAS strains grown under varied environmental conditions)

Table 4. Cytokine production by A549 and HEp-2 cells after two hours of stimulation with GAS M1 and M49 strains at varied environmental conditions

GAS serotype	Cell type	Conditions	Cytokine production in pg mL^{-1} ± SD (p values*)	
			IL-8	IL-6
M49	A549	Normal$^\Delta$	46.9 ± 29.42	2.00 ± 0.28
		0.3 M NaCl	96.25 ± 26.94 (> 0.05)	4.95 ± 1.20 (> 0.05)
		0.6 M NaCl	173 ± 31.11 (> 0.05)	1.8 ± 0.28 (> 0.05)
		25°C	10.2 ± 3.68 (> 0.05)	19.8 ± 4.24 (< 0.05)
		30°C	32.7 ± 0.565 (> 0.05)	ND (< 0.05)
		41°C	24.55 ± 0.92 (> 0.05)	9.1 ± 1.27 (< 0.05)
	HEp-2	Normal$^\Delta$	49.95 ± 3.32	184.05 ± 24.11
		0.3 M NaCl	51.2 ± 7.21 (> 0.05)	305.45 ± 31.04 (< 0.05)
		0.6 M NaCl	29.7 ± 9.76 (> 0.05)	204.40± 11.88 (> 0.05)
		25°C	122.6 ± 2.73 (< 0.05)	317.4 ± 12.73 (< 0.05)
		30°C	1.45 ± 0.64 (< 0.05)	82.8 ± 30.41 (> 0.05)
		41°C	104.0 ± 7.21 (< 0.05)	136.35 ± 9.12 (> 0.05)
M1	A549	Normal$^\Delta$	77.1 ± 15.27	ND
		0.3 M NaCl	84.9 ± 1.84 (> 0.05)	2.25 ± 0.64 (< 0.05)
		0.6 M NaCl	17.45 ± 0.00 (< 0.05)	1.4 ± 0.283 (< 0.05)
		25°C	9.95 ± 1.77 (< 0.05)	ND
		30°C	ND (< 0.05)	ND
		41°C	ND (< 0.05)	ND
	HEp-2	Normal$^\Delta$	21.00 ± 2.55	120.65 ± 28.50
		0.3 M NaCl	29.95 ± 9.97 (> 0.05)	124.80 ± 49.92 (> 0.05)
		0.6 M NaCl	8.55 ± 2.192 (< 0.05)	203.4 ± 9.33 (> 0.05)
		25°C	91.15 ± 8.70 (< 0.05)	133.7 ± 7.21 (> 0.05)
		30°C	11.9 ± 1.98 (> 0.05)	181.15 ± 15.77 (> 0.05)
		41°C	16.65 ± 5.02 (> 0.05)	55.45 ± 14.78 (> 0.05)

$^\Delta$Normal conditions: 37°C and 0.1 M NaCl; *P values were calculated by unpaired t-test between control sets (cytokine production by A549 and Hep-2 cells when infected with GAS strains grown under normal conditions) and respective experimental sets (cytokine production by A549 and Hep-2 cells when infected with GAS strains grown under varied environmental conditions)

Figure 1. Growth patterns of GAS M49 (A) and M1 (B) at different salt concentrations (Normal, 0.3, 0.6 & 0.9 M NaCl).
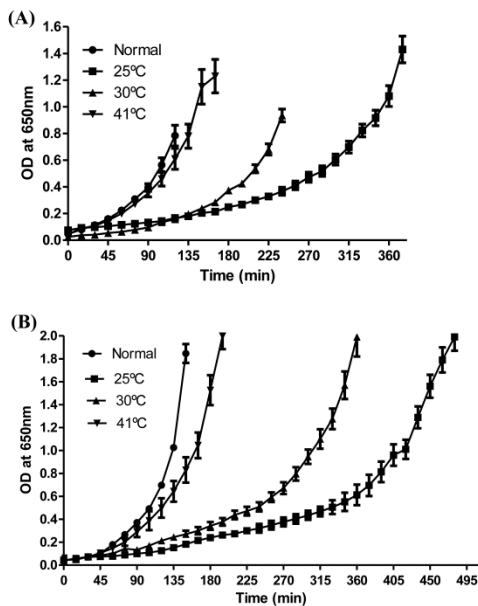
Figure 2. Growth patterns of GAS M49 (A) and M1 (B) at different temperatures (25, 30, 37 & 41ºC).
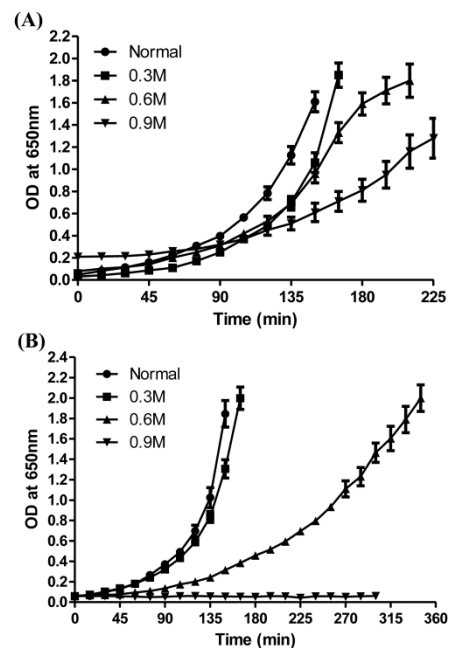
In the case of M1 serotype, CFU counts decreased as the salt concentration was increased from 0.1 to 0.6 M NaCl and decreased as the temperature deviated from 37°C. In the case of M49 serotype, CFU counts also decreased as the salt concentration was increased from 0.1 to 0.6 M NaCl, but increased as the temperature deviated from 37°C (Table 2).

Cell culture infection assays

All M1 strains were less infective with A549 cells than with M49 strains (data not shown). Amongst all M1 strains, the M1.27 strain was found to be more invasive than M1.tss and M1.13tss strains. Furthermore, the M1.tss and M1.13tss strains poorly invaded the A549 cell lines (data not shown). The strains selected for this study were M1 (subtype M1.27) and M49 (subtype M49.4) because they showed the highest rate of infectivity with A549 and HEp-2 cell lines. Infection efficiencies under different environmental conditions by both M1 and M49 strains were compared in this study.

The efficiency of infection of A549 and HEp-2 cells by both M1 and M49 strains decreased at temperatures above the control (37°C) and increased at temperatures below the control. In general, as the salt concentrations increased, the infection efficiency decreased with some exceptions. As shown in Table 3, the M1 strain grown at 25°C infected A549 and HEp-2 cells with minimum efficiency (0%) and when grown in 0.6 M NaCl, the M1 strain infected A549 cells with maximum efficiency (3.61%). Another exception was observed when the M49 strain (grown at 0.3 M NaCl) infected HEp-2 cells with minimum efficiency (0.046%) and infected A549 cells with maximum efficiency (12.2%) when grown at 25°C (Table 3).

The M1 strain infected A549 cells with less efficiency under different conditions (0, 0.0072, 0.003% at 25°C, 30°C, and 41°C, respectively; 0.0044% at 0.3 M NaCl) compared to control conditions (37°C and 0.1 M NaCl, with invasion efficiency of 0.066%) ($p < 0.05$) (Table 3). Infection efficiency of the M1 strain with A549 cells at 0.6 M NaCl was exceptionally high (50 fold higher) ($p \leq 0.03$) (Table 3). The M49 strain infected HEp-2 more often than A549 cells ($p < 0.05$) under normal conditions (Table 3). The highest infection efficiency of M49 with A549 cells was at 25°C (almost fourfold higher than normal conditions) and with HEp-2 cells at 30°C (more than twofold higher), and differences were found to be significant ($p \leq 0.05$) (Table 3).

Temperature of 41°C and 0.3 M NaCl were unfavorable conditions for both the serotypes of GAS. The invasion of A549 by M1 was 0.003% at 41°C and 0.0044% at 0.3M NaCl, while for M49 it was 0.34% at 41°C and 2.42% at 0.3M NaCl. The HEp-2 cells showed that M1 did not infect at 41°C and at 0.3 M NaCl; the efficiency was 0.035%. The infection efficiency for M49 was 0.052% at 41°C and 0.046% at 0.3 M NaCl (Table 3). Although infection efficiency of both the GAS strains with A549 and HEp-2 cells was found to be variable under different environmental conditions, clear trends could not be established.

Cytokine production

No significant increase in production of the proinflammatory cytokines TNF-α, IL-1β, IL-10, and IL-12p70 by A549 and HEp-2 cells was observed when infected with M1 and M49 strains (Table 4). Additionally, minimal amounts (< 1 pg mL^{-1}) of cytokine were observed with controls (untreated cells) (data not shown). Only IL-6 and IL-8 were significantly produced and therefore have been described in this study (Table 4). Under all growth conditions, M49 stimulated IL-8 production by A549 and HEp-2 cells with higher efficiency compared to the M1 serotype (Table 4).

Production of IL-6

HEp-2 cells produced significantly more IL-6, ranging from 82.8 (± 30.4) to 317.4 (± 12.7) pg mL^{-1}, than A549 cells (0 to 19.8 ±4.2 pg ml^{-1}) when infected by M49 under all environmental conditions (Table 4). HEp-2 cells infected by M1 released IL-6 ranging from 55.45 (± 14.8) to 203.4 (± 9.3) pg mL^{-1} in all tested conditions (Table 4). However, the M1 strain infected A549 cells did not produce IL-6 at 25°C, 30°C, 37°C, 41°C, and much less production (1.4 ± 0.2 to 2.25 ± 0.6 pg mL^{-1}) was observed under salt stress conditions (Table 4).

Production of IL-8

IL-8 was produced in significant but variable amounts by both A549 and HEp-2 cells when stimulated by M1 and M49 under the conditions described. Production of IL-8 by A549 infected with M49 strain was 46.9 (± 29.4), 96.3 (± 27), and 173 (± 31.11) pg mL^{-1} at normal conditions, 0.3 M, and 0.6 M NaCl concentrations, respectively (Table 4). Under similar conditions, HEp-2 cells produced less IL-8 in comparison to A549 cells: 50 (± 3.32), 51.2 (± 7.2), and 29.7 (± 9.8) pg ml$^{-1,}$ respectively (Table 4). When

exposed to the M49 strain, A549 cells released 173 ± 31.1 pg mL^{-1} of IL-8 at 0.6 M NaCl, and at 25ºC, the HEp-2 cells produced 122.6 ($\pm$ 12.7) pg mL^{-1} of IL-8 (Table 4).

The M1 strain infected A549 cells produced IL-8 in amounts of 77.1 ($\pm$ 15.3), 84.9 ($\pm$ 1.8), 17.5 ($\pm$ 0) pg mL^{-1} at normal, 0.3 M, and 0.6 M NaCl, respectively (Table 4). A smaller amount of IL-8 (10 $\pm$ 1.7 pg mL^{-1}) was released at 25°C, and none was detected at 30ºC and 41°C (Table 4). Maximum IL-8 production (91.1 $\pm$ 8.7 pg mL^{-1}) was observed at 25°C when the M1 strain infected HEp-2 cells as compared to (21.0 $\pm$ 2.5 pg mL^{-1}) normal conditions (37°C) (p < 0.05) (Table 4).

Discussion

There are more published studies regarding the epidemiology of GAS from technologically developed countries than from developing countries such as India. The present study focused on determining the prevalent M types of GAS infecting patients of a North Indian population. A total of 1,047 clinical samples were collected from different patients in northern India between 2004 and 2010. One hundred and sixty isolates were found to be β-hemolytic (15.28%) and out of these, 92 isolates were identified as GAS. We found a high prevalence of GAS (8.79%) as compared to the previous studies from different regions of India (3% and 2.8% in the northern part and 4.5% in the southern part) [10,17,30]. A potential reason for the discordant data may be explained by our selection of clinical samples collected from patients with symptoms suggestive of GAS infection. We recovered GAS from 45 cases of pharyngitis (most frequently from schoolchildren) and from 47 cases of impetigo [31]. Patients in India usually do not seek medical assistance when they have a sore throat. If they do visit a clinician, they will often be treated empirically, and cultures are usually not obtained. On the other hand, skin lesions are cultured for pathogens and are treated with antimicrobial agents. Although we also detected groups C, D, F, and G (data not shown) beta streptococci in this study, the incidence was considerably less than GAS, and these were not included in the present study. The higher incidence of GAS compared to other groups of streptococci has not been confirmed in some previously published reports [32].

Currently, more than 150 different M serotypes of GAS have been identified [9]. Horizontal and lateral gene transfers among different GAS strains are responsible for this vast GAS heterogeneity [33,34].

The heterogenic distribution of GAS in Indian populations has been previously reported [10,13-19,35]. A study by Sagar *et al.* [16] based on samples collected from Punjab state between 2000 and 2004 found M49 to be the predominant serotype. The prevalence of M49 followed by M82 and M74 from Chennai, South India, was also reported previously [35]. Our study, which included GAS collected from patients from New Delhi, National Capital Region, and Jaipur, Rajasthan, resulted in data showing that type M1 was most prevalent, followed by M49, M42, and M48. In addition, we detected eight M types: M1, M49, M74, M77, M86, M100, M104, and M112 in this study that were reported previously [16]. However, this study reports 11 M types including M8, M15, M42, M48, M66, M78, M114, M116, and Mst.1389 that were isolated for the first time in patients from India. The difference in GAS M type prevalence in this study compared to earlier Indian reports results from patient selection, pathogen change over time, and different geographical regions. This underscores the need for continuing surveillance of M types [36].

We observed that M42 and M48 were not only different from previously reported M types in India, but also from the other Asian countries such as Japan, Taiwan, and Hong Kong, where M28 and M12 were reported to predominate [37-39]. In contrast to these countries, the most common serotype in Australia was M14, in Italy M12, in Spain M4, and in Germany and the United States, M1 prevailed, followed by M12 [2,12,20,40-42]. The knowledge of the geographic distribution of GAS serotypes in developing and industrialized countries is clinically important to the research and development of GAS vaccines. It has been suggested that serotype M49, one of the prevalent M types isolated in the study, should be included in the experimental vaccine [7].

Molinari and Chhatwal [43] reported that infection efficiency of GAS in cell lines depends on various factors such as anatomical site, serotype, and cell type. In this study, we made an attempt to study infectious behavior of GAS grown under different environmental conditions using prevalent GAS M types. It has been documented that M1 is the most infectious and most circulating serotype in the human population worldwide [12]. We also found that M1 and M49 were the most prevalent and the second most prevalent serotypes in the North Indian population. Therefore, we used these two serotypes for infection studies with A549 and HEp-2 cells. For this purpose, infection efficiency of all M1 (n = 20) and M49 (n = 8) strains

grown at normal conditions (0.1 M NaCl, 37°C) were checked with A549 cells (data not shown here). One highly infectious strain belonging to each of M1 and M49 serotypes was selected to check its infectivity with A549 and HEp-2 cells and corresponding proinflammatory immune response by host cells under different environmental conditions.

We found that the M1 strain was less infectious than the M49 strain under all conditions with both the cell lines (A549 and HEp-2), except at the 0.6 M NaCl condition. Both strains (M1 and M49) were more infectious when they interacted with HEp-2 cells compared to A549 cells under most environmental conditions (Table 3). These results indicate that serotype and cell type are important factors that decide the variability in infection efficiency of GAS isolates [43].

Environmental factors may also play a crucial role in variations in infectious behavior of GAS because pathogenic bacteria control gene expression and virulence factor production by complex regulatory circuits in response to alterations in the host milieu [23]. For example, virulence factor synthesis is influenced by nutrient availability, pH, osmolarity, growth phase, oxygen tension, CO_2 concentrations, iron levels, temperature, and levels of iron and glucose [44]. In the present study, we attempted to show how the infectious behavior of a GAS strain changes when it infects the same cell line (A549 and HEp-2) under different environmental conditions (such as salt stress at 0.3 and 0.6 M NaCl and temperatures of 25°C, 30°C, and 41°C).

We found that infection efficiency was highly variable in both the strains under different environmental conditions (Table 3). It is interesting to note that both the serotypes M49 and M1 infected A549 cells and HEp-2 cells at the highest rate when they were grown at 25°C and 30°C, respectively (Table 3). It may explain the reason why the frequency of GAS infection in humans is higher during the transition time from summer to winter or vice versa; in this region (Indian subcontinent), the temperature is generally around 25-30°C. Furthermore, it was observed that high temperatures (41°C) are not favorable for pathogenesis of GAS, indicating that it is unable to cope with the feverish condition in the human body.

In general (except in cases when M1 was grown at 0.6 M NaCl and interacted with A549), salt stress condition were not favorable for both the GAS isolates to infect A549 and HEp-2 cells because infection efficiencies were observed to be less at higher salt concentrations (0.3 and 0.6 M NaCl) in comparison to the optimum condition (0.1 M NaCl) (Table 3).

We hypothesize that the differences in the environmental stimuli may affect the infectious behavior of GAS and in turn may also regulate the immune response by the host. Increased production of proinflammatory cytokines by A549 and HEp-2 cells in response to GAS infection was significantly increased compared to the control, indicating that bacterial infection stimulates human cells for immune response (Table 4). IL-6 and IL-8 up-regulate activity by macrophages/monocytes and polymorphonuclear cells and leukocyte chemotaxis [45-48]. Greater production of IL-6 and IL-8 and lower production of IL-10 by A549 and HEp-2 cells in all conditions was observed in this study (Table 4), which suggests that in the first phase of infection, human cells respond immunologically to up-regulate activity of macrophages/monocytes, inflammatory cells, and polymorphonuclear cells to phagocytize and clear bacteria from the cellular environment. IL-6 and IL-8 are also known to enhance the activity of B-cells for antibody production. As we have not studied these activities, this area warrants further investigation. Overall, the greater production of IL-6 and IL-8 and lower production of IL-10 result in a proinflammatory type environment by GAS-infected A549 and HEp-2 cells.

Overall, A549 and HEp-2 cells produced all cytokines in higher amounts (Table 4) when they were stimulated by the M49 serotype compared to when they were stimulated by the M1 serotype, indicating that the M49 serotype was more aggressive and potent in inducing immune responses. On the basis of our present observations, we suggest that different serotypes of GAS may have different potential to infect and induce the human cells for immune response (Tables 3 and 4).

We hypothesize that environmental factors may regulate the infectious behavior of GAS by affecting its infection efficiency and potential to induce immune response in human cells. We tried to correlate the infection efficiency of GAS and immune response by human cells under different environmental conditions. Although clear correlation was not observed due to other variable factors (different environmental conditions), it was observed that for the same serotype and cells, immune response by the host cells was inversely related to infection efficiency of GAS – for example, IL-8 production by A549 cells decreased with the increase in infection efficiency of the M49 serotype (Tables 3 and 4). Continued research will

further improve our understanding about the pathogenesis of different strains of GAS serotypes and host response.

Acknowledgements

References

1. Cunningham MW (2000) Pathogenesis of group A streptococcal infections. Clin Microbiol Rev 13: 470-511.
2. Aberti S, Garcia-Rey C, Dominguez MA, Aguilar L, Cercenado E, Gobernado M, García-Perea A (2003) Spanish Surveillance Group for Respiratory Pathogens. Survey of *M* gene sequences from pharyngeal *Streptococcus pyogenes* isolates collected in Spain and their relationship with erythromycin susceptibility. J Clin Microbiol 41: 2385-2390.
3. Podbielski A, Melzer B, Lutticken R (1991) Application of the polymerase chain reaction to study the M protein(-like) gene family in beta-hemolytic streptococci. Med Microbiol Immun 180: 213-227.
4. Pruksakorn S, Sittisombut N, Phornphutkul C, Pruksachatkunakorn C, Good MF, Brandt E (2000) Epidemiological analysis of non-M-typeable group A streptococcus isolates from a Thai population in northern Thailand. J Clin Microbiol 38: 1250-1254.
5. Seppala H, He Q, Osterblad M, Huovinen P (1994) Typing of group A streptococci by random amplified polymorphic DNA analysis. J Clin Microbiol 32: 1945-1948.
6. Stanley J, Desai M, Xerry J, Tanna A, Efstratiou A, George R (1996) High-resolution genotyping elucidates the epidemiology of group A streptococcus outbreaks. J Infect Dis 174: 500-506.
7. Steer AC, Law I, Matatolu L, Beall BW, Carapetis JR (2009) Global *M* type distribution of group A streptococci: systematic review and implications for vaccine development. Lan Infect Dis 9: 611-616.
8. Beall B, Facklam R, Thompson T (1996) Sequencing *M*-specific PCR products for routine and accurate typing of group A streptococci. J Clin Microbiol 34: 953-958.
9. Enright MC, Spratt BG, Kalia A, Cross JH, Bessen DE (2001) Multilocus sequence typing of *Streptococcus pyogenes* and the relationships between *M* type and clone. Infect Immun 69: 2416-2427.
10. Dey N, McMillan DJ, Yarwood PJ, Joshi RM, Kumar R, Good MF, Sriprakash KS, Vohra H (2005) High diversity of group A streptococcal *M* types in an Indian community: the need to tailor multivalent vaccines. Clin Infect Dis 40: 46-51.
11. Gardiner DL, Goodfellow AM, Martin DR, Sriprakash KS (1998) Group A streptococcal Vir types are M-protein gene (*emm*) sequence type specific. J Clin Microbiol 36: 902-907.
12. Johnson DR, Kaplan EL, VanGheem A, Facklam RR, Beall B (2006) Characterization of group A streptococci (*Streptococcus pyogenes*): correlation of M-protein and M-gene type with T-protein agglutination pattern and serum opacity factor. J Med Microbiol 55: 157-164.
13. Menon T, Lloyd C, Malathy B, Sakota V, Jackson D, Beall B (2008) *M* type diversity of β-haemolytic streptococci recovered in Chennai, India. J Med Microbiol 57: 540-542.
14. Navaneeth BV, Ray N, Chawda S, Selvarani P, Bhaskar M, Suganthi N (2001) Prevalence of beta hemolytic streptococci carrier rate among school children in Salem. Ind J Ped 68: 985-986.
15. Sagar V, Bakshi DK, Nandi S, Ganguly NK, Kumar R, Chakraborti A (2004) Molecular heterogeneity among north Indian isolates of group A streptococcus. Lett Appl Microbiol 39: 84-88.
16. Sagar V, Kumar R, Ganguly NK, Chakraborti A (2008) Comparative analysis of M type pattern of group A streptococcus throat and skin isolates from India and their association with closely related SIC, a streptococcal virulence factor. BMC Microbiol 8: 150.
17. Kumar R, Vohra H, Chakraborty A, Sharma YP, Bandhopadhya S, Dhanda V, Sagar V, Sharma M, Shah B, Ganguly NK (2009) Epidemiology of group A streptococcal pharyngitis figure impetigo: a cross-sectional & follow up study in a rural community of northern India. Indian J Med Res 130: 765-771.
18. Dhanda V, Vohra H, Kumar R (2011) Virulence potential of Group A streptococci isolated from throat cultures of children from north India. Indian J Med Res 133: 674-680.
19. Dhanda V, Vohra H, Kumar R (2011) Group A Streptococcus virulence factors genes in north India & their association with *emm* type in pharyngitis. Indian J Med Res 133: 110-115.
20. Hu MC, Walls MA, Stroop SD, Reddish MA, Beall B, Dale JB (2002) Immunogenicity of a 26-valent group A streptococcal vaccine. Infect Immun 70: 2171-2177.
21. Kelley J (1990) Cytokines of the lung. Am Rev Respir Dis 141: 765-788.
22. Teti G, Mancuso G, Tomasello F (1993) Cytokine appearance and effects of anti-tumor necrosis factor alpha antibodies in a neonatal rat model of group B streptococcal infection. Infect Immun 61: 227-235.
23. Rubens CE, Smith S, Hulse M, Chi EY, Belle G (1992) Respiratory epithelial cell invasion by group B streptococci. Infect Immun 60: 5157-5163.
24. Nakamura T, Hasegawa T, Torii K, Hasegawa Y, Shimokata K, Ohta M (2004) Two-dimensional gel electrophoresis analysis of the abundance of virulent exoproteins of group A streptococcus caused by environmental changes. Arch Microbiol 181: 74-81.
25. Rubens CE, Raff HV, Jackson JC, Chi EY, Bielitzki J, Hillier S (1991) Pathophysiology and histopathology of group B streptococcal sepsis in *Macaca nemestrina* primates induced after intraamniotic inoculation: evidence for bacterial cellular invasion. J Infect Dis 164: 320-330.
26. Bazziacalupo M, Fani R (1996) The use of RAPD for generating specific DNA probes for microorganisms. In Clapp J, editor. Method in Molecular Biology, vol. 50. Totowa, New Jersey: Hamana Press Inc. 155-175.
27. Johri AK, Padilla J, Malin G, Paoletti LC (2003) Oxygen regulates invasiveness and virulence of group B streptococcus. Infect Immun 71: 6707-6711.

28. Mikamo H, Johri AK, Paoletti LC, Madoff LC, Onderdonk AB (2004) Adherence to, invasion by, and cytokine production in response to serotype VIII group B streptococci. Infect Immun 72: 4716-4722.

29. Jimenez R, Ramırez R, Carracedo J, Aguera M, Navarro D, Santamarıa R, Perez R, Castillo DD, Aljama P (2005) Cytometric bead array (CBA) for the measurement of cytokines in urine and plasma of patients undergoing renal rejection. Cytokine 32: 45-50.

30. Dhakal R, Sujatha S, SParija SC, Bhat BV (2010) Asymptomatic colonization of upper respiratory tract by potential bacterial pathogens. Ind J Pedia 77: 775-778.

31. Nandi S, Kumar R, Ray P, Vohra H, Ganguly NK (2001) Group A streptococcal sore throat in a periurban population of northern India: A one-year prospective study. Bull WHO 79: 528-533.

32. Bramhachari PV, Kaul SY, McMillan DJ, Shaila MS, Karmarkar MG, Sriprakash KS (2010) Disease burden due to Streptococcus dysgalactiae subsp. equisimilis (group G and C streptococcus) is higher than that due to *Streptococcus pyogenes* among Mumbai school children. J Med Microbiol 59: 220-223.

33. Sumby P, Porcella SF, Madrigal AG, Barbian KD, Virtaneva K, Ricklefs SM, Sturdevant DE, Graham MR, Vuopio-Varkila J, Hoe NP, Musser JM (2005) Evolutionary origin and emergence of a highly successful clone of serotype M1 group A Streptococcus involved multiple horizontal gene transfer events. J Infect Dis 192: 771-782.

34. Musser JM, Shelburne SA III (2009) A decade of molecular pathogenomic analysis of group A Streptococcus. J Clin Invest 119: 2455-2463.

35. Menon T, Whatmore AM, Srivani S, Kumar MP, Anbumani N, Rajaji S (2001) *M* types of *Streptococcus pyogenes* in Chennai. Ind J Med Microbiol 19: 161-162.

36. Dale JB, Shulman ST (2000) Dynamic epidemiology of group A streptococcal serotypes. Lancet 359: 889.

37. Ho PL, Johnson DR, Yue AW, Tsang DN, Que TL, Beall B, Kaplan EL (2003) Epidemiologic analysis of invasive and noninvasive group a streptococcal isolates in Hong Kong. J Clin Microbiol 41: 937-942.

38. Kao CH, Chen PY, Huang FL, Chen CW, Chi CS, Lin YH, Shih CY, Hu BS, Li CR, Ma JS, Lau YJ, Lu KC, Yu HW (2005) Clinical and genetic analysis of invasive and non-invasive group A streptococcal infections in central Taiwan. J Microbiol Immun Infect 38: 105-111.

39. Tanaka D, Gyobu Y, Kodama H, Isobe J, Hosorogi S, Hiramoto Y, Karasawa T, Nakamura S (2002) *emm* Typing of group A streptococcus clinical isolates: identification of dominant types for throat and skin isolates. Microbiol Immunol 46: 419-423.

40. Brandt CM, Spellerberg B, Honscha M, Truong ND, Hoevener B, Lutticken R (2001) Typing of *Streptococcus*

41. Espinosa LE, Li Z, Barreto D, Jaimes EC, Rodriguez RS, Sakota V, Facklam RR, Beall B (2003) M protein gene type distribution among group A streptococcal clinical isolates recovered in Mexico City, Mexico, from 1991 to 2000, and Durango, Mexico, from 1998 to 1999: overlap with type distribution within the United States. J Clin Microbiol 41: 373-378.

42. McGregor KF, Bilek N, Bennett A, Kalia A, Beall B, Carapetis JR, Currie BJ, Sriprakash KS, Spratt BG, Bessen DE (2004) Group A streptococci from a remote community have novel multilocus genotypes but share *M* types and housekeeping alleles with isolates from worldwide sources. J Infect Dis 189: 717-723.

43. Molinari G, Chhatwal GS (1998) Invasion and survival of *Streptococcus pyogenes* in eukaryotic cells correlates with the source of the clinical isolates. J Infect Dis 177: 1600-1607.

44. Mekalanos JJ (1992) Environmental signals controlling expression of virulence determinants in bacteria. J Bacteriol 174: 1-7.

45. Bogdan C, Vodovotz Y, Nathan C (1991) Macrophage deactivation by interleukin 10. J Exp Med 174: 549-1555.

46. Cassatella MA, Meda L, Bonora S, Ceska M, Constantin G (1993) Interleukin 10 (IL-10) inhibits the release of proinflammatory cytokines from human polymorphonuclear leukocytes. Evidence for an autocrine role of tumor necrosis factor and IL-1 beta in mediating the production of IL-8 triggered by lipopolysaccharide. J Exp Med 178: 2207-2211.

47. Puliti M, Von Hunolstein C, Verwaerde C, Bistoni F, Orefici G, Tissi L (2002) Regulatory role of interleukin-10 in experimental group B streptococcal arthritis. Infect Immun 70: 2862-2868.

48. Kasama T, Strieter RM, Lukacs NW, Lincoln PM, Burdick MD, Kunkel SL (1995) Interleukin-10 expression and chemokine regulation during the evolution of murine type II collagen-induced arthritis. J Clin Invest 95: 2868-2876.

pyogenes strains isolated from throat infections in the region of Aachen, Germany. Infection 29: 163-165.

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