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Differential Induction of Th1 versus Th2 Cytokines by Group A Streptococcal Toxic Shock Syndrome Isolates

ANNA NORRBY-TEGLUND,[1] ROBERT LUSTIG,[2] AND MALAK KOTB[3]*

Research Service, Veterans Affairs Medical Center, Memphis, Tennessee 38104[1]; Department of Pediatrics, University of Tennessee—Memphis, Memphis, Tennessee 38103[2]; and Departments of Surgery and of Microbiology and Immunology, University of Tennessee—Memphis, Memphis, Tennessee 38163[3]

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The majority of group A streptococcal (GAS) isolates from patients with streptococcal toxic shock syndrome (STSS) and necrotizing fasciitis (NF) express numerous virulence factors, including several superantigens (SAgs). Purified SAgs are potent inducers of inflammatory (Th1) cytokines that contribute to the pathogenesis of severe infections. However, GAS-infected individuals are likely to be exposed to a mixture of GAS SAgs as well as other virulence factors produced by the bacteria, and therefore, our goal was to characterize the mitogenic and cytokine induction profiles of this mixture. All GAS isolates tested had brisk mitogenic activity and induced potent cytokine responses, with higher frequencies of Th1 than Th2 cytokine-producing cells. The mitogenic activity produced in culture supernatants of three selected clinical GAS isolates was significantly different, but no marked difference was found in their overall cytokine induction profiles. However, significant differences ($P < 0.0062$) were noted in the induction of Th2 cytokines between GAS supernatants and recombinant streptococcal pyrogenic exotoxin A (rSpeA), suggesting that the presence of other SAgs and/or the production of additional virulence factors may alter the overall cytokine induction profile of SAgs. A significant individual variation in the level of proliferative and cytokine responses to the same GAS culture supernatants or to rSpeA was noted. Individuals with higher frequencies of cells producing Th2 cytokines mounted lower levels of Th1 cytokine responses, and vice versa. Furthermore, quantification of the intensity and cell area of interleukin-1β (IL-1β)-producing cells by image analysis revealed that individuals with higher Th2 responses had significantly lower IL-1β production ($P < 0.0001$) than the individual with a strong Th1 response. Differences in the ability to induce Th1 versus Th2 cytokines, as well as the individual variations in cytokine responses to streptococcal SAgs, may play a central role in determining the severity of invasive GAS infections.

Group A streptococci (GAS) can cause a wide spectrum of human diseases ranging from uncomplicated noninvasive to severe invasive infections, such as streptococcal toxic shock syndrome (STSS) and necrotizing fasciitis (NF) (3, 34, 43). The pathogenesis of severe GAS infections has been shown to involve the action of superantigens (SAgs) produced by the bacteria (4, 14, 17, 25, 27, 28, 33, 35, 42). SAgs are microbial proteins that stimulate very powerful immune responses by bypassing conventional rules for antigen processing and presentation (23). SAgs bind without prior cellular processing to the Vβ region of the T-cell receptor and to major histocompatibility complex class II molecules on the antigen-presenting cells (23). Cross-linking the two cell types results in potent stimulation and massive production of inflammatory cytokines (13, 17, 18, 24, 29). Although cytokines are required for a functional immune response, their overproduction may result in the pathogenic conditions seen in STSS, including fever, hypotension, and multiorgan failure (5, 6, 11, 37).

Cytokines are often classified as inflammatory or regulatory based on their biological effects, and the fine-tuned balance between these subsets is essential for a controlled and functional immune response. The inflammatory cytokines include, among others, Th1-type cytokines (interleukin-2 [IL-2], tumor necrosis factor beta [TNF-β], and gamma interferon [IFN-γ]), whereas the Th2 cytokines (IL-4, IL-5, and IL-10) are usually included among the regulatory cytokines. The classification of T-helper cells into different populations, i.e., Th0, Th1, and Th2, is based on the expression of different cytokine patterns (1, 26, 31, 38). It has been shown that Th1 and Th2 cytokines can promote different types of immune responses (9, 26, 44). The Th0 response represents an intermediate stage, where production of both Th1 and Th2 cytokines can be seen (36). The differentiation of the subsets is regulated by several factors, including cytokines such as IL-10, which stimulates Th2 cytokine production, and IL-12, which is a strong promoter of Th1 responses (1, 39). Expression of IL-4 or IFN-γ promotes differentiation of Th2 or Th1 cells, respectively, and inhibits differentiation of the other subset, thereby resulting in further polarization of the cytokine responses (1, 12, 31, 39).

In previous studies the cytokine induction profile of purified GAS SAgs was investigated, and they were found to induce a relatively large number of cells to express inflammatory Th1-type cytokines but very few cells to produce regulatory Th2 cytokines (2, 29). A similar pattern of cytokine production was reported for purified staphylococcal SAgs (2), and it was suggested that overproduction of Th1 cytokines might be an important factor contributing to the pathogenesis of severe invasive infections by gram-positive bacteria.

Attempts to implicate a certain strain and/or SAg in severe invasive GAS disease (8, 10, 14, 27, 35, 42) revealed that several strains producing a variety of SAgs, including SAgs that have not yet been characterized (42), can trigger these diseases. Therefore, in vitro and in vivo responses to defined, purified SAgs may not be representative of the actual disease situation, where the patient is exposed to a mixture of virulence factors. Indeed, it has been shown that other streptococcal virulence factors, such as streptolysin O, can synergize with

* Corresponding author. Mailing address: Veterans Administration Medical Center, Research Service 151, 1030 Jefferson Ave., Memphis, TN 38104. Phone: (901) 448-7247. Fax: (901) 577-7273.

SAgs to further enhance the cytokine response (13). Guided by these findings, we decided to examine the cytokine induction profile of GAS culture supernatants containing secreted SAgs as well as other extracellular virulence factors. The results showed that the GAS supernatants and purified SAg differ in their relative induction of Th1 versus Th2 cytokines. Furthermore, we observed a marked individual variation, both in mitogenic and Th1 versus Th2 cytokine responses, to various GAS supernatants and purified SAg. This study underscores the importance of host factors in regulating immune responses to GAS virulence factors and in contributing to disease pathogenesis.

MATERIALS AND METHODS

Expression of rSpeA. Recombinant SpeA (rSpeA) was expressed and purified according to the manufacturer's (Novagen, Madison, Wis.) recommendations as a His fusion protein from *Escherichia coli* BL21 containing pET15b-*speA* (16), kindly provided by B. Kline and C. Collins, University of Miami, Miami, Fla. The N-terminal His tag was removed by digestion of the fusion protein with 1 U of thrombin/mg of rSpeA for 16 h at room temperature with the thrombin cleavage capture kit (Novagen). Before being used, the digested rSpeA was treated with polymyxin B agarose (Boehringer Mannheim, Indianapolis, Ind.) for 6 h at room temperature to adsorb any contaminating endotoxin. Thrombin-digested rSpeA contained four more amino acids on the amino terminus than native SpeA (16). However, the superantigenic activity of rSpeA was comparable to that previously reported for native SpeA with regard to mitogenic activity, cytokine-inducing capacity (29), and Vβ specificity (19).

Clinical isolates. GAS strains of serotype M3T3 isolated from sterile sites of three patients with STSS and NF, kindly provided by D. E. Low, Mount Sinai Hospital, Toronto, Canada, were included in the study. The presence of the genes encoding SpeA, SpeC, SpeF, and SSA was detected by PCR with primer pairs specific for each gene, as previously described (25, 28, 40).

Preparation of GAS culture supernatant. The three GAS isolates were cultured overnight in 10 ml of Todd-Hewitt broth (Difco, Detroit, Mich.) supplemented with 1.5% yeast extract (Difco). The bacteria were removed by centrifugation, and proteins in the supernatants were precipitated by adding 95% ice-cold ethanol (1 part supernatant to 3 parts ethanol) and incubating the mixture for 24 h at −20°C. The precipitates were dissolved in 1 ml of distilled H_2O and dialyzed for 24 h against distilled H_2O. The dialysates were filter sterilized and stored at −20°C. Each GAS supernatant was tested at different dilutions for its ability to induce T-cell proliferation, and the optimal concentration (1:100 dilution) was then used in cytokine cultures. Todd-Hewitt broth alone without streptococci, treated in a manner identical to that of the GAS supernatant, was included as a negative control and did not elicit any proliferative or cytokine responses.

SDS-gel electrophoresis and Western blotting. Expression of genes encoding SpeA, SpeF, and SSA was detected by Western blotting. Protein samples, 0.1 μg of rSpeA, rSpeF, or rSSA, and 100 μl of lyophilized culture supernatant prepared from clinical isolates were dissolved and boiled for 5 min in loading buffer (60 mM Tris-HCl [pH 6.8], 2% sodium dodecyl sulfate [SDS], 5% 2-mercaptoethanol, 5% glycerol) for analysis by SDS-gel electrophoresis (10% total acrylamide; 2.7% bisacrylamide). Proteins were electroblotted onto nitrocellulose membranes, and the blots were blocked overnight with 6% nonfat dry milk in Tris-buffered saline (TBS) (50 mM Tris [pH 7.5], 150 mM NaCl). The blots were then incubated for 2 h with either anti-SpeA, anti-SpeF, or anti-SSA antibody (kindly provided by P. Schlievert, Minneapolis, Minn.; S. Holm, Umeå, Sweden; and R. R. Rich, Houston, Tex., respectively) diluted 1:3,000, 1:5,000, and 1:1,000, respectively, in 0.1% nonfat dry milk in TBS, washed, and then incubated with secondary antibody (anti-rabbit immunoglobulin conjugated to horseradish peroxidase) (Fisher Biotech, Pittsburgh, Pa.) diluted 1:10,000 in 0.1% nonfat dry milk in TBS. Binding was detected by chemiluminescence with luminol reagents (Amersham ECL kit). The processed blots were exposed to X-ray films, and the autoradiograms were analyzed.

PBMC preparation and proliferation assay. Peripheral blood mononuclear cells (PBMC) were isolated from healthy individuals by Ficoll-Hypaque gradient centrifugation. The cells (10^6/ml) were cultured in RPMI 1640 medium supplemented with 25 mM HEPES, 4 mM L-glutamine, 100 U of penicillin-streptomycin/ml, and 10% fetal bovine serum (FBS) (RPMI complete medium). The cells were stimulated with various dilutions of GAS supernatants, different concentrations of rSpeA, 10 ng of SpeF (kindly provided by S. Holm) per ml, or 10 ng of rSSA (kindly provided by R. R. Rich) per ml at 37°C in 5% CO_2 and 95% humidity. After 3 days, the cells were pulsed for 6 h with 1 μCi of [^{3}H]thymidine (specific activity, 6.7 Ci/mmol; DuPont, Wilmington, Del.) per well, harvested onto glass fiber filters, and counted in a liquid scintillation counter (Packard, Downers Grove, Ill.). All samples were assayed in triplicate, and the data are presented as mean [^{3}H]thymidine uptake (in counts per minute) ± standard deviation (SD).

Analysis of cytokine production at a single-cell level. PBMC (10^6/ml) were cultured in RPMI complete medium supplemented with 10% FBS which had been treated with polymyxin B agarose (Boehringer Mannheim) to adsorb any residual endotoxin. PBMC were cultured with medium alone, a 1:100 dilution of GAS supernatant, or 10 ng of rSpeA/ml and harvested after 0, 8, 12, 24, 36, 48, and 72 h of culture. Harvested cells were washed with Hanks balanced salt solution (HBSS) (Life Technologies, Grand Island, N.Y.) and applied to individual slots on adhesion glass slides (Marienfeld Laboratory Glassware, Mergenthelm, Germany) as previously described (32). The cells were allowed to adhere to the slides for 10 min at room temperature and were then fixed with freshly prepared 2% formaldehyde (Fisher Scientific Co., Fair Lawn, N.J.) in phosphate-buffered saline for 20 min, followed by five washes in HBSS. The slides were blocked with 2% FBS in HBSS for 5 min at 37°C, after which 0.1% saponin (Sigma, St. Louis, Mo.) in HBSS was added to permeabilize the cells and allow intracellular staining (32). The primary cytokine-specific monoclonal antibodies (MAbs) (2 μg/ml) were added to the permeabilized cells, incubated at 37°C for 30 min, and washed repeatedly in HBSS-saponin. The following cytokine-specific MAbs were used: IL-1β (H34) (murine IgG1 MAb from J. Kenney, Syntex, Palo Alto, Calif.), IL-2 (MQ2-17H12), IL-4 (MP4-25D2), IL-6 (MQ2-6A3), IL-10 (19F1), TNF-α (all rat IgG MAbs were from J. Abrams, DNAX, Palo Alto, Calif.) (MP9-20A4), TNF-β (LTX-21) (murine IgG2b; Biosource International, Camarillo, Calif.), and IFN-γ (murine IgG2a; Genzyme, Cambridge, Mass.). In order to reduce nonspecific binding of the secondary antibody, the slides were incubated with 2% goat serum in HBSS-saponin for 30 min at room temperature, after which the biotinylated secondary antibodies goat anti-mouse IgG1, IgG2a, and IgG2b (Caltag Laboratories, San Francisco, Calif.) diluted 1:300 or goat anti-rat IgG (Vector Laboratories, Burlingame, Calif.) diluted 1:100 in HBSS-saponin were added. Human AB serum (5%) was added to both the primary and the secondary antibodies to avoid nonspecific Fc-receptor interactions. After 30 min of incubation at room temperature the slides were thoroughly washed with HBSS-saponin and incubated for 30 min at room temperature with the avidin-alkaline phosphatase solution (Vectastain-ABC-AP kit; Vector Laboratories). The color reaction was developed by adding alkaline phosphatase substrate (Vector Laboratories) supplemented with levamisole (Vector Laboratories) to block endogenous alkaline phosphatase. The reaction proceeded for 30 min and was then stopped by washing with HBSS, and the cells were counterstained with Mayer's hematoxylin solution (Sigma). Duplicate slots were prepared for each staining, and at least 1,000 cells per slot were counted by direct microscopy. Assessment of all cytokine-producing cells, except IL-1β, was facilitated by a local dense juxtanuclear staining, representing accumulation of the cytokines to the Golgi organelle (2). IL-1β-producing cells showed a more diffuse cytoplasmic staining, which is consistent with previous findings (2). The data are presented as mean percent cytokine-producing cells ± SD.

SAg-induced IL-1β production was further quantified with the Image 2.0 analysis system (Oncor, Gaithersburg, Md.). The analysis was performed at the single-cell level and measured the cell area (mm^2) and the integrated density of stain over the entire cell (intensity). At least 100 positively stained cells per field, which constituted 10 to 15% of the total cells per field, were analyzed.

Statistical evaluation. Analysis of variance (ANOVA; single factor) or a two-tailed Student's *t* test was used to determine the significance of the data. A *P* value of <0.05 was considered significant. Post hoc analysis (Tukey-Kramer) was performed to validate the statistical significance as determined by ANOVA.

TABLE 1. Characterization of SAg genotype and expression of clinical GAS isolates

GAS supernatant	Presence of genes encoding[a]:				Expression of[b]:		
	SpeA	SpeC	SpeF	SSA	SpeA	SpeF	SSA
5635	+	−	+	+	−	+	+
5693	−	−	+	+	−	+	+
6044	+	−	+	+	+	+	+

[a] The presence (+) of genes encoding various SAgs was detected by PCR with specific primer pairs for each gene (25, 28, 40).

[b] Spe production was determined by Western blot analysis of supernatants with antisera specific for SpeA, SpeF, and SSA. +, expressed; −, not expressed.

RESULTS

Differences in mitogenic activity elicited by GAS culture supernatants. Culture supernatants, containing secreted SAgs and other virulence factors, were prepared from three M3T3 GAS isolates obtained from STSS and NF patients. The three isolates contained superantigenic activity in their culture supernatants, as evidenced by their mitogenic profiles and their ability to stimulate T cells in a Vβ-specific manner (19). Analysis for the presence of the genes encoding various SAgs revealed that all three isolates harbored the genes for at least two known SAgs (Table 1), and subsequent determination of SAg expres-

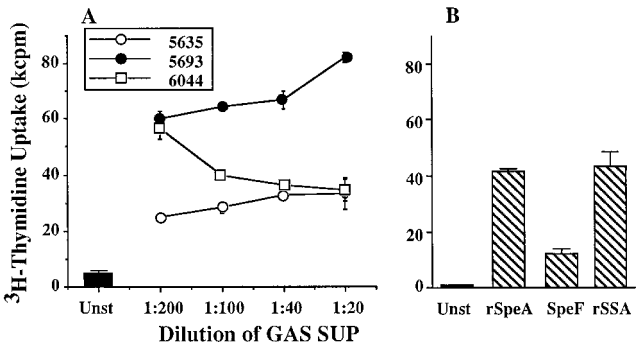

FIG. 1. Mitogenic activity of culture supernatant prepared from clinical GAS isolates. PBMC prepared from one healthy individual (IND.1) were stimulated with various dilutions of culture supernatants (SUP) prepared from the GAS isolates 5635, 5693, and 6044 (A) or with 10 ng of rSpeA, SpeF, or rSSA per ml (B). The proliferative response was assessed after 3 days of culture, and the data are presented as mean [^{3}H]thymidine uptake (in counts per minute) ± SD of triplicate samples. ANOVA revealed significant differences in mitogenic activity among the supernatants ($P < 0.0001$). The differences were validated by Tukey-Kramer with an alpha level of 0.01, which verified that 5693 > 6044 > 5635 is significantly different. The response of the same individual to the three GAS isolates was highly consistent on repeated testing, with the response to supernatants being 5693 > 6044 > 5635. Unst, unstimulated.

sion showed that all three isolates produced SpeF and SSA whereas SpeA was only produced by isolate 6044 (Table 1).

Comparison of the mitogenic activity of the three GAS supernatants revealed that the three isolates differed significantly in their potencies ($P < 0.0001$) (Fig. 1A). All three isolates induced patterns of proliferative response that were indicative of superantigenic activity, inasmuch as the responses plateaued over a wide concentration range (Fig. 1A). The level of proliferative response was comparable to that obtained in cultures with rSpeA, SpeF, and rSSA (Fig. 1B). Interestingly, strain 5693, which expressed SpeF and SSA but not SpeA (Table 1), was a more potent inducer of proliferative response than strain 6044, which expressed all three SAgs (Fig. 1 and Table 1). Furthermore, culture supernatants from isolates 5635 and 5693 also differed significantly in their mitogenic activity ($P < 0.0001$), despite having identical known-SAg expression profiles. Lower mitogenic activity induced by higher concentrations of 6044 supernatant could be due to overstimulation of cells or increased concentration of cytotoxic substances in the supernatant. However, addition of GAS supernatant to phytohemagglutinin-stimulated cells failed to reduce the response at concentrations as high as 1:40, but inhibition of the phytohemagglutinin response was observed at a 1:20 dilution of the GAS supernatant (data not shown).

Differences in Th1 and Th2 cytokine production profiles induced by GAS supernatants and purified rSpeA. First, we compared the general cytokine induction profile of GAS culture supernatants with that of recombinant SAg, namely, rSpeA. Production of Th1 (IL-2, TNF-β, and IFN-γ), Th2 (IL-4 and IL-10), and other sepsis-associated cytokines (IL-1β, IL-6, and TNF-α) elicited by the three GAS supernatants or rSpeA was studied by immunohistochemical staining at 0, 8, 12, 24, 36, 48, and 72 h after stimulation (Fig. 2A and B). Initial analysis comparing the cytokine induction profiles of all three isolates revealed no significant difference among them (Fig. 2A). However, a comparison of the cytokine profiles induced by GAS supernatants and purified SAg showed interesting differences. As expected, the frequencies of producer cells and peak production times differed for the various cytokines studied; however, several common features were noted. The frequency of the Th1 cytokines, IL-2, TNF-β, and IFN-γ, was higher than that of the Th2 cytokines, IL-4 and IL-10, following stimulation with either rSpeA or GAS supernatants (Fig. 2B). The kinetics of Th1 cytokine induction in response to either GAS supernatants or rSpeA were very similar (Fig. 2B). By contrast, a significant difference in the induction of the Th2 cytokines, IL-4 and IL-10, was noted between GAS supernatants and rSpeA ($P < 0.0062$). While rSpeA induced very low levels of IL-4 and IL-10, these cytokines were readily detectable in cells stimulated with GAS supernatants (Fig. 2B). These data indicate that the relative production of Th1 and Th2 cytokines is different for purified SAg and a mixture of SAgs present in crude GAS supernatants. Both GAS supernatants and rSpeA induced high frequencies of IL-1β-producing cells that peaked between 8 and 24 h; however, the frequency of IL-1 producer cells induced by GAS supernatants was significantly higher than that induced by rSpeA ($P < 0.0001$) (Fig. 2B). Similarly, stimulation by GAS supernatants induced a significantly higher frequency of cells to produce IL-6 than that seen following rSpeA stimulation ($P < 0.007$) (Fig. 2B). GAS supernatants and rSpeA induced comparable TNF-α responses (Fig. 2B).

Interestingly, the production of IL-4, IL-10, IL-6, and TNF-α by GAS supernatants showed a biphasic response, which may reflect the known complex interaction of the cytokine network and/or differential peak production time by different cell populations producing these cytokines (Fig. 2A and B).

Individual variation in proliferative and cytokine responses induced by GAS supernatants and rSpeA. Previous studies revealed that different individuals mount different levels of cytokines in response to the same stimulus (20, 29). To investigate individual differences in cytokine and proliferative responses to different GAS supernatants and rSpeA, we analyzed the responses of three healthy individuals to these stimuli. Regardless of whether GAS supernatants or rSpeA was used to stimulate PBMC from these individuals, the proliferative response of individual 1 was consistently higher than that of individual 2 or 3 ($P < 0.0001$), and individual 2 was consistently the lowest responder (Fig. 3). The most pronounced difference in response was noted following stimulation with supernatant from isolate 6044, which induced 10- and 7-fold higher proliferative responses in individual 1 than in individuals 2 and 3, respectively (Fig. 3).

The individual variation was also evident in cytokine responses induced by GAS supernatants or rSpeA. Figure 4 shows the peak values of Th1 and Th2 cytokine production by the three individuals tested following stimulation with the GAS supernatant of isolate 5693 or with rSpeA. The results illustrate the marked individual variations in the different cytokine responses. In correlation with the proliferative responses, individual 1 mounted the highest level of Th1 cytokine responses, inasmuch as the frequency of cells producing TNF-β and IFN-γ was higher in cells from that individual than from individuals 2 and 3 (Fig. 4). A marked difference in the relative induction by GAS supernatants of Th1 and Th2 cytokine responses in different individuals was noted (Fig. 4). With regard to the response to GAS supernatants, the highest Th1 responder was the lowest Th2 responder, and vice versa (Fig. 4). However, this reciprocal relation was not seen when cells were stimulated with rSpeA, presumably because we could not detect Th2 cytokine responses to the purified SAg. All three individuals mounted significantly lower IL-4 responses to rSpeA than to the GAS supernatants, whereas the TNF-β and IFN-γ responses were comparable for all three individuals (Fig. 4).

The individual variation was also evident with regard to the TNF-α response, which following stimulation with all three

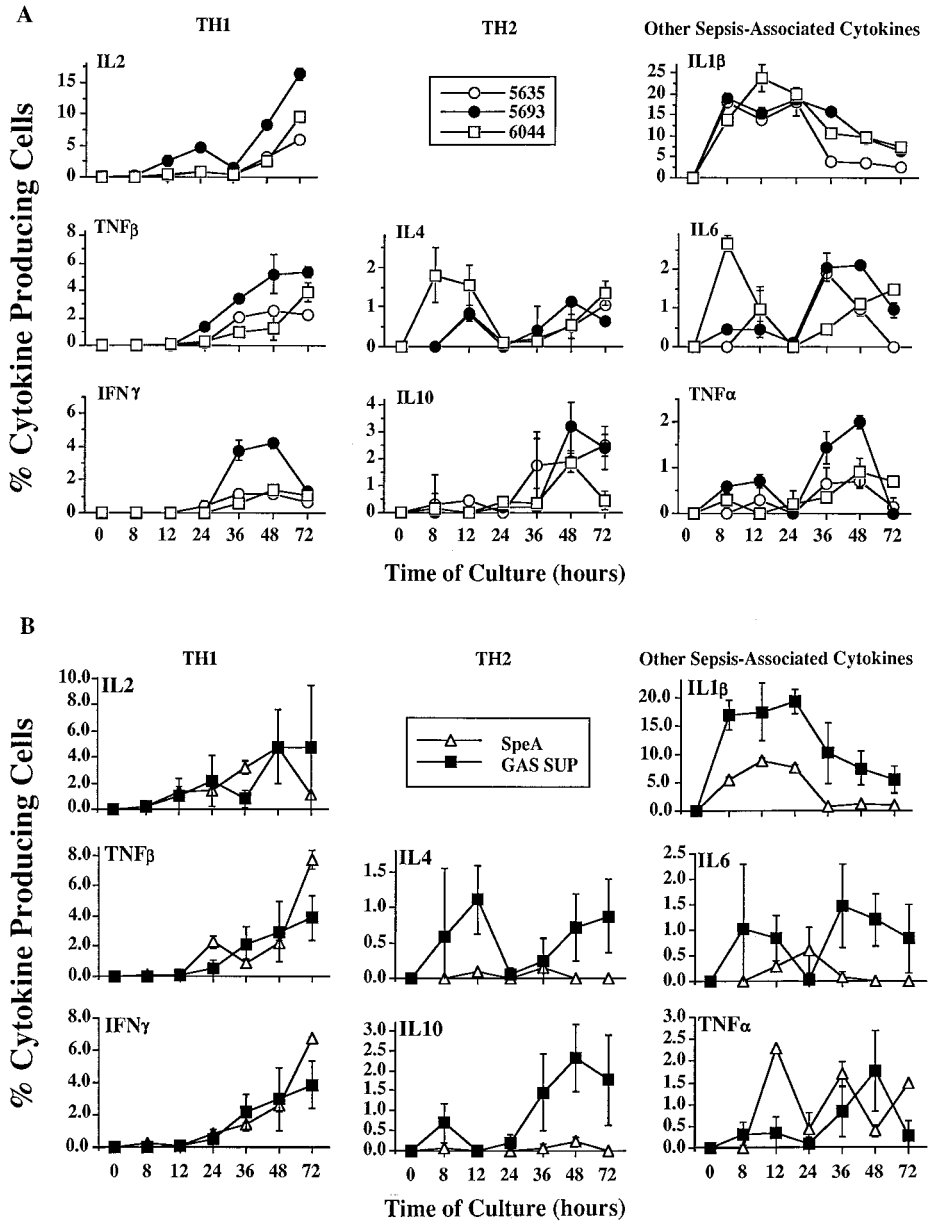

FIG. 2. Differences in induction of Th1 and Th2 cytokines by GAS supernatants and rSpeA. PBMC were prepared from one healthy individual (IND 3) and stimulated with a 1:100 dilution of culture supernatants (SUP) prepared from the GAS strains 5635, 5693, and 6044 or with 10 ng of rSpeA/ml. Cytokine production was analyzed by intracellular immunohistochemical staining following 0, 8, 12, 24, 36, 48, and 72 h of stimulation. Duplicate samples of each stimulus for each cytokine were evaluated, and frequencies of cytokine-producing cells were determined by direct microscopy. The figure shows mean percent cytokine-producing cells ± SD of the various stimuli minus percent cytokine-producing cells in unstimulated cultures. (A) Cytokine induction profile of supernatants prepared from strains 5635, 5693, and 6044; (B) cytokine production of GAS supernatants (mean percent of all three GAS supernatants) compared to that of rSpeA. ANOVA revealed that GAS supernatants were significantly more potent inducers of IL-4, IL-10, IL-6, and IL-1 than was rSpeA ($P < 0.0062$). The difference was validated by Tukey-Kramer with an alpha level of 0.01, which verified that GAS supernatants > rSpeA is significantly different. Todd-Hewitt broth alone (1:100 dilution) without streptococci, treated in a manner identical to that of the other supernatants, was included as a negative control and did not elicit any proliferative or cytokine responses.

GAS supernatants was markedly higher in individual 1 than in individual 2 or 3 (Fig. 5). Further support for the individual variation was obtained by quantification by computerized image analysis of GAS supernatant-induced IL-1β production. As summarized in Table 2, there was a significant difference among the three individuals ($P < 0.0001$) following stimulation with GAS supernatants. The difference was highly consistent for all three GAS supernatants, as evidenced by the results of the post hoc analysis, which showed that individual 1 had a significantly higher intensity and the largest cell area of IL-1β-producing cells, followed by individual 2, while individual 3 showed the weakest intensity and the smallest area of IL-1β-producing cells regardless of which GAS supernatant was used to stimulate the cells (Table 2).

DISCUSSION

Recent studies have provided strong evidence for the role of SAgs in severe GAS infections (14, 25, 27, 30, 35, 42). However, these studies also indicated that a wide variety of SAgs

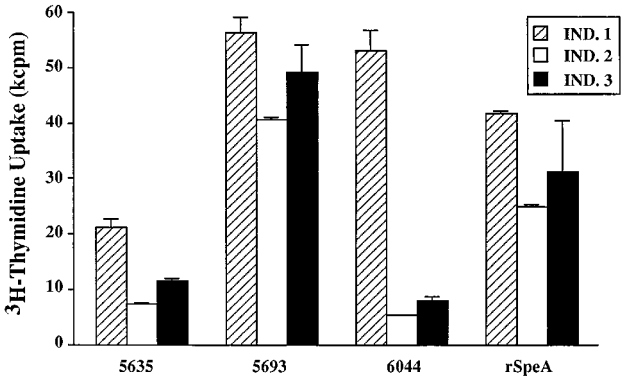

FIG. 3. Individual variation in proliferative responses induced by culture supernatants prepared from clinical GAS isolates. PBMC prepared from three healthy individuals (IND.1, 2, and 3) were stimulated with 10 ng of rSpeA/ml or with 1:200 dilutions of supernatants prepared from the GAS isolates 5635, 5693, and 6044. The proliferative response was assessed after 3 days of culture, and the data are presented as mean [^{3}H]thymidine uptake (in counts per minute) ± SD of triplicate samples. ANOVA revealed significant differences in GAS supernatant- or rSpeA-induced proliferative responses among the individuals ($P < 0.0001$). The difference was validated by Tukey-Kramer with an alpha level of 0.01, which verified that IND.1 > IND.2 = IND.3 is significantly different. The relative difference in the responses of the three individuals to rSpeA or to the supernatants tested was consistent on repeated testing.

can potentially trigger disease in the infected host. In attempting to decipher the mechanism by which GAS SAgs may contribute to the disease process, one must, therefore, consider that GAS-infected hosts are most likely to be exposed to a mixture of SAgs as well as to other extracellular virulence factors produced by the bacteria.

This study was conducted to characterize host proliferative and cytokine responses to a mixture of virulence factors, including SAgs, produced by clinical GAS isolates and to compare these responses to those elicited by purified GAS SAgs.

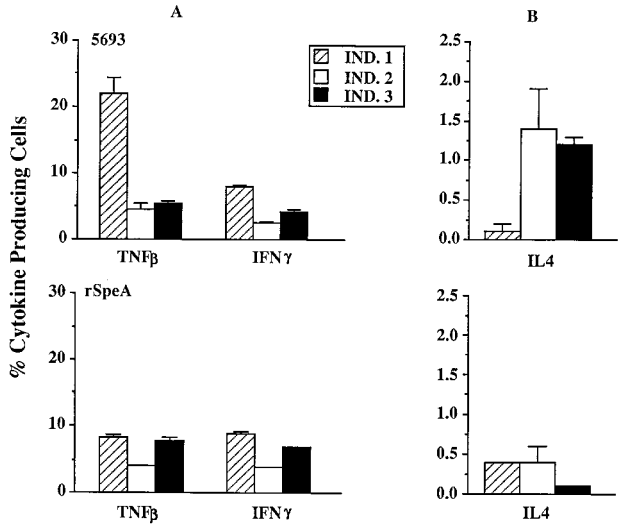

FIG. 4. Individual variation in Th1 (A) and Th2 (B) cytokine responses to GAS supernatants or rSpeA. PBMC prepared from three healthy individuals (IND.1, 2, and 3) were stimulated with a 1:100 dilution of SUP prepared from GAS isolate 5693 or with 10 ng of rSpeA/ml. The cytokine production was assessed by intracellular immunohistochemical staining after 0, 8, 12, 24, 36, 48, and 72 h of stimulation, and the results are presented as mean percent cytokine-producing cells ± SD minus percent cytokine-producing cells in unstimulated cultures. The figure shows the peak value of cytokine production for each individual.

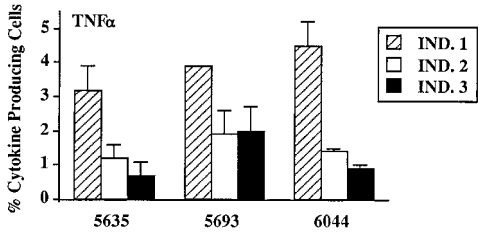

FIG. 5. Individual variation in TNF-α expression induced by GAS supernatants. PBMC prepared from three healthy individuals (IND.1, 2, and 3) were stimulated with a 1:100 dilution of supernatants prepared from GAS isolates 5635, 5693, and 6044. Production of TNF-α was assessed by intracellular immunohistochemical staining after 0, 8, 12, 24, 36, 48, and 72 h of stimulation, and the results are presented as mean percent cytokine-producing cells ± SD minus percent cytokine-producing cells in unstimulated cultures. The figure shows the peak value of TNF-α production for each individual.

We selected three clinical GAS strains isolated from patients with STSS and NF. All three isolates were of the M3T3 serotype, and two had identical known Spe genotypes. Analysis of SAg expression revealed that all three GAS supernatants produced the SAgs SpeF and SSA, and one of the isolates also expressed SpeA. Culture supernatants from all three isolates were found to be highly potent inducers of proliferative and inflammatory cytokine responses. The results showed that although two of the isolates apparently had the same SAg profile, they differed in their mitogenic activity. This may be attributed to differences in the type and/or amount of SAg produced by the isolate. However, isolate 6044, which expressed SpeA, SpeF, and SSA, elicited a lower mitogenic response than isolates 5635 and 5693, which expressed SpeF and SSA only. This apparent contradiction can be explained in light of recent data showing that most invasive GAS isolates produce novel, yet-uncharacterized SAgs, which are likely to contribute to the mitogenic activity produced by these isolates (19).

Despite differences in the production of mitogenic activity among the three GAS supernatants tested, there was no sig-

TABLE 2. Individual variation in IL-1β production induced by GAS culture supernatants

Stimulus	IND[a]	Quantification of IL-1β-producing cells[b]			
		Intensity (mean ± SE)[c]	*P* value[d]	Area (mean mm^2 ± SE)[c]	*P* value[d]
5635	1	20,348 ± 654		162 ± 4.4	
5635	2	17,213 ± 843		138 ± 6.7	
5635	3	16,188 ± 456	0.0001	128 ± 3.2	0.0001
5693	1	22,613 ± 604		178 ± 4.2	
5693	2	20,316 ± 878		152 ± 6.8	
5693	3	18,131 ± 398	0.0001	141 ± 2.9	0.0001
6044	1	27,550 ± 590		216 ± 4.6	
6044	2	21,283 ± 904		165 ± 6.8	
6044	3	20,080 ± 464	0.0001	154 ± 3.6	0.0001

[a] IND, individual.

[b] PBMC from three healthy individuals were stimulated with a 1:100 dilution of culture supernatants prepared from the clinical GAS isolates 5635, 5693, and 6044. The cells were harvested after various times of culture, fixed, and stained for cytokines by immunohistochemical staining. The values shown are following 8 h of stimulation.

[c] The integrated density of stain (intensity) and cell size (area) of IL-1β-producing cells were determined by the Oncor Image 2.0 analysis system.

[d] ANOVA was used to determine significant differences. The ANOVA was validated by Tukey-Kramer with an alpha level of 0.01, which verified that IND 1 > IND 2 = IND 3 is significantly different.

nificant difference in their cytokine induction profiles. Cytokines can be divided into inflammatory and regulatory groups, and it has been shown that an imbalance in the production of these two groups can lead to pathology in the host (1, 21, 41). Previous studies reported that defined streptococcal SAgs induce a Th1 type of response, with high levels of the inflammatory cytokines TNF-β and IFN-γ and only very low levels of the Th2 cytokines IL-4 and IL-5 (2, 29). However, as shown in this study, GAS supernatants, which contain a mixture of SAgs, were significantly more potent inducers of Th2 cytokines than was the defined GAS SAg, rSpeA ($P < 0.0062$). Thus, it appears that a mixture of SAgs, combined with other virulence factors, induces a different cytokine induction profile than do defined SAgs, and this profile may reflect more closely the in vivo situation.

Results from this study showed that a particular individual consistently mounted significantly higher proliferative and inflammatory cytokine responses against all three GAS supernatants and rSpeA than the other two individuals. Individual differences in cytokine responses have been noted both among healthy individuals and patients with varying manifestations of invasive GAS diseases (data not shown). Together these observations are consistent with our hypothesis that the host contributes significantly to the magnitude and relative balance between inflammatory and regulatory cytokine responses.

In addition, marked individual differences in the relative induction of Th1 and Th2 cytokines were noted. The individual who mounted the highest inflammatory cytokine response was the lowest producer of Th2 cytokines, and the reverse was also true. These findings are in strong agreement with a large number of studies showing that the Th2 cytokine IL-4 inhibits the generation of Th1 cytokines (15, 22, 31, 38). Further support for the hypothesis that there were differential Th1 and Th2 responses among the individuals was obtained by image analysis of IL-1β-producing cells following stimulation with GAS supernatants. The analysis revealed that the individual with the strongest Th1 response and the lowest Th2 response had a significantly higher intensity and larger area of IL-1β-producing cells than individuals with a lower Th1 and higher Th2 response. This is in agreement with a recent study by Chizzolini et al. (7), which showed that Th1 cells induced high IL-1 production whereas Th2 cells induced high IL-1 receptor antagonist production by monocytes. We believe that differences among individuals in Th1 versus Th2 responses may contribute to differences in susceptibility and/or severity of invasive GAS infections. Although this hypothesis remains to be proven, a recent study by Walley et al. (41) showed that in a murine animal model, the severity and mortality of sepsis were related to the balance of inflammatory and regulatory cytokines. Further support for this hypothesis was obtained from our recent studies of the levels of cytokines in patients with severe and nonsevere invasive infection caused by genotypically identical M1T1 GAS strains. The results revealed that during the acute phase, patients with severe invasive GAS infection (i.e., STSS) had significantly higher levels of inflammatory cytokines than patients with nonsevere infection ($P > 0.0006$ to 0.026) (30a). Most importantly, the responses of the patients during convalescence (>4 weeks postdischarge) reflected the same difference seen during the acute phase (30a). Together the data underscore the role of host immunogenetic factors in regulating the inflammatory cytokine responses to GAS SAgs and modulating the severity of the systemic manifestations in invasive infections. Further studies should identify those factors and reveal the underlying molecular and cellular mechanisms affecting the pathogenicity of streptococcal diseases.

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