INFECTION AND IMMUNITY, June 1997, p. 2119–2126 Vol. 65, No. 6
0019-9567/97/$04.00+0

Keratinocyte Proinflammatory Responses to Adherent and Nonadherent Group A Streptococci

BEI WANG,[1] NATIVIDAD RUIZ,[1] ALICE PENTLAND,[2,3]† AND MICHAEL CAPARON[1]*

Departments of Molecular Microbiology,[1] Dermatology,[2] and Molecular Biology and Pharmacology,[3] Washington University School of Medicine, St. Louis, Missouri 63110-1093

Received 13 January 1997/Returned for modification 18 February 1997/Accepted 27 March 1997

The gram-positive bacterium *Streptococcus pyogenes* (group A streptococcus) is the causative agent of a wide variety of suppurative infections of cutaneous tissues. Previous analyses have demonstrated that the M protein of *S. pyogenes* is an adhesin that directs the attachment of the streptococcus to keratinocytes in the skin. In this study, we have examined keratinocyte function in response to *S. pyogenes* and found that adherent versus nonadherent streptococci promote distinct patterns of expression of several proinflammatory molecules and keratinocyte cell fate. When analyzed by a quantitative reverse transcriptase PCR method, infection of cultured HaCaT keratinocytes with adherent, but not nonadherent, streptococci resulted in increased expression of mRNA for the cytokines interleukin-1α (IL-1α), IL-1β, and IL-8 but neither infection induced expression of tumor necrosis factor alpha. In contrast, both adherent and nonadherent *S. pyogenes* induced expression of IL-6 and each promoted synthesis and release of prostaglandin E_2 (PGE_2). However, considerably greater levels of IL-6 expression were stimulated by adherent streptococci relative to nonadherent streptococci and the kinetics of PGE_2 release in response to nonadherent streptococci was delayed compared to the response to adherent streptococci. Staining with the fluorescent probe ethidium homodimer-1 revealed that keratinocyte membranes were rapidly damaged upon infection with adherent streptococci but were not damaged by nonadherent streptococci. Finally, treatments which inhibited streptococcal metabolism completely blocked the ability of adherent streptococci to elicit responses. These data suggest that expression of an adhesin is a strategy used by *S. pyogenes* to modulate keratinocyte responses during infection of the skin and implicate additional streptococcal products in these signaling interactions.

The keratinocyte, the major cell lineage in the epidermis, represents the interface between the internal tissues of the host and the external environment. In this capacity, it acts as a critical first line of defense against microbial infection. Recent evidence suggests that keratinocytes do not simply serve as a passive barrier to infection but play an active role in host defense. Of relevance to the initial stages of a bacterial infection, keratinocytes can elaborate an array of proinflammatory molecules like prostaglandins (20) and various cytokines, including interleukin-1α (IL-1α), IL-1β, IL-6, IL-8, and tumor necrosis factor alpha (TNF-α) (1, 16). However, the mechanisms by which keratinocytes sense the presence of bacteria and the inductive cues for expression of proinflammatory signaling molecules in the epidermis have not been well characterized.

The gram-positive bacterium *Streptococcus pyogenes* (group A streptococcus) is one of the most important pathogens that interact with keratinocytes in the cutaneous tissues. *S. pyogenes* can cause numerous serious suppurative and inflammatory infections of the skin that range from relatively mild and self-limiting impetigo to more destructive infections (erysipelas, ecthyma, cellulitis, and pyoderma) (39) to life-threatening necrotic infections (necrotizing fasciitis and myositis) (2). Systemic complications can accompany infection of the skin (scarlet fever, toxic shock syndrome, and sepsis) (2), as well as postinfection sequelae in both the cutaneous tissues (guttate psoriasis) (40) and other organs (acute glomerulonephritis) (39). For the most part, the contribution of any specific streptococcal virulence factor to the pathogenesis of any of these diseases is poorly understood.

Since the initial stage of all streptococcal infections of the skin likely involves interaction with keratinocytes, the outcome of this encounter may play an important role in influencing the subsequent course of disease. To begin to address this issue, the factors which promote the recognition of keratinocytes by *S. pyogenes* have been examined and it has been shown that a specific surface-exposed protein of *S. pyogenes*, known as the M protein (7), is an adhesin that directs intimate attachment of the streptococcus to the keratinocyte (25, 30). Structure-function studies have shown that a region of M protein, known as the C repeat domain, is important for the recognition of keratinocytes (24, 30). In addition, membrane cofactor protein (MCP or CD46), a surface protein found on many lineages of nucleated cells, including keratinocytes (18), can bind to M protein in a C repeat-dependent fashion (24), and soluble derivatives of MCP effectively inhibit adherence of *S. pyogenes* to keratinocytes (24). These data implicate MCP as a receptor for adherence of *S. pyogenes* to keratinocytes.

The function of MCP is to regulate the alternative pathway of complement activation at the cell surface (18), and the protein is also the receptor essential for the attachment and internalization of measles virus (6). A number of MCP-promoted signaling events are associated with infection by measles virus (6, 15), suggesting that M protein-MCP interactions could modulate the responses of keratinocytes. Other pathogenic bacteria have been reported to modulate the responses of mucosal epithelial cells through adhesin-receptor interactions (11, 13).

To gain insight into the molecular mechanisms by which keratinocytes respond to bacterial infection in the skin and how adhesin-receptor interactions and other virulence proper-

* Corresponding author. Phone: (314) 362-1485. Fax: (314) 362-1232. E-mail: caparon@borcim.wustl.edu.

† Present address: Department of Dermatology, University of Rochester, Rochester, NY 14642.

ties of *S. pyogenes* can potentially influence these responses, we have examined prostaglandin E_2 (PGE_2) production and the expression of several proinflammatory cytokines by keratinocytes in response to infection with adherent *S. pyogenes* and well-defined nonadherent mutants. The resulting data demonstrate that distinct patterns of cytokine gene expression, prostaglandin production, and membrane damage are induced by adherent versus nonadherent streptococci and implicate other virulence factors in addition to adhesin-receptor interaction in streptococcal-keratinocyte signaling events.

MATERIALS AND METHODS

Bacterial strains and media. *S. pyogenes* JRS4 produces a serotype 6 M protein and is a spontaneous streptomycin-resistant derivative of D471 from the Rockefeller University collection (34). Insertional inactivation of the gene which encodes M protein (*emm6.1*) in JRS4 generated JRS145 (4). Reintroduction of *emm6.1* into JRS145 produced JRS236 (29). Strain JRS4 transformed with the plasmid pLZ12 (28) generated JRS4(pLZ12). *Escherichia coli* DH5α (Gibco/BRL) was used in molecular cloning experiments and for production of plasmid DNA. *S. pyogenes* strains were grown in Todd-Hewitt medium (BBL) supplemented with 0.2% yeast extract (Difco). *E. coli* was cultured in Luria-Bertani medium. When required, ampicillin was added to *E. coli* cultures to a final concentration of 100 μg/ml.

Infection of keratinocytes. The HaCaT human keratinocyte line (3) binds *S. pyogenes* in an M protein-dependent and MCP-inhibitable manner identical to that of normal human keratinocytes (24, 30). HaCaT cells were cultured in 75-cm^2 flasks or on glass coverslips in 24-well plates in HaCaT medium (24) for 3 to 4 days before use in a humidified atmosphere of 7% CO_2 at 37°C. Various *S. pyogenes* strains from overnight broth cultures were washed twice in Dulbecco's modified Eagle's medium (DMEM) or phosphate-buffered saline (PBS; pH 7.4), resuspended in HaCaT medium to equivalent concentrations (optical density at 600 nm, 0.05), and used to infect HaCaT cultures as described previously (24). In selected experiments, erythromycin (final concentration, 10 μg/ml) or chloramphenicol (final concentration, 20 μg/ml) was added to the suspensions of streptococci immediately prior to infection of the HaCaT cells. These antibiotic concentrations are greater than 100-fold higher than the MICs of erythromycin and chloramphenicol for these strains. Adherence phenotypes of unsupplemented and antibiotic-treated streptococcal suspensions were confirmed by using streptococci labeled with ^{35}S as described elsewhere (38). Microscopic examination revealed that both adherent and nonadherent streptococci multiplied approximately to the same degree during the 8-h period of infection, that the adherent bacteria remained attached to the cell surface during the full period of infection, and that nonadherent streptococci remained in suspension with very few, if any, bacteria in contact with the cell surface. Where indicated, additional controls included mock infection of cell cultures with medium alone or with latex beads (Sigma catalog no. SD-6A) that were washed twice in DMEM prior to infection or with uninoculated Todd-Hewitt medium (10% [vol/vol] in DMEM). Total cellular RNA was isolated from infected and mock-infected keratinocyte cultures at various time points after infection by selective binding to a silica membrane (RNeasy; Qiagen) in accordance with the recommendations of the manufacturer.

RNA standards for quantitative PCR. The PCR-based method of Jung et al. (13) for the quantitation of cytokine expression in epithelial cells was adapted for use in keratinocytes by construction of a standard molecule as follows. Oligonucleotide overlap extension and PCR amplification (12) were used to create a molecule in which priming sites for various cytokine messages flank a 30-bp spacer sequence. The spacer sequence consists of recognition sites for several restriction endonucleases, and the sequences of the priming sites for the various cytokine messages were derived from those of Jung et al. (13). The standard molecule contains priming sites for the proinflammatory cytokines that are known to be produced by keratinocytes, including IL-1α, IL-1β, IL-6, IL-8, and TNF-α. Priming sites for β-actin (13) were included as a control. The molecule was introduced adjacent to the T7 RNA polymerase promoter of pGEM-3Z (Promega), and the resulting plasmid was designated pMBW1. The nucleotide sequence of the insert in pMBW1 was confirmed by the dideoxy-chain termination method using a modified T7 DNA polymerase (Amersham Life Science). The order of the priming sites and the location of the T7 polymerase promoter are shown in Fig. 1.

Production of standard RNA. To generate standard RNA, pMBW1 was linearized by digestion with the restriction endonuclease *Hin*dIII and the standard sequence was transcribed in vitro by using T7 RNA polymerase in accordance with the recommendation of the manufacturer (Gibco/BRL). Template DNA was removed by DNase (Promega) treatment, followed by purification of the RNA transcript by using a silica membrane (RNeasy; Qiagen). The 368-nucleotide standard RNA molecule was further subjected to oligo(dT) chromatography (Quick mRNA isolation kit; Stratagene), and the concentration of the final product was determined by measurement of A_{260}.

PCR amplification for quantitation of cytokine mRNA. Increasing numbers of standard RNA molecules were mixed with a constant amount of total cellular RNA from each sample to be assayed and subjected to reverse transcription as follows. Depending on the specific cytokine message analyzed, standard RNA molecules (from 50 to 10^7 molecules) were mixed with a constant amount of total cellular RNA (0.3 to 0.6 μg) in a reaction which contained 10 mM Tris-HCl (pH 8.3); 50 mM KCl; 5 mM $MgCl_2$; 1 mM each dATP, dCTP, dGTP, and dTTP; 10 U of RNasin Inhibitor (Promega); 0.05 μg of oligo$(dT)_{12-18}$ (Gibco/BRL); and 25 U of reverse transcriptase (Superscript II; Gibco/BRL) in a final volume of 10 μl. Following 60 min at 37°C, the reaction was terminated by heating at 95°C for 10 min and the mixture was placed on ice. Cytokine-specific primers which annealed to sites derived from both the cytokine mRNA and the standard RNA molecule were used in a PCR for each sample in an individual reaction mixture that contained primers (10 pmol of each); 50 μM each dATP, dCTP, dGTP, and dTTP; and 0.5 U of *Taq* DNA polymerase (Gibco/BRL) in a final volume of 50 μl using the buffer supplied by the supplier (Gibco/BRL) at the recommended final concentration. Amplification involved 35 cycles of 1 min of denaturation at 95°C, 1 min of annealing at 55°C (IL-1α, IL-1β, IL-6, and TNF-α) or 60°C (IL-8 and β-actin), and 2 min of extension at 72°C.

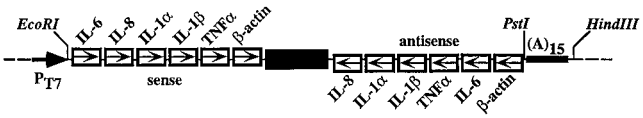

FIG. 1. Template for generation of standard RNA. The DNA template for preparation of standard RNA is contained in plasmid pMBW1, whose relevant features are shown here. Transcription in vitro from the T7 promoter (P_{T7}) generates an RNA molecule that contains annealing sites for sense and antisense oligonucleotide primers specific for the indicated keratinocyte mRNAs. The annealing sites for each message flank a 30-bp spacer segment, and the standard RNA terminates with a poly(A) tail of 15 A residues [$(A)_{15}$]. Amplification of the standard RNA by reverse transcription PCR yielded a product with a size different from that of the corresponding target RNA. The sizes of the products for the target and standard RNAs with each specific primer pair are shown in Table 1. From the initiation codon of the T7 promoter to the end of the $(A)_{15}$ segment, the standard molecule itself is 368 nucleotides long and was inserted between the *Eco*RI and *Hin*dIII sites of pGEM-3Z to construct pMBW1. The spacer segment region contains recognition sites for (from left to right) *Sal*I, *Sac*I, *Kpn*I, and *Sma*I.

Quantitation of PCR products. The PCR products were separated on a 2% regular agarose gel. Table 1 indicates the sizes of the PCR products from the target RNA and standard RNA for each cytokine-specific primer. PCR products were initially visualized by using ^{32}P-end-labeled primers and a phosphorimager (Molecular Imager; Bio-Rad) or by staining with ethidium bromide and exposure to UV illumination and band intensity quantitated by densitometry (IS1000; Innotech). However, since the two methods yielded equivalent results, subsequent analyses utilized only the ethidium bromide staining method. The number of molecules of target cytokine mRNA present in each sample was calculated as described previously (13). Briefly, the ratio of the band intensities of PCR products from the standard RNA and target RNA was plotted against the starting number of standard RNA molecules by using a double-logarithmic scale. When the ratio of band intensities equals 1, the number of target RNA molecules in the sample is equivalent to the number of standard RNA molecules (13). When ethidium bromide staining was used, the value obtained from the plot was multiplied by the ratio of the target band length to standard band length to correct for the difference in staining intensity due to the size difference between the standard and target products. A representative assay and standard curve are illustrated in Fig. 2. The data in the text are shown as the number of transcript molecules present in 1 μg of RNA and represent the mean value obtained from three independent infections, each of which typically differed by less than 10% from the stated mean.

TABLE 1. Sizes of PCR products of keratinocyte cytokine and β-actin cDNAs

mRNA species	Size of PCR product (bp)[a]	
	Standard RNA	Target RNA
IL-1α	169	420
IL-1β	186	388
IL-6	307	628
IL-8	184	289
TNF-α	185	355
β-Actin	213	661

[a] Templates for PCR were cDNAs derived from total cellular RNA (target RNA) or from in vitro transcription of the standard molecule (standard RNA; see Fig. 1). The primers and reaction conditions used are described in the text.

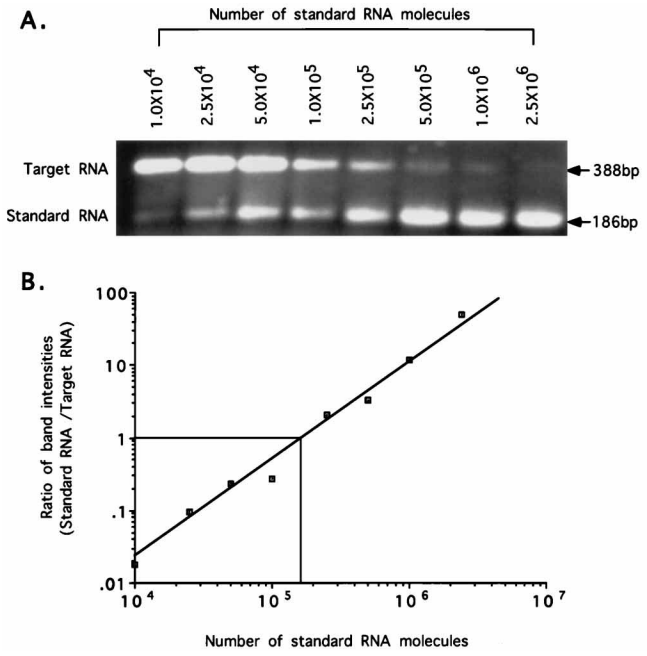

FIG. 2. Representative reverse transcriptase PCR analysis for quantitation of cytokine mRNA. (A) Various numbers of standard RNA molecules (as indicated at the top) were added to separate reaction mixtures with a constant amount (375 ng) of cellular RNA from a keratinocyte culture infected with *S. pyogenes* JRS4 in the presence of erythromycin. The standard and target RNAs in each reaction mixture were amplified by reverse transcriptase PCR with IL-1β specific primers. The 388-bp target and 186-bp standard PCR products were separated on a 2% agarose gel and visualized by staining with ethidium bromide. (B) The intensities of the bands from the gel shown above were determined by densitometry, and the ratio of standard RNA to target RNA was plotted against the number of standard RNA molecules added to each reaction mixture. At a ratio of 1.0, the numbers of target and standard molecules are equivalent (1.6×10^5 molecules in this example), which indicates 4.3×10^5 IL-1β molecules per μg of cellular RNA. To correct for differences in the binding of ethidium bromide due to differences in product size, this value was then multiplied by the ratio of the size of the target product to the size of the standard product to obtain the final concentration of 9.0×10^5 IL-1β molecules per μg of cellular RNA. Jung et al. (13) have described the method more completely.

Measurement of PGE_2. At various time points, the media from control and infected HaCaT cultures were collected and centrifuged (5 min, 15,000 × *g*) to remove bacteria and the resulting supernatants were frozen at −20°C until assayed. The concentrations of PGE_2 in these samples were determined by an enzyme-linked immunosorbent assay (Caymen Chemical catalog no. 514010) in accordance with the recommendations of the manufacturer. The data shown represent the mean and standard error from duplicate determinations of each sample from a single experiment that was representative of at least three independent experiments.

Analysis of membrane damage. Confluent, 2-day-old HaCaT cultures were infected with *S. pyogenes* strains as described above. At various time points, the supernatants were removed and the wells were washed once in PBS (pH 7.4). A 0.25-ml aliquot of a solution of calcein AM (1.2 μM) and ethidium homodimer-1 (EthD-1; 0.75 μM) in PBS (Live/Dead Eukolight; catalog no. L-3224; Molecular Probes) was added to each well. After 45 min of incubation at room temperature in the dark with gentle rocking, each well was washed once with PBS and the coverslips were removed and mounted on microscope slides. The samples were examined under a fluorescence microscope (Laborlux 12; Leitz) for staining of the keratinocytes with calcein AM, which stains cells with undamaged membranes, or with EthD-1, which stains the DNA of cells whose membranes are damaged (11). The mean percentage of EthD-1-stained cells per microscope field was calculated from at least five randomly chosen fields representing the analysis of a minimum of 1.8×10^3 total cells. The data presented were derived from a single experiment representative of at least five independent experiments.

RESULTS

Cytokine gene transcription following infection of keratinocytes with adherent or nonadherent *S. pyogenes*. Keratinocytes, the principal cell lineage of the skin, have the capacity to produce several proinflammatory cytokines. Basal numbers of transcripts for IL-1α, IL-1β, and IL-8 were detected in uninfected HaCaT keratinocytes (Table 2). The basal numbers of transcripts for IL-6 and TNF-α in these cells were below the level of detection of this assay (Table 2).

Examination of the expression of this panel of cytokine genes in response to infection with an *S. pyogenes* strain that expresses the M protein adhesin (JRS4), and consequently had the capacity to adhere to keratinocytes, revealed a considerable increase in the number of cytokine gene transcripts detected. For IL-1α, IL-1β, and IL-8, this amounted to 7-, 26-, and 84-fold increases, respectively (Table 2), after only 2 h of exposure to the adherent streptococci. For IL-6, the increase in the numbers of transcripts detected was particularly dramatic given the low basal level of transcription for this cytokine gene in uninfected cells (Table 2). In contrast, no stimulation of expression of TNF-α was observed in response to adherent streptococci, as was a minimal increase in transcription of a noncytokine (β-actin) gene included for comparison (Table 2).

Analysis of a derivative of the adherent *S. pyogenes* strain which was incapable of adhering to keratinocytes due to the deletion of the gene which encodes the adhesin (JRS145) revealed a distinctly different pattern of cytokine gene expression. Similar to infection with adherent JRS4, infection with nonadherent JRS145 provoked a considerable increase in the number of IL-6 transcripts detected at 2 h following initiation of infection (Table 2). However, only moderate increases in the numbers of transcripts of the genes for IL-1β and IL-8 were observed and there was no increase in the number of IL-1α mRNA molecules (Table 2). Also, like the adherent strain, the nonadherent mutant did not stimulate a detectable level of TNF-α expression and had no effect on the expression of β-actin (Table 2).

The stimulation of cytokine gene transcription caused by infection with adherent and nonadherent streptococci followed similar time courses. For IL-6 transcription stimulated by both adherent and nonadherent *S. pyogenes*, the maximal increase was observed 2 h following the initiation of infection. After 4 and 6 h, IL-6 levels had declined considerably relative to the levels observed at 2 h but were still considerably elevated compared to those of uninfected keratinocytes (Table 2). For each of the other cytokines stimulated in response to infection with the adherent *S. pyogenes* strain (IL-1α, IL-1β, and IL-8; Table 2), the maximal response was observed at 2 h, had declined when measured at 4 h, but remained relatively constant at 6 h. The number of TNF-α transcripts was not stimulated above detectable levels at any later time point, and the number of β-actin transcripts remained constant (Table 2).

Comparison of the transcript levels achieved after infection of keratinocytes with adherent JRS4 and nonadherent JRS145 demonstrated that, overall, adherence has a dramatic stimulatory effect on cytokine gene transcription. Adherence stimulated a 9- to 26-fold-enhanced level of transcription for each of the stimulated cytokines after 2 h of infection, including a greater than 15-fold increase for IL-6 (Table 2). Cytokine gene expression was also enhanced at each later time point for infection with adherent relative to nonadherent streptococci. For comparison, there was a minimal (less than twofold) increase in the number of β-actin transcripts detected after infection with adherent versus nonadherent streptococci at any time point tested (Table 2). In additional controls for these experiments, reintroduction of the gene encoding the adhesin into the adhesin-deficient mutant generated a strain (JRS236) that regained the abilities both to adhere to HaCaT keratinocytes (30) and to stimulate IL-1α and IL-1β transcription to

TABLE 2. Cytokine expression patterns in uninfected and infected keratinocytes

Postinfection time and cytokine	No. of transcripts/μg (ratio)[a]			JRS4/JRS145 ratio
	Uninfected keratinocytes	JRS4 (M^+)[b]-infected keratinocytes	JRS145 (M^-)[b]-infected keratinocytes	
2 h				
IL-1α	1.1×10^6	8.0×10^6 (7.3)	3.2×10^5 (0.3)	24.3
IL-1β	5.4×10^5	1.4×10^7 (25.9)	1.6×10^6 (2.9)	8.9
IL-6	<2,000	5.3×10^7 (>25,000)	1.6×10^6 (>1,600)	15.6
IL-8	1.1×10^6	8.3×10^7 (75.5)	6.0×10^6 (5.4)	14.0
TNF-α	<200	<200	<100	
β-Actin	3.4×10^7	7.2×10^7 (1.1)*	4.5×10^7 (1.3)*	0.85*
4 h				
IL-1α	1.1×10^6	1.1×10^6 (1.0)*	1.8×10^5 (0.2)	5.0
IL-1β	5.0×10^5	3.5×10^6 (7.0)	9.6×10^5 (1.9)	3.7
IL-6	<2,000	1.0×10^5 (>48)	9.4×10^4 (>45)	5.9
IL-8	9.3×10^5	1.7×10^7 (18.3)	2.8×10^6 (3.1)	1.1
TNF-α	<200	<200	<200	
β-Actin	3.3×10^7	2.7×10^7 (0.8)*	2.7×10^7 (0.7)*	1.1*
6 h				
IL-1α	1.1×10^6	2.7×10^5 (2.5)	4.7×10^5 (0.5)	5.0
IL-1β	5.2×10^5	3.8×10^6 (7.3)	9.8×10^5 (1.8)	4.0
IL-6	<2,000	3.9×10^4 (>19)	2.0×10^4 (>10)	1.9
IL-8	9.6×10^5	2.9×10^7 (30.2)	3.1×10^6 (3.4)	8.9
TNF-α	<200	<200	<200	
β-Actin	3.1×10^7	2.4×10^7 (0.8)*	2.7×10^7 (0.8)*	1.0*

[a] Infected/uninfected. Values used to derive ratios are significantly different by Student's two-tailed *t* test ($P < 0.005$), unless marked with an asterisk.
[b] Adhesin phenotypes are in parentheses.

levels equivalent to those stimulated by the original adherent parental strain, JRS4 (data not shown). Cytokine expression profiles following mock infection with either latex beads or uninoculated streptococcal culture medium (see Materials and Methods for details) were identical to those of uninfected keratinocyte cultures at 2, 4, and 6 h (data not shown).

Adherence is required but not sufficient for enhanced cytokine expression. To address whether the stimulation of cytokine gene transcription was a direct response of M protein adhesin-keratinocyte receptor signaling, keratinocytes were infected with adherent JRS4 in the presence of erythromycin to block nascent streptococcal protein synthesis. In initial experiments, it was found that the concentration of erythromycin used had no effect on the ability of adhesin-proficient *S. pyogenes* to adhere to keratinocytes (data not shown), had no effect on the viability of the keratinocytes, and had no effect on the ability of adherent, erythromycin-resistant strain JRS236 to stimulate expression of cytokine genes (data not shown). However, erythromycin treatment completely blocked the ability of adherent, erythromycin-sensitive JRS4 to trigger expression of the panel of proinflammatory cytokines, including IL-1α, IL-1β, IL-6, and IL-8, at 2 h after infection (Table 3). Infection with a fourfold excess of JRS4 in the presence of erythromycin revealed a modest increase in the number of transcripts for IL-1α detected at 2 h, which was similar to the increase in β-actin transcripts observed at this time point in the absence of erythromycin (2.5-fold; Table 2). This concentration of streptococci was in excess of that present at 2 h when erythromycin was not added to the culture. These data suggest that while adherence has a stimulatory effect on the triggering of cytokine responses, some other product(s) of streptococcal metabolism must participate in streptococcal-keratinocyte signaling interactions.

Adherence stimulates PGE_2 synthesis. In addition to cytokines, keratinocytes have the capacity to synthesize proinflammatory products of arachidonic acid metabolism, including PGE_2 (20). When PGE_2 generation by keratinocytes in response to infection with *S. pyogenes* was compared in adherent and nonadherent streptococci, it was observed that both promoted the release of PGE_2 but that the patterns of stimulation were very different. Adherent strain JRS4 promoted an initial vigorous response in which the levels of PGE_2 increased at a high rate for up to 4 h after infection (Fig. 3). After this time, PGE_2 continued to be released by the infected keratinocytes, although at a slower rate (Fig. 3). In contrast, infection with nonadherent JRS145 lacked the strong initial response promoted by the adherent strain and levels at 6 h postinfection were approximately threefold lower than those released in response to adherent JRS4 (Fig. 3). However, by 24 h, the concentration of PGE_2 released in response to JRS145 ap-

TABLE 3. Cytokine expression patterns in keratinocytes in response to metabolism-inhibited JRS4

Cytokine	No. of transcripts/μg		Ratio[b]	% Inhibition[c]
	Uninfected keratinocytes	JRS4-infected keratinocytes + Em[a]		
IL-1α	1.1×10^6	6.7×10^5	0.6	92
IL-1β	5.4×10^5	8.8×10^5	1.6	94
IL-6	<2,000	<2,000	1.0	>99
IL-8	1.1×10^6	3.6×10^6	3.3	96
TNF-α	<200	<200	1.0	
β-Actin	3.4×10^7	4.0×10^7	1.2	+44

[a] HaCaT keratinocytes infected by incubation with JRS4 for 2 h in the presence of 10 μg of erythromycin (Em) per ml.
[b] Infected/uninfected.
[c] % Inhibition = $100 \times [1 - (\text{transcripts } \mu g^{-1} \text{ JRS4 + Em/transcripts } \mu g^{-1} \text{ JRS4})]$. The number of transcripts per microgram obtained with JRS4-infected keratinocytes is taken from the 2-h time point of Table 2.

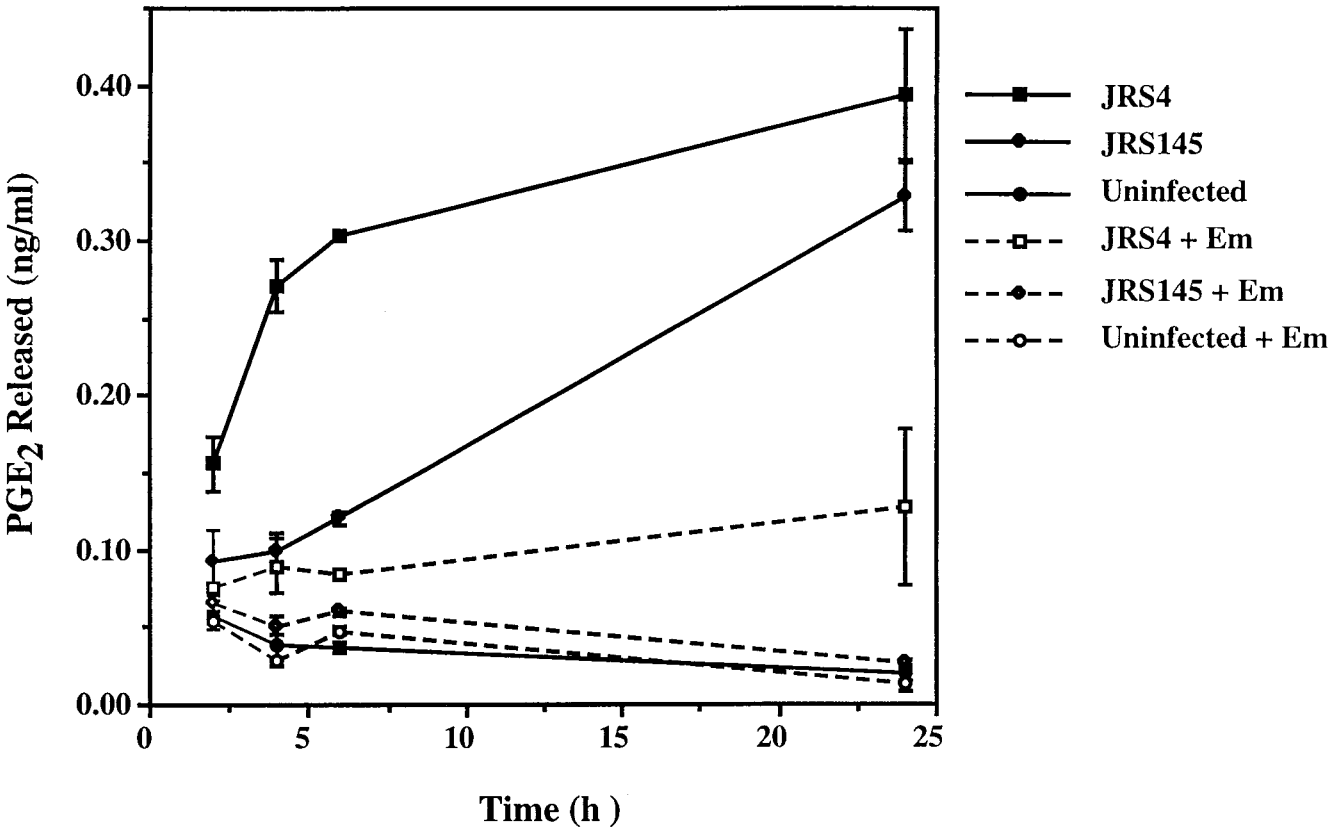

FIG. 3. Kinetics of prostaglandin production differ during infection by adherent versus nonadherent *S. pyogenes*. The level of PGE_2 released during infection of HaCaT keratinocytes with adherent (JRS4) or nonadherent (JRS145) *S. pyogenes* was determined by a quantitative enzyme immunoassay and compared to the level released by uninfected keratinocytes. Also shown are the levels of PGE_2 released in response to infection with *S. pyogenes* in the presence of erythromycin (Em), which was used to block streptococcal protein synthesis. The lower limit of sensitivity of this assay is 0.04 ng of PGE_2 per ml.

proached the level obtained with JRS4 (Fig. 3), indicating that while the nonadherent strain could promote the release of PGE_2 from keratinocytes, the response was delayed relative to that caused by the adherent strain. Infection in the presence of erythromycin eliminated both the rapid initial increase in the release of PGE_2 in response to JRS4 and the delayed response to JRS145 (Fig. 3).

Adherence promotes keratinocyte membrane damage. To gain additional insight into keratinocyte responses to adherent and nonadherent *S. pyogenes*, the membrane integrities of keratinocytes infected with the two *S. pyogenes* strains were compared. Staining with a probe whose fluorogenic cleavage product can be retained only by cells with intact membranes (calcein AM) (10) revealed that virtually all of the keratinocytes in cultures infected with adherent JRS4 began to lose the ability to cleave calcein AM to its green fluorescent product by 1 h after infection and that by 4 h, virtually all keratinocytes lacked fluorescence (data not shown). Simultaneous staining with a high-affinity fluorescent DNA probe that can pass only through compromised membranes (EthD-1) (10) indicated that, in parallel with the loss of staining with the vital probe, the membranes in virtually all of the keratinocytes in cultures infected with adherent JRS4 lost their ability to exclude the probe by 4 h after infection (Table 4). From 1 to 3.5 h after infection with adherent JRS4, diffuse EthD-1 staining of the cytoplasm was observed which progressed to intense staining of the nuclei beginning approximately 3.5 h after infection. These results contrasted markedly with those of infection with the nonadherent mutant JRS145, in which nearly all of the keratinocytes retained calcein AM staining (data not shown) and very few possessed nuclei stained with EthD-1, even 8 h after infection (Table 4). Reintroduction of the adhesin gene into the mutant restored the membrane-damaging phenotype (JRS236 infection in Table 4), and addition of chloramphenicol to block nascent streptococcal protein synthesis completely blocked the ability of a chloramphenicol-sensitive adherent strain to damage membranes (JRS4 infection + Cm in Table 4) but did not block the ability of this strain to damage membranes following introduction of a plasmid to render it chloramphenicol resistant (pLZ12 infection + Cm in Table 4). A 2.5-fold decrease in the initial inoculum of adherent JRS4 did not result in fewer EthD-1-stained keratinocytes at any time point examined. However, in the presence of chloramphenicol, a 16-fold increase in the initial inoculum resulted in only a moderate (<5%) increase in the number of keratinocytes stained with EthD-1 at 8 h. This concentration of streptococci is in excess of the number present due to multiplication of the standard inoculum in the absence of chloramphenicol at this time point.

DISCUSSION

Through its developmental program of differentiation leading to cornification and generation of the stratum corneum, the principal role of the keratinocyte is to provide a physically tough barrier between the host and the environment. Its efficiency in this regard is illustrated by the fact that most pathogens, including *S. pyogenes*, have almost no ability to penetrate

TABLE 4. Adherent *S. pyogenes* damages membranes of HaCaT keratinocytes

Keratinocyte treatment[b]	Avg % of cells stained with EthD-1 postinfection ± SD[a]			
	3 h	4 h	6 h	8 h
JRS4 [M$^+$] infection	<10	96.0 ± 1.6	95.5 ± 1.3	97.1 ± 1.4
JRS236 [M$^+$] infection	ND[d]	99.4 ± 0.3	98.5 ± 1.1	98.3 ± 0.9
JRS145 [M$^-$] infection	<0.3	5.5 ± 1.3	5.1 ± 1.9	5.9 ± 1.2
No infection	<0.3	<0.3	<0.3	<0.3
JRS4 infection + Cm[c]	ND	ND	ND	1.1 ± 1.1
JRS4(pLZ12) infection + Cm	ND	ND	ND	98.7 ± 0.8
No infection + Cm	ND	ND	ND	<0.3

[a] The number of cells stained with EthD-1 at the indicated times following infection was determined by fluorescence microscopy. The data are averages derived from at least five microscopic fields.
[b] Adhesin phenotypes are in brackets.
[c] Chloramphenicol (Cm) was added at a final concentration of 20 μg/ml.
[d] ND, not done.

an intact stratum corneum. However, far from being passive, the keratinocyte has the capacity to play an active role in host defense once its barrier function has been breached. The present study has examined how keratinocytes respond to streptococcal infection and has shown that distinct patterns of membrane damage and expression of proinflammatory molecules are induced by adherent versus nonadherent *S. pyogenes*.

Certain features of keratinocyte cytokine production are somewhat unusual, including high basal rates of IL-1 production, preferential production of IL-1α relative to IL-1β, and expression of significant numbers of IL-1 receptors in the absence of stimulation (16). The inductive cues for the release of IL-1 from keratinocytes are poorly understood; however, since neither IL-1α nor IL-1β can be actively secreted by keratinocytes, it is felt that the signal likely involves perturbation of the cellular membrane (16). Once released, IL-1 can act as an autocrine or paracrine factor to further up-regulate keratinocyte production of other cytokines, including IL-1 itself, to initiate inflammatory processes (1, 16). Consistent with this model was the observation that stimulation of IL-1 transcription was observed to occur in parallel with the membrane damage caused by adherent streptococci and was not promoted by the nonadherent mutant. Alternatively, rather than being a product of membrane damage, induction of IL-1 expression may be the signal which triggers damage, as occurs with the toxicity of *Bordetella pertussis* for respiratory epithelial cells (22). Also, while IL-1α is active in the absence of processing, keratinocytes lack the ability to process pro-IL-1β to its active form (16). It is interesting that the cysteine protease secreted by *S. pyogenes* can activate pro-IL-1β (14), suggesting that the bacterium has the ability to modulate the inflammatory response at multiple levels.

One role for keratinocyte-derived IL-1 is to stimulate keratinocyte expression of other cytokines, including IL-6 and IL-8 (1, 16). The latter two cytokines are involved in the amplification of local inflammatory responses through effects that include the recruitment of phagocytic cells (IL-8) and the stimulation of growth and/or activation of lymphocytes and keratinocytes (IL-6) and stimulation of acute-phase protein synthesis (IL-6) (26, 36). Elevated levels of IL-6 can be demonstrated in a number of pathological skin conditions, including psoriasis (8), a disease that can be associated with streptococcal infection (40). A variety of other stimuli besides IL-1 can activate transcription of IL-6 through several distinct signal transduction pathways (36). Since IL-6 was the only gene examined whose expression was effectively stimulated by nonadherent streptococci, these data may suggest that the pathway leading to expression of IL-6 in response to *S. pyogenes* may be different than those for the other genes. Similarly, the observation that adherent bacteria were considerably more effective stimulators of IL-6 expression may suggest that the signal transduction pathway activated by adherent bacteria differs from that activated by nonadherent streptococci.

The activation of distinct pathways of PGE_2 release by adherent versus nonadherent *S. pyogenes* is suggested by the kinetics of the respective responses. PGE_2 is the major prostaglandin associated with inflammation, and its production from arachidonic acid is controlled at the level of the two isoforms of prostaglandin endoperoxide synthase (cyclooxygenase), the key rate-limiting enzyme in its biosynthesis (41). The two isoforms (COX-1 and COX-2) are activated by different pathways, each of which produces a characteristic and different temporal pattern of PGE_2 synthesis (41). The distinct temporal patterns of PGE_2 release in response to adherent and nonadherent streptococci may suggest triggering of different COX pathways. Alternatively, the same pathway may be utilized but adherent streptococci are more efficient at delivery of a higher concentration of the signaling molecule. Of interest is speculation that the use of nonsteroidal anti-inflammatory drugs, which act primarily by inhibiting both COX isoforms, is associated with invasive streptococcal disease (35). Should further epidemiological studies support this association, additional studies on the induction of prostaglandin synthesis in response to streptococcal infection would be of considerable interest.

It has been previously reported that attachment of other bacteria can enhance epithelial cell cytokine responses (11); however, the mechanisms responsible are unknown. In *Escherichia coli*, the interaction of certain pilus adhesins with their receptors may directly trigger transmembrane signaling events which activate IL-6 expression (11). The cytoplasmic tail of the MCP receptor recognized by streptococcal M protein contains consensus sequences for phosphorylation by kinases and for nuclear targeting (33), suggesting that it can transduce signals. However, while attachment of *S. pyogenes* promoted keratinocyte signaling, experiments with metabolism-inhibited streptococci demonstrated that attachment by itself was not sufficient to trigger signaling. In this case, the role of attachment may be to increase the concentration of another streptococcal product(s) at the local environment of the keratinocyte surface, as has been proposed to explain the reduced ability of toxin inhibitors to neutralize the toxicity of streptolysin S for certain cultured cells (23). In addition to streptolysin S, *S. pyogenes* produces lipoteichoic acid and several other secreted toxins that are known to affect the function of other cell types (9, 17,

21, 37) that are candidates for participation in signaling interactions with keratinocytes.

Cytotoxic activity of *S. pyogenes* has been reported for some cell types but not for others (5, 23, 32). For example, both adherent and nonadherent *S. pyogenes* strains lysed primary human tonsillar epithelial cells but not buccal epithelial cells (5). By using a streptococcal mutant with a defined defect in adhesion, we have shown that adherence resulted in membrane damage in keratinocytes. This result was somewhat surprising, given that keratinocytes are relatively resistant to killing by the cutaneous pathogen *Haemophilus ducreyi* after extended periods of incubation, even though this organism is highly toxic for other cell types (27). Similar to the signaling responses examined, adherence was not by itself sufficient to cause membrane damage, which demonstrates that this effect is also multifactorial.

To date, one streptococcal regulatory gene has been described which controls the expression of the M protein adhesin and it has been shown that the regulatory circuitry is responsive to several environmental cues in vitro (4, 19, 31). These data suggest that *S. pyogenes* has the capacity to modulate its adhesive potential during infection. The observation that adherence versus nonadherence induces distinct patterns of responses in keratinocytes suggests that *S. pyogenes* may regulate expression of its adhesin to modulate how keratinocytes orchestrate inflammatory responses. Because keratinocyte signaling is influenced by streptococcal products in addition to the adhesin, the coordinate expression of different subsets of streptococcal virulence molecules may induce distinct patterns of response in keratinocytes and influence the eventual outcome of the infection, whether impetigo or a more serious invasive infection. Further analysis of the interaction of host and pathogen regulatory networks, including identification of the specific pathways of cytokine induction and prostaglandin synthesis, the analysis of levels of biologically active proinflammatory molecules stimulated, and the identification of streptococcal signaling molecules, will contribute to our understanding of the pathogenesis of streptococcal disease.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant AI38273 from the National Institutes of Health. M.C. is an Established Investigator of the American Heart Association.

REFERENCES

1. **Ansel, J., P. Perry, J. Brown, D. Damm, T. Phan, C. Hart, T. Luger, and S. Hefeneider.** 1990. Cytokine modulation of keratinocyte cytokines. J. Invest. Dermatol. **94:**101S–107S.
2. **Bisno, A. L., and D. L. Stevens.** 1996. Streptococcal infections of skin and soft tissues. N. Engl. J. Med. **334:**240–245.
3. **Boukamp, P., R. T. Petrussevska, D. Breitkreutz, J. Hornun, A. Markham, and N. E. Fusenig.** 1988. Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. J. Cell Biol. **106:**761–771.
4. **Caparon, M. G., R. T. Geist, J. Perez-Casal, and J. R. Scott.** 1992. Environmental regulation of virulence in group A streptococci: transcription of the gene encoding M protein is stimulated by carbon dioxide. J. Bacteriol. **174:**5693–5701.
5. **Caparon, M. G., D. S. Stephens, A. Olsén, and J. R. Scott.** 1991. Role of M protein in adherence of group A streptococci. Infect. Immun. **59:**1811–1817.
6. **Dörig, R. E., A. Marcil, A. Chopra, and C. D. Richardson.** 1993. The human CD46 molecule is a receptor for measles virus (Edmonston strain). Cell **75:**295–305.
7. **Fischetti, V. A.** 1989. Streptococcal M protein: molecular design and biological behavior. Clin. Microbiol. Rev. **2:**285–314.
8. **Grossman, R. M., J. Kraeger, D. Yourish, G. Piperno, D. P. Murphy, L. T. May, T. S. Kupper, P. B. Sehgal, and A. B. Gottleib.** 1989. Interleukin-6 is expressed in high levels in psoriatic skin and stimulates proliferation of cultured human keratinocytes. Proc. Natl. Acad. Sci. USA **86:**6367–6371.
9. **Hackett, S. P., and D. L. Stevens.** 1992. Streptococcal toxic shock syndrome: synthesis of tumor necrosis factor and interleukin-1 by monocytes stimulated with pyrogenic exotoxin A and streptolysin O. J. Infect. Dis. **165:**879–885.
10. **Haugland, R. P.** 1992. Fluorescent dyes for assessing vital cell functions, p. 172–180. *In* K. D. Larison (ed.), Handbook of fluorescent probes and research chemicals. Molecular Probes, Eugene, Oreg.
11. **Hedges, S. R., W. W. Agace, and C. Svanborg.** 1995. Epithelial cytokine responses and mucosal cytokine networks. Trends Microbiol. **3:**266–270.
12. **Ho, S. N., H. D. Hunt, R. M. Horton, J. K. Pullen, and L. R. Pease.** 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene **77:**51–59.
13. **Jung, H. C., L. Eckmann, S.-K. Yang, A. Panja, J. Fierer, E. Morzycka-Wroblewska, and M. F. Kagnoff.** 1995. A distinct array of proinflammatory cytokines is expressed in human colon epithelial cells in response to bacterial invasion. J. Clin. Invest. **95:**55–65.
14. **Kapur, V., M. W. Majesky, I. L. Li, R. A. Black, and J. M. Musser.** 1993. Cleavage of interleukin 1β (IL-1β) precursor to produce active IL-1β by a conserved extracellular cysteine protease from *Streptococcus pyogenes*. Proc. Natl. Acad. Sci. USA **90:**7676–7680.
15. **Krantic, S., C. Gimenez, and C. Rabourdin-Combe.** 1995. Cell-to-cell contact via measles virus hemagglutinin-CD46 interaction triggers CD46 downregulation. J. Gen. Virol. **76:**2793–2800.
16. **Kupper, T. S.** 1990. The activated keratinocyte: a model for inducible cytokine production by non-bone marrow-derived cells in cutaneous inflammatory and immune responses. J. Invest. Dermatol. **94:**146S–150S.
17. **Leung, D. Y., M. Gately, A. Trumble, B. Ferguson-Darnell, P. M. Schlievert, and L. J. Picker.** 1995. Bacterial superantigens induce T cell expression of the skin-selective homing receptor, the cutaneous lymphocyte-associated antigen, via stimulation of interleukin 12 production. J. Exp. Med. **181:**747–753.
18. **Liszewski, M. K., T. W. Post, and J. P. Atkinson.** 1991. Membrane cofactor protein (MCP or CD46): newest member of the complement activation gene cluster. Annu. Rev. Immunol. **9:**431–455.
19. **McIver, K. S., A. S. Heath, and J. R. Scott.** 1995. Regulation of virulence by environmental signals in group A streptococci: influence of osmolarity, temperature, gas exchange, and iron limitation on *emm* transcription. Infect. Immun. **63:**4540–4542.
20. **Miller, C. C., P. M. Hale, and A. P. Pentland.** 1994. Ultraviolet light injury increases prostaglandin synthesis through a tyrosine kinase-dependent pathway. J. Biol. Chem. **269:**3529–3533.
21. **Müller-Alouf, H., J. E. Alouf, D. Gerlach, J.-H. Ozegowski, C. Fitting, and J.-M. Cavaillon.** 1994. Comparative study of cytokine release by human peripheral blood mononuclear cells stimulated with *Streptococcus pyogenes* superantigenic erythrogenic toxins, heat-killed streptococci, and lipopolysaccharide. Infect. Immun. **62:**4915–4921.
22. **Nixon Heiss, L., S. A. Moser, E. R. Unanue, and W. E. Goldman.** 1993. Interleukin-1 is linked to the respiratory epithelial cytopathology of pertussis. Infect. Immun. **61:**3123–3128.
23. **Ofek, I., D. Zafriri, J. Goldhar, and B. I. Eisenstein.** 1990. Inability of toxin inhibitors to neutralize enhanced toxicity caused by bacteria adherent to tissue culture cells. Infect. Immun. **58:**3737–3742.
24. **Okada, N., M. K. Liszewski, J. P. Atkinson, and M. Caparon.** 1994. Membrane cofactor protein (MCP or CD46) is a keratinocyte receptor for the M protein of the group A streptococcus. Proc. Natl. Acad. Sci. USA **92:**2489–2493.
25. **Okada, N., A. Pentland, P. Falk, and M. Caparon.** 1994. M protein and protein F act as important determinants of cell-specific tropism of *Streptococcus pyogenes* in skin tissue. J. Clin. Invest. **94:**965–977.
26. **Oppenheim, J. J., C. O. C. Zachariae, N. Mukaida, and K. Matsushima.** 1991. Properties of the novel proinflammatory supergene "intercrine" cytokine family. Annu. Rev. Immunol. **9:**617–648.
27. **Palmer, K. L., W. E. Goldman, and R. S. Munson, Jr.** 1996. An isogenic haemolysin-deficient mutant of *Haemophilus ducreyi* lacks the ability to produce cytopathic effects on human foreskin fibroblasts. Mol. Microbiol. **21:**13–19.
28. **Perez-Casal, J., M. G. Caparon, and J. R. Scott.** 1991. Mry, a *trans*-acting positive regulator of the M protein gene of *Streptococcus pyogenes* with similarity to the receptor proteins of two-component regulatory systems. J. Bacteriol. **173:**2617–2624.
29. **Perez-Casal, J., E. Maguin, and J. R. Scott.** 1993. An M protein with a single C repeat prevents phagocytosis of *Streptococcus pyogenes*: use of a temperature sensitive shuttle vector to deliver homologous sequences to the chromosome of *S. pyogenes*. Mol. Microbiol. **8:**809–819.
30. **Perez-Casal, J., N. Okada, M. G. Caparon, and J. R. Scott.** 1995. Role of the conserved C-repeat region of the M protein of *Streptococcus pyogenes*. Mol. Microbiol. **15:**907–916.
31. **Podbielski, A., J. A. Peterson, and P. P. Cleary.** 1992. Surface protein-CAT reporter fusions demonstrate differential gene expression in the *vir* regulon of *Streptococcus pyogenes*. Mol. Microbiol. **6:**2253–2265.
32. **Quinn, R. W., and P. N. Lowry.** 1967. Effect of *Streptococcus pyogenes* on tissue cells. J. Bacteriol. **93:**1825–1831.
33. **Russell, S. M., B. E. Loveland, R. W. Johnstone, B. R. Thorley, and I. F. C. McKenzie.** 1992. Functional characterisation of alternatively spliced CD46 cytoplasmic tails. Transplant. Proc. **24:**2329–2330.

34. **Scott, J. R., P. C. Guenther, L. M. Malone, and V. A. Fischetti.** 1986. Conversion of an M^- group A streptococcus to M^+ by transfer of a plasmid containing an M6 ne. J. Exp. Med. **164:**1641–1651.
35. **Seachrist, L.** 1995. The once and future scourge. Could common anti-inflammatory drugs allow bacteria to take a deadly turn? Science **148:**234–235.
36. **Sehgal, P. B.** 1990. Interleukin-6: molecular pathophysiology. J. Invest. Dermatol. **94:**2S–6S.
37. **Standiford, T. J., S. A. Arenberg, J. M. Danforth, S. L. Kunkel, G. M. Van Otteren, and R. M. Streiter.** 1994. Lipoteichoic acid induces secretion of interleukin-8 from human blood monocytes: a cellular and molecular analysis. Infect. Immun. **62:**119–125.
38. **St. Geme, III, J. W.** 1994. The HMW1 adhesin of nontypeable *Haemophilus influenzae* recognizes sialylated glycoprotein receptors on cultured human epithelial cells. Infect. Immun. **62:**3881–3889.
39. **Wannamaker, L. W.** 1970. Differences between streptococcal infections of the throat and skin. N. Engl. J. Med. **282:**23–30.
40. **Whyte, H. J., and R. D. Baughman.** 1964. Acute guttate psoriasis and streptococcal infection. Arch. Dermatol. **89:**350–356.
41. **Williams, C. S., and R. N. DuBois.** 1996. Prostaglandin endoperoxide synthase: why two isoforms? Am. J. Physiol. **270:**G393–G400.

Editor: V. A. Fischetti