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An Experimental Model of Cutaneous Infection Induced by Superantigen-Producing *Staphylococcus aureus*

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Skin infections caused by *Staphylococcus aureus*, such as erysipelas, are commonly occurring, painful, and costly for society. Despite the high prevalence of this condition, little is known about the host immune responsiveness and bacterial virulence factors during *S. aureus* dermatitis. We present here a mouse model of infectious dermatitis in which *S. aureus* is inoculated by an intracutaneous injection to the shaved back of NMRI mice. Visible skin inflammation, characterized by redness and swelling, was noted 48 h after inoculation of staphylococci in mice that received 2×10^8 colony-forming units of *S. aureus*. Microscopic evaluation revealed a dermal and subcutaneous infiltrate rich in macrophages and neutrophilic granulocytes already within 6 h after inoculation. A sparse influx of T lymphocytes was noted somewhat later. Bacterial cultures from skin revealed high numbers of staphylococci early after inoculation, with a successive decline during 2 wk follow-up. Total white blood cell count as well as the number of polymorphonuclear leukocytes peaked 2 d after bacterial inoculation. Also, serum interleukin-6 levels peaked within 2 d, with a 10-fold increase compared to non-infected control mice, indicating a systemic reaction to skin infection. The role of toxic shock syndrome toxin 1 in the pathogenesis of the dermatitis was assessed using isogenic *S. aureus* strains. Even though the gross inflammatory skin reaction was similar for mice infected with either of the strains, it was apparent that bacteria secreting toxic shock syndrome toxin 1 preferentially triggered influx of T lymphocytes to the skin. In addition, mice inoculated with staphylococci producing toxic shock syndrome toxin 1 showed a weight decrease during the experiment whereas mice inoculated with the isogenic strain showed a weight increase. This model of staphylococcal dermatitis will enable future in-depth studies regarding the host–bacterium relationship.

Key words: dermatitis/mice/*S. aureus*/superantigen. J Invest Dermatol 114:1120–1125, 2000

Staphylococcal skin infections are commonly seen in both healthy and immunocompromised human beings. *Staphylococcus aureus* is the causative agent in up to 75% of primary pyodermas (Trilla and Miro, 1995). Certain skin and soft tissue infections may lead to severe complications such as sepsis. Risk factors for secondary infection with *S. aureus* are pre-existing abnormalities in skin architecture such as tissue injuries, burn wounds, exudative dermatitis, as well as certain underlying diseases characterized by general immunosuppression, e.g., diabetes mellitus and neoplasms (Trilla and Miro, 1995). Interestingly, during inflammatory skin diseases such as atopic dermatitis, the involved dermis is known to be colonized with superantigen-releasing *S. aureus* and recent studies implicate a role for these superantigens in the pathogenesis of this condition (Leung *et al*, 1995). Despite the high prevalence of cutaneous infections, little is known about the role of host immune responsiveness and bacterial virulence factors during *S. aureus* dermatitis. This fact is even more puzzling as both rabbit (Johnson *et al*, 1961) and murine (Noble, 1965) models of staphylococcal skin infections were established already 40 y ago. In this study we describe a model of skin infection mediated by superantigen-secreting *S. aureus*. The availability of such a model will enable an understanding of the pathogenesis of infectious dermatitis as well as establishing efficient therapeutic regimens.

MATERIALS AND METHODS

Mice Male NMRI mice, 5–6 wk old, were purchased from B&K Universal (Sollentuna, Sweden). They were housed in the animal facility of the Department of Rheumatology, University of Göteborg, under standard conditions of light and temperature, and were fed standard laboratory chow and water *ad libitum*.

Bacterial strains NMRI mice were inoculated intracutaneously with *S. aureus* LS-1 strain, being harboured naturally on, for example, the skin of many mice (Bremell *et al*, 1990). This bacterial strain has been shown to produce large amounts of toxic shock syndrome toxin 1 (TSST-1) and is coagulase and catalase positive. To compare superantigen-producing *versus* non-producing bacteria, *S. aureus* strains isogenic for TSST-1 production were used. Strain RN 4282 (provided by R.P. Novick, Public Health Research Institute, New York) is a clinical isolate of *S. aureus* that produces TSST-1, and RN 6938 is a mutant of RN 4282 that does not express TSST-1 (Sloane *et al*, 1991).

The bacteria were kept frozen at −20°C in 5% bovine serum albumin (BSA) and 10% dimethylsulfoxide (C_2H_6OS) in phosphate-buffered saline (PBS), pH 7.4, until used. Before use, the bacterial solution was thawed and washed in PBS. Viable count was used to check the number of live bacteria in each bacterial solution.

Experimental protocol Two groups of NMRI mice (groups A and B), with 15 and nine mice in groups A and B, respectively, were inoculated

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Abbreviations: TSST-1, toxic shock syndrome toxin 1.

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intracutaneously with *S. aureus* LS-1 strain during a neurolept-analgesia (Dormicum-Hypnorm). The intracutaneous injections containing live bacteria were given at four different sites on the shaved back of each animal. Each mouse was inoculated with 0.1 ml of saline containing either 0 (control), 2×10^6, 2×10^7, or 2×10^8 colony-forming units (cfu) of *S. aureus*.

The mice were followed clinically and sacrificed by cervical dislocation at different time intervals. In group A, mice were sacrificed after 6 h, 48 h, 1 wk, and 2 wk, and skin samples were taken from the injection sites for histopathologic and immunohistochemical evaluation. In group B, mice were sacrificed after 48 h, 1 wk, and 2 wk, and skin samples corresponding to the injection sites, as well as blood samples, were taken for bacterial analysis. Another group (group C) of 22 NMRI mice was inoculated intracutaneously with 1.2×10^8 cfu *S. aureus* and followed during 2 wk. Blood samples were taken before inoculation with bacteria, after 42 h, 6 d, and 14 d, and analyzed regarding total white cell counts, granulocyte counts, and levels of interleukin-6, immunoglobulins (IgG1, IgG2a, IgG3, and IgM), and specific antibodies against TSST-1 and against bacterial cell walls. Forty-two hours, 6 d, and 2 wk after bacterial inoculation three mice were sacrificed and bacterial skin growth was assessed. All the mice were followed clinically for local inflammatory reaction, signs of sepsis, and weight development. To assess the role of TSST-1, 20 NMRI mice were inoculated at two different sites on the shaved back with 2×10^8 cfu (0.1 ml) *S. aureus* of either the TSST-1-producing or the nonproducing substrain. Skin biopsies as well as blood samples were obtained after 48 h, and histopathologic, immunohistochemical, bacteriologic, and serologic analyses were performed.

Histopathologic examination Skin samples were examined histopathologically after routine fixation and staining with hematoxylin and eosin. Microscopic evaluation was made to characterize the inflammatory infiltrates. At least eight skin sections per mouse were analyzed.

Immunohistochemical technique and examination Immunohistochemical analysis was performed regarding the occurrence of $CD11b^+$, $CD4^+$, and $CD8^+$ cells. Briefly, skin samples were frozen in isopentane prechilled by liquid nitrogen and kept at –70°C until cryosectioned. All sections were fixed in cold acetone for 5 min and washed in PBS. The sections were incubated overnight in a humid atmosphere at +4°C with unlabeled rat monoclonal antibodies diluted in PBS containing 1% BSA. After washes, endogenous peroxidase was depleted by treatment with 0.3% H_2O_2 for 5 min. Biotin-labeled rabbit antirat Ig diluted in PBS–BSA was used as secondary antibody (Vector Lab, Burlingame, CA). Binding of biotin-labeled secondary antibodies was detected by stepwise incubation with avidin–biotin–peroxidase complexes (DAKO, Denmark) and 3-amino-9-ethyl-carbazole containing H_2O_2. Primary antibodies used for staining were Mac-1 (M1/70) (Springer *et al*, 1979), anti-CD8 (53.6.7) (Ledbetter and Herzenberg, 1979), and anti-CD4 (H129.19) (Pierres *et al*, 1984). All the sections were counterstained with Meyer's hematoxylin.

Bacterial culture Skin and blood samples from mice were used for bacterial analysis. After disinfection with 70% alcohol, skin samples corresponding to the injection sites were deposited in sterile plastic bags, homogenized, and suspended in 10 ml PBS. Skin suspensions were plated in appropriate dilutions on agar plates containing 5% horse blood and incubated at 37°C for 24 h. The numbers of cfu per skin sample and per 100 μl blood were counted and the bacterial colonies were tested for coagulase and catalase activity.

Serological analyses

IL-6 assay Cell line B13.29, which is dependent on interleukin-6 (IL-6) for growth, has been described previously (Lansdorp *et al*, 1986; Aarden *et al*, 1987; Helle *et al*, 1988). For IL-6 determinations, the more sensitive subclone B9 was used. B9 cells were harvested from tissue culture flasks, seeded into microtiter plates (Nunc, Roskilde, Denmark) at a concentration of 5000 cells per well, and cultured in Iscove's medium supplemented with 5×10^{-5} M 2-mercaptoethanol, 10% fetal bovine serum (Seralab, Sussex, UK), and gentamycin (50 μg per ml), and L-glutamin and serum samples were added. [^{3}H]thymidine was added after 68 h of culturing and the cells were harvested 4 h later. The samples were tested in 2-fold dilutions and compared with a recombinant IL-6 standard. B9 cells were previously shown not to react with several recombinant cytokines, including IL-1α, IL-1β, IL-2, IL-3, IL-5, granulocyte–macrophage colony stimulating factor, tumor necrosis factor, and α and γ interferon. There was only weak reactivity with IL-4 (Helle *et al*, 1988).

Immunoglobulins Total serum levels of IgG1, IgG2a, IgG3, and IgM were measured by the radial immunodiffusion technique (Mancini *et al*, 1965). Antisera and immunoglobulin standards specific for IgG1, IgG2a, IgG3, and IgM were purchased from Sigma.

Antibodies to cell walls of S. aureus Serum levels of IgG and IgM antibodies specific for *S. aureus* cell wall constituents were estimated by an enzyme-linked immunosorbent assay (ELISA) using methylated BSA (10 μg per ml) to precoat the wells and 100 μl of whole, formalin-treated (4%, 20 min) *S. aureus* LS-1 cells (10^8 per ml) to coat wells. All sera were serially diluted in 0.5% PBS–BSA and incubated in wells. To measure the level and class specificity of anti-cell-wall antibodies bound to the solid phase, affinity-purified and biotinylated $F(ab')_2$ fragments of goat antimouse IgG and IgM (Jackson Laboratories), diluted 1:3000 in PBS–Tween 20, were added to wells followed stepwise by 0.5 μg per ml of Extravidin-horseradish peroxidase (Sigma) and 2.5 mg per ml of the enzyme substrate 2,2-azino-bis-(3-ethylbenzothiazoline sulfonic acid) (Sigma) in citrate buffer (pH 4.2) containing 0.0075% H_2O_2. The A_{414} was measured in a Spectra Max Plus spectrophotometer (Molecular Devices). All optical density values were converted to antigen-specific arbitrary units with calibration curves based on the optical density values obtained from serial dilutions of a reference pool of sera. The calibration curves were constructed with a computer program based on weighted logit-log models (Russell *et al*, 1986; Lue *et al*, 1988).

Anti-TSST-1 antibodies Serum levels of IgG antibodies to TSST-1 were estimated by an ELISA using 0.5 μg per ml of highly purified TSST-1 (Toxin Technology, Sarasota, FL) as a solid phase coating. The subsequent steps were similar to those described above.

In all the serological analyses described, sera sampled from the mice before inoculation of *S. aureus* were used as controls.

Hematological analysis Mice were bled from the tail into heparinized tubes. Total white blood cell counts were determined in a hemacytometer (Toa Medical Electronics, Kobe, Japan). Blood smears were prepared and stained by the May–Grunewald–Giemsa method for differential counts.

Statistical analysis Student's paired *t* test was used for comparisons of weight differences and hematological and serological parameters. All comparisons regarding the TSST-1 isogenic strains were made by Mann–Whitney U test. p values below 0.05 were considered statistically significant. If not otherwise stated all numerical values are provided as means ± SD.

RESULTS

Clinical evaluation Irrespective of the inoculation dose, none of the animals inoculated intradermally with *S. aureus* displayed any general symptoms compatible with sepsis. Visible inflammation, characterized by redness and swelling of the skin, was noted 48 h after the inoculation only at sites injected with 2×10^8 cfu of *S. aureus*. In mice inoculated with the staphylococcal strains isogenic for TSST-1 there was a weight loss in the group receiving superantigen-producing staphylococci (–2.6%) compared to the superantigen nonproducing group, which gained weight (0.6%) (n.s.).

Microscopic evaluation In contrast to the clinical features, clear histopathologic signs of inflammatory response were noted in skin locations inoculated also with 2×10^7 cfu. Microscopic evaluation revealed a dense infiltrate of neutrophilic granulocytes with visible bacteria in deep dermis and subcutaneous fat already after 6 h. In addition, abscess formation in the superficial musculature was noted. Surrounding these abscesses, a diffuse infiltrate of neutrophilic granulocytes and mononuclear cells in dermis dominated, but sparse perivascular infiltrates were also seen (**Fig 1**). Involvement of epidermis was not noted, except in a few cases where the injection needle had damaged and contaminated the epidermis and upper dermis. The inflammatory changes peaked at 48 h and started to disappear 2 wk after the inoculation (**Fig 2**).

In mice that received inoculation of either the TSST-1-producing strain or the nonproducing isogenic counterpart, the histopathologic examination 48 h after inoculation of *S. aureus* revealed a central necrosis and a dense infiltrate of neutrophils and macrophages around a micro-abscess, mostly situated in subcutis.

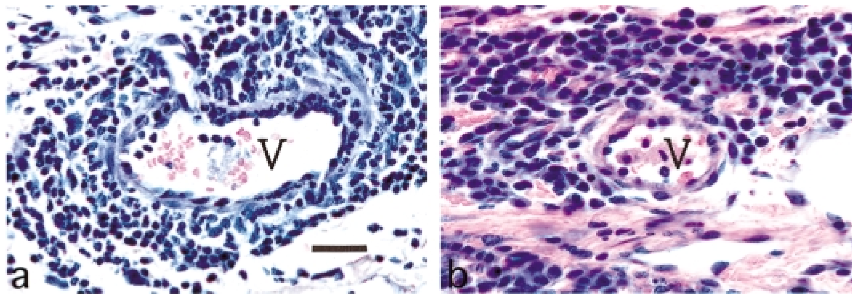

Figure 1. Types of infiltrating cells during cutaneous *S. aureus* infection. Micrograph shows a mixed perivascular infiltrate with neutrophilic granulocytes and mononuclear cells 48 h after inoculation with 2×10^8 cfu of *S. aureus* (*a*), and a perivascular infiltrate with mononuclear cells 1 wk after the inoculation (*b*). V, blood vessel. *Scale bar*: 40 μm.

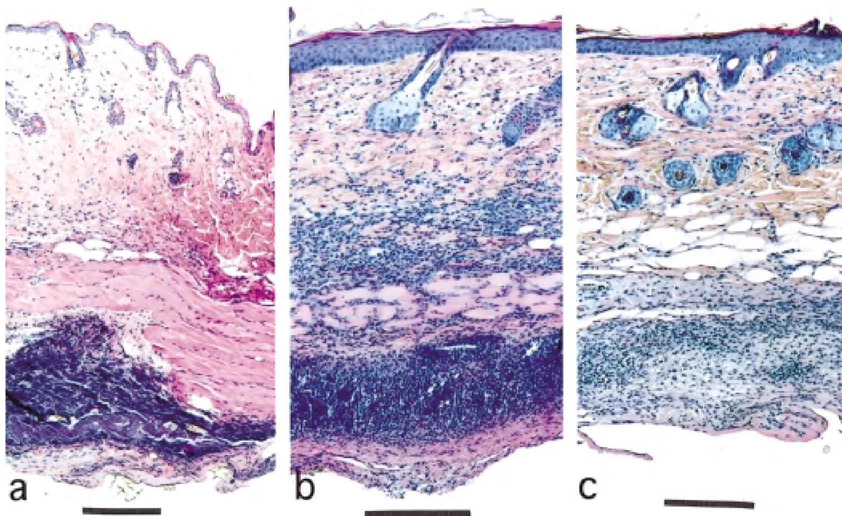

Figure 2. Early and persistent infiltration of inflammatory cells during *S. aureus* dermatitis. Micrograph shows the inflammatory infiltrate in skin inoculated with 2×10^8 cfu of *S. aureus* after 6 h (*a*), 48 h (*b*), and 1 wk (*c*). *Scale bars*: 200 μm.

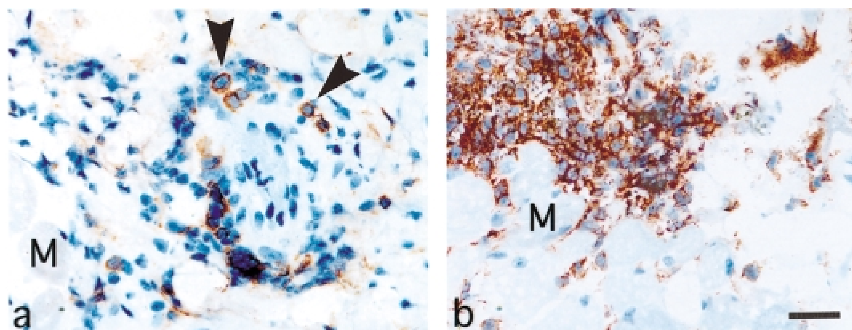

Figure 3. Overwhelming influx of macrophages during *S. aureus* dermatitis. Micrograph shows sparse numbers of $CD4^+$ cells (*a*) and a dense infiltrate of $CD11^+$ cells (*b*) in skin 48 h after inoculation with 2×10^8 cfu of *S. aureus*. M, superficial muscles. *Arrowheads* show $CD4^+$ cells. *Scale bar*: 20 μm.

Bacterial colonies were frequently seen. There were no clearcut differences regarding the characteristics of inflammatory infiltrates between the two groups studied.

Immunohistochemical analysis Immunohistochemical analysis was performed to analyze the occurrence of $CD11b^+$ cells (phagocytic cells), $CD4^+$ T cells, and $CD8^+$ T cells. The results revealed a dense infiltrate of $CD11b^+$ cells 6 h and 48 h after the bacterial inoculation (**Fig 3*b***), with almost total disappearance of these cells within 2 wk after bacterial inoculation (**Table I**). In seven out of 15 mice relatively sparse numbers of $CD4^+$ and $CD8^+$ T cells were noted (**Fig 3*a***).

In mice inoculated with the TSST-1-producing strain RN 4282, six out of 10 mice (60%) showed the occurrence of dermal $CD4^+$ T cells, compared to two of 10 (20%) with the nonproducing isogenic RN 6938 strain. In contrast, $CD8^+$ T cells were frequently found in both study groups (RN 4282, 60%; RN 6938, 90%).

Bacterial cultures Bacterial cultures from mouse skin in group B revealed growth of high numbers of catalase- and coagulase-positive bacteria 48 h after inoculation. In contrast, only few surviving bacteria were seen 2 wk after the inoculation procedure (**Fig 4**). Blood samples obtained for bacterial culture were negative at all time intervals.

In mice inoculated with strains isogenic for TSST-1 production, no major differences were found with regard to bacterial growth. Bacterial cultures from skin samples 2 d after the inoculation revealed growth of $2.1 \times 10^7 \pm 1.0 \times 10^7$ cfu in the group receiving TSST-1^+ bacteria and $2.0 \times 10^7 \pm 1.6 \times 10^7$ cfu in the group receiving the isogenic TSST-1^- strain (n.s.). Blood analyses of staphylococcal growth 48 h after the inoculation (>20 cfu) showed the presence of *S. aureus* in two of 10 and one of 10 cases, respectively (n.s.).

Serological analyses

IL-6 Serum levels of IL-6 were significantly increased after inoculation of *S. aureus*. IL-6 levels peaked to levels exceeding 400 pg per ml 2 d after inoculation ($p < 0.0001$) and significant production of IL-6 was visible even 6 d after bacterial inoculation

Table I. Local influx of CD11b+ cells at different time intervals after intracutaneous inoculation of *S. aureus*

No. of bacteria inoculated	Occurrence of CD11b+ cells in skin											
	6 h			48 h			1 wk			2 wk		
	<10	10–25	>25	<10	10–25	>25	<10	10–25	>25	<10	10–25	>25
0	3/4	1/4	0/4	3/4	1/4	0/4	4/4	0/4	0/4	3/3	0/3	0/3
2×10^7	0/4	0/4	4/4	2/4	0/4	2/4	2/4	2/4	0/4	3/3	0/3	0/3
2×10^8	0/4	0/4	4/4	0/4	0/4	4/4	3/4	0/4	1/4	3/3	0/3	0/3

The data represent the number of skin samples expressing CD11b+ cells divided by the total number of animals tested.

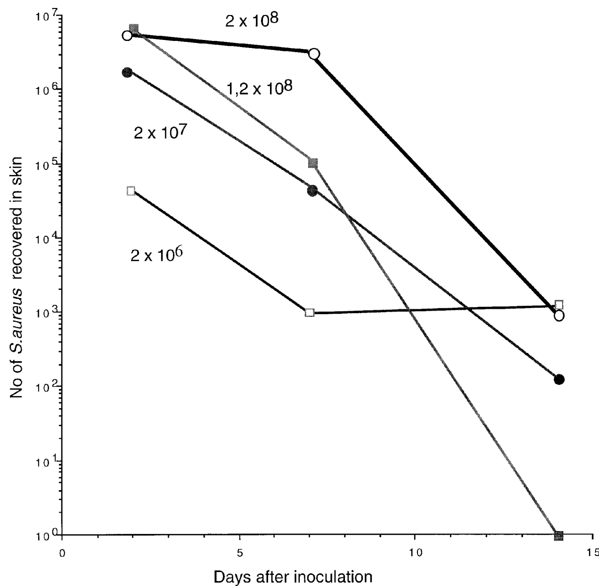

Figure 4. Variation of bacterial load as a function of time. Number of *S. aureus* in skin samples recovered after varying time intervals (n = 3 for each time period and inoculation).

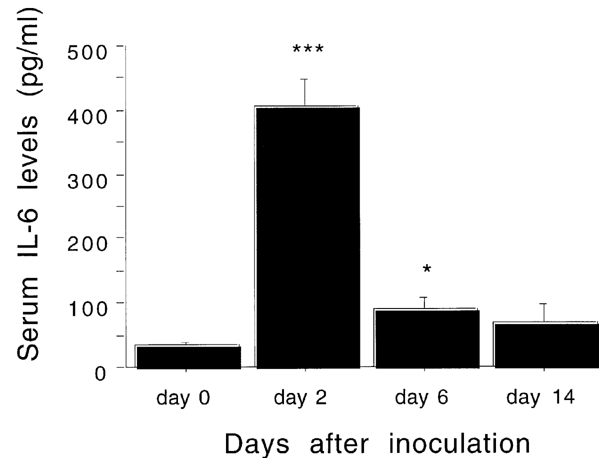

Figure 5. Increased systemic IL-6 production during *S. aureus* dermatitis. Serum levels of IL-6 (mean ± SEM) (n = 11–20) peaked to levels exceeding 400 pg per ml 2 d after inoculation ($p < 0.0001$) and significant production of IL-6 was visible even 6 d after bacterial inoculation ($p < 0.014$).

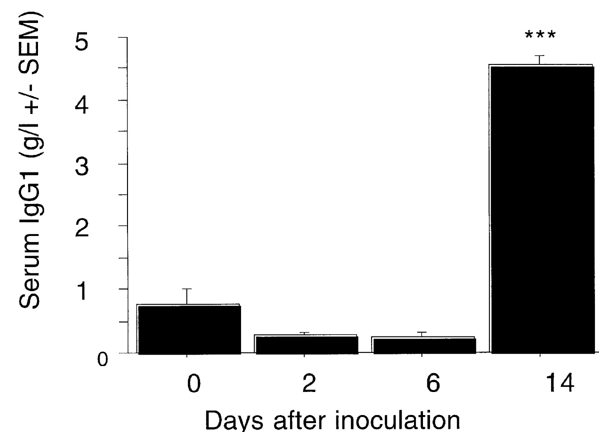

Figure 6. Late upregulation of IgG1 levels. Serum levels of IgG1 in NMRI mice (n = 11–21) after a single intracutaneous injection with 1.2×10^8 cfu *S. aureus*.

($p < 0.014$) (**Fig 5**). In mice inoculated with strains isogenic for TSST-1 production, no major differences were found regarding serum levels of IL-6.

B lymphocyte responses Since IL-6 is an efficient B lymphocyte differentiating factor, we decided to analyze serum levels of immunoglobulins and specific antibodies to staphylococcal components. Serum levels of IgG1 were increased 18-fold, with levels exceeding 4.5 g per l after 2 wk (**Fig 6**). Serum levels of IgG2a, IgG3, and IgM were increased only to a minor degree (results not shown). Also, serum levels of IgG class anti-*S. aureus* specific antibodies were significantly increased 2 wk after bacterial inoculation (**Fig 7**). The levels of IgM anti-*S. aureus* antibodies showed a slight but not statistically significant increase 2 wk after bacterial inoculation (data not shown). No significant increase of serum antibody levels of IgG or IgM class specific for TSST-1 was noted (data not shown).

Hematologic analysis The number of white blood cells increased significantly 2 d after bacterial inoculation and decreased 2 wk later to levels below the original values. These findings were accounted for by an increase in polymorphonuclear granulocytes (**Fig 8**). In mice inoculated with strains isogenic for TSST-1, there were no significant differences regarding total number or percentage of granulocytes 2 d after inoculation of *S. aureus*.

DISCUSSION

In this report, we present a murine model of staphylococcal dermatitis. A similar type of model has been described before (Noble, 1965) but present knowledge in the field of immunology and bacterial genetics enables an in-depth understanding of the pathogenesis of infectious dermatitis and consequently a delineation of the host–bacterium relationship with the aim of establishing efficient therapeutic regimens. In our model, healthy mice were inoculated intracutaneously with different amounts of *S. aureus*. We have shown that mice injected intracutaneously with high numbers of staphylococci (10^8 cfu) displayed clinical signs of local inflammation within 48 h but lacked clinical or bacteriologic signs of sepsis. Earlier

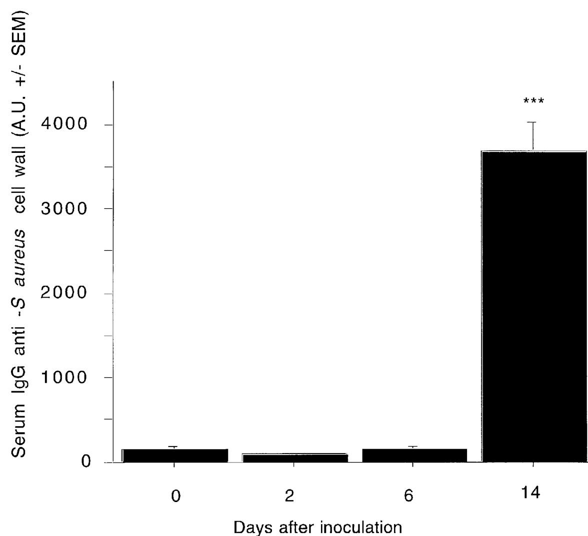

Figure 7. Specific antibody responses to bacterial components. Serum levels of IgG antibodies to *S. aureus* cell wall (arbitrary units, mean ± SEM) were significantly increased 2 wk after bacterial inoculation ($p < 0.0001$), $n = 16$–22.

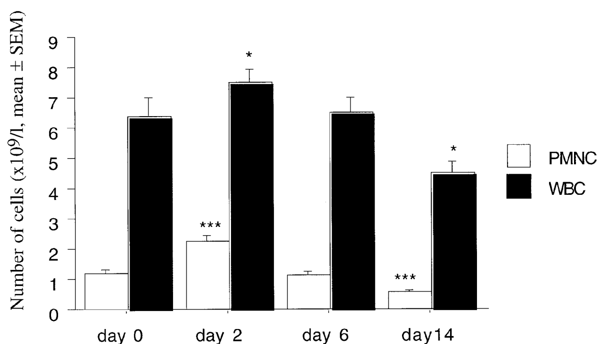

Figure 8. Systemic host response to dermally residing bacteria. The total numbers of white blood and polymorphonuclear cells show a similar pattern with a significant increase 2 d after bacterial inoculation ($p < 0.05$ and $p < 0.001$ respectively), normalization after another 4 d, and a decrease after 2 wk.

findings from our laboratory have shown that intravenous injection of 10^7 cfu *S. aureus* resulted in 75% of the mice displaying arthritis, with detectable sepsis in a small minority of the mice (Bremell *et al*, 1991). Taken together, these data indicate that intact skin is a very efficient barrier against invading pathogens. Signs of arthritis were not found in any mouse group dermally inoculated with bacteria, a finding in line with data presented earlier and showing that subcutaneous deposition of *S. aureus* was inefficient in inducing systemic infection (Fisher and Robson, 1963; Bremell *et al*, 1991). In contrast to the clinical findings, both microscopic evaluation and bacterial culturing from exposed skin samples showed clear signs of inflammatory response and high amounts of live bacteria, respectively, within the first week after inoculation with 2×10^7 cfu of *S. aureus*. The microscopic picture revealed an abundance of granulocytes but also the presence of many mononuclear infiltrating cells in the infected skin. In this respect it is not astonishing that during *S. aureus* infection granulocytes home early to the infected site, being of utmost importance for the host defense (Verdrengh and Tarkowski, 1997). Indeed, early on during the infectious process we were able to see a significant increase of circulating polymorphonuclear granulocytes, a prerequisite for efficient homing to the site of inflammation, whereas later the numbers of these cells diminished. Interestingly, depletion of granulocytes with monoclonal antibody RB6-8C5 markedly aggravates the infectious dermatitis, giving rise also to marked bacteremia (Mölne *et al*, submitted). Further examination of the skin samples using an immunohistochemical technique revealed infiltrates rich in $CD11b^+$ cells, many of them being mononuclear, i.e., belonging to the monocyte/macrophage lineage. In addition, somewhat later (48 h to 1 wk), a sparse number of $CD4^+$ and $CD8^+$ cells was noted. We have recently shown that $CD4^+$ T cells, especially those expressing $V\beta11^+$ T-cell receptor, are important mediators of *S. aureus* triggered septic arthritis and sepsis (Abdelnour *et al*, 1994). The latter finding indicates that at least the arthritic component of infection caused by the *S. aureus* strain used in this study is superantigen mediated.

Serological analyses showed an early increase of IL-6 production, with a peak 2 d after bacterial inoculation. IL-6 is produced by macrophages and T lymphocytes and is known to promote B-cell differentiation. As could be expected, serum IgG levels as well as specific antibodies to staphylococcal cell walls were significantly increased 2 wk after the inoculation. In this regard we have recently shown that neutralization of IL-6 profoundly decreases B-cell production of Ig in response to staphylococcal cell walls (Abdelnour and Tarkowski, 1993).

One of the important tasks of this study was to assess the role of superantigens (here TSST-1) in our model of infectious dermatitis. To this end, *S. aureus* strains isogenic for TSST-1 production were inoculated intracutaneously in healthy NMRI mice. TSST-1, just like all other staphylococcal superantigens, will readily differentiate Th1 cells *in vitro*, ultimately leading to tissue damage (Zhao *et al*, 1995). In the case of staphylococcal dermatitis there were non major differences with respect to gross inflammatory skin reaction between the isogenic strains. Bacteria secreting TSST-1, however, preferentially triggered influx of $CD4^+$ T lymphocytes to the skin. In addition, mice inoculated with TSST-1-producing staphylococci showed a weight decrease during the experiment whereas mice inoculated with the isogenic strain showed a weight increase. With respect to potential similarities between animal models of superantigen-mediated pathologies and human diseases it may be stated that in certain cases there is a direct parallel in causality and disease manifestations (e.g., toxic shock syndrome) although certain aspects of immunologic responses may differ (Michie and Cohen, 1998). In other cases (e.g., psoriasis, rheumatoid arthritis) *in situ* expansion of certain T-cell receptor $V\beta$ families supports (but does not formally prove) the possibility that these diseases may be superantigen triggered or driven. Interestingly, in the case of rodents we have recently shown that both mice (Bremell *et al*, 1990) and rats (Bremell *et al*, 1994) may develop spontaneous infection on both skin and joints with superantigen-producing *S. aureus* strains. This fact provides a natural prerequisite for superantigen-mediated pathologies in these animals. We believe that our model of staphylococcal dermatitis will enable further studies regarding the host–bacterium relationship in this commonly encountered condition.

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