Correlation between Bioluminescence and Bacterial Burden in Passively Protected Mice Challenged with a Recombinant Bioluminescent M49 Group A Streptococcus Strain

Meru Sheel,1,2 Manisha Pandey,1 Michael F. Good,1 and Michael R. Batzloff1*

Australian Centre for Vaccine Development, Queensland Institute of Medical Research, PO Royal Brisbane Hospital, Brisbane, Queensland 4029, Australia,1 and Queensland University of Technology, Gardens Point, Queensland 4001, Australia2

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Streptococcus pyogenes, also known as group A streptococcus (GAS), is a human pathogen which infects people of all age groups. Previous work has reported that conformationally constrained chimeric peptide J8 protects against GAS challenge. In the current study, we demonstrate the utility of bioluminescent imaging as a rapid technique for monitoring bacterial dissemination following the passive transfer of rabbit anti-J8 antibodies to naïve mice and subsequent challenge with recombinant GAS strain M49, an emm type shown to be associated with poststreptococcal glomerular nephritis.

Streptococcus pyogenes, commonly referred to as group A streptococcus (GAS), is a human pathogen which infects people of all age groups. GAS-associated diseases range from mild bacterial pharyngitis and pyoderma to severe invasive diseases, such as necrotizing fasciitis. However, of most concern are the postinfectious sequelae of rheumatic fever (RF), rheumatic heart disease (RHD), and poststreptococcal glomerular nephritis (PSGN), which are highly prevalent in developing countries and among the indigenous populations of developed nations (5).

To date, the majority of GAS vaccine development has focused on the M protein (18, 22). The M protein is a coiled-coil surface protein that is highly variable at the amino terminus and that becomes conserved toward the carboxyl terminus. Several research groups have identified the potential of vaccine candidates based on the conserved region (3, 4, 28). We have previously described a conformationally constrained chimeric peptide, J8 (14), which has been shown to protect against GAS challenge (1). Following active immunization of mice with J8 conjugated to diphtheria toxoid (DT), survival postchallenge correlated with the J8-specific serum IgG titers in both inbred (B10.BR) and outbred (Quackenbush) mice (1).

With a view to understanding the mechanism of protection offered by the J8 peptide, we recently used a passive transfer model (26). Earlier studies showed that high antibody titers were necessary for the protection of mice against GAS challenge. In order to achieve significant titers and to elucidate the role of antibodies in protection, we used purified IgG containing J8-DT antibodies raised in rabbits (26).

To measure the level of protection, experimental animals such as mice and rabbits are often challenged with the pathogenic organism. Conventional challenge mouse models involved aseptic removal of infected organs to measure the numbers of CFU (2, 27). In contrast, in vivo bioluminescent imaging (BLI) has been reported to be a noninvasive real-time technique which can reduce the number of animals required per experiment (7, 10, 15, 16). For example, using the bioluminescent M49 GAS strain, Park et al. demonstrated the dissemination of the GAS strain through the nasal-associated lymphoid tissue (NALT) of intranasally infected mice (27).

To further investigate the mechanism of J8-DT antibody-mediated protection, we used BLI after passive transfer of rabbit IgG into naïve mice followed by GAS challenge. We demonstrate for the first time that the passive transfer of J8-DT-specific IgG significantly decreases the dissemination of GAS in peritoneal organs. We independently confirmed that a titer of J8-DT IgG apparently lower than that required during several active immunization studies with mice was sufficient for protection. Finally, we also corroborate the capability of BLI in comparison with that of the traditional microbiological methods. We believe that BLI is a powerful tool in experimental GAS vaccinology.

MATERIALS AND METHODS

Bacterial strains and culture media. Recombinant bioluminescent GAS strain M49 (Xenogen), which is resistant to kanamycin, was passaged in mice to enhance its virulence, as described previously (1). This operation did not affect the strain’s bioluminescence property. The GAS strain was grown at 37°C in liquid medium containing Todd-Hewitt broth (THB; Oxoid, Australia) supplemented with 1% neopeptone and 0.5% yeast extract (Difco) or on blood agar plates consisting of the liquid medium described above, 2% agar, and 2% horse blood.

Immunization of rabbits and IgG preparation. Rabbits received a primary immunization and five boosts (according to the primary immunization schedule) with J8-DT–alum every 21 days. All immunizations were given intramuscularly, and a total of 500 μg J8-DT was administered. The rabbits were bled terminally to collect hyperimmune sera (Institute of Medical and Veterinary Sciences, South Australia, Australia). Protein G columns (GE Healthcare) were used to purify IgG from immunized rabbit sera (IRS), according to the manufacturer’s instruction. Purified rabbit IgG containing J8-DT antibodies (J8-DT RλG) was used for passive transfer experiments. Normal rabbit IgG (nRλG; Zymed, Australia) was used as a negative control during the passive transfer.

Opsonophagocytosis (indirect bacterial assay). Opsonophagocytosis assays were performed as described previously (1, 14). Briefly, GAS M49 was grown overnight at 37°C in 5 ml liquid medium. GAS M49 was then serially diluted in phosphate-buffered saline (PBS), and a 10−5 dilution was used as the inoculum. Pooled IRS (50 μl) or normal rabbit serum (NRS; 50 μl) was incubated with an
equal volume of the GAS inoculum at room temperature for 30 min. Nonopsonic heparinized human blood (400 μl) was added, and the mixture was further incubated with end-to-end rotation (mixing) at 37°C for 3 h. Fifty microliters of this mixture was then plated in quadruplicate on blood agar plates. The number of CFU on each plate was determined after overnight incubation at 37°C. The percent reduction in the number of CFU was calculated as \[ \frac{1}{\text{CFU in the presence of IRS}} \frac{\text{CFU in the presence of NRS}}{100}. \]

Passive transfer and serum collection. Both the intraperitoneal and the intravenous routes of passive transfer were applied. For intraperitoneal passive transfer, cohorts of naïve inbred BALB/c (H-2d) mice (n = 6) were intraperitoneally administered 500 μl of purified J8-DT RIgG (1 mg/ml). A positive control group of naïve BALB/c mice (n = 6) was administered 200 μl of M49 antiserum. The M49 antiserum was generated from mice previously infected with GAS M49 and contained a titer of serum IgG to whole GAS M49 of 1,600. For intravenous passive transfer, cohorts (n = 10) were intravenously administered 200 μl purified J8-DT RIgG (1 mg/ml). Mice to which nRIgG was passively transferred at 26 h and 2 h prior to GAS challenge were also included as a negative control group.

The antibody titers in the recipient mice after passive transfer were determined by enzyme-linked immunosorbent assay (ELISA), as described before (1). Blood/serum samples were collected 2 h after the second administration of IgG, followed by challenge with GAS M49. All animal experiments were conducted in accordance with the guidelines of the National Health and Medical Research Council (NHMRC; Australia) and were approved by the Queensland Institute of Medical Research Animal Ethics Committee.

Intraperitoneal bacterial challenge. Mice were challenged with GAS strain M49 as described previously (1). Briefly, passaged recombinant GAS M49 was grown overnight at 37°C in several liquid 5-ml cultures. The overnight cultures were pooled and were then washed twice with and resuspended in the liquid medium. The bacterial inoculum was prepared by measuring the optical density at 600 nm and plating 10-fold dilutions on blood agar plates. After the overnight incubation at 37°C, the numbers of CFU were determined. The same predetermined dose of GAS was administered intraperitoneally to mice from all the experimental cohorts.

Bioluminescent imaging of mice. Following challenge, the mice were imaged for 5 min with an IVIS 100 charge-coupled-device camera system (Xenogen) at 2, 24, and 72 h postchallenge. The anesthetized mice were euthanized, imaged ventrally, and dissected to image the exposed organs for bacterial dissemination. Photon emission was determined with Xenogen’s Living Image Software package (version 2.50).

Organ collection and CFU determination. After the mice were sacrificed, their spleens and livers were aseptically removed and imaged in vivo for 5 min by using the IVIS system. Subsequently, the spleens were homogenized in PBS, and 10-fold dilutions were plated in duplicate. Spleen homogenates were plated on blood agar and kanamycin (200 μg/ml). The plates were incubated overnight at 37°C, and the numbers of CFU were determined (2).

RESULTS

Immunogenicity and in vitro efficacy of J8-DT in rabbits. Intramuscular and subcutaneous hyperimmunization of rabbits with J8-DT–alum induced a strong serum IgG response with a J8-specific endpoint titer of up to 10^6 (Fig. 1). The immunizations also induced DT-specific antibodies.

Opsonophagocytosis assays were performed to determine the in vitro efficacy of hyperimmune sera from rabbits immunized with J8-DT–alum (IRS). The numbers of GAS M49

FIG. 1. J8-specific serum IgG response in rabbits immunized with J8-DT–alum and DT-alum. Rabbits were immunized on day 0, followed by boosts at weeks 3, 6, 9, 12, and 17. The J8-specific IgG titers of rabbits immunized with J8-DT–alum intramuscularly (n = 2) (A) and subcutaneously (n = 1) (B) are shown. The DT-specific IgG titers of rabbits immunized with DT-alum intramuscularly (n = 2) (C) and subcutaneously (n = 1) (D), as determined by ELISA, are shown. The results are means ± standard errors of the means, where n is >1. Differences in the means were not significant (P > 0.05), as calculated by the t test.
CFU were reduced by 91% (200 CFU) in the presence of pooled IRS compared to the number in the presence of pooled NRS (2,340 CFU). By itself, in the absence of human blood and rabbit sera, the control inoculum grew 32 times (2,080 CFU).

**Protective efficacy of rabbit J8-DT IgG.** The *in vivo* efficacy of the hyperimmune serum was determined by passive transfer experiments. Purified IgG from IRS (J8-DT RlgG) and positive control M49 antiserum were intraperitoneally passively transferred to mice, and the mice were subsequently challenged with $10^8$ CFU/ml GAS M49 to monitor them for the development of infection (n = 2 each day). Naïve mice succumbed to infection overnight. A detectable level of bioluminescence was observed from control mice, indicative of the dissemination of GAS. Color scales indicate photon emission (photons s$^{-1}$ m$^{-2}$) during the 5-min exposure time. A different scale is used for organs from naïve mice at 24 h (minimum, 100,000; maximum, 400,000). The crosses indicate that the mice succumbed to infection.

![Bioluminescence imaging of mice](image.png)

**FIG. 2.** Detection of M49 GAS by BLI. (A) Bioluminescence emission from the peritoneal cavity of mice; (B) dissected livers and spleens from infected mice at 2, 24, and 72 h postchallenge. J8-DT RlgG and M49 antiserum were intraperitoneally passively transferred to mice, and the mice were subsequently challenged with $10^8$ CFU/ml GAS M49 to monitor them for the development of infection (n = 2 each day). Naïve mice succumbed to infection overnight. A detectable level of bioluminescence was observed from control mice, indicative of the dissemination of GAS. Color scales indicate photon emission (photons s$^{-1}$ m$^{-2}$) during the 5-min exposure time. A different scale is used for organs from naïve mice at 24 h (minimum, 100,000; maximum, 400,000). The crosses indicate that the mice succumbed to infection.

CFU were reduced by 91% (200 CFU) in the presence of pooled IRS compared to the number in the presence of pooled NRS (2,340 CFU). By itself, in the absence of human blood and rabbit sera, the control inoculum grew 32 times (2,080 CFU).

Protective efficacy of rabbit J8-DT IgG. The *in vivo* efficacy of the hyperimmune serum was determined by passive transfer experiments. Purified IgG from IRS (J8-DT RlgG) and positive control M49 antiserum were administered intraperitoneally to naïve mice prior to challenge with GAS M49. Bioluminescent imaging of mice at 2, 24, and 72 h postchallenge (Fig. 2A and B) demonstrated the dissemination of GAS through the peritoneal cavity and organs. The flux (bioluminescence, in photons per second) observed from the spleens of naïve mice was increased compared to that observed from mice that received J8-DT RlgG or M49 antiserum (Fig. 3A). In addition, the spleens from mice administered J8-DT RlgG or M49 antiserum also had a reduced GAS burden, as determined from the CFU counts (Fig. 3B). Naïve mice did not show a decreased GAS burden and succumbed to infection by day 2.
It is possible that the antibodies may have directly interacted with or caused the clumping of GAS M49 when they were administered by the same route (homologous system), as in the experiment described above. To confirm that this reduction in the numbers of CFU in the mice to which J8-DT RIgG was administered intraperitoneally was not a result of clumping of GAS with the antibodies circulating in the peritoneum, we separated the administration of IgG and GAS to different sites, utilizing intravenous passive transfer, followed by an intraperitoneal GAS challenge (heterologous system). J8-DT RIgG purified from IRS was intravenously administered to naive mice. The presence of rabbit J8-specific antibodies in the recipient mice was confirmed by ELISA. A J8-specific titer (geometric mean, 63,000) and a DT-specific titer (geometric mean, 620,837) were observed in the sera of the recipient mice was calculated by an unpaired t test. The crosses indicate that the mice succumbed to infection before 72 h.

In the previous experiment with intraperitoneal passive transfer, the mice were imaged at 2, 24, and 72 h postchallenge; however, the naive mice had succumbed to infection by 48 h after challenge. Therefore, in subsequent experiments, the mice were monitored for up to 24 h postchallenge. After the passive transfer and challenge with GAS M49, the mice were monitored for up to 24 h postchallenge. Therefore, in subsequent experiments, the mice were imaged at 2, 24, and 72 h postchallenge; after the passive transfer and challenge with GAS M49, the mice were imaged to monitor the bacterial burden (data not shown). Following whole-body imaging, the mice were aseptically dissected to visualize the GAS burden in the spleen and the liver (Fig. 4A). The GAS burden in the spleen was determined by using both flux emission and the traditional CFU method (Fig. 4B and C). Mice to which J8-DT RIgG was passively transferred had a very low bacterial burden. Therefore, in subsequent experiments, the mice were monitored for up to 24 h postchallenge; after the passive transfer and challenge with GAS M49, the mice were imaged to monitor the bacterial burden (data not shown). Following whole-body imaging, the mice were aseptically dissected to visualize the GAS burden in the spleen and the liver (Fig. 4A). The GAS burden in the spleen was determined by using both flux emission and the traditional CFU method (Fig. 4B and C). Mice to which J8-DT RIgG was passively transferred had a significantly reduced (P < 0.05) GAS burden compared to that of mice administered the nRIgG control. A significant positive correlation (P < 0.05) between the flux and the numbers of CFU was also observed in both passive transfers (Fig. 5). A basal level of flux of $10^6$ photons s$^{-1}$ m$^{-2}$ was observed from the spleens, which were either uninfected (unpublished observation) or which had a very low bacterial burden.

**DISCUSSION**

In this study, we have been able to demonstrate the protective efficacy of J8-DT antibodies both in vitro and in vivo utilizing bioluminescent imaging, in addition to the traditional CFU assay. Moreover, we report for the first time passive protection against a recombinant GAS strain (serotype M49) in mice with lower J8-DT antibody levels compared to those detected in previous studies (1, 26).

Active immunization of outbred and inbred mice with J8-DT induced a strong J8-specific serum IgG response, which has been shown to be efficacious in protection assays performed both in vivo and in vitro. More specifically, immunization enhanced the survival of mice after infection with GAS and also generated sera capable of opsonizing several GAS strains (1). More recently, we have also demonstrated active and passive protection against GAS strain M1 and the possible role of J8-DT antibodies in immunotherapy (26). Human sera containing antibodies to peptide p145, which contains the J8 epitope, were also capable of opsonizing GAS M5 in the presence of neutrophils (4).

Rabbits have traditionally been used to generate polyclonal sera to antigenic vaccine candidates for laboratory studies (13, 21). Likewise, the species is also frequently used in toxicology studies prior to clinical testing of vaccine candidates. Expanding from our work with mice, we wished to determine whether our vaccine formulation (J8-DT–alum) was immunogenic in an alternate animal species, such as rabbits. The hyperimmune rabbit serum generated was also used for the passive transfer studies. We found J8-DT–alum to be immunogenic following immunization by the intramuscular or subcutaneous route. Moreover, sera from rabbits immunized with J8-DT–alum were opsonic against GAS, suggesting that the rabbit antibodies to J8-DT have functional activity similar to the activities of antibodies to J8-DT from mice and humans (1, 4).

The passive transfer of antibodies has been shown to protect mice against intracellular bacteria such as Francisella tularensis (29). A recent review summarized the role of immune serum and the associated passive protection that it offers against other intracellular bacteria, such as Ehrlichia chaffeensis (6, 19), and extracellular bacteria, including Streptococcus pneumoniae and Haemophilus influenzae (6). The transfer of immune murine serum to mice also protected against Schistosoma mansoni infections, in which the levels of protection transferred were dependent on the time of administration of the antibodies (20). Passive protection on the transfer of rabbit IgG to mice before S. mansoni infection was also reported (21).

Studies have reported on the decomplementation (adsorption against mouse erythrocytes) of rabbit sera before passive transfer into mice to reduce the immune response to foreign rabbit sera (24). Alternatively, the use of rabbit IgG in passive transfer studies has been preferred over the use of hyperimmune rabbit sera due to the reduced cross-reactivity (11, 20, 25). In a previous study, we have demonstrated the protective potential of rabbit J8-DT IgG against GAS M1 infection (26), which is associated with RF and RHD (12). We now show that the passive transfer of anti-J8-DT IgG is also protective against the skin isolate GAS M49. Serotype M49 has often been referred to as a “nephritogenic GAS strain,” commonly associated with poststreptococcal glomerular nephritis (23). We confirmed that the protection due to passive transfer was antibody mediated and that a direct antigen-antibody interaction at the point of injection was not responsible for this protection.
Taken together, these data highlight the potential of J8-DT-specific antibodies to protect against multiple GAS strains. Instead of monitoring mice for survival postchallenge, we used BLI as a rapid assay to test the \textit{in vivo} efficacy of our vaccine candidate. The use of BLI has facilitated the investigation of disease progression and the distribution of other pathogens through the peritoneal cavity and anatomical organs, such as the spleen (7, 15). In this study, we have compared BLI in parallel with traditional microbiological techniques, such as the determination of the numbers of CFU.

We monitored the dissemination of GAS to peritoneal organs, such as the spleen, over a period of time. Interestingly, GAS had disseminated from the site of administration to the spleen as rapidly as 2 h from the time of challenge and was also found to be circulating in the blood (data not shown). The GAS burden determined from the flux emitted from each spleen correlated with the numbers of CFU. A strong correlation between the flux emitted and the numbers of CFU indicates that BLI can be effectively used to measure the bacterial load \textit{in vivo} (15), highlighting the potential of BLI in future experimental GAS vaccine studies.

The level of J8-specific antibody titers required for protection in this study \((6.3 \times 10^5)\) differed from the titer of \(\sim 10^6\) that was previously observed in active immunization experiments (1, 26). One possible explanation is that the purified rabbit antibodies may have a higher affinity and a higher avidity to the antigen (9), and thus, an apparently lower level of IgG is required in passive protection.

Our findings have important implications in therapeutic applications of immune sera. As an example, intravenous immunoglobulin (IVIG) is currently used for clinical intervention against invasive streptococcal infections, including toxic shock syndrome (17). However, due to the various levels of neutralizing activity against GAS superantigens, the outcomes of these treatments have varied. It has been suggested that a simple prescreening of IVIG batches to test for GAS-specific antibodies would improve the results of IVIG treatment (8, 30). In view of that, spiking of IVIG with humanized J8-DT antibodies or monoclonal antibody preparations alone may result in an improved efficacy of the treatments. Our passive transfer studies could hence be extended to therapeutic use for the IVIG treatment of streptococcal diseases.

In conclusion, we have demonstrated the immunogenicity of the peptide conjugate J8-DT–alum following subcutaneous
and intramuscular immunization in rabbits. The J8-DT-specific antibodies have been shown to be efficacious against a recombinant GAS M49 strain (nephritogenic serotype), by BLI and CFU assays, signifying the role of the vaccine candidate in providing protection against different streptococcal strains and further highlighting its potential for use in passive immunotherapy.

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