A rat model of *Staphylococcus aureus* chronic osteomyelitis that provides a suitable system for studying the human infection

M. E. POWER, M. E. OLSON, P. A. G. DOMINGUE and J. W. COSTERTON

Department of Biological Sciences, University of Calgary, 2500 University Drive NW, Calgary, Alberta, Canada, T2N 1N4

Summary. Chronic osteomyelitis was produced by inoculating *Staphylococcus aureus* into rat tibia. The infection was characterised grossly by bone deformation and histopathologically by inflammation and the presence of coccal organisms sequestered within the bone tissue. Further observations by scanning electronmicroscopy demonstrated bacteria in microcolonies surrounded by dehydrated amorphous material that was considered to be glycocalyx. Transmission electronmicroscopy, when aided by antibody stabilisation, revealed extensive glycocalyx production within the tibia. These findings indicate that the rat model of chronic *S. aureus* osteomyelitis mimics the human infection with respect to the sessile mode of growth of bacteria within the bone. Serum antibody levels were assayed by ELISA and immunoblotting procedures. After an initial increase, ELISA titres remained relatively stable, apparently indicating the establishment of chronic osteomyelitis, whereas in immunoblotting an increase in titre over the course of infection was observed. Whole-cell ELISA revealed less subtle differences in antibody titre than did immunoblotting with cell-wall antigen. We found that mid-range antigens, including an antigen implicated as protein A, featured prominently in the immune response in this model of infection.

Introduction

Osteomyelitis is an infection that is often typified by chronic morbidity.¹ In man, the bacteria have been shown to persist within glycocalyx-enclosed microcolonies adherent to the bone and to prosthetic devices.^{2,3} Bacterial glycocalyces have been implicated in providing a protective structure for the bacteria, since bacteria in the adherent or sessile mode of growth demonstrated increased resistance to antibiotics⁴ and to host humoral and cellular immune responses.^{5,6} Therefore, the predominance of the sessile mode of growth in osteomyelitis makes it an important consideration when implementing an animal model to study the pathology and aspects of host defence mechanisms.

The rabbit model of chronic osteomyelitis has been well characterised⁷ and is similar to the human condition.⁸ The rat model developed by Rissing *et al.*^{9,10} has not been examined as fully with regard to both the mode of growth of the infecting bacteria and the resultant host immune response. However,

this model has several advantages over the rabbit model, including the greater convenience of rats for extensive antibiotic trials.⁹

The purpose of this study was to determine the mode of growth of *S. aureus* in the rat model of chronic osteomyelitis and to follow the humoral immune response over the course of infection. We present preliminary results obtained from histopathological, electronmicroscopic and immunological measurements that suggest that this model is ideally suited to study of the human infection.

Materials and methods

Bacteria

S. aureus strain 2645, a phage non-typable isolate from a case of chronic osteomyelitis (Dr M. K. Dasgupta, University Hospital, University of Alberta, Edmonton), was maintained in vials at -70°C in Tryptic Soy Broth (TSB; Difco Laboratories, Detroit, MI, USA) containing glycerol (Difco) 5%. Before use, the organism was grown on Tryptic Soy Agar (TSA; Difco) overnight at 37°C to check for purity.

Bacterial inoculum

S. aureus strain 2645 was grown in TSB overnight at 37°C with shaking at 100 rpm (orbital incubator; New Brunswick Scientific, New Brunswick, NJ, USA); 1 ml was added to 10 ml of TSB and incubated for 5 h at 37°C as above to obtain exponential phase bacteria which were harvested by centrifugation for 10 min at 3000 g and washed three times with sterile saline, 0.85%. The cell pellet was resuspended to 2×10^8 cfu/ml in sterile saline. Bacterial numbers were confirmed by standard plate counts.

Rats

Forty adult male Sprague-Dawley rats (~400 g) were housed in standard shoebox cages with corn-cob bedding. They were fed standard rodent chow pellets and fresh tap water *ad libitum*. Care, housing and all manipulations of animals were conducted in accordance with the Guidelines for the Care and Use of Experimental Animals (Canadian Council of Animal Care, Ottawa, Ontario).

Experimental model

The rat osteomyelitis model of Rissing *et al.*⁹ was used with slight modifications. Briefly, rats were anaesthetised with halothane (MTC Pharmaceuticals, Cambridge, Ontario, Canada) 4% and the left hind leg was shaved and scrubbed with povidone-iodine (Betadine; Perdue Fredrickson Co., Toronto, Ontario) and isopropyl alcohol 70% before surgery. The medial metaphyseal region of the left tibia was surgically exposed. The cortex was penetrated with a 4000-rpm drill (Teledyne Emesco, NY, USA) equipped with a 1-mm bit and 5 µl of arachidonic acid (Sigma Chemical Co., St Louis, MI, USA) 50 µg/ml, which acts as a sclerosing agent,¹⁰ was injected into the marrow cavity with a 10-µl Hamilton microsyringe (Hamilton Co., Reno, NE, USA), followed by 5 µl of the standardised 5-h culture. To facilitate recovery of *S. aureus*, a 2-cm length of sterile PE-90 tubing (Becton Dickenson, Parsippany, NJ, USA) was inserted into the medulla of four animals. The bone apertures in all animals were sealed with sterile bone wax (Ethicon Sutures Ltd, Peterborough, Ontario, Canada). The incision was closed with 3-0 vicryl sutures (Ethicon) and the epithelial layers were closed with tissue adhesive (Vetbond; Animal Care Products/3M, St Paul, MN, USA).

The rats were monitored daily for signs of wound infection or open incisions. At appropriate times, randomly chosen animals were killed by intraperitoneal overdose of sodium pentobarbital (Somnitel; MTC Pharmaceuticals). Blood was collected by cardiac puncture and the resulting sera were pooled and stored at -70°C. Tibiae from the infected animals were removed aseptically for bacteriological, histopathological and electronmicroscopic examination. In some animals the right tibiae were recovered and used as uninfected controls.

Quantitative bone counts

Tibiae were suspended in 20 ml of sterile phosphate-buffered saline (PBS, pH 7.2) and homogenised in Waring blenders three times for a total of 6 min followed by 5 min of low intensity (50–60 Hz) sonication (Bransonic 220; Bransonic Cleaning Equipment, Danbury, CT, USA) to disrupt microcolonies. Homogenates were serially diluted and plated in duplicate on TSA plates and incubated at 37°C for 36 h. Colonies were counted and bacterial numbers represented as cfu/tibia. Bacteria recovered from infected tibiae were phage typed (Dr C. Pai, Department of Microbiology, Foothills Hospital, Calgary, Alberta, Canada) and examined by biochemical identification tests (API Staph Trac; API Products, St Laurent, Quebec, Canada).

Histological examination

Freshly isolated tibiae were cut transversely with a sterile hacksaw blade into 1-cm lengths. Bone lengths were fixed for 24 h at room temperature in neutral buffered formalin (NBF) 10% and were decalcified with formic acid 17.6% in NBF. The tibiae were dehydrated and embedded in plastic (Polysciences JB4 Embedding Kit; Polysciences Inc., Warrington, PA, USA) and the blocks were sectioned to 4-µm thickness. Mounted sections were stained with Lee's methylene blue-basic fuchsin rapid stain¹¹ and examined by light microscopy.

Antibody production

An overnight culture was harvested, washed and resuspended in formalin 3.7% in PBS (pH 7.2) for 24 h. Cells were washed, resuspended in PBS to 10^8 cfu/ml, emulsified in an equal volume of Freund's incomplete adjuvant and injected intramuscularly into a New Zealand White rabbit; one dose was given each week for 4 weeks followed by three bi-weekly injections. Blood was collected by ear vein puncture and the serum was stored at -70°C for use in glyocalyx stabilisation and immunoblotting tests.

Electronmicroscopy

Freshly isolated tibiae were cut longitudinally and rinsed with sterile PBS to remove bone marrow and loosely associated bacteria. For transmission electron-microscopy (TEM), the infected portion of the bone was scraped with a sterile scalpel blade and the scrapings were fixed for 2 h at room temperature in glutaraldehyde 5% in 0.1 M cacodylate buffer (pH 6.2) containing ruthenium red 0.15%. The samples were mixed in Bacto-agar (Difco) 4%, washed five times in wash buffer (cacodylate buffer containing ruthenium red 0.05%) and post-fixed in OsO₄ 2% in wash buffer for 2 h at room temperature. Samples were dehydrated and embedded in Spurr low viscosity resin (Electron Microscopy Sciences, Quebec, Canada). Blocks were sectioned on an LKB Ultratome II and stained with uranyl acetate and lead

citrate. Grids were reinforced with evaporated carbon and examined by TEM at an accelerating voltage of 50 kV (Model H-600 electronmicroscope; Hitachi, Tokyo, Japan).

Longitudinally cut tibiae meant for scanning electron-microscopy (SEM) were rinsed as for TEM and fixed in glutaraldehyde 5% in filter-sterilised cacodylate buffer. Samples were washed five times in the sterile cacodylate buffer and dehydrated in a graded ethanol series followed by an ethanol-freon 113 series and allowed to dry in air. The bone pieces were mounted on stubs and sputter-coated with gold:palladium (60:40) in a Hummer 1 sputter coater. Specimens were examined by SEM at an accelerating voltage of 20 kV (Model S-450 scanning electronmicroscope; Hitachi).

The animals implanted with the PE-90 foreign body were killed at 4 weeks. In two animals, the tubing was scraped and the tibiae were homogenised and cfus quantified as stated previously. In the remaining two animals, the tubing was gently removed from the medullary cavity and cut longitudinally for TEM preparation. Pieces of tubing were scraped and the scrapings were incubated for 1 h at 37°C with heat-depleted hyperimmune rabbit serum. Following this incubation step, which served to bind and stabilise the bacterial glycocalyx,¹² the samples were washed three times in PBS and processed for TEM as described above. Control cells, grown overnight in TSB, were processed with and without hyperimmune rabbit serum for TEM.

Enzyme-linked immunosorbent assay (ELISA)

A microtitration-plate modification of the technique of Engvall and Perlman¹³ was used. Briefly, formalised *S. aureus* strain 2645 whole cells (10^7 cfu/well) were prepared as for antibody preparation, suspended in carbonate buffer (pH 9.6) and incubated for 18 h at room temperature in 96-well microtitration plates (Linbro/Titertek; Flow Laboratories, McLean, VA, USA). Plates were washed with PBS containing Tween 20 0.05% (PBS-Tween, pH 7.2) and blocked for 30 min with bovine serum albumin 1% in PBS-Tween; 100- μ l volumes of serum from the hyperimmunised rabbit and pooled sera from infected and control rats (serially diluted from 1 in 2 in PBS-Tween) were added in triplicate to wells and incubated for 2 h at 37°C. Plates were washed thoroughly, goat anti-rat or anti-rabbit IgG-alkaline phosphatase conjugates (Sigma) diluted 1 in 5000 in PBS-Tween were added and plates were re-incubated for 2 h at 37°C. After five washes of 5 min each with PBS-Tween, the enzyme substrate *p*-nitrophenyl phosphate (Sigma) in diethanolamine buffer 10% (pH 9.8) was added and the reactions were allowed to develop for 30 min at room temperature in the dark. The reaction was stopped with 1.0 M NaOH and the optical density (OD) determined at 405 nm in a Titertek Multiscan ELISA reader (Flow Laboratories). The antibody titre was considered to be the last dilution that gave an $OD_{405} > 0.2$ after background OD_{405} was subtracted.¹⁴ Serum samples were assayed in triplicate

on three separate days and results are presented as the average OD_{405} .

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

Crude cell walls were prepared from an overnight 100-ml culture. Cells were harvested, washed and resuspended in 20 ml of sterile PBS. The bacteria were broken by shaking for 15 min in a Braun Cell Homogenizer (Fisher Scientific, Pittsburg, PA, USA) with 50 g of acid-washed glass beads (0.1 mm; Fisher Scientific) and cooled with a stream of CO₂ every 30 s. The disrupted cells were centrifuged at 1500 *g* for 5 min to remove glass beads and whole cells. The supernate was centrifuged at 27 000 *g* for 30 min; the pellet was resuspended and the process repeated. The pellet of the final 27 000-*g* centrifugation was resuspended in 1 ml of PBS. The protein concentration was determined by the method of Lowry *et al.*¹⁵

Crude cell-wall preparations were mixed with an equal volume of sample buffer containing 2-mercaptoethanol 5% and sodium dodecyl sulphate (SDS) 2% and denatured for 10 min at 100°C. Samples were run on 12% gels at 150 V (MiniProtein II; Bio-Rad Laboratories, Mississauga, Ontario, Canada) with the discontinuous buffer system of Laemmli.¹⁶ To ensure constancy in antigen presentation and concentration, the samples were loaded at 200 μ g of protein/gel into a single-lane preparative well.

Antigens were transferred electrophoretically by the method of Towbin *et al.*,¹⁷ as modified by Burnette,¹⁸ to nitrocellulose membrane (0.2 μ m; Bio-Rad) at a constant voltage of 40 V for 18 h at 4°C. The membrane was blocked with skimmed milk 5% in 0.2 M Tris-HCl buffer (pH 7.4) containing Tween 20 0.05% for 1 h at 37°C. Strips of the nitrocellulose 5 mm wide were cut (strip blots) and incubated separately in infected and normal rat sera (serial two-fold dilutions from 1 in 20 in Tris-Tween) or hyperimmune rabbit serum (diluted 1 in 100 in Tris-Tween) for 2 h at 37°C. After five washes with Tris-Tween buffer, the strips were incubated with goat anti-rat or anti-rabbit IgG-alkaline phosphatase conjugate (Sigma) diluted 1 in 2000 in Tris buffer, for 2 h at room temperature. The strips were then thoroughly washed with five changes of Tris-Tween over a period of 45 min and developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Sigma). Protein A exhibits significant molecular weight heterogeneity, which is influenced by the preparation methods¹⁹ and the strains used.²⁰ Therefore, we exploited its ability to bind avidly but non-immunologically to the F_c portion of IgG from certain animal species.²¹ This allowed us to locate protein A *in situ*, in the nitrocellulose-immobilised preparations of *S. aureus* strain 2645.^{20, 22} Nitrocellulose strips were probed with a rabbit-raised, affinity-purified, IgG-alkaline phosphatase conjugate directed against human IgG. The latter was unlikely to share antigenicity with the staphylococcal envelope, and the conjugate was

used at a 1 in 500 dilution as this was optimal for the non-immune binding.

Results

Quantitative bacterial counts

Bacteria recovered from all the infected tibiae were identical to the original inoculum as determined by API Staph Trac and phage typing criteria. Whole-tibia homogenates allowed quantitative recovery of bacteria (table). Over the entire course of the study, three of the infected animals showed no sign of gross bone deformation and cultures of their tibiae were sterile, indicating that these animals had cleared the infection. These rats, killed at 1, 4 and 6 weeks, were subsequently excluded from the study and are not included in the table. The bacterial counts in those animals that succumbed to infection remained high and relatively stable although numbers began to drop near the end of the study. The first 2 weeks of infection appeared to be a critical phase of bacterial establishment and growth. Between weeks 2 and 3, bacterial numbers seemed to reach a plateau which was interpreted as evidence of successful establishment of a chronic infection. The foreign body appeared to have no significant effect on bacterial numbers and the cfu counts obtained from two animals in which PE-90 tubing was implanted are included in the table.

Gross morphology

Infected tibiae showed the expected deformation with substantial cortical thickening. Sinus tracts with draining purulent material were obvious in c. 60% of infected tibiae. Localised areas of bone destruction were observed throughout the study samples at all time points.

Table. Numbers of bacteria (expressed as mean log values) recovered from rat tibiae

Number of weeks after infection	Mean numbers of <i>S. aureus</i> ($\log_{10}$ cfu) recovered from	
	infected tibiae ($\pm$ SD)	control tibiae
1	6.16 $\pm$ 0.78 (5)*	0 (3)
2	7.61 $\pm$ 0.88 (5)	0 (3)
3	5.73 $\pm$ 0.74 (5)	0 (3)
4	5.85 $\pm$ 0.51 (7)	0 (3)
6	5.54 $\pm$ 0.32 (5)	0 (3)
12	4.84 $\pm$ 0.40 (3)	0 (3)

* (), Number of animals per sampling group.

Histological appearances

Bacterial microcolonies were observed consistently within sequestrae of dead bone (fig. 1, arrowhead). Throughout the study, polymorphonuclear leucocytes (PMNLs; fig. 1, arrow) were found in close proximity to the bacteria indicating an inflammatory response.

Electronmicroscopy

Microcolonies were observed adherent to the bone matrix generally associated with areas of cortical erosion (fig. 2). Frequently, large portions of the bone surface were occluded by bacteria enclosed in dehydrated glycocalyx.

By TEM, bacteria were difficult to locate in bone scrapings because large amounts of PMNLs and amorphous bone material were present. Occasionally, bacteria were seen adherent to bone material and in these cases strands of fibrillar material bridged between the bacterial cells and the bone material (fig. 3, arrow). The implanting of PE-90 tubing more readily facilitated recovery of bacteria because the bacteria adhered tenaciously to the tubing and its surface was easily scraped. These bacteria had a tendency to exhibit architectural deformities and a close association with collapsed glycocalyx material (fig. 4A). Subsequent antibody stabilisation of the PE-90 scrapings prevented the dehydration and collapse of the fibrous exopolysaccharide. This confirmed the association of extensive glycocalyx with the bacterial cells and the encompassing of entire microcolonies (fig. 4B; arrowhead). Control bacteria grown in TSB were generally smaller and had more regular cell walls with condensed glycocalyx material adjacent to the cells (fig. 4C). Antibody stabilisation demonstrated a less extensive glycocalyx than that seen *in vivo* (fig. 4D; arrow).

ELISA

The titre of the hyperimmune rabbit serum was 5120 (average of three tests). The ELISA titres of the sera from the rat model (fig. 5) are presented as the average OD₄₀₅ of three separate runs and represent a generalised response because pooled sera were used throughout the study. Control sera and sera taken 1 week after infection had little detectable IgG response to the whole-cell antigen. An increase in IgG titre was observed over the course of infection. A maximum titre of 640 was observed at 6 weeks and at 12 weeks this had diminished only slightly. The major increase in

Fig. 1. Methylene blue—basic fuchsin stained section of a decalcified tibia recovered 4 weeks after infection. Bacteria are observed within a sequestrum of dead bone (arrowhead). Large numbers of PMNLs (arrow) have infiltrated the area adjacent to the sequestered bacteria. Bar = 10 μ m.

Fig. 2. Scanning electronmicrograph of staphylococci protruding from a crevice in the bone partially occluded by dehydrated glycocalyx material. Infected tibiae contained an abundance of these pitted areas that appear to indicate cortical erosion. Bar = 5 μ m.

OD₄₀₅ occurred after the first month of infection as judged by data obtained with the lowest serum dilution.

Immunoblotting

Representative results of blots performed at least three times are shown in figs 6 and 7. Separated crude cell-wall preparations, when stained with Coomassie Blue, showed a large number of protein bands (data not shown), many of which were recognised by hyperimmune rabbit sera in immunoblotting (fig. 6, lane 1). The IgG probe was bound by a 54-Kda component; this, by implication, was protein A (fig. 6, lane 2). In contrast to the rabbit serum, normal and rat sera collected 1 week after infection recognised only faintly (at a 1 in 20 dilution) two components of 75 and 54 Kda (fig. 6, lanes 3 and 4 respectively). Semi-quantitative strip blots depicted a significant increase in antibody titre and in the number of antigens recognised by the sera as the infection progressed. By 3 weeks,

33- and 36-Kda antigens were strongly recognised to a dilution of 1 in 2560 (fig. 7A). Also, a significant banding in the 54–95-Kda range was observed. At 4 and 6 weeks, antigens at 129 and 17 Kda were newly recognised and an increase in titre to the previously seen antigens occurred (data not shown). By 12 weeks, no major new antigens were detected; however, changes in minor banding and a further increase in titre were apparent (fig. 7B). An overall strengthening in titre to most antigens occurred particularly to mid-range and 17-Kda antigens. These results differ from those of the whole-cell ELISA in that this technique disclosed dramatically increased titres to specific, prominent antigens whereas the ELISA results represent an average titre to the whole cell.

Discussion

Our aim was to evaluate a rat model of chronic osteomyelitis. The model was established successfully in approximately 93% of animals as demon-

Fig. 3. Transmission electronmicrograph of a scraping of infected tibia. Fibrils are apparent, bridging between the organism and the amorphous bone material (arrow). Bar = 1 μ m.

strated bacteriologically and histologically. Bacterial growth as a biofilm was demonstrated readily. This is significant since the natural appearance of bacteria in human osteomyelitis is in the form of adherent glycocalyx-enclosed microcolonies or biofilms.^{2,3} Bacterial biofilms have been associated with the refractoriness of chronic infections in that the glycocalyx material and the size of the microcolonies are thought to be involved in increased bacterial resistance to host defence factors and to antimicrobial agents.⁵

SEM has enabled visualisation of microcolonies of *S. aureus* in both the rabbit⁸ and rat²³ models of osteomyelitis. Additional techniques, including TEM, have been used to visualise the glycocalyxes that serve as the matrices of these microcolonies. The dehydration steps in TEM and SEM preparation condense bacterial glycocalyx material. Specific antibody has been shown to stabilise the glycocalyx of *S. aureus* grown *in vitro*, preventing its collapse upon dehydration by cross-linking the polysaccharide molecules and rendering them more rigid.²⁴ We were able to use this technique to

demonstrate significantly increased glycocalyx production by *S. aureus* strain 2645 *in vivo* as compared with those grown *in vitro*.

The biofilm mode of growth also may be responsible for the relatively limited antibody response to *S. aureus* in chronic osteomyelitis. It may be that biofilms reduce immune stimulation by inhibiting opsonophagocytosis.²⁵ There has been considerable controversy as to whether or not there are increased antibody titres to staphylococcal antigens such as peptidoglycan, lipoteichoic acid or cell walls^{14,26,27} in chronic staphylococcal osteomyelitis. Two advantages of the rat model were that there was minimal F_c binding of IgG to protein A²¹ and little pre-existing immunity before infection, enabling more accurate monitoring of increases in antibody titre associated with infection. Our results clearly showed that an increase in ELISA titre to whole-cell antigen occurred, that it was a limited response with a maximum titre reached at 6 weeks, and that it remained stable for the remainder of the study. The finding of a continued elevated titre underlines the chronicity of infection.

Significantly, immunoblotting detected more subtle increases in antibody response to particular components of the bacterial cell wall over the course of infection than did ELISA, as the titre in the immunoblots increased through to 12 weeks. This no doubt reflects the use of different antigen preparations in addition to the basic differences in methodologies. It is possible to hypothesise that the mid-range antigens, which were strongly recognised to a very high titre by immunoblotting, are present in relatively small amounts on the cell surface and thus do not contribute greatly to the whole-cell ELISA titre, yet are highly immunogenic in infection and, therefore, figured prominently in immunoblotting. Also, these antigens may not be surface-exposed to a great extent, at least in the *in-vitro* phenotype; thus, antibodies would bind to a relatively lower degree in the ELISA assay. Our current work is aimed not only at answering these questions but also at characterising the significant antigens involved.

Sera from infected rats recognised a more limited number of antigens than the hyperimmune rabbit serum. In contrast to homologous blotting between hyperimmune rabbit serum and *in-vitro* antigen, heterologous blotting, as represented by the reaction between sera from infected rats and the *in-vitro* antigen, revealed fewer antigenic bands. Presumably, due to phenotypic plasticity,²⁸ the antibodies induced during infection did not react with all of the *S. aureus* antigens expressed *in vitro*. Sera from

Fig. 4. Transmission electronmicrographs of bacteria recovered from PE-90 tubing implanted in rat tibiae conventionally prepared (A) and treated with specific antiserum (B), and of bacteria grown in TSB broth conventionally prepared (C) and treated with specific antiserum (D). Antibody stabilisation enabled visualisation of glycocalyx material which was much less extensive in bacteria grown *in vitro* (D; arrow) than in those grown *in vivo* (B; arrowhead) where it enclosed entire microcolonies. Bar = 1 μ m.

Fig. 5. Whole-cell ELISA titration curves of infected rat serum. Results presented as OD_{450} of normal rat serum ($\bigcirc$ --- $\bigcirc$), and serum from infected rats collected after 1 ($\diamond$ — $\diamond$), 3 ($\bullet$ — $\bullet$), 4 ($\square$ — $\square$), 6 ($\blacksquare$ — $\blacksquare$) and 12 ($\triangle$ — $\triangle$) weeks examined at various serum dilutions. The 0.2 OD value cut-off for titre is indicated by the interrupted line (see text).

infected animals recognised their homologous antigens with greater affinity, both in terms of titre and bands visualised with *S. aureus* grown *in vivo* rather than *in vitro* (unpublished data).

Our strip immunoblotting technique enabled us to detect increased antibody titre to individual antigens. This technique may be valuable in the identification of important antigens in an infection and in following the course of infection. However, immunoblotting is a technique that has only recently been used to study staphylococci and many antigens are as yet uncharacterised. Recognition of high and middle molecular-weight antigens correlates well with results reported elsewhere.²⁹ Because

Fig. 6. Immunoblots of *S. aureus* crude cell walls separated by SDS-PAGE, electrophoretically-transferred and probed with: a 1 in 100 dilution of hyperimmune rabbit serum (lane 1); 1 in 500 dilution of rabbit anti-human IgG conjugate to probe for protein A (2); a 1 in 20 dilution of normal rat serum (3); and a 1 in 20 dilution of rat serum collected 1 week after infection. Numbers refer to molecular weights (Kda).

protein A varies in molecular weight,^{19,20} we employed an in-situ probe to locate this molecule in our strain and found that it was recognised as a prominent antigen in this model.

We suggest that the rat model is a good system for studying chronic osteomyelitis and for recognising the staphylococcal antigens involved in human infection.

We thank Liz Middlemiss and Joyce Nelligan for expert assistance with the electronmicroscope and photography. M.E.P. was supported by the Alberta Heritage Foundation for Medical Research.

Fig. 7. Strip immunoblots of *S. aureus* crude cell wall separated by SDS-PAGE, electrophoretically-transferred and probed with serial doubling dilutions of pooled infected rat serum at dilutions from 1 in 20 (lane 1) to 1 in 10 240 (10). **A**, 3 week serum; **B**, 12 week serum. Numbers refer to molecular weights (Kda).

REFERENCES

- Waldvogel FA, Papageorgion PS. Osteomyelitis: the past decade. *N Engl J Med* 1980; **303**: 360-370.
- Gristina AG, Oga M, Webb LX, Hobgood CD. Adherent bacterial colonisation in the pathogenesis of osteomyelitis. *Science* 1985; **228**: 990-993.
- Marrie TJ, Costerton JW. Mode of growth of bacterial pathogens in chronic polymicrobial human osteomyelitis. *J Clin Microbiol* 1985; **22**: 924-933.
- Nickel JC, Ruseska I, Wright JB, Costerton JW. Tobramycin resistance of *Pseudomonas aeruginosa* cells growing as a biofilm on urinary catheter material. *Antimicrob Agents Chemother* 1985; **27**: 619-624.
- Costerton JW, Cheng K-J, Geesey GG *et al.* Bacterial biofilms in nature and disease. *Annu Rev Microbiol* 1987; **41**: 435-464.
- Peterson PK, Wilkinson BJ, Kim Y, Schmeling D, Quie PG. Influence of encapsulation on staphylococcal opsonization and phagocytosis by human polymorphonuclear leukocytes. *Infect Immun* 1978; **19**: 943-949.
- Norden CW. Experimental osteomyelitis: I. A description of the model. *J Infect Dis* 1970; **122**: 410-418.
- Mayberry-Carson KJ, Tober-Meyer B, Smith JK, Lambe DW, Costerton JW. Bacterial adherence and glycocalyx formation in osteomyelitis experimentally induced with *Staphylococcus aureus*. *Infect Immun* 1984; **43**: 825-833.
- Rissing JP, Buxton TB, Weinstein RS, Shockley RK. Model of experimental chronic osteomyelitis in rats. *Infect Immun* 1985; **47**: 581-586.
- Rissing JP, Buxton TB, Fisher J, Harris R, Shockley RK. Arachadonic acid facilitates experimental chronic osteomyelitis in rats. *Infect Immun* 1985; **49**: 141-144.
- Bennett HS, Wyrick AD, Lee SW, McNeil JH. Science and art in preparing tissues embedded in plastic for light microscopy, with special reference to glycol methacrylate, glass knives and simple stains. *Stain Technol* 1976; **51**: 71-97.
- Mackie EB, Brown KN, Lam J, Costerton JW. Morphological stabilisation of capsules of group B streptococci types Ia, Ib, II, and III with specific antibody. *J Bacteriol* 1979; **138**: 609-617.
- Engvall E, Perlman P. Enzyme-linked immunosorbent assay, ELISA. III. Quantitation of specific antibodies by enzyme-labelled anti-immunglobulin in antigen-coated tubes. *J Immunol* 1972; **109**: 129-135.
- Jacob E, Arendt DM, Brook I, Durham LC, Falk MC, Schaberg SJ. Enzyme-linked immunosorbent assay for detection of antibodies to *Staphylococcus aureus* cell walls in experimental osteomyelitis. *J Clin Microbiol* 1985; **22**: 547-552.
- Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin-phenol reagent. *J Biol Chem* 1951; **193**: 265-275.
- Laemmli UK. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature* 1970; **227**: 680-685.
- Towbin E, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* 1979; **76**: 4350-4354.
- Burnette WN. "Western blotting" electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide

- gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal Biochem* 1981; **112**: 195–203.
19. Björk I, Petersson B-A, Sjöquist J. Some physiochemical properties of protein A from *Staphylococcus aureus*. *Eur J Biochem* 1972; **29**: 579–584.
 20. Cheung AL, Bayer AS, Peter J, Ward JI. Analysis by gel electrophoresis, western blot, and peptide mapping of protein A heterogeneity in *Staphylococcus aureus* strains. *Infect Immun* 1987; **55**: 843–847.
 21. Forsgren A, Ghetie V, Lindmark R, Sjöquist J. Protein A and its exploitation. In: Easmon CSF, Adlam C (eds) *Staphylococci and staphylococcal infections*, vol 2. London, Academic Press Inc., 1983: 429–480.
 22. Domingue PAG, Lambert PA, Brown MRW. Iron depletion alters surface-associated properties of *Staphylococcus aureus* and its association to human neutrophils in chemiluminescence. *FEMS Microbiol Lett* 1989; **59**: 265–268.
 23. Buxton TB, Horner J, Hinton A, Rissing JP. *In vivo* glycocalyx expression by *Staphylococcus aureus* Phage type 52/52A/80 in *S. aureus* osteomyelitis. *J Infect Dis* 1987; **156**: 942–946.
 24. Caputy GG, Costerton JW. Morphological examination of the glycocalyxes of *Staphylococcus aureus* strains Wiley and Smith. *Infect Immun* 1982; **36**: 759–767.
 25. Baltimore RS, Mitchell M. Immunological investigations of mucoid strains of *Pseudomonas aeruginosa*: comparison of susceptibility to opsonic antibody in mucoid and non-mucoid strains. *J Infect Dis* 1980; **141**: 238–247.
 26. Tuazon CU. Teichoic acid antibodies in osteomyelitis and septic arthritis caused by *Staphylococcus aureus*. *J Bone Joint Surg* 1982; **64**: 762–765.
 27. Jacob E, Durham LC, Falk MC, Williams TJ, Wheat LJ. Antibody response to teichoic acid and peptidoglycan in *Staphylococcus aureus* osteomyelitis. *J Clin Microbiol* 1987; **25**: 122–127.
 28. Brown MRW, Williams P. The influence of environment on envelope properties affecting survival of bacteria in infections. *Annu Rev Microbiol* 1985; **39**: 527–556.
 29. Bell JA, Pennington TH, Petrie DT. Western blot analysis of staphylococcal antibodies present in human sera during health and disease. *J Med Microbiol* 1987; **23**: 95–99.