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Experimental Staphylococcal Infection in the Mouse

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**Presented for the Degree of Doctor of Philosophy
in the Faculty of Science, University of Glasgow**

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We cannot separate one aspect of Wisdom from another, neither the philosophic from the scientific, nor the wisdom that is in the heart from the wisdom that issues from the hands, in other words, the Ideal from the practical.

N. Sri Ram of India.

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OBJECTS OF THE RESEARCH

Staphylococcus aureus is known to cause several types of infection, both superficial and systemic, in man. Relatively little, however, is known of the mechanisms of its pathogenicity. The organism produces many biologically active extracellular products which have been the subject of much research but only two of these, i.e. the epidermolytic toxin and the enterotoxin have been directly associated with specific disease symptoms.

A major drawback to staphylococcal research has been that laboratory animals exhibit high natural resistance to experimental infection by Staph. aureus. However, a very successful animal model for studying Toxic Epidermal Necrolysis, a staphylococcal disease of infants and children, has been developed in the neonatal mouse. But there is still a requirement for a generally applicable experimental animal system for the other more common staphylococcal infections, i.e. of the pyogenic type. Clinical evidence indicates that man is in general, most susceptible to staphylococcal infections in infancy. For these reasons the objects of this research were:-

1. To find an experimental system in the mouse for the study of pyogenic staphylococcal infections.
2. To test whether susceptibility to staphylococcal infection is related to age in the mouse and if so, to determine whether this applies to certain other bacteria.
3. To examine several strains of Staph. aureus, including fresh clinical isolates, of known haemolysin and enzyme patterns for differences in virulence.

4. To study the growth of strains of Staph. aureus in-vivo.
5. If possible to identify the role of particular virulence factors in the mechanism of pathogenicity.

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LIST OF ABBREVIATIONS

CCY	=	casein (acid-hydrolysed) - casein (trypsin-hydrolysed) - yeast extract medium
c.f.u.	=	colony forming units
CGD	=	chronic granulomatous disease
CNS	=	central nervous system
CRF	=	coagulase reactive factor
DNA	=	deoxyribonucleic acid
DOCR	=	deoxycholate residue
DTH	=	delayed-type hypersensitivity
FAT	=	fluorescent antibody test
Fc	=	crystallisable fragment of the immunoglobulin molecule
FITC	=	fluorescein isothiocyanate isomer I
H and E	=	haematoxylin and eosin stain
HU	=	haemolytic unit
IgG	=	immunoglobulin G
k	=	growth rate constant
LEC	=	lymphocyte enriched cell suspension
LU	=	leucocidal units
MDT	=	mean doubling time
MEC	=	macrophage enriched cell suspension
MSA	=	mannitol salt agar
NCTC	=	National Collection of Type Cultures
PBS	=	phosphate buffered saline
PMN	=	polymorphonuclear
RBC	=	red blood cells
RES	=	reticuloendothelial system

RPHA	=	reversed passive haemagglutination
SCI	=	specific cell-mediated immunity
TEN	=	Toxic Epidermal Necrolysis
WPEC	=	whole peritoneal exudate cell suspension

INTRODUCTION

A. The Pathogenicity of Staphylococcus aureus

I. General remarks

As a result of the work of Ogston (1882) Staphylococcus aureus became recognised as a major pathogen. Very few micro-organisms have its ability to produce such a diverse array of both superficial and systemic infections. (Table I). Yet Staph. aureus is commonly considered as part of the normal microflora of the body. Newborn infants are colonised rapidly (Hurst, 1965; Evans et. al., 1973; Deacon and Smith, 1973 and Kwong et. al., 1973) and the organism is found with great frequency on the skin and in the nasopharynx of normal healthy adults. Indeed, it is well established that a normal individual may carry and excrete many millions of staphylococci.

The interrelationships between local carriage, contiguous infection at a local site and metastasis are more difficult to understand (Fig. 1). However, It seems the staphylococcus readily takes advantage of any imbalance of the host-parasite relationship in its favour causing both local and systemic disease.

2. Host defence mechanisms in relation to Staph. aureus infections

Skin and mucous membranes: The skin is an excellent mechanical barrier to bacterial infection. When the integrity of the epithelium is broken a portal of entry is created through which organisms may pass. Most commonly it is in this way that staphylococci invade skin and subcutaneous tissues. Wounds, surgical incisions, burns, cutaneous viral infections, e.g. varicella and exfoliative dermatitis are particularly susceptible to staphylococcal infections (Penikett, Knok and Liddell, 1958; Cluff et. al., 1968; Hambreaus, 1973 and Wald, Levine and Togo, 1973). Also when the skin barrier is bypassed

TABLE I

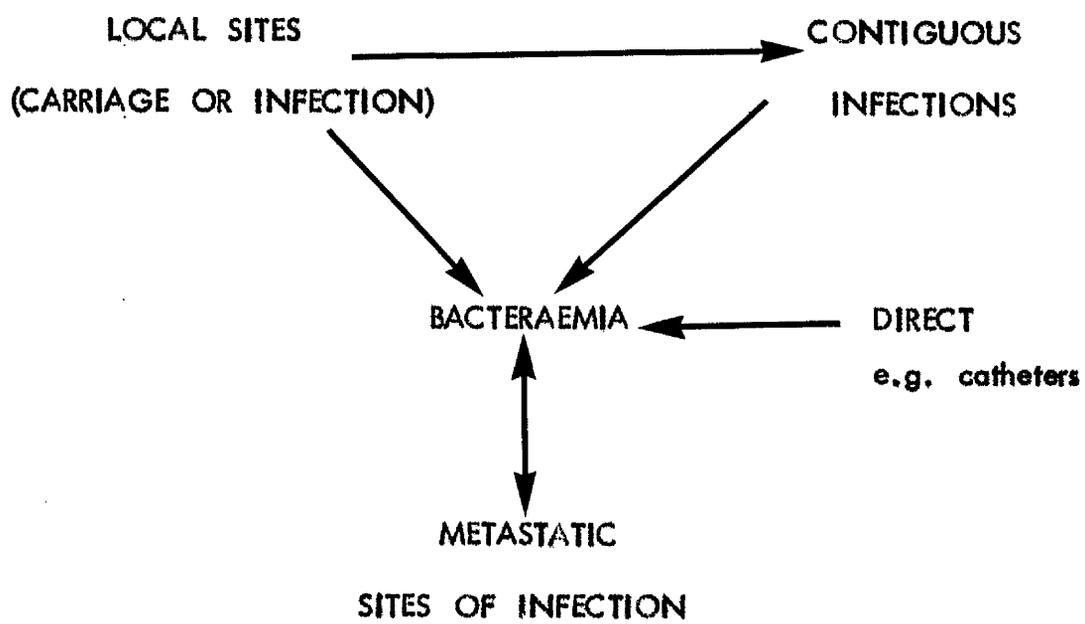
Spectrum of staphylococcal infectionsA. Local Infection Sites

- | | |
|---------------------------|--|
| 1. Skin | folliculitis, furunculosis, boils, caruncles, impetigo, Toxic Epidermal Necrolysis |
| 2. Eye | conjunctivitis, deep orbital infections |
| 3. Mouth, nose and throat | sinusitis, pharyngitis, parotitis, botryomycosis, mastoiditis |
| 4. Gastrointestinal tract | enterocolitis |
| 5. Urogenital tract | cystitis, prostatitis, prostatic abscess, cervicitis, salpingitis, pelvic abscess, Bartholinitis |

B. Metastatic Infection Sites

- | | |
|---------------------|---|
| 1. Bones and joints | osteomyelitis, septic arthritis |
| 2. Lungs | secondary pneumonia |
| 3. Skin and muscle | abscesses |
| 4. Heart | endocarditis, myocarditis, pericarditis |
| 5. Kidney | abscesses, pyelonephritis, glomerulonephritis |
| 6. CNS | abscesses, meningitis |

Figure 1: Interrelationships of carriage and infection



by injection without attention to aseptic techniques, e.g. by drug addicts, Staph. aureus can cause serious local infection occasionally followed by bacteraemia and metastatic abscesses. On rare occasions immunisation by jet-gun has allowed entry of staphylococci from the skin with fatal outcome (Kassanoff, Nahamias and Abrutyn, 1971).

The ciliated epithelium of the upper respiratory tract is normally efficient in coping with inhaled bacteria. When the normal function of this is impaired, e.g. by influenza virus the lower respiratory tract may be invaded by bacteria including Staph. aureus. Secondary staphylococcal pneumonia also occurs commonly in individuals with mucoviscidosis (cystic fibrosis), where viscid pulmonary secretions in the lung may account for the increased ability of the staphylococci to survive.

Natural cellular and humoral immunity: Non-specific cellular immunity is primarily a function of the phagocytic cells of which there are basically two types (i) the polymorphonuclear (PMN) leucocytes and (ii) the mononuclear monocytes (macrophages). These cells are present in all tissues of the body, in circulating blood and in certain filtering organs such as lymph nodes, liver and spleen. When they encounter bacteria either randomly or by directed movement (chemotaxis), they may ingest them by phagocytosis and kill them by means of bactericidal factors. Normal phagocytes have several bactericidal factors as follows:-

- (1) Acid
- (2) Phagocytin
- (3) Muramidase (lysozyme)
- (4) Cationic proteins
- (5) Myeloperoxidase
- (6) Hydrogen peroxide
- (7) Halides

Defects in non-specific immunity can lead to increased susceptibility to staphylococcal infection. When chemotaxis is impaired or when leucocytes are incapable of responding properly to chemotactic and phagocytic stimuli, recurrent staphylococcal infections occur (Mandell, 1972; Clark et. al., 1973).

Among the deficiencies in intracellular bactericidal factors by far the most important in relation to staphylococcal disease is hydrogen peroxide which acts synergistically with myeloperoxidase and halides to kill bacteria. Individuals with chronic granulomatous disease (CGD) were shown to be virtually incapable of producing H_2O_2 and such individuals suffer from severe recurrent infections by staphylococci and other catalase-producing bacteria. (Quie et. al., 1967).

Other known leucocyte malfunctions are not so important in staphylococcal disease (Quie and Davis, 1973). However, the importance of the non-specific cellular immune response in general is underlined by the fact that general granulocytopenia whether congenital, associated with leukaemia or artificially induced, leads to increased susceptibility to staphylococcal infection (Cluff et. al., 1968).

Specific Cellular Immunity (SCI) involves primarily thymus dependent (T) lymphocytes which during development, become sensitised to particular antigens. Associated with SCI is delayed-type hypersensitivity (DTH), a form of immune allergy mediated by the sensitised small lymphocytes (see p. 43).

In general, individuals with defective SCI do not normally suffer from severe staphylococcal infections. From this we can conclude that SCI is

not of prime importance in protecting against staphylococcal infection. Indeed, there appears to be some evidence that SCI, or more accurately DTH, is an important contributing factor in recurrent staphylococcal infection (Mudd, Taubler and Baker, 1970). This is discussed more fully in Section C2.

Humoral immunity is important in protecting against staphylococcal infection, for instance patients with deficiencies of humoral immunity are more susceptible than normal subjects to staphylococcal infection. Antibodies probably have two main functions:-

- (i) the promotion of phagocytosis by opsonisation, and
- (ii) antitoxic activity once an infection is established.

Thus staphylococci most often express their pathogenic potential in a host whose immune mechanisms are impaired.

3. Other predisposing factors

The presence of foreign bodies in any site tend to increase susceptibility to staphylococcal infection. Intravenous catheters, sutures, vascular grafts or pacemakers, prostheses used in various types of surgery and valves used in the treatment of hydrocephalus are examples of such foreign bodies whose prolonged presence in the tissue may lead to infection.

Staphylococcal superinfections may develop in patients being treated with penicillin for infection by another organism because many strains of Staph. aureus produce penicillinase. In these patients the normal microflora is suppressed. Also, prophylactic use of antibiotics prior to gastro-intestinal surgery may lead to staphylococcal enterocolitis (Tisdale, Fenster and Klatskin, 1960 and Garrod, 1972).

Certain other diseases, e.g. diabetes mellitus, alcoholism, coronary artery disease, chronic lung disease and various malignant tumours are associated with increased susceptibility to staphylococcal infection.

Table 2 summarises the factors which most often predispose to staphylococcal infections.

Staph. aureus therefore, is responsible for a wide range of localised and generalised infections.

It is notable that the more serious and often fatal staphylococcal infections occur in infants and children (Cluff et. al., 1968; Quie et. al., 1967 and Lyell, Dick and Alexander, 1969). In particular, staphylococcal impetigo, osteomyelitis and Toxic Epidermal Necrolysis are almost exclusively diseases of children.

There is little doubt that certain strains of Staph. aureus are associated with particular types of infection. Also for reasons which are only partly understood individuals vary considerably in susceptibility to infection. There has been much debate as to whether Staph. aureus is best considered as a "true" pathogen or as an "opportunistic" pathogen. Neither term adequately describes this group of coagulase positive cocci; on occasion, strains of high virulence can cause outbreaks of infection in certain human populations; more commonly the organism escapes from its normally parasitic niche to penetrate the impaired defences and establish an opportunistic infection.

4. Epidemiology of Staph. aureus

It is well established that staphylococcal infections are a continuing problem in the hospital environment despite the availability of anti-

TABLE 2

Factors predisposing to staphylococcal infection

<u>Predisposing Factor</u>	<u>Possible Resulting Infection</u>
1. Skin injury e.g. burns surgical incisions	Initially local with consequent bacteraemia and metastasis
2. Impaired respiratory function e.g. viral infection cystic fibrosis	Secondary pneumonia
3. Leucocyte defects (i) granulocytopenia (ii) defects in chemotaxis (iii) defects in phagocytosis (iv) defects in intracellular killing	Local and metastatic Cold abscesses, pneumonia Abscesses Recurrent deep skin abscesses
4. Humoral immunity defects	Local and metastatic
5. Presence of foreign bodies	Local and metastatic
6. Therapeutic and prophylactic use of antibiotics	Superinfections including secondary pneumonia and enterocolitis
7. Other illnesses which reduce host resistance e.g. diabetes mellitus chronic artery disease alcoholism	Local and metastatic

staphylococcal antibiotics. Also in nurseries, outbreaks of staphylococcal infection are not infrequent. In such situations it is essential to have some means of differentiating and identifying strains of Staph. aureus. The following section outlines the principal methods of typing strains of Staph. aureus which are commonly used as epidemiological tools.

Serotyping: Cowan (1939) was the first to identify strains of Staph. aureus by serotyping with absorbed antisera. He found three main groups, subsequently designated, Cowan I, II, and III. Since then advances in immunochemistry have allowed further subdivision of the Cowan types.

Pillet and his colleagues developed a serotyping system producing what are known as the International Types. Per Oeding in 1952 reported a new system based on patterns of multiple type-specific antigens. This system was refined by Haukenes in the early 1960's.

It is obvious from the literature that serotyping of staphylococci is a very complex subject and that the different systems are not sufficiently reproducible for general laboratory use.

Bacteriophage typing: Bacteriophages lytic for staphylococci were first isolated by Callow in 1922. Since then many phages have been obtained and some of them selected and standardised for use in phage typing. The practical aspects of phage typing have been established since 1952 (Williams and Rippon, 1952) and are accepted as being of primary importance in epidemiological and clinical investigations. The "basic set" of phages for typing of Staph. aureus of human origin falls into four main groups as follows:-

<u>Group Number</u>	<u>Phages</u>
I	29, 52, 52A, 79, 70
II	3A, 3C, 55, 71
III	6, 42E, 47, 53, 54, 75, 77, 83A, 85
IV	42D
Unassigned	81, 187

Distinct from this, is a system for typing strains of Staph. aureus of animal origin in particular, bovine strains.

Patterns of phage types have emerged which suggest a correlation with disease potential, e.g. strains of Staph. aureus lysed by the group I phages of 80, 81, 52, 52A are often associated with outbreaks of sepsis in hospitals and strains lysed by the group II phage 71 are associated with superficial skin infections both in adults (Impetigo) and in children (Toxic Epidermal Necrolysis and Pemphigus Neonatorum). (Parker, Tomlinson and Williams, 1955; Lowney et. al., 1967; Samuels, 1967 and Lyell, Dick and Alexander, 1969). Also, strains lysed by group III phages 6, 47 have been associated with food poisoning outbreaks. However, the correlation is not complete and it must be remembered that the strains of staphylococci being typed in this way are usually from specialised environments, e.g. an outbreak of sepsis in a hospital.

B. Virulence Factors and Disease

Many pathogenic micro-organisms produce virulence factors which are thought to contribute either directly or indirectly to their pathogenicity. However, it must be stressed that because an organism considered to be a

pathogen produces a product, does not mean that this product must be involved in pathogenicity, i.e. a virulence factor. Indeed, there are relatively few bacterial products which have been proved definitively to be virulence factors. This is especially true of the staphylococcus.

I. Toxins and enzymes

Staphylococci produce a wide range of extracellular products including some toxins and enzymes. Many, if not all, of these have been studied to determine whether or not they are virulence factors and, if so, their role in pathogenicity. Several, especially the toxins, have been isolated in highly purified form and much information is available about their structure, antigenicity, specificity and biological activity. (Arbuthnott, 1970; Abramson, 1972; Jeljaszewicz, 1972; Woodin, 1972; Bergdoll, 1972; Möllby and Wadström, 1973).

This discussion will refer mainly to work done in vivo since, by definition, pathogenicity is the ability to produce disease and, as stressed at the beginning of this section a specific product must be shown to be active in vivo before it can be considered a true virulence factor.

Coagulase: Loeb in 1903 was the first to describe the action of staphylocoagulase on human plasma. It is now commonly used to differentiate the potentially pathogenic strains of staphylococci from the non-pathogens.

There are two types of coagulase:-

- (i) bound coagulase (clumping factor) which acts directly on fibrinogen, and
- (ii) free coagulase which requires a plasma co-factor called "coagulase reacting factor" (CRF).

The use of coagulase production as the criterion for potential pathogenicity assumes that it is directly related to virulence. Several workers have found that coagulase-positive strains are indeed virulent for mice while coagulase negative are less so (Alami, Kelly and Race, 1968; Gorrill, Klyhin and McNeil, 1966). This seems to be related to the ability of the coagulase-positive strains to survive for longer periods in the tissues of the infected animals (Smith and Dubos, 1956), and not to the ability of coagulase-positive strains to resist phagocytosis (Foster, 1962). These in-vivo findings are in agreement with classical in-vitro studies of phagocytosis and intracellular killing of coagulase-positive and coagulase-negative strains. (Rogers and Melly, 1960; Melly, Thomson and Rogers, 1960). These studies showed that in the presence of human serum, both coagulase-positive and negative strains are phagocytosed equally by human and rabbit PMN leucocytes but that coagulase-positive strains can survive intracellularly for much longer periods.

By contrast, experiments with rabbits and mice showed that coagulase-negative mutants were as virulent as the parent strain and, conversely, a coagulase-positive mutant was avirulent when administered by the intravenous, subcutaneous and intradermal routes (Kapral and Li, 1960; Li and Kapral, 1962; Karas and Kapral, 1962). Although the coagulase test has proved immensely valuable in practice, justification for accepting coagulase production as a unique determinant of pathogenicity remains questionable. A further point is that both coagulase-positive and negative strains used in the experiments described above were tested for clot production in human plasma. It is known that plasma from different species gives varying results

in the coagulase test, due to differing amounts of CRF which is a necessary accessory factor for clotting. Mouse and fowl plasma for example, contain very small amounts of CRF compared to human. Gorrill (1951) noted that when mouse plasma was used in the coagulase test to differentiate staphylococcal strains, there was complete correlation between coagulase positivity and virulence for the mouse. Also, when mouse plasma was used to test strains from humans, sheep and cows, the animal strains gave strong coagulase reactions while weak reactions were given by human strains. The animal strains were more virulent for the mouse (Smith, 1963). Coagulase testing of strains with plasma from the host species may be more valid as an indicator of pathogenic potential for that species.

The importance of coagulase as a virulence factor may be related to the ability of the organism to become lodged and establish a focus of infection, e.g. an abscess. Rammelkamp and Lebovitz (1956) point out that the plasma of children contains only low levels of CRF and that abscesses and localised staphylococcal infection is rarer in children than in adults and invasion of the blood stream is more common than in older age groups. Also, in experimental infections with coagulase-positive strains injected i.v. often death results from the formation of kidney abscesses (Gorrill, 1951, 1958; Foster, 1963; Rogers, 1956; Alami, Kelly and Race, 1968).

The fact that coagulase-negative strains cause clinical diseases such as endocarditis, urinary tract infections and eye infections (Shulman and Nahamias, 1972) is in itself an argument against coagulase production as a necessary determinant of virulence. Also, since there is a variation in

virulence amongst the coagulase-positive strains It is most likely that coagulase, although a contributing factor in pathogenicity, is not an overriding determinant.

Alpha haemolysin: The ability of strains of staphylococci to produce toxins is relatively easily measured *in vitro* and may provide a useful indication of virulence in differentiating potentially pathogenic strains. It is, however, only an indication and cannot be used determinately.

Alpha-toxin (α -lysin) is excreted by the majority of strains of Staph. aureus (Elek and Levy, 1950; Jeljaszewicz, 1960 a,b). It has many biological activities, the most important of which are its haemolytic, dermonecrotic and lethal effects in both laboratory animals and man (Jeljaszewicz, 1972). Those properties have lead many investigators to study α -toxin as a factor in staphylococcal disease.

There is little doubt that α -toxin is produced by staphylococci growing in vivo in rabbits, mice and guinea pigs (Gladstone and Glencross, 1960; Kapral, Keogh and Taubler, 1965; Foster, 1967; Bartell et. al., 1968; Takeuchi and Suto, 1974 and Anderson, 1974). Tissue necrosis has been linked directly and indirectly with its production *in-vivo* (Foster, 1967; Goshi et. al., 1961, b and 1963), as has increased invasiveness (Kimura, 1971; Takeuchi and Suto, 1974).

One approach which has been used by several groups is to select mutants lacking the ability to produce α -lysin but this was not accompanied by loss of virulence (Foster, 1963 and Kimura, 1971). In a more recent

systematic study, a series of mutants of a mouse-virulent strain was produced which lacked as far as possible only one product (van der Vijver, van Es-Boon and Michel, 1975 a). The virulence of these mutants was compared with the wild type in mice challenged subcutaneously and intravenously (van der Vijver, van Es-Boon and Michel, 1975 b). The α -lysin deficient mutants did not cause dermonecrosis in local lesions which developed after subcutaneous challenge and had reduced ability to multiply in the kidneys of mice challenged intravenously. This is in agreement with some work referred to earlier.

Another approach to measuring the contribution of α -lysin to the virulence of strains is to measure the efficacy of antitoxic immunity in protecting against infection with living staphylococci. In reviewing the work from several laboratories it is difficult to compare results because of the use of (i) different species of experimental animal (ii) different strains of Staph. aureus (iii) different challenge routes, and (iv) different methods of challenge. Nevertheless it seems that α -antitoxic immunity is more important in protecting against the local effects of infection on challenge with living organisms (Koenig, Melly, and Rogers, 1962 a; Goshi, Cluff and Norman, 1963; Agarwal, 1967 a,b,c; Ekstedt and Yoshida, 1969; Easmon and Glynn, 1975 a). It also protects against death, although persistent infection may still develop in 'immunised' experimental animals (Ekstedt, 1972).

Beta haemolysin: β -toxin (β -lysin) a hot-cold haemolysin, is produced mostly by strains of Staph. aureus of animal origin. It is active against sheep, bovine, goat and human red blood cells. The toxin has been shown to be a phospholipase C acting specifically on sphingomyelin (Doery et. al., 1965; Wiseman and Caird, 1967).

In purified form β -lysin is dermonecrotic and lethal for rabbits, guinea pigs and mice (Gow and Robinson, 1969; Wadström and Möllby, 1972) but very little in-vivo work has been done to correlate the production of this toxin with pathogenicity. Kimura (1971) showed that mutant strains of Staph. aureus producing β -lysin but not α -lysin were lethal but less invasive in mice challenged intraperitoneally than mutants producing α -lysin. It has been shown to be produced during experimental infections in mouse skin. (Takeuchi and Suto, 1974).

Delta haemolysin: The haemolytic activity of δ -toxin (δ -lysin) for mammalian RBC is relatively non-species specific. It also has some leucocidal activity and 0.5 mg of a partially purified preparation was shown to cause an acute inflammatory response. (Gladstone and van Heynigen, 1957; Gladstone, 1966).

The production of δ -lysin correlates well with potential pathogenicity of strains of Staph. aureus, in fact, equally as well as α -lysin (Elek and Levy, 1950; Jeljaszwick, 1960 a,b). In vivo lethal doses of α or δ -lysin (15 - 20 HD₅₀ and 200 HD₅₀ respectively) are produced in the peritoneal cavity of mice and rabbits within 2 h of inoculation if the inoculum is large enough (2×10^8 or more). If α -lysin is not produced, or is neutralised by antitoxin, δ -lysin is of prime importance (Kapral, 1974) and, in combination with α -lysin, has been shown to have dermonecrotic activity in-vivo. Mutant strains of Staph. aureus lacking δ -lysin had reduced dermonecrotic activity in local lesions which developed after subcutaneous injection (van der Vijver, van Es-Boon, and Michel, 1975 a,b).

Other haemolysins (γ and ϵ): Comparatively little is known about these two haemolysins: γ -toxin (γ -lysin) is now generally accepted as distinct from the other haemolysins (Taylor and Bernheimer, 1974) but the existence of an ϵ -toxin (ϵ -lysin) distinct from δ -lysin is still controversial. Raised levels of neutralising anti γ antibody have been detected in sera of patients with staphylococcal bone disease (Taylor and Plommet, 1973).

Epidermolytic toxin: Epidermolytic toxin is produced by certain strains of Staph. aureus (originally thought to be exclusively phage group II but now non group II strains have been found). These strains are normally associated with superficial infections of the skin of children (Impetigo, Pemphigus Neonatorum and Toxic Epidermal Necrolysis of Ritter's type). (Parker, Tomlinson and Williams, 1955; Lyell, 1967; Lowney et. al., 1967; Lyell, Dick and Alexander, 1969). Epidermolytic toxin is a diffusible protein which with the exception of enterotoxin, is the only exotoxin shown to be responsible for specific symptoms of a staphylococcal disease. TEN or Scalded Skin Syndrome is a disease of infants and children characterised by intra-epidermal splitting and extensive exfoliation of the epidermis. Several groups of workers have been able to reproduce these symptoms in neonatal and adult mice and in man by subcutaneous injection of phage group II strains or purified epidermolytic toxin. (Melish and Glasgow, 1970; Melish and Glasgow and Turner, 1971; Arbuthnott et. al., 1972; Kapral and Miller, 1972; Elias et. al., 1974 a,b). The toxin causes intra-epidermal splitting at the level of the stratum granulosum and electron microscopy of toxin-treated mouse skin showed that the toxin causes the development of fluid filled spaces between adjacent cells of the stratum granulosum and

that desmosomal splitting is a secondary effect. (McLay, Arbuthnott and Lyell, 1975).

Epidermolytic toxin has a definite role in the pathogenesis of TEN and bullous impetigo and as such is a virulence factor. The elucidation of its action on skin is an example of the value of a good experimental animal model. This will be discussed more fully in Section D.

Leucocidin: Panton-Valentine (P-V) leucocidin is produced by many strains of Staph. aureus. It specifically kills PMN leucocytes and macrophages of rabbit and man but no other cell type. It consists of two protein components, the F (fast) component and the S (slow) component, so named because of their relative rates of migration on a carboxymethylcellulose column. The F and S components act synergistically to produce their cytotoxic effect.

The leucocidin is produced in vivo by organisms growing in cellophane sacs implanted in the peritoneal cavity of the mouse, rabbit, rat and guinea pig (Gladstone and Glencross, 1960) and in humans raised levels of anti-leucocidin are associated with infection (Gladstone et. al., 1962 a,b). Attempts to protect rabbits by immunising with toxoid containing about 50% leucocidin have been partially successful but without any correlation between degree of protection and anti-leucocidin titres (Souckova-Stepanova et. al., 1965). Large doses of leucocidin injected into rabbits caused a marked but brief granulocytosis (Gladstone, 1966). Measured in this way leucocidin is relatively non-toxic. Also, mutants lacking leucocidin did not differ in virulence from the wild type strain when injected either subcutaneously or intravenously into mice. It remains, therefore, to be shown that leucocidin

in itself is a virulence factor. We should remember, however, that Staph. aureus produces leucocidin in combination with other cytotoxic factors.

Enterotoxins: Five serologically distinct staphylococcal enterotoxins have been identified and designated A, B, C, D and E. (Bergdoll, 1972). These are produced mainly by phage group III strains. In adults the symptoms of staphylococcal food poisoning are vomiting and diarrhoea which usually develop 1 - 6 hours after ingestion of the food contaminated with enterotoxin (Dack, 1956). 1 µg of the enterotoxin is thought to be the minimal amount required to produce the disease symptoms in man. Many laboratory animals including rhesus monkeys, cats, dogs, rabbits, rats and mice have been used to study the effects of the enterotoxin. Animals appear to be more resistant than man, on a weight basis, to the effects of enterotoxin, but young rhesus monkeys have proved to be extremely valuable in enterotoxin research (Surgalla, Bergdoll and Dack, 1953). Intra-gastric administration of enterotoxins to monkeys produces acute gastroenteritis which mimics very closely the disease in humans and has permitted investigation of the emetic action and histopathological effects of this toxin (Sugiyama and Hyama, 1964, 1965; Kent, 1966; Merrill and Sprinz, 1968). Thus the role of this toxin in the pathogenesis of food poisoning is well established although its mode of action is not known.

Extracellular enzymes: Many strains of Staph. aureus produce exoenzymes as well as producing toxins. Although none of these have been linked directly with pathogenicity some have important biological activities which may contribute to the pathogenic process. The following enzymes have been studied more extensively in vitro than in vivo.

Hyaluronidase hydrolyses the cell cementing substance hyaluronic acid and for this reason it is sometimes termed 'spreading factor'. It is produced by both coagulase-positive and coagulase-negative strains of staphylococci and is produced in vivo (Schmidt, 1965; Ivanova, 1968).

Heat stable deoxyribonuclease (DNase) hydrolyses the 5' phosphodiester bond of DNA. Its production correlates well with coagulase production. It, too, has been demonstrated in vivo in lesions in mice (Kalyuk, 1971; Takeuchi and Suto, 1974).

Proteases, including fibrinolysin and gelatinase, are produced by many strains of staphylococci both coagulase-positive and negative. As with the other enzymes they have been detected in vivo (Takeuchi and Suto, 1974).

Table 3 summarises the important staphylococcal products, their biological activities and possible roles in pathogenicity.

Thus the pathogenic mechanisms of Staph. aureus are undoubtedly multifactorial and the contribution of individual factors is very difficult to assess.

2. Surface components

Capsules: Capsules possessed by certain bacteria have long been established as virulence factors in that they confer upon that bacterium the ability to resist phagocytosis. Examples are the polysaccharide capsule of Strep. pneumoniae, the hyaluronic acid capsule of Strep. pyogenes and the protein capsule of B. anthracis (Smith, 1968). Certain strains of Staph. aureus have been reported as capsulated. Yoshida reported that 18% of fresh clinical

The major extracellular products of *Staphylococcus aureus*

Product	Assayed using	Biological activities	Proposed role in virulence
Coagulase	Human or rabbit plasma	Clotting of plasma	Confers ability to resist phagocytosis and to lodge in tissues
α -toxin	Rabbit red blood cells	Haemolytic, lethal, dermonecrotic	Potential of invasiveness. Faster multiplication in tissues. Direct cause of death.
β -toxin	Sheep red blood cells	Sphingomyelinase, lethal, dermonecrotic	Potential of invasiveness. Direct cause of death.
δ -toxin	Horse and human red blood cells	Haemolytic, cytotoxic dermonecrotic	Potential of invasiveness
γ -and ϵ -toxins	Horse and human red blood cells	Haemolytic, cytotoxic	?
Epidermolytic toxin	Neonatal mouse	Epidermolytic	Necrolytic agent in Toxic Epidermal Necrolysis.
Enterotoxin	Antitoxin (RPHA)* and monkeys	Emetic	Causative agent of staphylococcal food poisoning.
Leucocidin	Human and rabbit leucocytes	Leucocidal, granulocytotoxic	Potential of local spread.
Proteinases	Appropriate protein substrate	Hydrolytic for proteins	Potential of local spread.
DNAase	DNA	Nuclease	?
Hyaluronidase	Hyaluronic acid	Hydrolysis of hyaluronic acid	Potential of local spread.

* RPHA = Reversed passive haemagglutination

isolates studied were capsulated (Yoshida, 1971). However, the methods used for demonstrating the presence of capsules are controversial; "true" capsules are not always distinguished from 'pseudocapsules' or extracellular slime layers. There are three main methods for demonstrating staphylococcal capsules:-

- (i) Negative staining by making wet mounts of the organism in India ink; the capsule appears as an unstained halo around the organism (Duguid, 1951; Mudd and De Courcy, 1965).
- (ii) the specific capsular reaction "quellung" in which cells are allowed to interact with homologous immune serum and an apparent capsular swelling phenomenon takes place if the organism possesses a capsule. (Price and Kneeland, 1954; Wiley, 1972).
- (iii) characteristic diffuse growth in serum-soft agar (Finkelstein and Sulkin, 1958; Koenig, 1962; Yoshida, 1971).

Methods (ii) and (iii) correlate fairly well (Yoshida and Naito, 1972).

In discussing the contribution of capsular material to virulence there is one capsulated strain of Staph. aureus which merits special mention; this is the Smith diffuse strain first described by Smith and Dubos (1956). The Smith strain is virulent for mice at lower challenge doses than for other strains of Staph. aureus studied previously (Koenig, 1962; Koenig, Melly and Rogers, 1962 a; Parker, Warner and Slipeitz, 1966; Yoshida and Ekstedt, 1968; Wiley and Maverakis, 1968). The capsule protects this strain from phagocytosis in-vitro (Morse, 1960) and in-vivo (Koenig, Melly and Rogers, 1962 a) which

probably accounts for the increased virulence. Other strains of Staph. aureus exhibit increased capsular slime production in high carbohydrate-high salt medium. Grown under these conditions the strains were more virulent both i.p. in mice. (Yoshida and Ekstedt, 1968) and intradermally in cows (Brock and Reiter, 1972; Brock, Turvey and Reiter, 1973). The latter group of workers studied two strains of Staph. aureus originally isolated from bovine mastitis; these showed enhanced virulence when grown in raw milk which also has a high carbohydrate content. This effect may be significant in the pathogenesis of staphylococcal mastitis.

Much work has been done to purify and characterise the capsular material and this has been reviewed by Ekstedt, 1972.

The immunising capacity of capsular material is uncertain although there is some evidence that it may have a protective effect. Mice immunised with a heat-killed vaccine prepared from a diffuse colonial variant of the Smith strain were protected from an otherwise lethal infection with the homologous organism (Koenig, Melly and Rogers, 1962 b). Also, mice which were more susceptible to the Smith strain than to another mucoid strain were shown to be frequent carriers of strains of Staph. aureus closely related antigenically to the mucoid strain and to have capsular antibodies to these but not to the Smith strain (Wiley and Maverakis, 1968).

Protein A: This antigen is found on the surface of the majority of strains of Staph. aureus but not Staph. epidermidis. (Kronvall et al., 1971). It has been isolated and characterised chemically by several groups of workers (Oeding and Grov, 1972; Sjöquist, Meloun and Hjelm, 1972). Protein A has

the ability to react non-specifically with the Fc fragment of immunoglobulin G of many species including man (Forsgren and Sjöquist, 1966; Lind, 1968; Lind and Mansa, 1968; Lind and Rhodes, 1970; Lind, Reyn and Birch-Anderson, 1972). This ability has been linked indirectly with pathogenicity. Dosset et. al., (1969) demonstrated that protein A on the surface of the bacterium could combine with the Fc fragment of IgG molecules, thus blocking the opsonic site and retarding phagocytosis. Purified protein A had been shown to inhibit phagocytosis and this has been confirmed more recently (Forsgren and Nordström, 1974). Also, protein A - human IgG complexes can elicit Arthus-type hypersensitivity reactions when injected subcutaneously in rabbits.

More directly, purified protein A injected intracardially caused severe anaphylactic shock and death in normal guinea pigs.

Mutant strains of Staph. aureus which lacked the ability to produce several factors including protein A were tested for virulence in mice (Forsgren, 1972). Mutants with reduced amounts of both protein A and a haemolysin or mutants with neither protein A nor coagulase showed only slightly reduced virulence when injected intravenously or subcutaneously (with cotton dust). However, mutants which had lost the abilities to produce protein A, nuclease, coagulase and fibrinolysin were significantly less virulent. Thus there is no direct demonstration of protein A as a virulence factor, and indeed a fairly comprehensive study of different phage groups of Staph. aureus "revealed no demonstrable or suspected pathogenic properties of protein A" (Lind, 1972).

Deoxycholate residue (DOCR): The importance of the inflammatory response in protecting against cutaneous infections with staphylococci was established

experimentally in guinea pigs (Miles, Miles and Burke, 1957). Suppression of inflammation led to increased severity of lesions for a given dose of bacteria. This was established also in man (Elek and Conen, 1957) and the mouse (James and McLeod, 1961). Noble (1965) developed a mouse model for studying cutaneous Staph. aureus infections by injecting staphylococci subcutaneously on plugs of cotton dust. Agarwal (1967 a,b,c) used this system to study the development of local inflammatory response as manifested by oedema and infiltration of leucocytes. The virulence of the strains of Staph. aureus was scored by the severity of the lesions produced and cotton dust was found to potentiate virulence by delaying the onset of the inflammatory response thus allowing initial rapid multiplication of the bacteria (Agarwal, 1967 a). Agarwal further postulated that virulent staphylococci also possessed a factor associated with the bacterial cells which suppressed the initial inflammatory response (Agarwal, 1967 b). This was confirmed by Hill (1968) who isolated the factor from cell walls of virulent strains in the logarithmic growth phase. The factor was found in the residue of cell walls treated with deoxycholate (DOCR) and suppressed the early inflammatory response, thereby enhancing lesions produced by otherwise ineffective doses of Staph. aureus strain PS 80. In vitro, DOCR inhibits leucocyte migration (Weksler and Hill, 1969). More recently it has been claimed to act on the release of kinins (Easmon, Hamilton and Glynn, 1973). Because it acts on the defence mechanisms of the host without direct damage to host tissues, it is classed as an 'impedin' (Glynn, 1972). Its isolation only from so-called virulent strains of Staph. aureus and its activity in potentiating infection suggests that it may contribute to pathogenicity.

3. Other factors

Adlam et. al., (1970 a) showed that in vivo grown staphylococci (designated V) were more virulent than organisms passaged in rabbits and then grown in broth (designated P) or organisms grown throughout in broth (designated O). They postulated from an in-vitro study of the strains that V survived more readily inside phagocytes. But this has been disproved in more recent work with these strains and in-vivo growth is alternatively suggested "to enhance the association with and penetration of staphylococci into polymorphs". (Pearce, Scragg and Kolawole, 1975). The nature of the "In vivo acquired virulence factor" is unknown.

Another surface antigen which may be important is teichoic acid which may act as a protective antigen (Ekstedt, 1965, 1966). Interestingly Martin, Crowder and White, 1968 found that all human adults tested gave immediate type hypersensitivity reactions to staphylococcal teichoic acid.

C. Immunopathology of Staphylococcus aureus

The immune responses of the body have evolved sufficiently to protect us from invasion by many micro-organisms but in some circumstances the reactions so elicited may damage the host and possibly be of advantage to the invader. This is true in the cellular response to the staphylococci and although a distinct entity from the immune deficiencies (see p.20) this effect may be of similar importance in staphylococcal pathogenicity. This section discusses the evidence for this view.

1. Inflammation and infection by Staph. aureus

Inflammation is the response or reaction of tissues to injury which

includes that caused by foreign material such as bacteria. The most important cells in this response are the phagocytic leucocytes of the non-specific immune system. Staph. aureus has been shown to elicit an inflammatory reaction (Foster, 1960), suppression of which leads to enhancement of infectivity in both humans (Elek and Conen, 1957) and experimental animals (James and McLeod, 1961; Noble, 1965 and Agarwal, 1967 a). Furthermore, as discussed earlier, some strains of Staph. aureus possess as an integral part of their cell wall an anti-inflammatory substance (DOCR) which may contribute to increased infectivity (Hill, 1968; Hill, 1969 and Easmon *et. al.*, 1973).

Easmon and Glynn (1975 a) have suggested that an early inflammatory response to local infection with Staph. aureus is the major protective factor. They concluded that α -antitoxic immunity was protective against dermonecrotic effects of Staph. aureus not only because of its specific neutralising power but also because the antigen-antibody reaction taking place locally in the tissue elicited a brisk inflammatory response which proved protective. Non-specific acute inflammation also protected against dermonecrosis.

It thus appears that acute inflammation is somewhat protective against the staphylococci; however, when the inflammatory response is very severe and prolonged, there may be further tissue damage and consequent enhancement of infectivity (Goshi, Cluff and Johnson, 1961 a).

2. Delayed-type hypersensitivity and staphylococcal disease

One of the main features of serious staphylococcal disease is that it is often recurrent and persistent (Cluff, 1965). This is, in general, untrue of most other bacterial infections where a clinical or sub-clinical infection

confers immunity, however short-lived. It has also been noted that persons suffering from recurrent staphylococcal infections usually show hypersensitivity of the delayed-type to staphylococcal antigens (Cluff, 1965). Paradoxically delayed-type hypersensitivity is associated with immunity (specific cell mediated immunity). It is helpful in trying to understand this apparent contradiction of roles to consider a definition of delayed-type hypersensitivity.

Delayed-type hypersensitivity is an allergy mediated by sensitised lymphocytes which, when they encounter the antigen in a venule, cause the antigen to combine with cell bound antibody. Migratory inhibitory factor (MIF) is then released. This factor stops the migration of monocytes which adhere to the endothelial lining of the venule. Gradually, the monocytes are activated by MIF and are transformed into macrophages which force themselves through the endothelium and vessel wall and release lysosomal hydrolases. The end result is tissue damage (Waksman, 1971).

Johanovsky (1958) was the first to report that delayed-type hypersensitivity to staphylococcal antigens can be transferred from sensitised animals to normal recipients by means of living cells (presumably sensitised lymphocytes). Increased susceptibility to staphylococcal infection was transferred simultaneously. He suggested that tissue damage caused by interaction between the antigens of the invading microbe and sensitised lymphocytes may provide conditions which promote staphylococcal infection.

In a similar study in rabbits, repeated infection of the skin was associated with the development of delayed-type hypersensitivity which in turn increased the infectivity of the organism in the skin of the sensitised

animal. Abscesses were induced with inocula which gave no lesions in normal animals. (Johnson, Cluff and Goshi, 1961). The in vitro studies of Lenhart and Mudd (1972) showed that peritoneal macrophages from hypersensitive rabbits had no increased bactericidal activity for staphylococci.

Delayed-type hypersensitivity has been reported in both mice (Taubler, 1968; Taubler, Grieb and Mudd, 1970; Modak, Banerjee and Basu Mallik, 1971; Easmon and Glynn, 1975 b) and in guinea pigs (Kowalski and Berman, 1971; Targowski and Berman, 1974) infected repeatedly with Staph. aureus. The hypersensitivity was transferable to normal animals by lymphoid cells.

An interesting study of the dissemination of Staph. aureus from the site of carriage into the bloodstream and localisation at a traumatised portion of bone in mice has shown that localisation of the organism at the injured bone occurred only in hypersensitive animals (Banerjee et. al., 1971).

It, therefore, appears that the ability of staphylococcal antigens to elicit a delayed-type hypersensitive response may contribute to virulence, especially in recurrent staphylococcal disease.

In conclusion it is evident that the pathogenicity of Staph. aureus is dependent not only on the many cellular and extracellular factors it produces but also on the immune responses of the host.

D. Experimental Animals in the Study of Pathogenicity

I. Some classical animal models

No attempt will be made in this introduction to discuss all the

investigations in animals which have contributed to our understanding of microbial pathogenicity in man. However, there is justification for recalling some of the milestones. Griffiths (1928) demonstrated the importance of the capsule of Strep. pneumoniae in virulence for mice. Toxins responsible for the main pathological effects of anthrax (Smith, Keppie and Stanley, 1955) and cholera (De, Ghose and Sen, 1960; Finkelstein, Norris and Datta, 1964) were demonstrated in laboratory animals. Virulence factors of Pasteurella pestis were elucidated in mice (Burrows, 1955). Also, experiments in more unusual animals, e.g. ewes and ferrets, have shown that bacteria and viruses show specific localisation during infection (Lowrie and Pearce, 1970; Basarub and Smith, 1969). The use of experimental animals for studying the growth of the more fastidious microbes has led to the development of acceptable animal models for diseases caused by these organisms. For example, H. Influenzae has been studied successfully in rabbits (Schneerson and Robbins, 1971) and rats (Smith et. al., 1973). Syrian hamsters have proved to be acceptable models for the disease produced by Mycoplasma pneumoniae; another species Mycoplasma arthritidis caused a disease resembling human arthritis in mice (Cole, Ward and Golightly-Rowland, 1973).

Staphylococcal infections have proved more difficult to reproduce in laboratory animals because they are very resistant to infection by the staphylococci. However, some animal systems have been developed and are reviewed in the next section.

2. Experimental systems for the study of staphylococcal diseases of man

Staphylococcal Toxic Epidermal Necrolysis (TEN): This disease when first described was thought to be uniquely a disease of human infants caused by phage group II

strains of Staph. aureus. The disease is characterised by extensive loosening of the epidermis accompanied by peeling. Histologically the splitting occurs within the dermis at the level of the stratum granulosum (see p.32).

Melish and Glasgow (1970) injected phage group II strains, isolated from cases of TEN into neonatal mice and reproduced all the symptoms as well as the histological features of the disease. This work involving the newborn mouse lead to more detailed studies of the products of the TEN strains of Staph. aureus and showed that an extracellular product of the bacterium was responsible for the disease symptoms (Arbuthnott et. al., 1971; Melish et. al., 1972). It was originally reported that mice more than six days old (with the exception of hairless mice more than twenty-one days old) were resistant (Melish et. al., 1972; Arbuthnott et. al., 1972 and Arbuthnott et. al., 1973). More recently, however, it has been reported that adult mice and hairy as well as glabrous skin sites are potentially susceptible to the toxin (Elias et. al., 1974 a,b)

This has proved to be the most successful animal model for staphylococcal disease since it almost exactly mimics the human disease in many respects. Consequently, it has allowed detailed study of the factors involved in the pathogenesis of Toxic Epidermal Necrolysis.

Impetigo: Dajani and Wannamaker (1970, 1971, 1972) have reported experimental impetigo in Syrian hamsters. The disease so produced resembles human impetigo in the gross appearance of the lesions, in progression of the various stages, and in the histopathology of the process. Use of this animal has facilitated the study of the interaction of staphylococci and group A

streptococci in impetigo and, perhaps more important, evaluation of various therapeutic regimens.

Mastitis: A technique of intramammary inoculation in the mouse was devised by Chandler in 1970 and has been used by other workers (Anderson, 1971; Anderson, 1972; Anderson and Mason, 1974; Anderson, 1974) to produce staphylococcal mastitis. Using the mouse, staphylococci from outbreaks of bovine mastitis were compared and their susceptibility to antibiotic therapy evaluated.

A study of a natural outbreak of staphylococcal mastitis in rabbits showed that the disease could be transmitted via suckling young (Adlam et. al., 1975).

Osteomyelitis: Rabbits have been used with some success in the study of experimental osteomyelitis (Andriole, Nagel and Southwick, 1974). The pathological and radiological features of the experimental disease closely resemble those of the human disease which is an important, though infrequent complication of open reduction and internal fixation of fractures of long bones. Suitable experimental infections should prove invaluable for evaluating various specific approaches to treatment.

Endocarditis: Experimental endocarditis was produced in rabbits by Garrison and Freedman (1970) and by another group (Linnemann, Watanakunakorn and Bakie, 1973). Similar infections in dogs (Hamburger et. al., 1967) was used to evaluate antibiotic therapy.

Superficial lesions: Noble (1965) injected live staphylococci on plugs of

cotton dust into the skin of mice and produced skin lesions resembling human abscesses. The technique was developed further by Agarwal (1967 a, b, c) and has been used more recently for comparison of cellular responses to staphylococcal infection (Hill, 1968; Hill, 1969; Weksler and Hill, 1969 and Easmon and Glynn, 1975 a, b).

The fact that normal adult laboratory animals are resistant to staphylococcal infections was in part the stimulus for the present study. Since many of the more serious staphylococcal infections in humans occur in children and the age dependence successfully reflected in the TEN investigations, our approach was based on a study of age susceptibility. Also, the pathogenicity of Staph. aureus is thought to be connected with immune reactions. In the neonatal animals these reactions are underdeveloped and may, therefore, simplify a study of experimental infection.

MATERIALS AND METHODS

A. Strains of Bacteria used

1. Staphylococci

The strains of staphylococci used in this investigation included clinical isolates from children and adults and a strain isolated from an outbreak of bovine mastitis. Some lyophilised reference strains were also obtained from the National Collection of Type Cultures (NCTC), Colindale, London. For convenience the strains were given laboratory numbers as shown in Table 4 which lists the strains and their origins.

2. Other organisms

All other organisms were obtained from the Department of Medical Microbiology, Royal Infirmary, Glasgow. All were clinical isolates and they included the following genera:-

Escherichia, Pseudomonas, Klebsiella and Streptococcus.

B. Culture and Maintenance

1. Challenge inocula

Fifty-ml amounts of sterile Nutrient Broth (Oxoid) were inoculated with 3 or 4 morphologically similar colonies of each strain from horse blood agar (see Appendix I). These were incubated for 18 - 24 h at 37°C after which each starter culture was used to inoculate 500-ml amounts of Nutrient Broth. The 500-ml cultures were incubated for 16 - 18 h at 37°C. The cultures were centrifuged in 250-ml centrifuge bottles at 17,000 g for 10 min. at 4°C in a High-speed MSE 25 refrigerated centrifuge (MSE Ltd., London).

Laboratory Number	Other Designation	Origin	Source	Reason For Selection
SM 1	NCTC 7121 "Wood 46"	Infected burn	National Collection of Type Cultures	Classical α toxinogenic strain
SM 5	NCTC 8530 Cowan I	Septic Arthritis	National Collection of Type Cultures	Known producer of Protein A
SM 6	...	Urinary Tract Infection	Dr. C. G. Gemmell, Paisley College of Technology.	Coagulase negative <u>Staph. epidermidis strain</u>
SM 9	...	Knee abscess of a two year old child	Dr. T. A. McAllister, Royal Hospital for Sick Children, Glasgow.	Fresh clinical isolate
SM 10	...	Sputum of a two year old child suffering from cystic fibrosis	Dr. T. A. McAllister, Royal Hospital for Sick Children, Glasgow.	Fresh clinical isolate
SM 14	...	Conjunctiva of a twenty-one year old woman suffering from Pyoderma gangraenosum	Dr. M. A. B. De Sousa, Western Infirmary, Glasgow.	Fresh clinical isolate
SM 15	BB strain	Bovine mastitis	Dr. C. Adlam, Wellcome Research Laboratories, Kent.	β toxinogenic strain
SM 16	NCTC 9789 PS 80	Hospital epidemic	National Collection of Type Cultures	Mouse virulent strain
SM 18	V8	Chronic furunculosis	Dr. C. Adlam, Wellcome Research Laboratories, Kent.	Leucocidin producing strain

The pellets were suspended in 250 ml sterile Peptone Water (10% w/v Oxoid) at 4°C aseptically. The centrifugation and washing procedure was repeated once. The washed pellets of cells were resuspended in the appropriate volume of Peptone Water at 4°C to a concentration of approximately 2×10^{10} organisms per ml measured by means of Opacity Tubes (Wellcome Laboratories, Kent). The number of viable organisms per ml was measured by the technique outlined in Section C1, p. 53.

Maintenance of staphylococcal suspensions: The suspensions prepared as above were dispensed aseptically in sterile 2-ml ampoules (Flow Laboratories, Ayrshire) which were then sealed and immersed in liquid nitrogen in a 'Vivostat' liquid nitrogen refrigerator (BOC (Cryoproducts) Ltd., Glasgow). Thirty ampoules of each strain were prepared. Frozen in this way the viability of suspensions of staphylococci was reduced by 30% in the first week but thereafter remained stable over a period of months. The advantage of this method of maintenance is that small aliquots of the same standard suspension of organisms are available for experiments carried out over a long time, one aliquot being used for each experiment, thereby obviating variation in the challenge inoculum as a variable in animal experiments.

Other bacterial suspensions: Nutrient Broth in 50-ml amounts inoculated with 3 or 4 morphologically similar colonies from horse blood agar medium were incubated for 18 h at 37°C after which the cultures were centrifuged at 3,000 g for 10 min. in sterile 50-ml centrifuge tubes (MSE Ltd., London). The pellets were resuspended in sterile Peptone Water at 4°C and washed twice with Peptone Water. The washed pellet of cells was resuspended to a known concentration which was diluted to contain the required number of

viable organisms for challenge.

The strains were maintained on slopes of Nutrient Agar (Oxoid) in bijoux bottles stored at 4°C.

2. Production of exotoxins

Haemolysins: A suspension (1 ml) of each strain of staphylococcus was used to inoculate 15 ml of semi-solid agar medium (see Appendix I) in petri dishes. These were incubated for 48 h at 37°C in an atmosphere of 20% (v/v) CO₂. To prepare crude culture supernatant fluid, cultures were frozen to -10°C, thawed and centrifuged. (10,000 g for 10 min. at 4°C using a Highspeed MSE 25 refrigerated centrifuge.

Leucocidin: Modified CCY medium (50 ml) (see Appendix I) was inoculated with 3 or 4 morphologically similar colonies of each staphylococcal strain. The cultures, in dimpled Ehrlenmeyer flasks, were incubated at 37°C for 20 h in an orbital shaking incubator (IH400, A. Gallenkamp and Co. Ltd., Glasgow) at 100 cycles per min. and were then centrifuged at 3,000 g for 10 min. at 4°C.

3. Measurement of growth rates

Nutrient Broth (100-ml) in a 250-ml dimpled Ehrlenmeyer flask was inoculated with 1 ml containing 2×10^9 colony forming units (c.f.u.) of each strain SM 1, 6, 9, 10, 14 and 15. This gave a starting $E_{660}^{1\text{cm}} \approx 0.03$. The flasks were incubated at 37°C with shaking (100 cycles per min. Samples (5 ml) were withdrawn aseptically from each flask at 30 min. intervals for 6.5 h. The $E_{660}^{1\text{cm}}$ was measured in a SP 800 spectrophotometer (Pye Unicam, Cambridge).

C. Viable Counting Methods

I. Challenge suspensions

As described in section BI, the staphylococcal strains and other bacteria were prepared for challenge by suspension in cold Peptone Water to a final concentration of 2×10^{10} organisms per ml by opacity. The actual viable count (c.f.u. per ml) was then estimated by a method described by Postgate (1969) suitable for bacterial suspensions known to contain approximately 10^9 c.f.u. per ml.

In this procedure 100 ml of sterile Peptone Water was added to each of two sterile 250-ml Erlenmeyer flasks numbered (1) and (2); 0.1 ml was removed from flask (2) to give a volume of 99.0 ml. Using 'Microcap' pipettes sterilised with 70% (v/v) ethanol and dried in air for 30 sec. 10 μ l of bacterial suspension was added to flask (1). After vigorous shaking to achieve adequate mixing 0.1 ml was transferred from flask (1) to flask (2). Aliquots (0.2 ml) of diluted suspension (from flask (2)) were spread evenly with a sterile glass spreader on each of ten dried plates containing 15 - 20 ml Nutrient Agar. If the plates were not sufficiently dried isolated colonies were not always obtained on incubation and accurate counting was impossible. Plates were incubated overnight at 37°C and the number of colonies on each was counted and an average value obtained. Best results were obtained with suspensions which gave 20 - 50 colonies per plate. The average count was multiplied by 5×10^7 to obtain the number of colony forming units per ml of the original suspension.

The main disadvantage of this method is that the approximate viable

count of the suspension must be known within fairly narrow limits. Also, the method was designed to count suspensions of organisms of viable count around 10^9 c.f.u. per ml. However, with appropriate adjustments to the second dilution volume, it is possible to count more dilute suspensions.

This procedure was used to estimate the viable count of suspensions used for challenge in animal experiments. (One ampoule was removed from the liquid nitrogen refrigerator, thawed and diluted for each animal experiment).

This method of viable counting was chosen in preference to the more commonly used method of viable counting first described by Miles, Misra and Irwin (1938) because it involved fewer dilution steps and consequently less error. The error in using measuring cylinders with volumes of 100 ml is well within the Poissonian error of the average count. The method is particularly suitable for counting staphylococci because diluted suspensions can be shaken vigorously to break up clumps of staphylococci which gives more accurate counts.

2. Bacteria grown in vivo

The number of staphylococci in homogenates of mouse tissue (see p. 65) was estimated by the method described by Miles, Misra and Irwin (1938). This method was used because the number of staphylococci in these homogenates was unknown and it was, therefore, essential to cover a much wider range than was possible by the Postgate method. Ten-fold dilutions were made of the homogenate in 1-ml volumes of phosphate buffered saline (PBS) pH 7.0 (see Appendix II) in an ice bath with good mixing between steps. Each dilution (0.02 ml) was dropped from a sterile graduated pipette) onto Mannitol

Salt Agar (MSA; Oxoid) in petri plates dried for 24 h at 37°C. After the drops had been completely absorbed into the agar the plates were incubated at 37°C for 24 - 48 h after which the first countable dilution was selected, counted and multiplied by the corresponding dilution factor to give c.f.u. per ml in the tissue homogenate. Where possible two or three dilutions were counted and average values calculated and recorded. MSA being selective for staphylococci was chosen to exclude the growth of other microbes in the mouse tissue homogenates especially the skin homogenate. Also it helped to differentiate between Staph. aureus and other organisms. In practice about 95% of the colonies had the typical appearance of Staph. aureus and were recorded as such.

D. Assay of Staphylococcal Products

I. Exotoxins (haemolysins)

Crude culture supernates (see p. 52) were assayed for haemolytic activity against erythrocytes from four species:- rabbit, sheep, horse and human.

Standardisation of erythrocyte suspensions: Fresh whole blood was washed three times in sterile saline and an approximately 2% (v/v) suspension made in Bernheimers buffer (see Appendix II). This suspension was standardised by haemolysing an aliquot with a few crystals of saponin. The lysate was diluted ten-fold with distilled water and the amount of haemoglobin contained in the dilution was measured in an SP 800 spectrophotometer at $E_{545 \text{ nm}}^{0.5 \text{ cm}}$ of 0.20. The suspension was then considered to be a standardised 2% suspension of erythrocytes.

Haemolysin assays: Serial doubling dilutions of 0.5 ml of the crude culture supernates were made in quadruplicate in 0.5-ml volumes of Bernheimers buffer containing albumin (see Appendix II). To each tube was added 0.5 ml of the standardised suspensions of sheep, rabbit, horse or human erythrocytes. The tubes were incubated in a water bath at 37°C for 1 h with periodic shaking to prevent sedimentation of unlysed erythrocytes and the haemolytic titre recorded in HU per ml as the reciprocal of the dilution which caused visible haemolysis. To estimate hot-cold haemolysis with sheep erythrocytes, tubes were reincubated at 4°C overnight and a second reading of the haemolytic titre recorded.

The general haemolytic patterns against the four red cell species described indicate the predominant toxin(s) present in culture supernates.

The haemolytic patterns were interpreted as follows:-

- (1) A high titre against rabbit indicated α -lysin.
- (2) An increase in titre against sheep after hot-cold incubation indicated β -lysin.
- (3) Low titres against all four species indicated γ or δ -lysin.

Production of α -toxin was confirmed by the α -antitoxin neutralisation test as follows:-

The dilution of commercial α -antitoxin (Wellcome Laboratories, Kent) which neutralised 8 HU of freshly prepared α -lysin from strain Wood 46 was determined. Each culture supernate was diluted to contain 8 HU per 0.2 ml (calculated from the titre against rabbit erythrocytes). Cultures which contained < 40 HU per ml were used

undiluted. To each 0.2 ml of culture supernate was added 0.5 ml of the diluted antiserum and the mixture incubated for 90 min. at 37°C. To each tube was then added 0.3 ml of a 3% (v/v) suspension of rabbit erythrocytes and the incubation continued for a further 60 min. Absence of haemolysis indicated neutralisation of α -lysin by the antiserum. Residual haemolysis indicated that an additional non-neutralisable haemolysin was present. It was not possible, however, in this assay to distinguish between γ - and δ -lysins.

2. Exoenzymes

Coagulase: Citrated rabbit plasma was diluted 1 in 5 using Palitsch buffer (see Appendix II). To a 4 x ½" test tube containing 0.4 ml of diluted plasma and 0.2 ml Palitsch buffer was added 0.2 ml of an overnight broth culture of each strain. The tubes were incubated at 37°C in a water bath for 8 h. Known positive and negative strains were included as controls.

Fibrinolysin: Nutrient Agar was sterilised in an autoclave (15 lb/in² pressure for 15 min.) and then cooled to 56°C. Fresh human plasma was added to a final concentration of 12% (v/v) and the mixture heated at 56°C for a further 15 - 30 min. during which time the plasma clotted and the mixture became opaque. Plates containing 15-ml amounts of heated plasma agar were poured and allowed to cool. Each fibrin plate was streak inoculated with three strains of the staphylococci to be tested. The plates were incubated at 37°C for 17 - 22h. Clearing of the medium around the streak of growth was recorded as a positive result.

Lipase (tributyrase): Tributyrin was added to molten sterile Nutrient Agar (10 - 15 ml) in a universal container at 56°C to a final volume of 1% (v/v).

The mixture was shaken very vigorously to disperse the lipid, poured into a sterile petri plate and allowed to cool. Each plate was streaked with three strains of staphylococci and incubated at 37°C for 24 h. Zones of clearing around the streak measuring > 0 and < 3 mm in diameter were recorded as +; zones measuring > 3 mm were recorded as ++.

Gelatinase: Gelatin (Oxoid) was added to Nutrient Agar to a final concentration of 10% (w/v). The gelatin agar was sterilised in an autoclave (15 lb/in² for 15 min.), cooled to 56°C and 10 - 15-ml amounts poured into petri plates. The cooled, dried plates were streaked with one strain per plate and incubated at 37°C for 3 days when they were flooded with acidic mercuric chloride (see Appendix II) which formed a white precipitate with unhydrolysed gelatin. A clear area around the streak of growth was recorded as a positive result.

DNase: Petri plates containing 10 - 15 ml sterile DNase Agar (Difco) were streaked (one strain per plate) and after 24 h incubation at 37°C were flooded with a 10% (v/v) solution of 1.0 M perchloric acid. Clearing around the streaks was recorded as a positive result.

Egg Yolk Factor: Concentrated Egg Yolk Emulsion (Oxoid) was added to sterile Nutrient Agar cooled to 56°C to a final concentration of 5% (v/v). Egg Yolk Agar was poured (10 - 15-ml amounts) into petri plates which were cooled, dried and streaked with each strain (three strains per plate). The plates were then incubated at 37°C for 7 days and dense opacity around the growth recorded as a positive result.

Phosphatase: Sterile Phenolphthalein Phosphate (0.1% w/v, Oxoid) was added to sterile Nutrient Agar to give a final concentration of 0.01% (w/v).

Petri plates containing 10 - 15 ml of the Phenolphthalein Phosphate Agar were cooled, dried and streak inoculated with three strains of staphylococci. After 3 - 5 days incubation at 37°C the plates were exposed to ammonia vapour in a fume cupboard. Streaks of growth which turned bright pink were recorded as positive.

3. Leucocidin

The microscopical method as described by Gladstone et. al., (1962) was used with minor modifications:-

Glassware: Microscope slides, coverslips, test tubes and pipettes used in the assay were thoroughly cleaned in a 5% (v/v) solution of Decon 90 (Decon Laboratories, Brighton) in hot tap water. Thereafter the glassware was rinsed between six and ten times in tap water and once in distilled water. Slides and coverslips were kept in 70% (v/v) alcohol.

Leucocyte preparation: Fresh human blood from a finger prick was collected into an equal volume of ice cold Hanks Basal Salt Solution pH 7.0 (see Appendix II) on ice. Using a Pasteur pipette a drop of the diluted blood was placed on a coverslip and incubated in a moist chamber at 37°C for 15 - 30 min. The clot which formed was removed using a pair of fine forceps and residual erythrocytes were removed by rinsing with warm sterile gelatin (0.5% w/v) in saline. (see Appendix II) Under these conditions leucocytes adhered to the coverslip.

Serial doubling dilutions of culture supernates (see p. 52) were made in 1-ml volumes in 0.5% (w/v) gelatin saline. A drop of each dilution was

placed on a microscope slide and a coverslip with adhering leucocytes was inverted onto the drop. Preparations were sealed with nail varnish and incubated at 37°C for 15 - 30 min. Control preparations were mounted in gelatin saline solution.

Using phase contrast microscopy, the leucocyte preparations were examined for the following features of cytological damage:-

- (i) complete destruction of the leucocytes
- (ii) stimulated movement of intracellular granules
- (iii) rounding of leucocytes with motionless granules
- (iv) round blebs protruding from the leucocyte membrane

The titre of leucocidal activity was recorded in leucocidal units (LU) as the reciprocal of the highest dilution of supernate which caused cytological damage under the conditions described.

4. Protein A

The ability of protein A to adsorb serum globulins non-specifically is the basis of the assay (Lind, 1968). The globulin is labelled with fluorescein isothiocyanate (FITC), a fluorochrome dye which allows assay by direct observation.

Preparation of FITC labelled human immunoglobulin: Human immunoglobulin was used because it has been shown to give high titre non-specific staining in the fluorescent antibody test (FAT), (Lind, Live and Mansa, 1970). Human γ -globulin concentrate was obtained by precipitating the γ -globulin fraction of normal serum with an equal volume of 32% (w/v) Na_2SO_4 . The precipitate was dissolved in one-half the original volume of distilled water and an equal

volume of 32% (w/v) Na_2SO_4 was added to reprecipitate the γ -globulin. This was dispersed in a small volume of phosphate buffered saline (PBS, see Appendix II) and dialysed at 4°C for 24 h against PBS. The protein concentration of the concentrate was measured by the biuret method (Mehl, 1944).

Conjugation of FITC to γ -globulin: To one volume of human γ -globulin concentrate was added two volumes of bicarbonate buffer (see Appendix II) in a 25-ml beaker which was then cooled to 0°C in an ice bath. FITC isomer I (BDH Chemicals Ltd., Poole) was added with continuous stirring over a period of 15 min. The correct amount of FITC was calculated such that FITC: γ -globulin = 1 : 20 (w/w). The mixture was stirred overnight in the cold room using a magnetic stirrer and unreacted FITC removed by passing the mixture through a column of Sephadex G25. The fractions containing the FITC - labelled γ -globulin (monitored at E_{280} and E_{495}) were bulked and an equal volume of 32% (w/v) Na_2SO_4 added. The resulting precipitate was collected by centrifugation (3,000 g for 20 min.) and the sediment dissolved in 4 ml distilled water and dialysed overnight against 0.1 M phosphate buffer pH 7.4 (see Appendix II).

Fluorescent antibody test (FAT): Smears of the staphylococcal strains were made from overnight broth cultures and heat fixed. The FITC - labelled conjugate was diluted ten-fold and applied to the smears which were then incubated at room temperature in the dark for 15 - 20 min. After thorough rinsing in distilled water (5 changes over a period of 30 min.), coverslips were placed over the smears and sealed. The preparations were then examined using a Leitz Orthoplan microscope fitted with a fluorescent

vertical illuminator according to Ploem (1967). A brilliant yellow-green fluorescent layer uniformly covering the surface of each bacterial cell was recorded as positive. A pale bluish hue, or no apparent fluorescence, was recorded as negative. A known positive strain (Staph. aureus Cowan I) and a known negative strain (Staph. aureus Wood 46) were included as controls.

E. Animals

I. Strains used

In most experiments the CD-1 random bred Albino (Charles Rivers U.K. Ltd., Kent) strain of mice were used. This strain was derived by caesarean section and barrier-maintained. A breeding nucleus of this strain was used to establish a large colony. Although not barrier-maintained, special attention was paid to husbandry and the animals were housed in a modern, well ventilated Animal House.

Theillers strain (Tuck and Son, Essex) and CFLP strain (Carworth Europe, Huntingdon) were used in some experiments.

2. Age groups of mice used

Neonatal mice: Pregnant animals in the breeding colony were checked for litters at 9.30 a.m. and again at 5.00 p.m. each day. Litters born during this period were recorded and only these were used for experiments requiring accurately aged animals between one and ten days of age. Mice of these ages were housed, along with lactating females, as individual litters. In general the CD-1 mothers proved to be very tolerant to the handling of their litters. However, as a control for maternal neglect a neonatal animal which

received sterile peptone water (10% w/v) was included in each litter selected for challenge. If the control mouse died we assumed that the mother had neglected the litter which was thereafter not included in the results.

Weanling mice: In addition to neonatal mice, 21-day old weaned animals were used. These were maintained in groups of 10 animals per cage.

3. Preparation of the mice for challenge

The mice were challenged subcutaneously and to facilitate the observation of the lesions which developed in mice older than 7 days, hair was removed from the dorsolateral region.

Young mice (7-, 8-, 9- and 10-day old) were depilated with cosmetic depilating cream (BUTO, Wright, Layman and Umney Ltd., London) which was safe, non-irritant and effective. Great care was taken to remove all the cream by washing the mice thoroughly. Because the hair of 21-day old mice was coarser and thicker, the dorsolateral area was first shaved, with small electric clippers (Andis T-liner clippers, Brookwick, Ward and Co. Ltd., London) and the shaved area completely depilated with BUTO cream. Again the mice were carefully washed after the removal of hair. All the mice were weighed just prior to challenge.

4. Challenge procedures

For neonatal mice, the appropriate dilution of organisms (see p. 53) was injected subcutaneously (0.05 ml) from a 26 G 0.5 in. needle fitted to a 1-ml syringe. The injection was made along the dorsal midline. It was noted that back-pressure resulted in leakage of small amounts of the inoculum from the injection site. In order to monitor how much of the inoculum

was lost in this way, twenty neonatal mice were injected with a suspension of Staph. aureus of known viability, and the small drop which leaked after injection was collected with a sterile 10 μ l capillary micropipette. The material thus collected was immediately expelled into 100 ml sterile Peptone Water in an ice bath and the number of viable organisms counted (see p. 53).

The technique for weanling 21-day old animals was the same as for the neonates except that a 25G 1 in. needle was used. No leakage of inoculum occurred with the older animals.

F. Assessment of Virulence

1. Death rates

The number of dead animals was recorded each day and these animals were removed.

2. Lesion assessment

Lesions were scored as present or absent but, because of the differences in the types of lesions produced by the different strains of staphylococci, no attempt was made to develop a graded scoring system. However, differences in lesion types were noted.

3. Skin specimens

In-vivo growth studies: The comparative virulence of two strains, namely SM 9 and SM 10, was assessed by monitoring their growth rate in the skin of 3- and 21-day old mice. Groups of 60 - 100 mice of each age group were challenged subcutaneously with 10^6 c.f.u. of either strain. At time

intervals after challenge mice, selected at random, were sacrificed and an area of skin containing the injection/lesion site was excised. This area measured approximately 2 cm^2 in 3-day old mice and approximately 4 cm^2 in 21-day old mice. Each piece of skin was placed in a sterile bijou bottle of known weight in an ice bath. The bottles together with the skin specimens were weighed again. The skin was chopped finely with two sterile scalpels and homogenised to an even suspension in 1 ml cold phosphate buffered saline pH 7 (see Appendix II) in 10-ml glass tissue grinders (Jencons Scientific Ltd., Herts.). The grinders were sterilised by immersion in 70% alcohol (v/v) and dried in a hot air oven. The number of viable organisms in each skin homogenate was counted by the method of Miles, Misra and Irwin (1938) (see p. 54).

Histology: Skin specimens were collected from 3-day old mice 18 h after challenge with 10^6 c.f.u. of either strain SM 9 or SM 10. After fixing in formal saline and mounting in paraffin, thin sections of the specimens were cut and stained with (i) haematoxylin and eosin, and (ii) Gram stain. Skin from mice injected with sterile Peptone Water was included as a control.

G. Cell Transfer Experiments

I. Peritoneal exudate cells

With minor modifications these cells were prepared by the method of Hackbarth, Reinartz and Sagik, (1973). Peritoneal exudate cells were prepared by injecting 3 - 5 ml of Hanks BSS (see Appendix II) intraperitoneally into adult CFLP mice, and recollecting as much fluid as possible from the

peritoneal cavity. This was termed the whole peritoneal exudate cell (WPEC) suspension. The total number of cells in this suspension was counted in a Neubauer chamber (approximately 5×10^6 cells per ml).

Macrophage and Lymphocyte enriched cell suspensions: The WPEC suspension was incubated for 30 min. at 37°C in a small sterile glass tissue culture vessel. The supernatant fluid, designated the lymphocyte-enriched cell (LEC) suspension was removed and the number of lymphocytes per ml was counted using a Neubauer chamber. A smear was also made, fixed and stained with haematoxylin and eosin to check the cell types. The macrophages (attached to the vessel) were washed three times with sterile Hanks BSS (see Appendix II) and finally resuspended in one-third of the volume of the original WPEC suspension. This suspension was designated the macrophage-enriched cell (MEC) suspension. The number of macrophages per ml was counted and a stained smear made as before was prepared to check the cell types. Each cell suspension contained approximately 2×10^6 cells per ml.

2. Cell transfer to neonatal mice

LEC, MEC and WPEC suspensions were injected intraperitoneally in 0.05-ml amounts (approximately 1×10^5 cells) into newborn mice. Three days later the mice were challenged subcutaneously as before with 10^6 c.f.u. of Staph. aureus strains SM 9 and SM 10.

RESULTS

A. Characteristics of the Strains of Staphylococci Grown in vitro

I. Haemolysin patterns

The culture supernates of each of fifteen strains of staphylococci were tested for haemolytic activity against rabbit, horse, human and sheep RBC. From the original fifteen strains, six with varied toxin patterns (SM 1, 6, 9, 10, 14 and 15) were selected. The culture supernate of each of these six strains was tested for neutralisability by α -antitoxin. (Table 5) From these patterns it can be seen that the principal toxins produced by the strains were as follows:-

SM 1 (Wood 46)	α
SM 6	non-haemolytic
SM 9	very weak α and β
SM 10	α
SM 14	α , β and weak δ
SM 15	β and weak δ

2. Enzyme patterns, leucocidin and protein A production

Each of strains SM 1, 6, 9, 10, 14 and 15 was tested for production of a series of enzymes which have been considered as possible virulence factors (Table 6).

Leucocidal activity of the CCY supernates (see p.59) was assayed and activities compared to strain V8 which is a known leucocidin-producing strain. Each CCY supernate was also tested for haemolytic activity against rabbit and human RBC. None of the supernates had a haemolytic titre

TABLE 5

Haemolytic patterns of staphylococcal strains

Strain no.	HU against rabbit RBC	HU against horse RBC	Hu against human RBC	HU against sheep RBC		Neutralisation by α -antitoxin
				37°C	4°C	
1	5,120	40	40	160	320	N*
6	10	10	10	10	10	NN†
9	80	80	160	40	80	NN
10	1,280	20	80 - 160	320	640	N
14	640	80 - 160	80	160	1,280	PN‡
15	1,280	160	80 - 160	1,280	20,000	PN

* N = neutralised

† NN = not neutralised

‡ PN = partially neutralised

TABLE 6

Enzyme production by staphylococcal strains

Strain no.	Coagulase	DNase	Lipase (tributylin)	Phosphatase	Proteases			Egg Yolk Factor
					Fibrinolysin	Gelatinase		
SM 1	+	+	++	+	+	+	+	+
SM 6	-	-	++	+	-	w*	-	-
SM 9	+	+	++	+	-	-	+	+
SM 10	+	+	++	+	+	+	+	+
SM 14	+	+	++	+	+	-	-	-
SM 15	+	+	+	+	-	+	+	+

* w = weak reaction

< 16 HU per ml, which meant that any cytotoxic effect observed with dilutions of more than 1 in 16 of the supernates was unlikely to be due to a haemolysin. Only one of the strains, SM 9, had marked leucocidal activity (Table 7).

Each of the strains was tested for protein A on the surface of washed cells using strain SM 5 (Cowan I) as a positive control. SM 1 (Wood 46 and SM 6 (Staph. epidermidis) proved to be the only two negative strains (Table 7).

From Tables 6 and 7 it is evident that the six selected strains vary in their enzyme patterns, ability to produce leucocidin and protein A and haemolysins. This group was chosen for the initial mouse virulence studies to establish whether a pattern of virulence might emerge which could be related to these in-vitro properties.

3. Antibiotic patterns of the strains

Each of strains SM 1, 6, 9, 10, 14 and 15 was tested against six of the most common antistaphylococcal antibiotics in use today. (Table 8) Of particular interest is the fact that the three recent clinical isolates (i.e. SM 9, SM 10 and SM 14) were penicillinase producers. Also strain SM 14 was a methicillin-resistant strain.

4. Growth rates in vitro

In vitro growth curves of strains SM 1, 6, 9, 10, 14 and 15 are shown in Fig. 2. For each strain in mid-log phase (between 1 and 2 h) the growth rate constant:

$$k = \frac{\log_{10} N_t - \log_{10} N_0}{0.301t}$$

TABLE 7

Leucocidin and protein A production by staphylococcal strains

Strain no.	Leucocidin LU* per ml	Protein A
SM 1	16	-
SM 6	8	-
SM 9	500	+
SM 10	8	+
SM 14	16	+
SM 15	8	+
V 8	1,000	...

* LU (Leucocidal Units) = Inverse of titre in assay using human leucocytes

TABLE 8

Antibiograms of the staphylococcal strains

	Strain No.					
	SM 1	SM 6	SM 9	SM 10	SM 14	SM 15
Penicillin	+*	+	-*	-	-	+
Cephaloridine	+	+	+	+	-	+
Tetracycline	+	+	+	+	-	+
Clindamycin	+	+	+	+	+	+
Methicillin	+	+	+	+	-	+
Fucidin	+	+	+	+	+	+

* + = sensitive

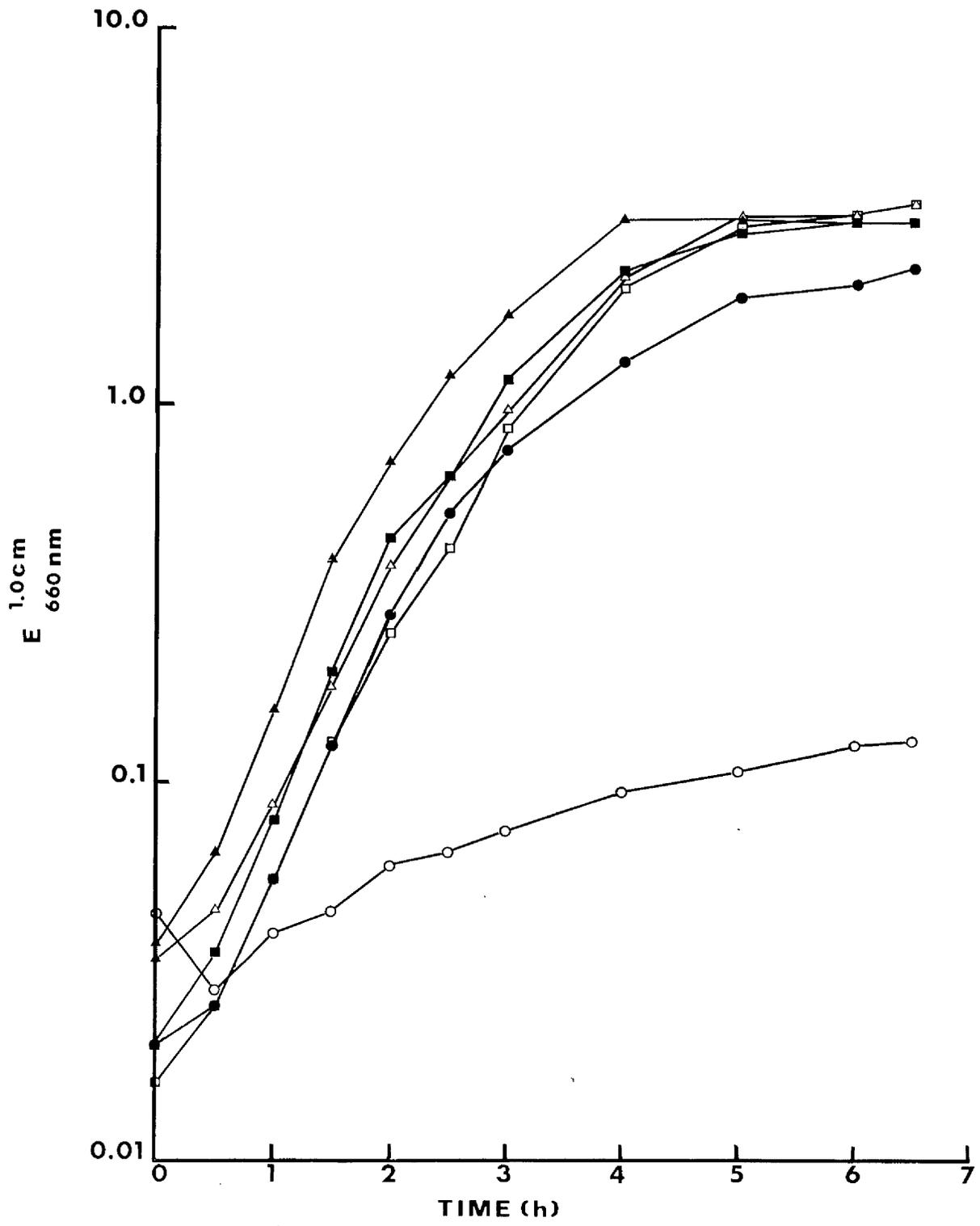
- = resistant

Figure 2: The in-vitro growth of staphylococcal strains

E_{660nm}^{1cm} was measured at intervals of 0.5 h

Symbols: E_{660nm}^{1cm} of strain

- : SM 1
- : SM 6
- ▲ : SM 9
- △ : SM 10
- : SM 14
- : SM 15



and the mean doubling time ($1/k$) were calculated (Table 9). These values show that strains SM 1, 9, 10, 14 and 15 multiply at very similar rates in vitro. SM 6 (Staph. epidermidis) however, grew at a much slower rate than the other strains. I will refer later to the possible relationship between in-vitro growth rate and virulence.

B. Assessment of Methods used in Animal Experiments

1. Viability of inocula maintained under liquid nitrogen

To maintain standard inocula for each mouse experiment, numerous small aliquots of a standard suspension of each strain were frozen. Before preparing and freezing all six strains, the viability of a suspension of a representative strain SM 9, was monitored just before freezing and at weekly intervals for six weeks thereafter (Table 10). The viability of the suspension fell by 31% on initial freezing but remained stable after this time. Such frozen suspensions were suitable therefore, for use as challenge inocula in animal experiments. Indeed, the viability of the suspensions has remained constant for two years.

2. Injection techniques

A small amount of the inoculum leaked out of the injection sites in 3-day old mice. The leaked fluid was collected as described previously (see p.53) from twenty 3-day old mice and the number of viable staphylococci determined. Leakage varied from 0.1% to 15% of the challenge inoculum (0.98×10^7 c.f.u.) and an average amounted to 5.5%.

3. Recovery of Staph. aureus from mouse tissue

Normal mouse tissue: It was planned to determine the presence and extent

TABLE 9

In-vitro growth rates of the staphylococcal strains

Strain no.	Growth rate constant (k) generations per h	Mean Doubling Time (1/k) min.
SM 1	2.5	24.3
SM 6	0.6	96.8
SM 9	2.2	27.6
SM 10	2.1	28.6
SM 14	2.5	24.3
SM 15	2.4	25.1

TABLE 10
Viability over a period of six weeks
of strain SM 9 stored in liquid nitrogen

Time (weeks)	Viable count (c.f.u. per ml)
0*	1.0×10^9
1	6.9×10^8
2	6.2×10^8
3	6.3×10^8
4	6.5×10^8
5	7.3×10^8
6	6.4×10^8

* before freezing

of multiplication of Staph. aureus in certain organs of experimentally infected mice. The organs selected were lung, spleen, liver, kidney and skin (injection site). In order to investigate the efficiency of recovery of staphylococci from these tissues, suspensions of SM 9 (10^4 c.f.u. and 10^8 c.f.u.) were added to homogenates from normal mice and viable counts were made of the mixtures (Table II). The recovery of 10^4 c.f.u. was never less than 50% from any homogenate and was particularly high from liver and spleen (70% and 80% respectively). The recovery of 10^8 c.f.u. was also satisfactory from all organ homogenates but again was very high from liver, kidney and skin (83%, 94% and 85% respectively). This experiment confirmed that Staph. aureus was recoverable with acceptable efficiency from normal mouse tissue.

Infected mouse tissue: In a pilot experiment in which 60 3-day old mice were challenged subcutaneously with 10^7 c.f.u. of strains SM 9 and SM 10, the numbers of staphylococci recoverable from various organs were estimated. A lethal dose was chosen for this experiment in order to follow the growth of organisms at the injection site and to trace any spread of the staphylococci into other organs of the body, i.e. lung, liver and kidney. Blood was also collected and examined for numbers of viable staphylococci. The results (Fig. 3) are expressed as Log_{10} number of organisms per mg (wet weight) tissue and per ml blood. Each point represents the average count for 4 mice.

Within a few minutes of challenge, organisms were detected in all organs, but the level of viable organisms in kidney and blood dropped to an almost undetectable level by 12 hours. At this time about 100 organisms per mg were detected in the liver. By contrast, in the first 12 hours the numbers

TABLE II

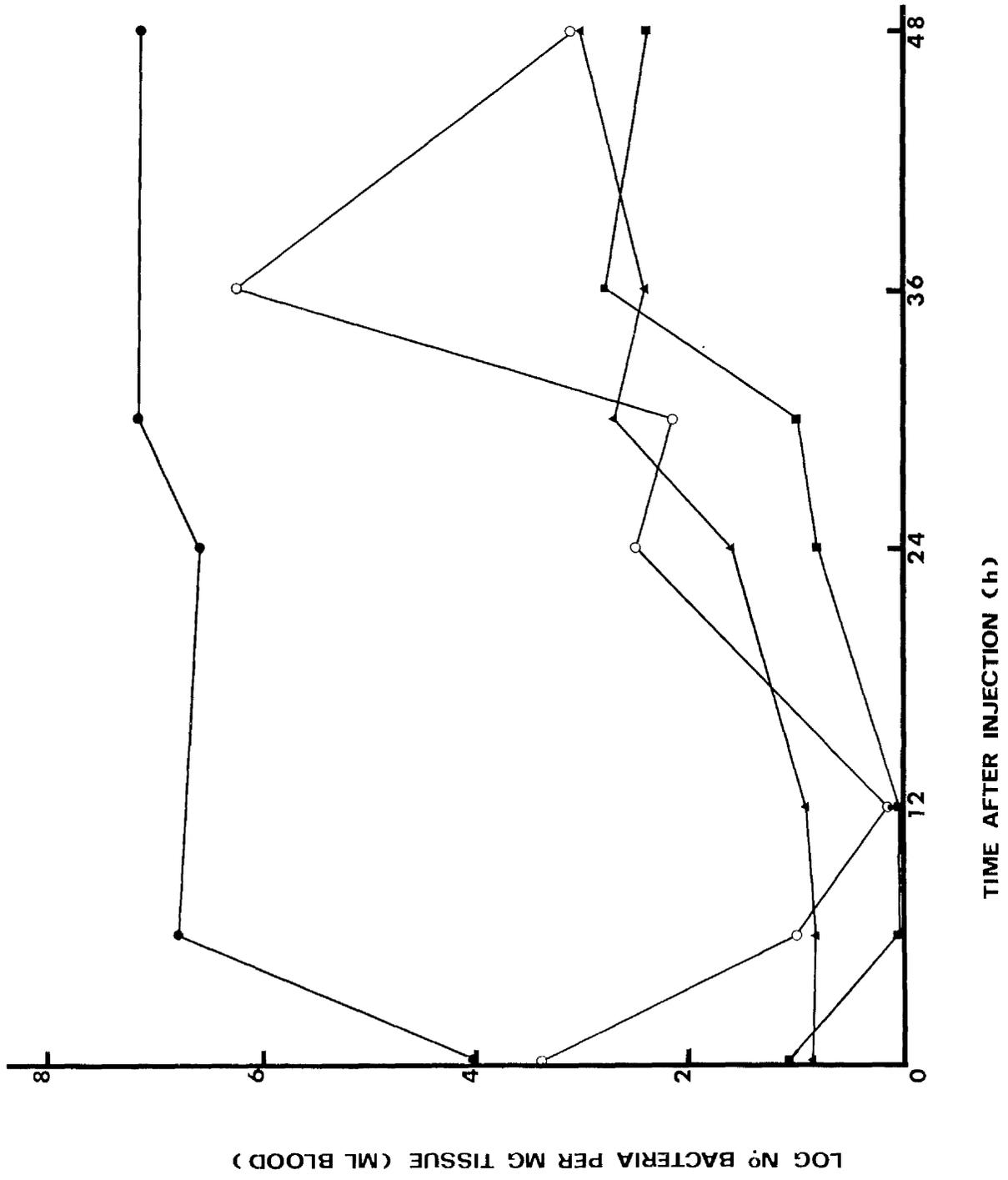
Recovery of strain SM 9
from mouse tissue homogenates

Organ	Percentage recovery of 10^4 c.f.u.	Percentage recovery of 10^5 c.f.u.
Spleen	60	59
Liver	80	83
Kidney	68	94
Lung	62	55
Skin	70	85

Figure 3: Numbers of staphylococci recovered from the organs of 3-day old mice challenged with 10^7 c.f.u. SM 9

Symbols: Log. no. of viable staphylococci recovered from a homogenate of

- : skin
- ▲ : liver
- : kidney
- : blood



of staphylococci in the injection site increased rapidly from 10^4 c.f.u. to 10^7 c.f.u. per mg. Between 12 and 36 hours the systemic spread of staphylococci was evidenced by the increasing numbers of organisms detected in the lung and kidney. In particular, it is notable that by 36 hours, the number of organisms per ml blood had increased to approximately 10^6 c.f.u. per ml producing a marked septicaemia. By this time the number of organisms in the skin had increased slightly and in general the mice appeared very ill, some being moribund. By 48 hours many mice had died and thereafter the death rate continued to increase.

For practical reasons in subsequent growth experiments involving large numbers of animals it was not possible to monitor all organs. Therefore, two organs were chosen; skin (injection site) and liver. Skin was chosen for two reasons:-

- (i) because of good recovery of organisms from skin homogenate, and
- (ii) because on subcutaneous challenge, lesions developed locally at the injection site and a study of the kinetics of growth at this site provided a means of investigating staphylococcal growth in-vivo.

Liver was chosen also for two reasons:-

- (i) because of good recovery of the organisms from homogenates, and
- (ii) because it was extremely difficult to obtain adequate volumes of blood aseptically from neonatal animals as an indication of systemic spread in the tissues of the host.

C. Virulence of the Strains for Mice of Different Ages

I. Staphylococcal strains

The natural high resistance of laboratory animals to Staph. aureus and the fact that man is most susceptible to serious staphylococcal infection in infancy were the two main reasons for studying the susceptibility of mice of different ages to experimental infection by the six selected strains. Strain SM 16 (PS 80) a virulent strain responsible for many outbreaks of hospital sepsis, was also included. In these early experiments, death and/or development of lesions locally at the injection site was recorded.

Susceptibility of 3-day, 10-day and 21-day old mice: Mice in these three age groups were challenged subcutaneously with doses of 10^4 , 10^6 and 10^8 c.f.u. of each strain. The results are summarised at 24 hours after injection (Table 12), 48 hours after injection (Table 13), 96 hours after injection (Table 14) and 10 days after injection (Table 15). In these tables the percentage mortality in an experimental group is represented by a circled figure; all the surviving animals in these groups developed lesions. In experimental groups where no deaths occurred, the percentage of animals which developed lesions is represented by a plain figure.

Differences in susceptibility between age groups: It should be emphasised that a challenge of 10^4 c.f.u. is relatively low for staphylococci. Three of the strains (SM 9, SM 14 and SM 16) produced lesions at 10^4 c.f.u. In each case the 3-day old mice were most susceptible (Tables 14 and 15). SM 14 also caused the death of a small percentage of the 3-day old and 10-day old mice at this dose.

With the exception of SM 6 (Staph. epidermidis), 10^6 c.f.u. of each strain caused mortality in the 3-day old groups ranging from 15 - 85%. Three

TABLE 12

Response of 3-day, 10-day and 21-day old mice
to strains at 24 hours after injection*

Age	Strain number						
	SM 1 10^4 10^6 10^8 c.f.u.	SM 6 10^4 10^6 10^8 c.f.u.	SM 9 10^4 10^6 10^7 c.f.u.	SM 10 10^4 10^6 10^8 c.f.u.	SM 14 10^4 10^6 10^8 c.f.u.	SM 15 10^4 10^6 10^8 c.f.u.	SM 16 10^4 10^6 10^8 c.f.u.
3-day	0 (25)	0 0 0	0 (1)	0 100 (40)	0 33 (44)	0 20 (85)	100 9 (100)
10-day	0 30 (70)	0 0 0	0 0 100	0 100 100	0 (33)	0 100 (100)	100 100 (100)
21-day	0 0 75	0 0 0	0 0 20	0 100 100	0 (35)	0 80 (15)	0 100 (100)

* Plain figures represent percentage of animals showing lesions in groups where no deaths occurred. Circled figures represent percentage mortality.

In these groups all surviving animals developed lesions.

TABLE 13

Response of 3-day, 10-day and 21-day old mice

to strains at 48 hours after injection*

Age	Strain number						
	SM 1 10^4 10^6 10^8 c.f.u.	SM 6 10^4 10^6 10^8 c.f.u.	SM 9 10^4 10^6 10^7 c.f.u.	SM 10 10^4 10^6 10^8 c.f.u.	SM 14 10^4 10^6 10^8 c.f.u.	SM 15 10^4 10^6 10^8 c.f.u.	SM 16 10^4 10^6 10^8 c.f.u.
3-day	0 (35)	0 0 (100)	50 (25)	0 (20)	0 (72)	0 (65)	100 (55)
10-day	0 0 (75)	0 0 (100)	0 100 100	0 100 100	0 0 (60)	0 (5)	100 100 (100)
21-day	0 0 (15)	0 0 20	0 20 50	0 100 100	0 0 (40)	0 5 (30)	0 100 (100)

* Plain figures represent percentage of animals showing lesions in groups where no deaths occurred. Circled figures represent percentage mortality.

In these groups all surviving animals developed lesions.

TABLE 14

Response of 3-day, 10-day and 21-day old mice
to strains at 96 hours after injection*

Age	Strain number						
	SM 1 10^4 10^6 10^8 c.f.u.	SM 6 10^4 10^6 10^8 c.f.u.	SM 9 10^4 10^6 10^7 c.f.u.	SM 10 10^4 10^6 10^8 c.f.u.	SM 14 10^4 10^6 10^8 c.f.u.	SM 15 10^4 10^6 10^8 c.f.u.	SM 16 10^4 10^6 10^8 c.f.u.
3-day	0 (40)	0 (100)	50 (48)	0 (50)	7 (7)	25 (85)	100 (100)
10-day	0 (0)	0 (75)	20 (100)	0 (100)	17 (5)	0 (5)	100 (7)
21-day	0 (0)	0 (15)	0 (30)	0 (100)	0 (0)	10 (100)	100 (100)

* Plain figures represent percentage of animals showing lesions in groups where no deaths occurred. Circled figures represent percentage mortality.

In these groups all surviving animals developed lesions.

TABLE 15

Response of 3-day, 10-day and 21-day old mice to strains at 10 days after injection*

Age	Strain number						
	SM 1 10^4 10^6 10^8 c.f.u.	SM 6 10^4 10^6 10^8 c.f.u.	SM 9 10^4 10^6 10^7 c.f.u.	SM 10 10^4 10^6 10^8 c.f.u.	SM 14 10^4 10^6 10^8 c.f.u.	SM 15 10^4 10^6 10^8 c.f.u.	SM 16 10^4 10^6 10^8 c.f.u.
3-day	0 (40)	0 0 (100)	60 (55) (100)	0 (50) (100)	7 (15) (85)	25 (85) (100)	100 (55) (100)
10-day	0 0 (75)	0 0 (100)	20 (100) (100)	0 (100) (100)	40 (5) (77)	10 (5) (100)	100 (7) (100)
21-day	0 0 (15)	0 0 (20)	0 (30) (70)	0 (100) (100)	0 (20) (50)	10 (100) (60)	100 (100) (100)

* Plain figures represent percentage of animals showing lesions in groups where no deaths occurred. Circled figures represent percentage mortality. In these groups all surviving animals developed lesions.

strains (SM 14, SM 15 and PS 80) at this dose produced a very low mortality in the 10-day old group. No 21-day old mouse died (Tables 14 and 15).

Each strain at 10^8 c.f.u. killed between 85 and 100% of 3-day old mice. Four of the strains (SM 1, SM 14 and SM 15 and PS 80) produced mortality (75 - 100%) in the 10-day old group and 15 - 100% in the 21-day old group (Tables 14 and 15).

It is evident from these results that the 3-day old mice were most susceptible to infection. This is made clear by the data summarised in Table 16 which shows the total number of animals dead in each age group at each challenge dose irrespective of staphylococcal strain. A total of 120 animals of each age were used.

Differences in susceptibility related to weight: The average weight of 3-day old mice was 2 g, of 10-day old mice was 6 g and of 21-day old mice was 10 g. This is a ratio for the three ages of 1 : 3 : 5. There was no evidence from the results either from lesion development or death that susceptibility was related to this ratio. In fact, for four of the strains (SM 1, SM 9, SM 10 and SM 15) there was less mortality within the 21-day old group injected with 10^8 c.f.u. than in the 3-day old group injected with 10^6 c.f.u. (a difference of a hundred fold in dose compared with a five fold difference in weight).

Susceptibility of 0 - 10-day old mice: In order to establish more precisely at what age mice became resistant to a single challenge dose (10^7 c.f.u.) a further study of age-related susceptibility was carried out, first with strain SM 9. Mice aged from newborn to ten days of age were challenged and

TABLE 16

Total number of mice dead in each age group*

Age (days)	10^4 c.f.u.	10^6 c.f.u.	10^8 c.f.u.
3	2	51	116
10	12	3	68
21	0	0	40

* 120 mice of each age were injected at each dose

Table 17 shows the percentage mortality at 5 days after challenge, by which time the pattern of results was established. The percentage mortality fell below 50% in mice aged four days and upwards.

A similar experiment was also carried out using 10^7 c.f.u. of strain SM 10. However, it was only possible to use twenty mice per age group. Percentage mortality was consistently less than 50% in mice aged seven days and older (Table 18).

These two experiments emphasise the value of liquid nitrogen as a method of storing aliquots of the same bacterial suspensions, since results from experiments performed on days (over a period of several months) on which adequate numbers of animals were available could be treated in a cumulative way.

Comparative virulence of the strains: The initial age-related studies (Tables 12 - 15) showed that the strains of staphylococci varied in their virulence for mice. In particular, SM 15 was highly virulent. Strains of Staph. aureus of bovine origin have previously been reported as particularly virulent for the mouse. (Chesbro, Taylor and Smith, 1972). However, in these experiments PS 80 and SM 14, both human derived strains, were also relatively virulent in that they produced lesions at 10^4 c.f.u. and caused high mortality rates at 10^8 c.f.u. in all ages of mice. Strains SM 9 and SM 10 were of similar virulence although they had very different toxin and enzyme profiles and produce different lesion types. For this reason these two strains were chosen for later comparative studies. Least virulent was strain SM 6 (Staph. epidermidis) confirming the validity in this system of the coagulase test as a criterion for

TABLE 17

Virulence of strain SM 9 at 10^7 c.f.u. for mice aged 0 - 10 days

Age of mouse (days)	No.* dead out of 10 animals challenged in											Total dead/ Total challenged
	Experiment no.											
	1	2	3	4	5	6	7	8	9	10	11	
0	10	10	10	10	...	10	10	10	70/70
1	10	10	9	9	...	10	10	58/60
2	10	9	10	9	9	10	57/60
3	7	4	9	5	2	10	10	10	10	8	9	81/110
4	2	6	2	1	5	6	22/60
5	3	0	1	10	5	1	3	1	24/80
6	0	0	1	1	2	3	7/60
7	1	0	0	1	5	7/50
8	0	0	0	0	5	5/50
9	0	0	0	0	0	0	0/60
10	0	0	0	0	0	0	0/60

* At 5 days after challenge

TABLE 18

Virulence of strain SM 10 at 10^7 c.f.u.

for mice aged 0 - 10 days

Age of mouse (days)	No.* dead out of 10 animals challenged in		Total dead/ Total challenged
	Experiment no.		
	1	2	
0	10	10	20/20
1	10	10	20/20
2	10	10	20/20
3	10	5	15/20
4	8	10	18/20
5	7	4	11/20
6	8	9	17/20
7	1	1	2/20
8	5	1	6/20
9	1	1	2/20
10	2	0	2/20

* At 5 days after challenge

pathogenicity. Lesions produced by this strain were very slight (a small area of inflammation with little evidence of pus formation).

Differences in virulence between the strains were seen most clearly, in terms of the number of deaths in 3-day old mice challenged with 10^6 c.f.u. of each strain. This dose produced mortality ranging from 0 - 85% (Fig. 4).

As indicated in Tables 12 - 15 the strains produced local lesions in all age groups of mice and these were of two types as judged by macroscopic appearance:-

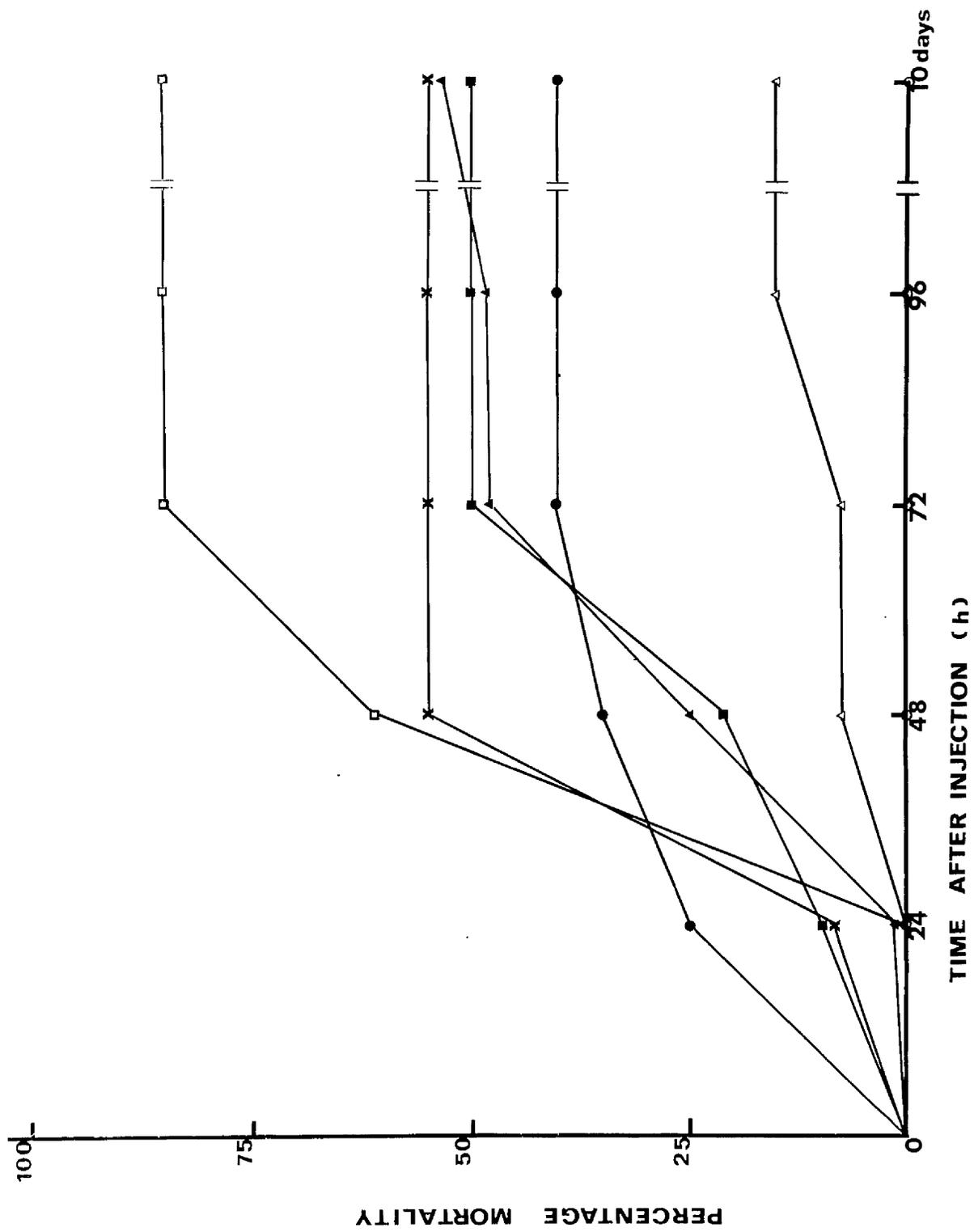
- (i) A localised abscess type: Pus formation was visible by 48 hours after injection and occasionally there was a central area of necrosis.
- (ii) A diffuse necrotic type: This was typically dark purple and grey when very severe. If an animal showing such a lesion survived the infection, pus often formed by 72 hours after injection.

Plates 1 and 2 show typical lesions of both types (i) at 48 hours after injection and (ii) at 24 hours after injection in 3-day old and 21-day old mice. Type (i) lesions (abscesses) were produced by strain SM 9 while type (ii) lesions (necrotic) were produced by strains SM 1, 10, 14, 15 and 16. This was in accordance with the toxin profiles since strain SM 9 was the only non-toxinogenic strain of Staph. aureus included in this study. It seemed possible, therefore, that the necrotic effect seen in vivo was due to the production of toxin in which case cell free culture filtrates of the toxinogenic strains might produce necrosis in the mice when injected subcutaneously.

**Figure 4: Comparative virulence of staphylococcal strains
assessed by mortality rates in 3-day old mice
challenged with 10^6 c.f.u. of each strain**

Symbols: Percentage of mice dead challenged with strain

- : SM 1
- : SM 6
- ▲ : SM 9
- : SM 10
- △ : SM 14
- : SM 15
- x : SM 16



**Plate 1: Macroscopic appearance of the lesions caused by
Staph. aureus in 3-day old mice.**

Symbol:

A: Abscess lesion-type (i) 48 h after injection

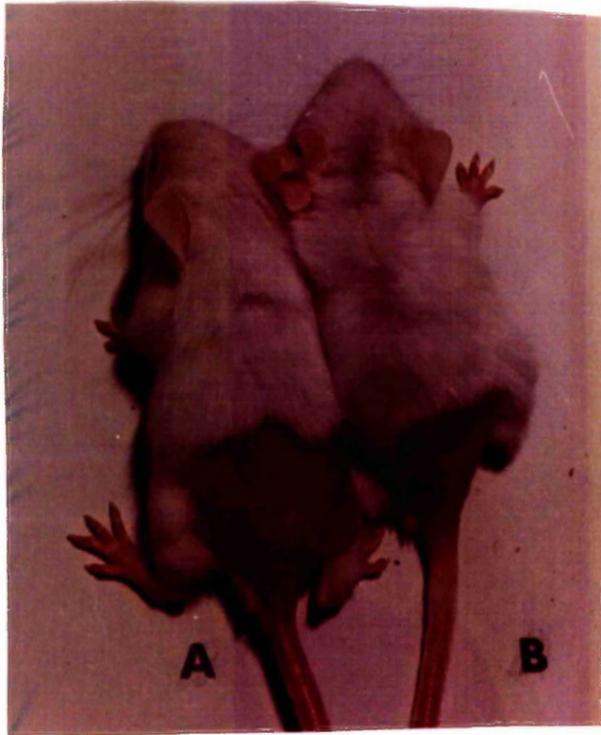
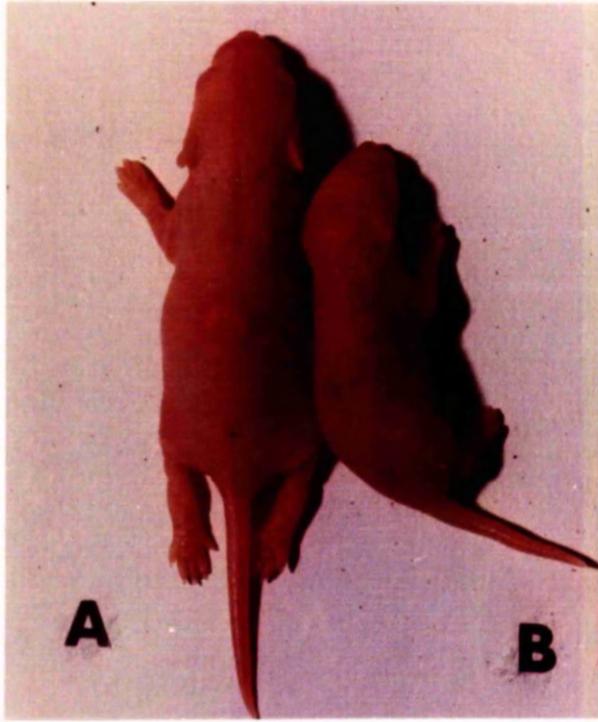
B: Necrotic lesion-type (ii) 24 h after injection

**Plate 2: Macroscopic appearance of the lesions caused by
Staph. aureus in 21-day old mice.**

Symbol:

A: Abscess lesion-type (i) 48 h after injection

B: Necrotic lesion-type (ii) 24 h after injection



2. Effects of extracellular staphylococcal products in vivo

Each of strains SM 1, 6, 9, 10, 14 and 15 were grown in two types of media and under conditions as follows:-

- (i) Nutrient Broth at 37°C for 18 hours (see p.49)
- (ii) Semi-solid agar at 37°C in 20% CO₂ for 48 hours (see p.52)

Supernate (0.05 ml) from each culture was sterilised by millipore filtration and injected subcutaneously into mice (3-day, 10-day and 21-day old). The results (Table 19) show the number of mice dead (circled figures) and the number which had developed lesions (plain figures) by 48 hours after injection. No mice injected with supernates from Nutrient Broth cultures died or developed lesions and this confirmed that Nutrient Broth was a suitable medium for culturing organisms for challenge, i.e. there was no preformed toxin associated with the cells. Semi-solid agar, on the other hand, is a medium which enhances toxin production and culture filtrates from the toxinogenic strains (SM 1, 10, 14, 15 and 16) grown in this medium were dermonecrotic and lethal. The lesions were similar to those produced by injection of whole organisms particularly at 10⁸ c.f.u. Filtrates from strains SM 6 and SM 9 grown in the same medium produced no observable effects, confirming that neither strain produced toxic factors under these conditions.

3. Other bacteria

To investigate whether the age-related susceptibility to staphylococci observed in the CD-1 mice might hold also for other organisms, representative strains of four other genera were tested in the same three age groups of mice. These were Escherichia coli, Pseudomonas aeruginosa, Klebsiella species and Streptococcus pyogenes; all were freshly isolated from clinical specimens.

TABLE 19

Effect of culture filtrates of staphylococcal strains*

Age	Strain number											
	SM 1		SM 6		SM 9		SM 10		SM 14		SM 15	
	NB [†]	SS [‡]	NB	SS	NB	SS	NB	SS	NB	SS	NB	SS
3-day	0	100	0	0	0	0	0	100	0	100	0	100
10-day	0	100	0	0	0	0	0	100	0	100	0	100
21-day	0	100	0	0	0	0	0	100	0	100	0	100

* Plain figures represent percentage of animals showing lesions in groups where no deaths occurred. Circled figures represent percentage mortality

† NB = supernate from nutrient broth culture

‡ SS = supernate from semi-solid agar culture

Tables 20 and 21 summarise the results 24 hours and 96 hours after challenge respectively. These tables show that there was some age-related susceptibility but, by contrast with the results for the staphylococcal strains, there was no difference in percentage mortality between 3-day old animals challenged with 10^6 c.f.u. and 21-day old animals challenged with 10^8 c.f.u. This suggests that any age-related susceptibility might have been, at least in part, due to weight differences between the two age groups.

The response to these four genera was different from that to the staphylococci in respect of the following:-

- (i) Mice injected with P. aeruginosa at 10^8 c.f.u. died very rapidly (12 - 24 hours).
- (ii) Animals in all age groups which survived showed no obvious lesions except a few of the mice challenged with Strep. pyogenes. This shows that the Staph. aureus strains have a characteristic property of multiplying in subcutaneous tissue.
- (iii) A less marked age-related susceptibility was observed in the CD-1 mice to these four organisms than to the staphylococci. However, of the four, the response to Klebsiella sp most resembled that to the staphylococcus.

D. Comparative Study of Strains SM 9 and SM 10

From the initial studies of the virulence of the six staphylococcal strains in mice, two strains namely SM 9 and SM 10 were shown to be of similar virulence as measured by the percentage mortality and lesion production. However, the two strains differed distinctly in three important features:-

TABLE 20

Response of 3-day, 10-day and 21-day old mice challenged with

Esch. coli, P. aeruginosa, Klebsiella sp. and Strep. pyogenes at 24 hours after challenge

Age	<u>Esch. coli</u> 10^4 10^6 10^8 c.f.u.	<u>P. aeruginosa</u> 10^4 10^6 10^8 c.f.u.	<u>Klebsiella sp.</u> 10^4 10^6 10^8 c.f.u.	<u>Strep. pyogenes</u> 10^4 10^6 10^8 c.f.u.
3-day	0	0	0	0
10-day	0	0	0	0
21-day	0	0	0	0

TABLE 21

Response of 3-day, 10-day and 21-day old mice challenged with

Esch. coli, P. aeruginosa, Klebsiella sp. and Strep. pyogenes at 96 hours after challenge

Age	Esch. coli $\left. \begin{matrix} 10^4 & 10^6 & 10^8 \\ & \text{c.f.u.} & \end{matrix} \right\}$	P. aeruginosa $\left. \begin{matrix} 10^4 & 10^6 & 10^8 \\ & \text{c.f.u.} & \end{matrix} \right\}$	Klebsiella sp. $\left. \begin{matrix} 10^4 & 10^6 & 10^8 \\ & \text{c.f.u.} & \end{matrix} \right\}$	Strep. pyogenes $\left. \begin{matrix} 10^4 & 10^6 & 10^8 \\ & \text{c.f.u.} & \end{matrix} \right\}$
3-day	0 0 60	0 100 100	70 100 100	0 0 100
10-day	0 0 90	0 66 100	12 84 100	20 0 100
21-day	0 0 0	0 0 100	0 24 100	0 0 0

- (i) SM 9 was only very weakly toxinogenic, while SM 10 was quite strongly toxinogenic producing mainly α -toxin.
- (ii) SM 9 was non-proteolytic, while SM 10 was proteolytic, producing both fibrinolysin and gelatinase, enzymes which have been associated in the past with staphylococcal virulence.
- (iii) SM 9 produced localised abscess-type lesions while SM 10 produced diffuse necrotic lesions. Because of their similar virulence and differences in other properties, these two strains were selected for a series of comparative studies.

I. Growth of in vivo in 21-day old and 3-day old mice

Our study of recovery of staphylococci from various organs of mice showed that both skin and liver were sites from which good recovery (80 - 95%) was possible. These were the sites selected for monitoring in-vivo multiplication of strains SM 9 and SM 10. Skin was the principal site because the progress of any lesions which developed could be monitored macroscopically. We used two age groups of mice, 3-day old and 21-day old because these two age groups responded very differently to the same challenge dose. The dose selected was 10^6 c.f.u. because this dose produced substantial mortality (50 - 55%) and severe lesions in neonates but had little or no observable effect on 21-day old mice. Large numbers of animals were challenged with 10^6 c.f.u. of either strain SM 9 or SM 10 and at intervals thereafter the number of viable staphylococci present in the skin and liver homogenates were counted (see p. 5).

Multiplication of staphylococci in the skin: Counts were expressed as \log_{10} as suggested by Moroney (1973).

The multiplication of the SM 9 in the skin of 3-day old mice is shown in Fig. 5. The arrow indicates the average number of viable organisms counted in a comparable area of skin from ten normal animals. Up to 48 hours, each time point represents the average number of viable staphylococci per injection site of 4 - 10 animals selected at random. By 48 hours after injection it was easy to distinguish between mice which were moribund and had severe lesions (50% at 10^6 c.f.u.) and those which were going to survive with milder lesions (50% at 10^6 c.f.u.). From 48 hours onwards animals in both categories were selected separately. In Fig. 5 the number of viable organisms in the lesions of moribund animals is represented by the solid line while that of survivors is represented by the broken line. It can be seen that the number of viable organisms per injection site of the moribund animals (i.e. the solid line) continued to rise slightly after 48 hours. However, in the injection site of the surviving animals the number of viable organisms had fallen to approximately 10^5 c.f.u. by 48 hours and this number decreased still further to the control level by 96 hours after challenge.

21-day old animals challenged with the same dose (10^6 c.f.u.) of SM 9 showed a different response (Fig. 6). Each time point represents the average count from four mice selected at random. The arrow indicates the average number of viable organisms in a comparable area of skin from 10 normal mice SM 9 multiplied only slightly in the first 48 hours post challenge, after which the number of viable organisms in the skin at the injection site fell to approximately the normal control level. No visible lesion was produced. Comparing the growth of SM 9 in the skin of the two age groups (Fig. 7) it is evident that, 1 hour after challenge, the number of viable staphylococci detectable in the injection site of both 3- and 21-day

Figure 5: Growth of strain SM 9 in the skin of 3-day old mice challenged with 10^6 c.f.u.

Symbols:

—●— : Log. no. of viable staphylococci recovered from a homogenate of skin taken from mice selected at random till 30 h and thereafter taken from mice which were moribund.

---●--- : Log. no. of viable staphylococci recovered from a homogenate of skin taken from mice which were not ill at 48 h or thereafter.

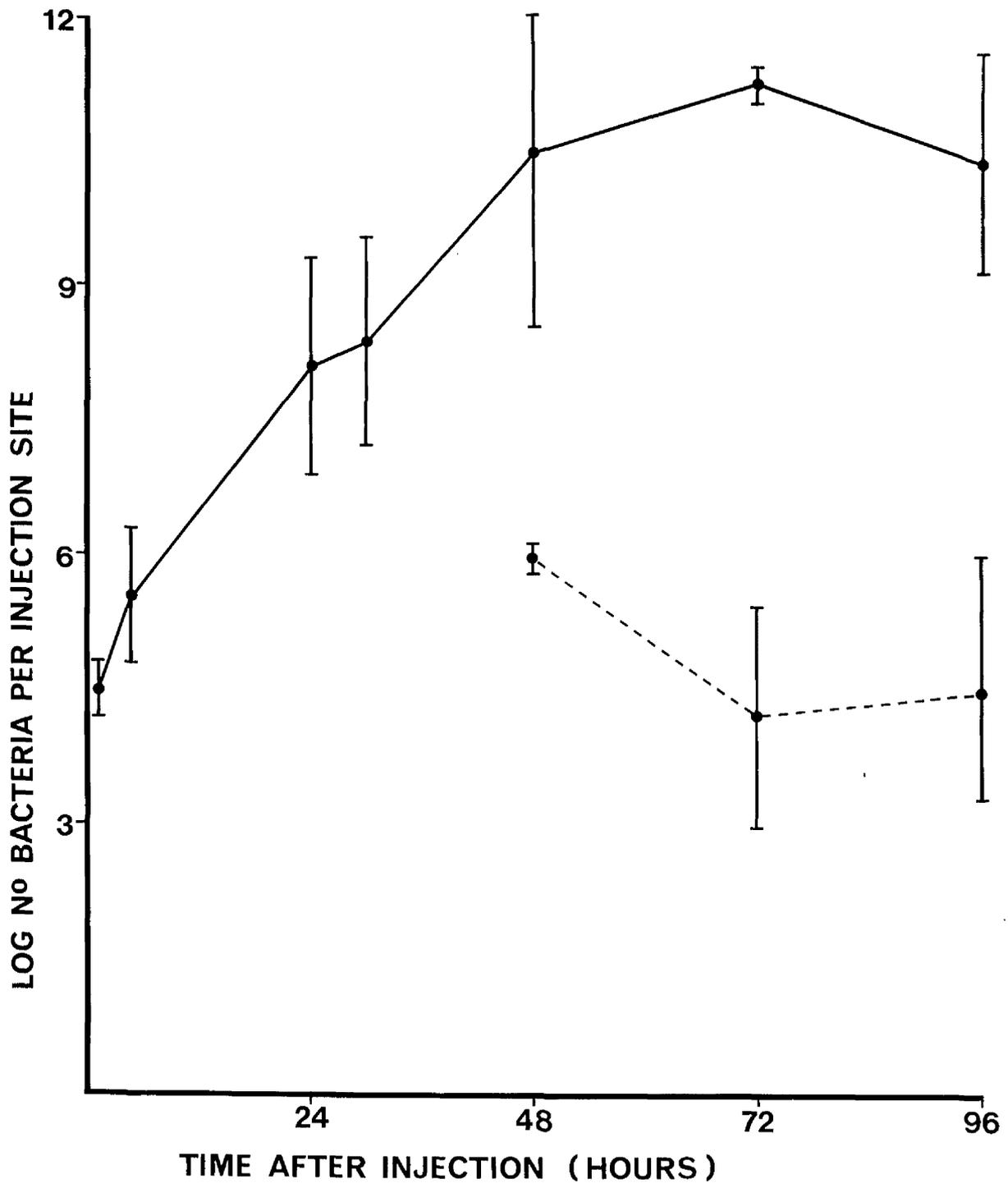
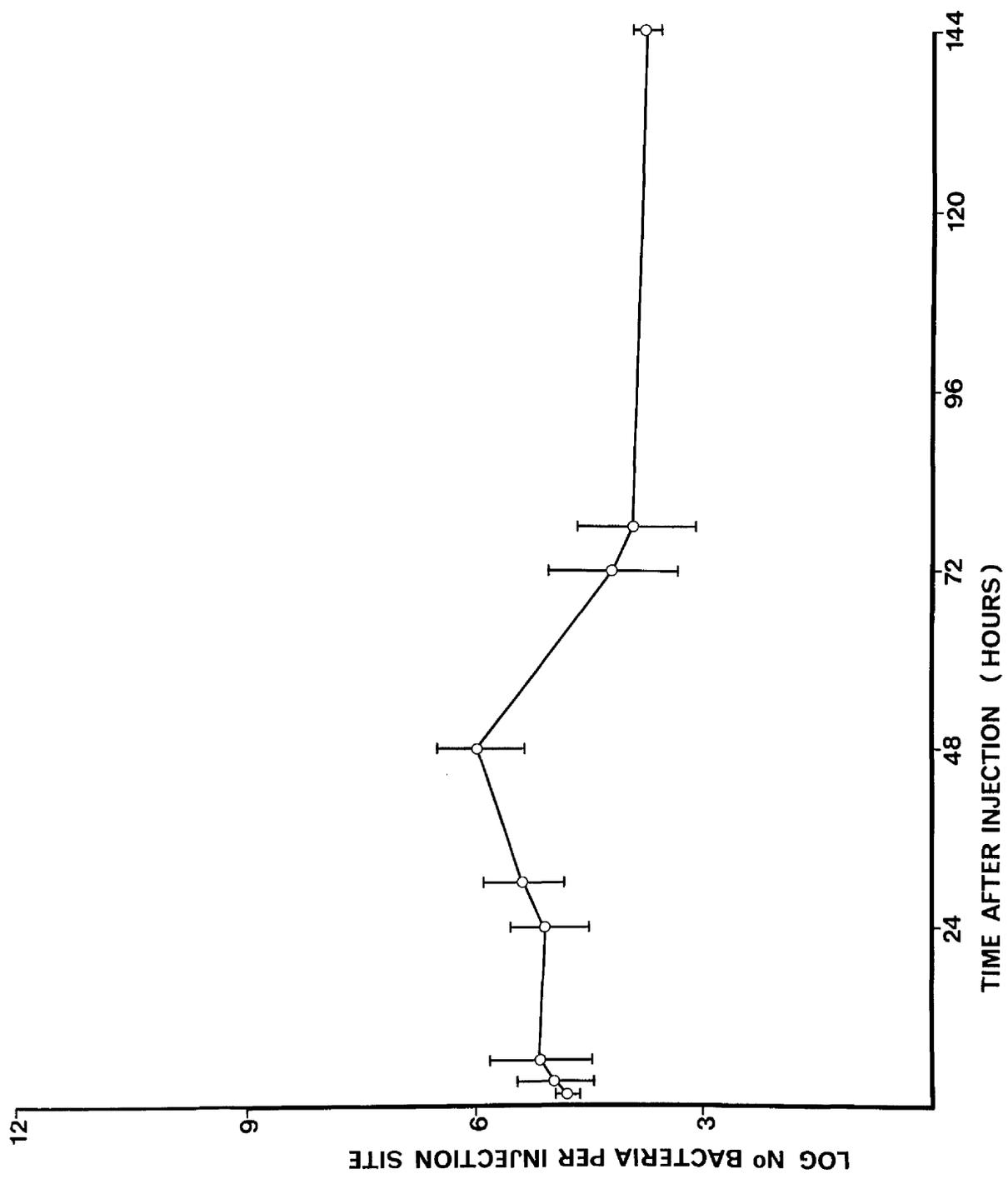


Figure 6: Growth of strain SM 9 in the skin of 21-day old mice challenged with 10^6 c.f.u.

Symbol:

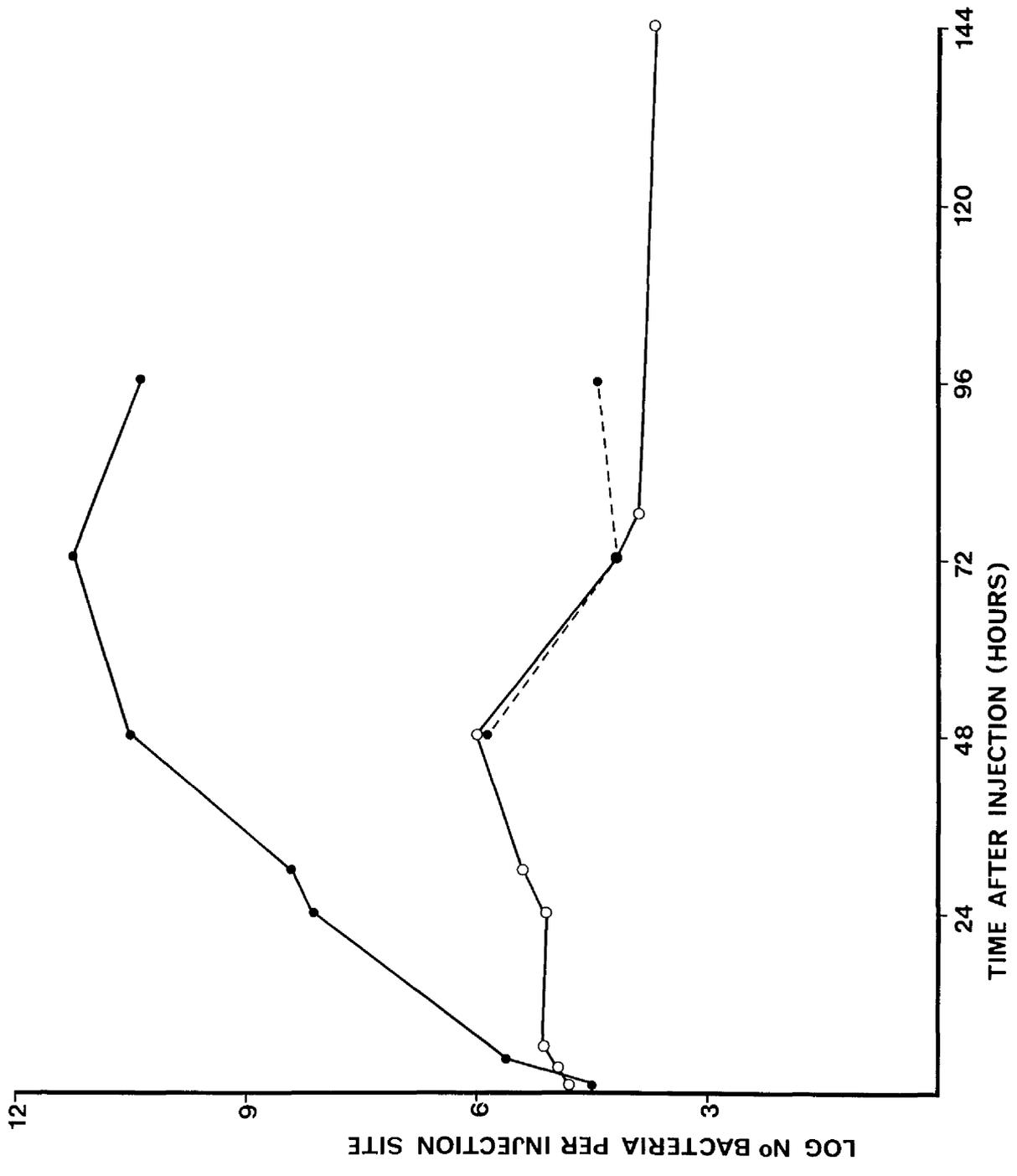
—○— : Log. no. of viable staphylococci recovered from a homogenate of skin



**Figure 7: Comparison of the growth of strain SM 9 in the skin of
3-day old and 21-day old mice challenged with 10^6 c.f.u.**

Symbols:

- : Log. no. of viable staphylococci recovered from a homogenate of skin taken from 3-day old mice selected at random till 30 h and thereafter from mice which were moribund.
- : Log. no. of viable staphylococci recovered from a homogenate of skin taken from 3-day old mice which were not ill at 48 h or thereafter.
- : Log. no. of viable staphylococci recovered from a homogenate of skin from 21-day old mice.



old animals was almost the same (10^5 c.f.u.). This represented 5 - 10% of the original challenge dose (10^6 c.f.u.). These remaining organisms were able to multiply rapidly in the skin of 60% of the 3-day old mice but scarcely at all in the 21-day old mice. The curve representing the number of staphylococci in the skin of the 40% of the 3-day old mice which survived (broken line) challenge followed very closely the curve representing the numbers of staphylococci in the skin of 21-day animals.

The growth of strain SM 10 followed a similar overall pattern. Fig. 8 shows the growth of SM 10 in the skin of 3-day old mice. As in Fig. 5 the solid line represents the numbers of staphylococci in the skin of the 60% of mice which developed severe lesions, became ill and died. Easily distinguishable at 30 hours after challenge were the mice which were going to survive. These were selected separately at each time point after 30 hours and the growth of SM 10 in their skin is represented by the broken line. As with SM 9 (Fig. 5) the viable numbers of staphylococci in the injection site of these surviving neonates decreased to the control level by 6 days after challenge.

In the skin of 21-day old mice (Fig. 9) there was only slight multiplication and after 48 hours the level of organisms in the injection site fell steadily to the control level. Comparing the curves for the two age groups (Fig. 10), it is clear that as with SM 9, approximately the same number of staphylococci were detectable 1 hour after challenge in the skin of both 3-day old and 21-day old mice. SM 10 was able to multiply rapidly in 60% of the neonates which died 30 - 36 hours after challenge. However, in the skin of 21-day old mice and similarly in the 40% of surviving neonates, SM 10 multiplied only slightly initially; by 6 days after challenge the viable count in the skin had fallen to the control level.

Figure 8: Growth of strain SM 10 in the skin of 3-day old mice challenged with 10^6 c.f.u.

Symbols:

- : Log. no. of viable staphylococci recovered from a homogenate of skin taken from mice selected at random till 24 h and thereafter taken from mice which were moribund.
- : Log. no. of viable staphylococci recovered from a homogenate of skin taken from mice which were not ill at 30 h or thereafter.

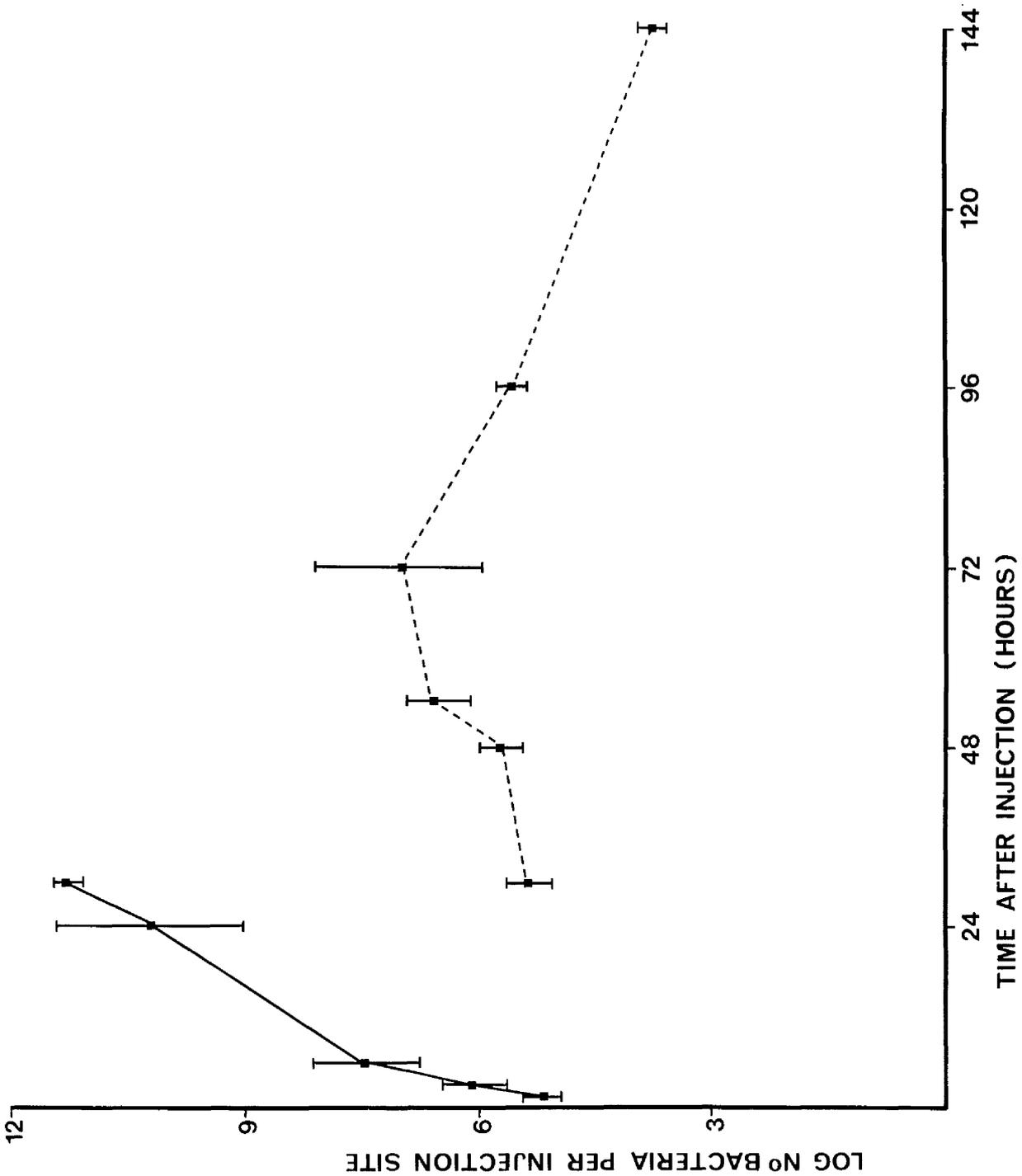


Figure 9: Growth of strain SM 10 in the skin of 21-day old mice challenged with 10^6 c.f.u.

Symbol:

—□— : Log. no. of viable staphylococci recovered from a homogenate of skin from 21-day old mice.

LOG N° BACTERIA PER INJECTION SITE

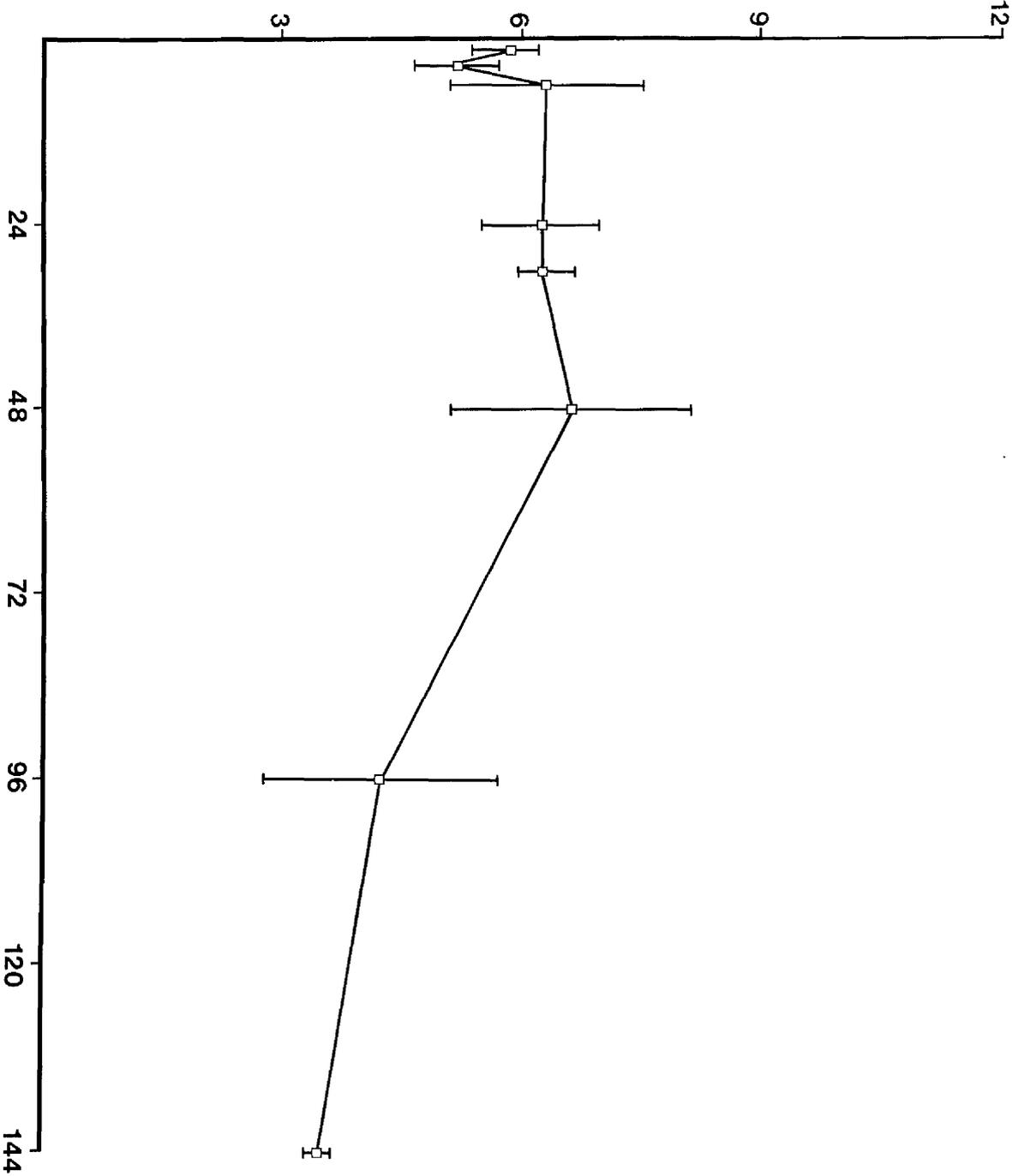
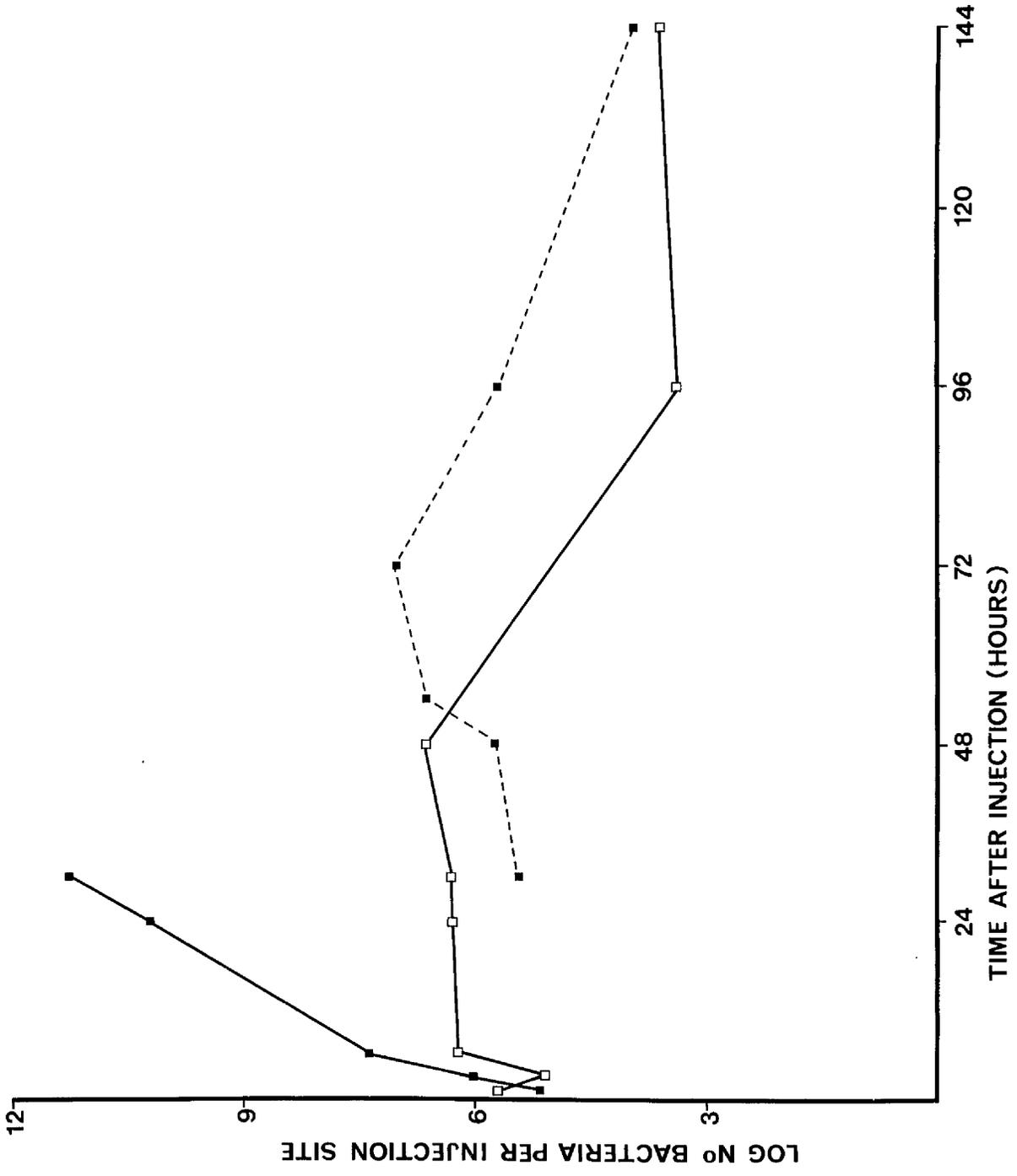


Figure 10: Comparison of the growth of strain SM 10 in the skin of 3-day old and 21-day old mice challenged with 10^6 c.f.u. of each strain.

Symbols:

- : Log. no. of viable staphylococci recovered from a homogenate of skin taken from 3-day old mice selected at random till 24 h and thereafter taken from mice which were moribund.
- : Log. no. of viable staphylococci recovered from a homogenate of skin taken from 3-day old mice which were not ill at 30 h or thereafter.
- : Log. no. of viable staphylococci recovered from a homogenate of skin taken from 21-day old mice.



Figs. 11 and 12 compare separately the curves of the two strains in each age group. The two curves for the growth of the SM 9 and SM 10 in the skin of 21-day old mice (Fig. 11) are very similar, suggesting that there was very little, if any, difference in the *in-vivo* behaviour of the two strains in the skin of 21-day old animals. In the skin of 3-day old mice, however, (Fig. 12) SM 10 was able to multiply faster than SM 9; the growth constants and the mean doubling times (MDT) measured between 4 hours and 24 hours after challenge are shown in Table 22. The table also shows the *in-vitro* growth rates of the two strains for comparison. For both strains, growth *in-vivo* was much slower than growth *in-vitro*. The mean generation time for SM 9 was 2.3 hours and 1.78 hours for SM 10. This compares with mean generation times of 27 and 28 min. respectively for those strains *in-vitro*. Also evident from this table is that although the strains grow at a very similar rate *in vitro*, SM 10 grows faster than SM 9 *in vivo*; k for SM 10 was 0.57 and for SM 9 was 0.44 *in vivo*. It is interesting to note, however, that both SM 9 and SM 10 multiply to the same level of approximately 10^{11} c.f.u./lesion site by which time animals are moribund; SM 10 reaches this number by 30 hours and SM 9 by 48 hours.

Presence of staphylococci in the liver: No staphylococci were detected in liver homogenates of 21-day old mice infected with either strain SM 9 or SM 10. In 3-day old mice the numbers of staphylococci detected in the liver homogenates varied substantially even in mice in which the number detected in the injection/lesion site was the same. A noteworthy feature of the results was, however, that the numbers of staphylococci in the livers of the 50 - 60% of 3-day old mice which became ill and died were markedly different in mice injected with SM 9 compared with SM 10. Table 23 shows that in the livers of mice injected with SM 9, organisms were detected 1 hour after challenge

Figure II: Comparison of the growth of strains SM 9 and SM 10 in the skin of 21-day old mice challenged with 10^6 c.f.u. of each strain.

Symbols:

—○— : Log. no. of viable staphylococci recovered from a homogenate of skin taken from mice challenged with strain SM 9.

—□— : Log. no. of viable staphylococci recovered from a homogenate of skin taken from mice challenged with strain SM 10.

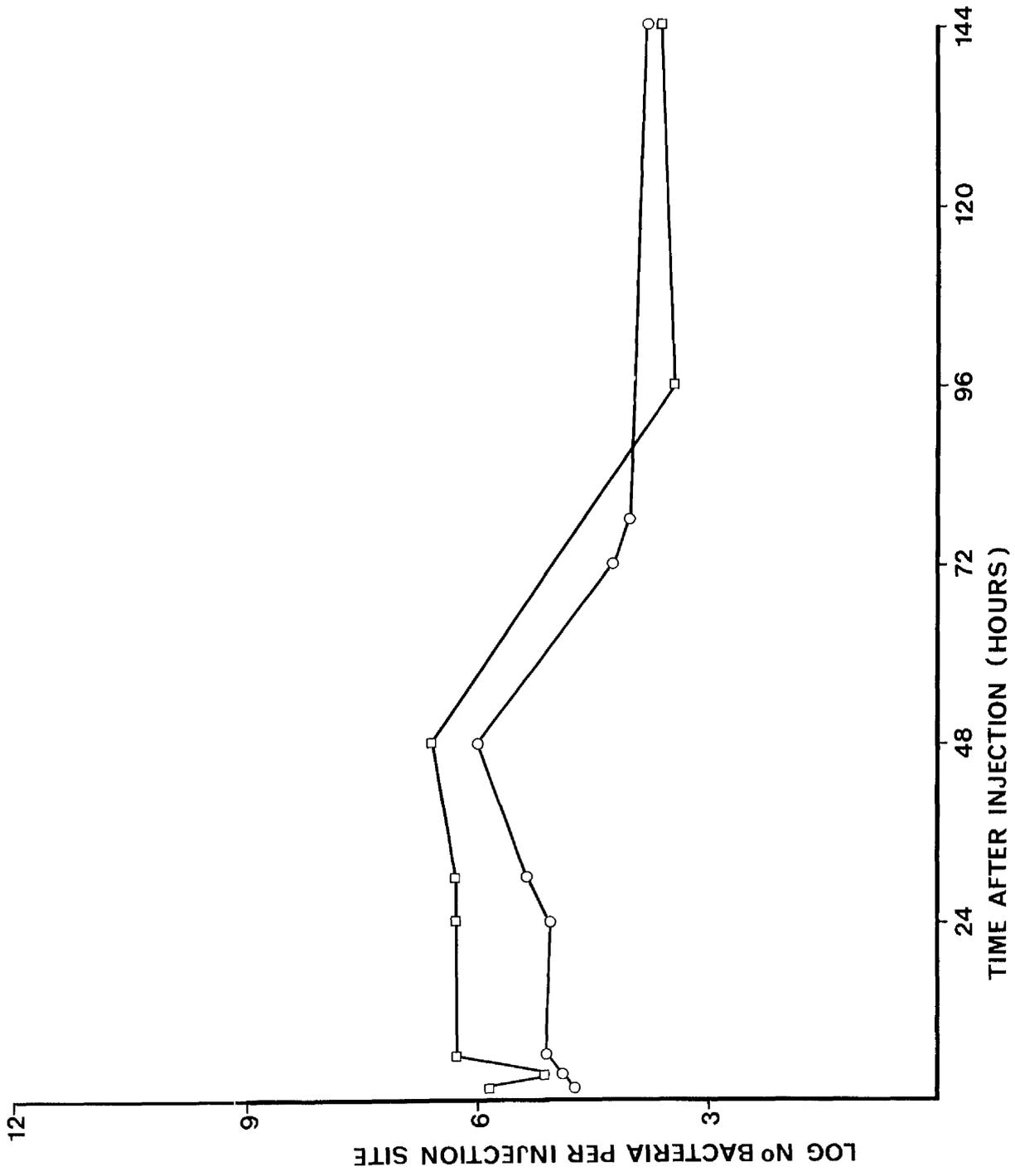


Figure 12: Comparison of the growth of strains SM 9 and SM 10 in the skin of 3-day old mice challenged with 10^6 c.f.u. of each strain.

Symbols:

- : Log. no. of viable staphylococci recovered from a homogenate of skin taken from mice challenged with 10^6 c.f.u. of strain SM 9 and selected at random till 30 h and thereafter from mice which were moribund.
- : Log. no. of viable staphylococci recovered from a homogenate of skin taken from mice challenged with 10^6 c.f.u. SM 9 which were not ill at 48 h or thereafter.
- : Log. no. of viable staphylococci recovered from a homogenate of skin taken from mice challenged with 10^6 c.f.u. of strain SM 10 and selected at random till 24 h and thereafter from mice which were moribund.
- : Log. no. of viable staphylococci recovered from a homogenate of skin taken from mice challenged with 10^6 c.f.u. of strain SM 10 which were not ill at 30 h or thereafter.

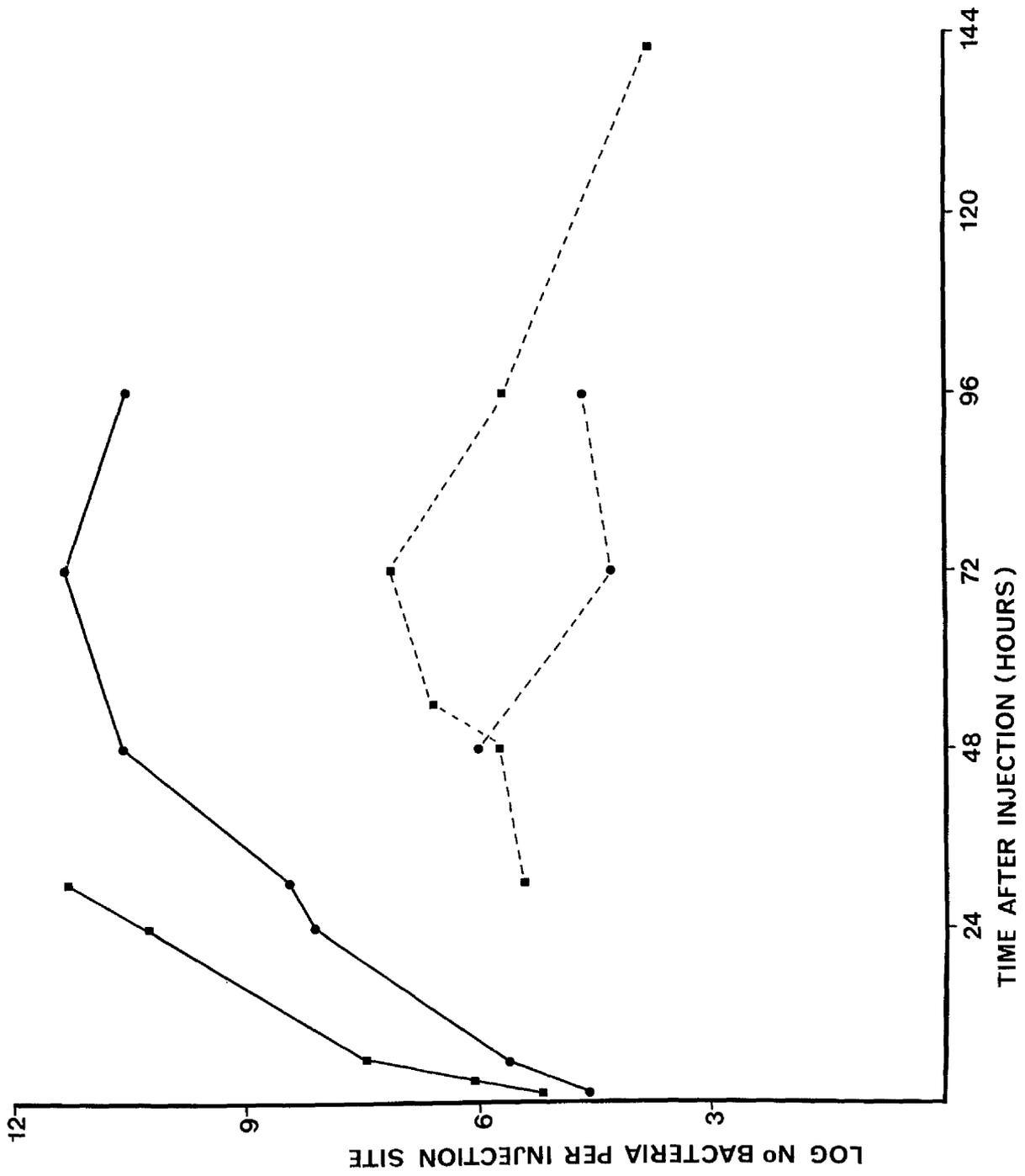


TABLE 22

Growth rates of strains SM 9 and SM 10

in-vivo and in-vitro

Strain no.	Growth rate constant (k) generations/h		Mean Doubling Time (l/k) mins.	
	In-vivo	In-vitro	In-vivo	In-vitro
9	0.436	2.17	137.40	27.64
10	0.568	2.10	105.60	28.57

TABLE 23

Recovery of staphylococci from liver tissue of 3-day old mice
challenged with 10^6 c.f.u. SM 9 and SM 10

Time after injection (h)	Average number per liver homogenate (4 mice)	
	SM 9	SM 10
1	7.1×10^2	37.0
4	0	3.4×10^2
24	0	6.3×10^4
30	62.5	1.0×10^4
48	3.5×10^4	...

after which time none were detected till 30 hours, by 48 hours there were on average 3.5×10^4 c.f.u. Counts in the lesion site had reached a level of 10^{10} c.f.u. by this time (Fig. 5) and animals were moribund and dying.

By contrast Table 23 shows that in the livers of 3-day old mice injected with SM 10, organisms were detected 1 hour after challenge and in increasing numbers thereafter to a level of 6.3×10^4 c.f.u. by 24 hours. Counts in the lesion site had reached a level of 10^{10} c.f.u. by 24 hours (Fig. 8) and mice were moribund and dying. These results indicate that staphylococci were detectable in the livers of moribund animals in high numbers (around 10^4 c.f.u.) and support the view that SM 10 was a more invasive strain than SM 9 as judged by its presence in the liver throughout the infection. However, both SM 9 and SM 10 were detectable in the liver in similar numbers just prior to death which occurs earlier with SM 10 than with SM 9. This is in accordance with the relative multiplication and final number of organisms in the skin.

2. The effect of antibiotic on growth in vivo

A pilot experiment was performed to investigate the effects of extracellular in-vivo products. Neonatal mice were challenged as before with SM 9 and SM 10 (10^6 c.f.u.) and 5 hours later the number of viable staphylococci in the injection site was monitored as before. 1 hour later, at 6 hours after challenge, a single dose (14 μ g) of Cephaloridine was injected intraperitoneally. Cephaloridine was chosen because both organisms were sensitive, it is well absorbed into the tissues and has been used with success in treating staphylococcal infection in man (Smith, 1971). The dose was chosen to kill all the viable staphylococci so as to allow any extracellular products present in the tissues at that time to exert observable effects. The

dose of Cephaloridine, calculated by weight, was twice the recommended adult human dose (3.5 mg per kg). At 3 hours and 45 hours after giving the antibiotic samples of the lesion sites were taken as before for bacterial counting.

The effect of the Cephaloridine on the numbers of SM 9 viable in the lesion site is shown in comparison with the numbers in untreated animals in Fig. 13. Just prior to administration of the antibiotic (first circled point) the number of staphylococci in the injection site of two mice was slightly higher than the corresponding point on the growth curve as measured in the previous experiments. At 9 hours (3 hours after the administration of Cephaloridine) although there had been a slight increase in the number of viable staphylococci the rate of multiplication of the organisms had been reduced compared with the average growth curve. After this time the antibiotic stopped further multiplication of SM 9. Also, none of the animals which received antibiotic died as compared with 8 out of 10 of the untreated control litter in this experiment.

The effect of Cephaloridine on the growth of SM 10 in the skin of 3-day old mice is shown in Fig. 14. At 5 hours after challenge with 10^6 c.f.u. SM 10, the average number of viable staphylococci per injection site of two mice (first circled point) lay exactly on the growth curve as measured in previous experiments. At 9 hours after challenge (3 hours after administration of Cephaloridine) the number of viable staphylococci per injection site had fallen sharply and by 48 hours it was reduced still further. One out of thirteen of the antibiotic-treated mice died as compared with all of the untreated control litter in this experiment.

Figure 13: The effect of cephaloridine on the growth of strain SM 9
in the skin of 3-day old mice challenged with 10^6 c.f.u.

Symbols:

- : Log. no. of viable staphylococci recovered from a homogenate of skin taken from mice selected at random till 30 h and thereafter taken from mice which were ill.
- : Log. no. of viable staphylococci recovered from a homogenate of skin taken from mice which were not ill at 30 h or thereafter.
- : Log. no. of viable staphylococci recovered from a homogenate of skin taken from mice
- : Log. no. of viable staphylococci recovered^{ine} on first challenge.
- ↑ : Time of cephaloridine administration.

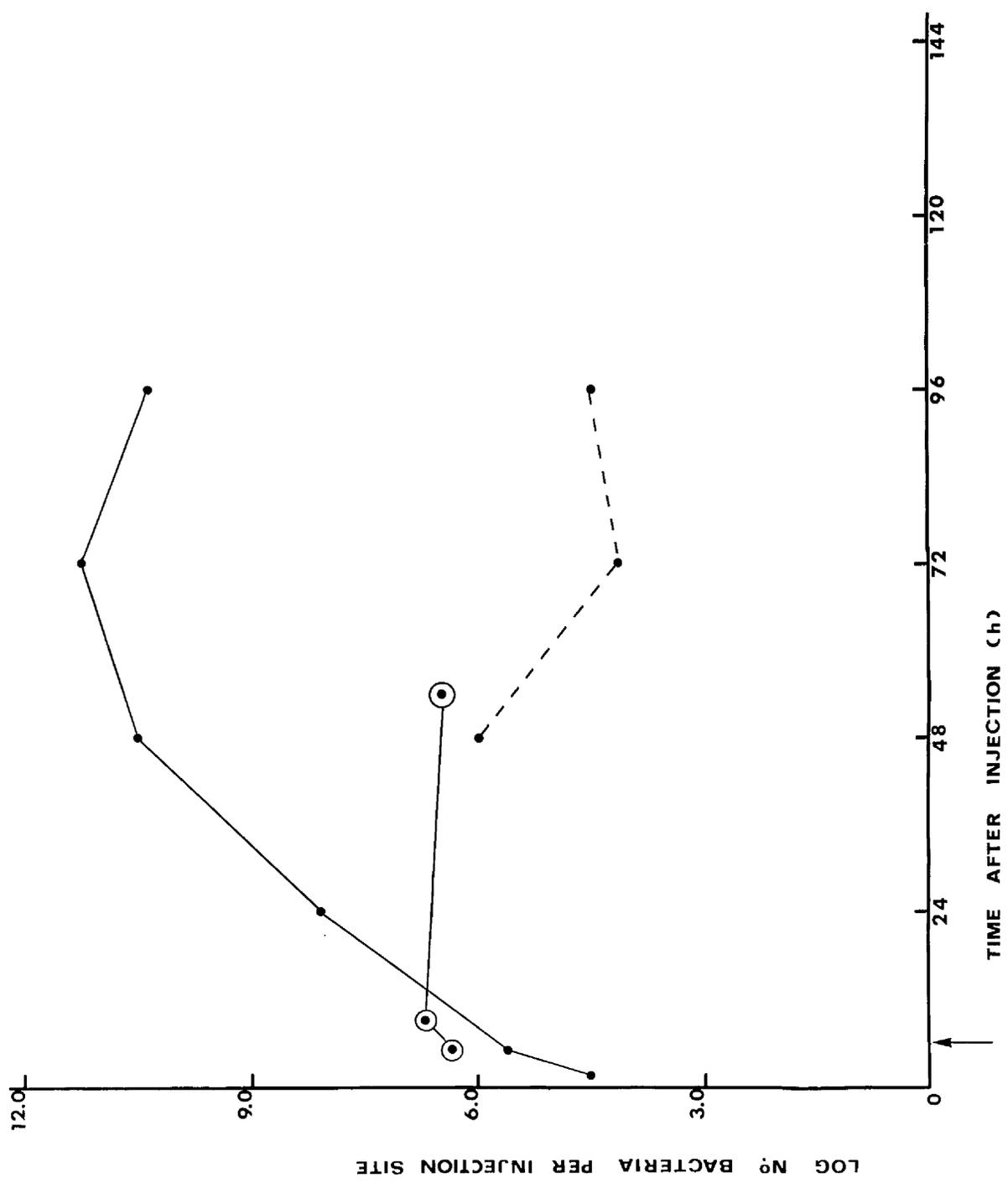
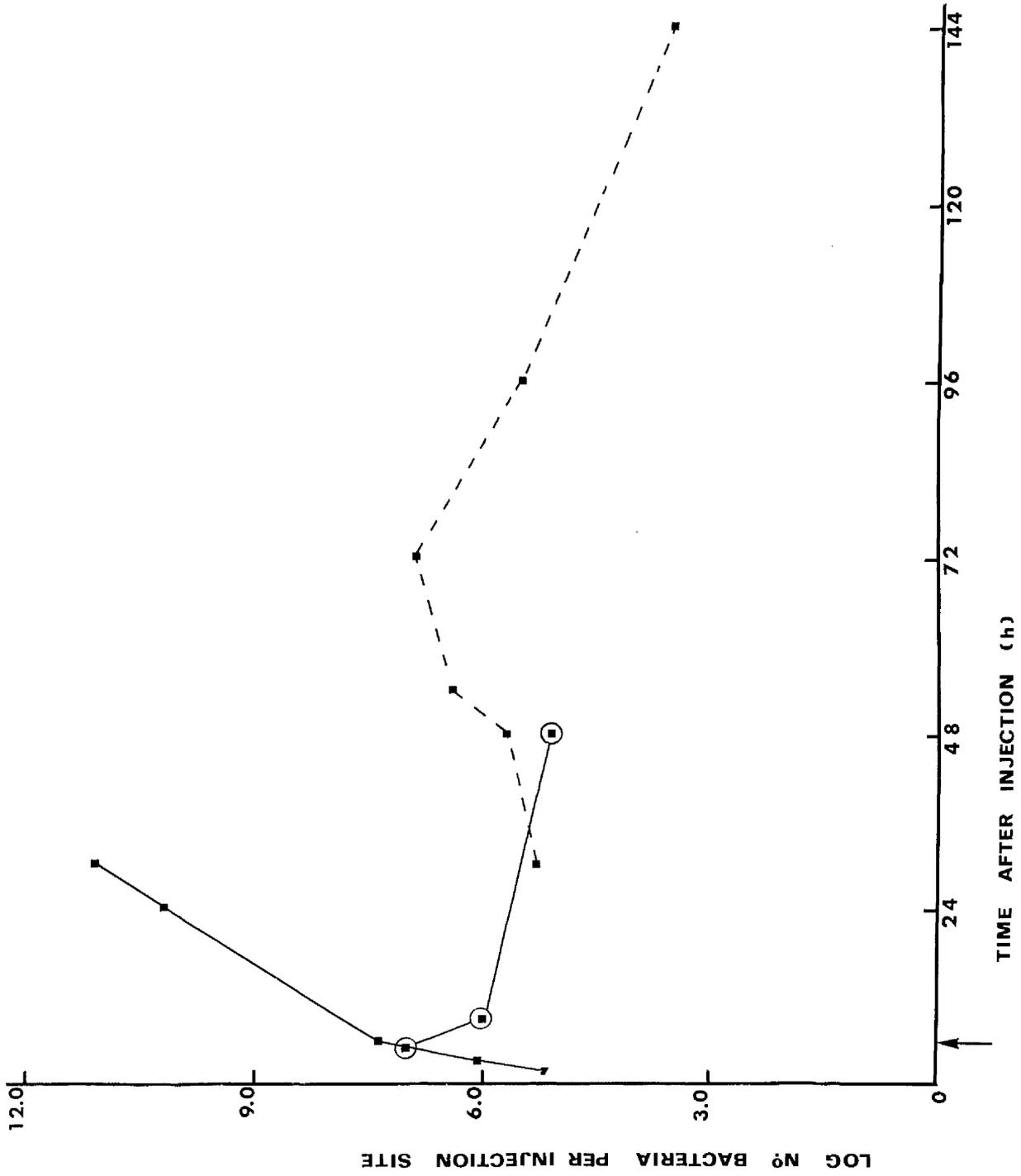


Figure 14: The effect of cephaloridine on the growth of strain SM 10 in the skin of 3-day old mice challenged with 10^6 c.f.u.

Symbols:

- : Log. no. of viable staphylococci recovered from a homogenate of skin taken from mice selected at random till 24 h and thereafter taken from mice which were moribund.
- : Log. no. of viable staphylococci recovered from a homogenate of skin taken from mice which were not ill at 30 h or thereafter.
- : Log. no. of viable staphylococci recovered from a homogenate of skin taken from mice which received a single dose of cephaloridine 6 h after challenge.
- ↑ : Time of administration of cephaloridine.



The 3-day old mice which had been challenged with SM 9 and SM 10 and subsequently treated with antibiotic not only survived the challenge but showed no lesions. Under these conditions, therefore, we were unable to demonstrate any effects of toxins which might have been produced in-vivo. However, these experiments confirm that multiplication of the staphylococci to a high level is necessary to produce lesions and death. It administration of the antibiotic was delayed till there were 10^8 organisms of each strain at the injection site (9 hours after challenge with SM 10 and 24 hours after challenge with SM 9) the outcome of the infection was unaltered, i.e. the mice died. However, the number of viable staphylococci in the lesion site about 20 hours after administration of the Cephaloridine was slightly less (approximately 10%) than in untreated control mice.

3. Histology of the lesions produced by SM 9 and SM 10

Macroscopically SM 9 produced a localised abscess and SM 10 produced a diffuse necrotic lesion. Histological examination of the skin of 3-day old mice 20 hours after challenge with 10^6 c.f.u. of either strain confirmed the macroscopic appearances. The skin sections were stained with -

- (i) Haematoxylin and eosin (H and E), and
- (ii) with Gram stain

Plate 3 shows normal mouse skin stained with H and E and plate 4 shows the same section stained by Gram stain. The stratum corneum (sc), epidermis (a) and dermis (d) are clearly visible.

Plate 5 shows the lesion produced by strain SM 9 stained by H and E and plate 6 by Gram stain. A classical abscess (A) can be seen beneath the

Plate 3: Histology of normal 3-day old mouse skin

Stain: haematoxylin and eosin

Symbols:

sc : stratum corneum

e : epidermis

d : dermis

Plate 4: Histology of normal 3-day old mouse skin

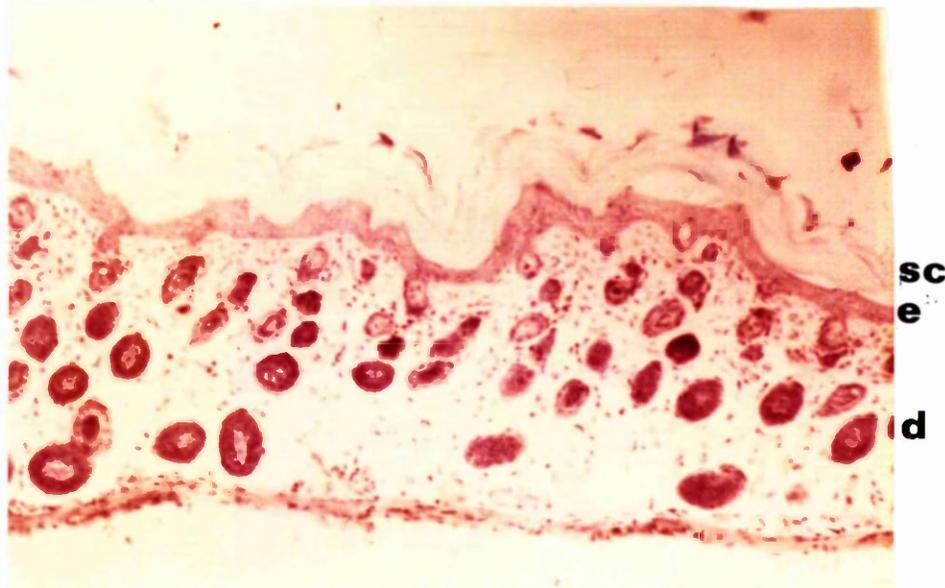
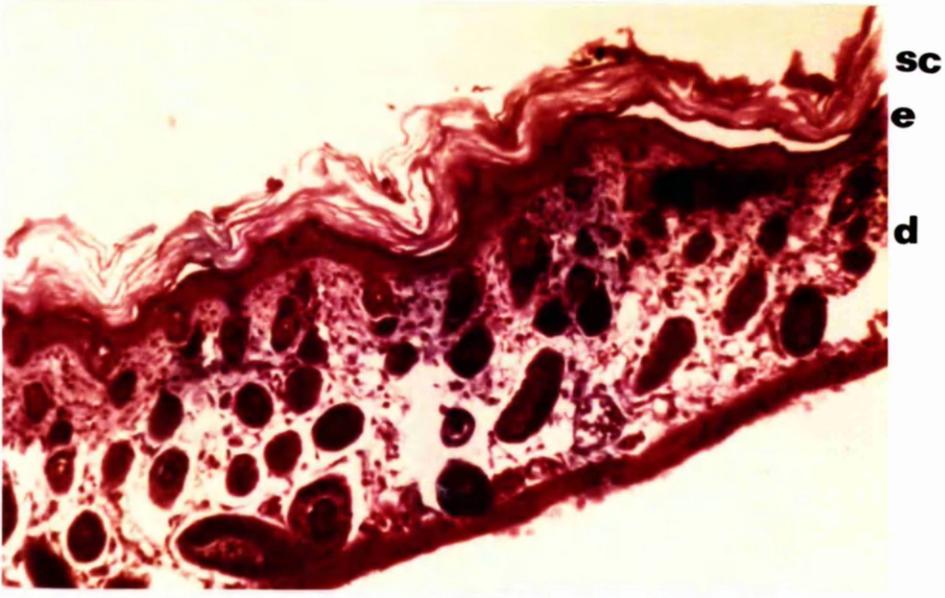
Stain: Gram

Symbols:

sc : stratum corneum

e : epidermis

d : dermis



**Plate 5: Histology of the lesion caused by strain SM 9
in the skin of 3-day old mice.**

Stain: haematoxylin and eosin

Symbol:

A : abscess

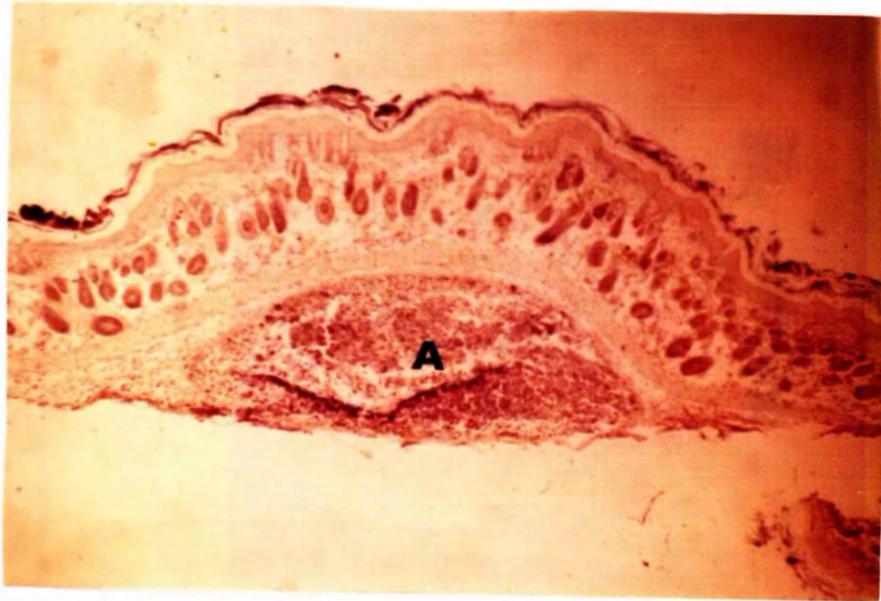
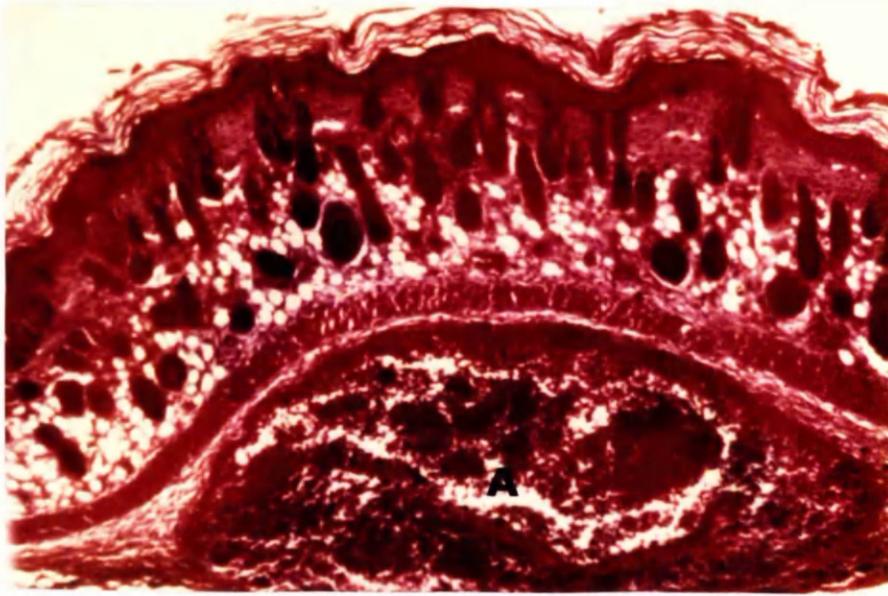
**Plate 6: Histology of the lesion caused by strain SM 9
in the skin of 3-day old mice.**

Stain: Gram.

**Staphylococci stained dark purple can
be seen within the abscess.**

Symbol:

A : abscess



dermis; this is characterised by localised infiltration of polymorphs and macrophages. The staphylococci can be seen stained dark purple (plate 6) within this structure. The dermis lying above the abscess has a normal appearance.

Plate 7 shows the lesion produced by strain SM 10 stained with H and E and plate 8 with Gram stain. The central area of the dermis is faintly stained and the cells appear disorganised and degenerate. This is an area of necrosis (N). The staphylococci stained dark purple (plate 8) are seen to have spread along the underside of the dermis.

These histological studies confirmed the differences in the lesion types produced by SM 9 and SM 10 as judged by their macroscopic appearance.

E. Cell Transfer Experiments

Whole peritoneal exudate cell (WPEC) suspension, macrophage-enriched cell (MEC) suspension and lymphocyte-enriched cell (LEC) suspension (1×10^5 cells of each) were injected intraperitoneally into 1-day old mice. Two days later (when the mice were 3-days old) each group was challenged with 10^6 c.f.u. of strain SM 9 and the cumulative mortality was recorded over a period of ten days. Control mice which had received Hanks BSS (see Appendix II) intraperitoneally were challenged in the same way. The results (number of mice dead and alive in each group) are summarised in Table 24. Using the χ^2 test each group was compared with the control group and p values are shown in Table 24. Because a multiple comparison was involved the results with LEC should be taken as showing a possibly significant effect. However, there was no significant difference between the survival of mice treated with either

**Plate 7: Histology of the lesion caused by strain SM 10
in the skin of 3-day old mice.**

Stain: Haematoxylin and eosin

Symbol:

N : necrosis

**Plate 8: Histology of the lesion caused by strain SM 10
in the skin of 3-day old mice.**

Stain: Gram.

**Staphylococci stained dark purple can
be seen below the dermis.**

Symbol:

N : necrosis

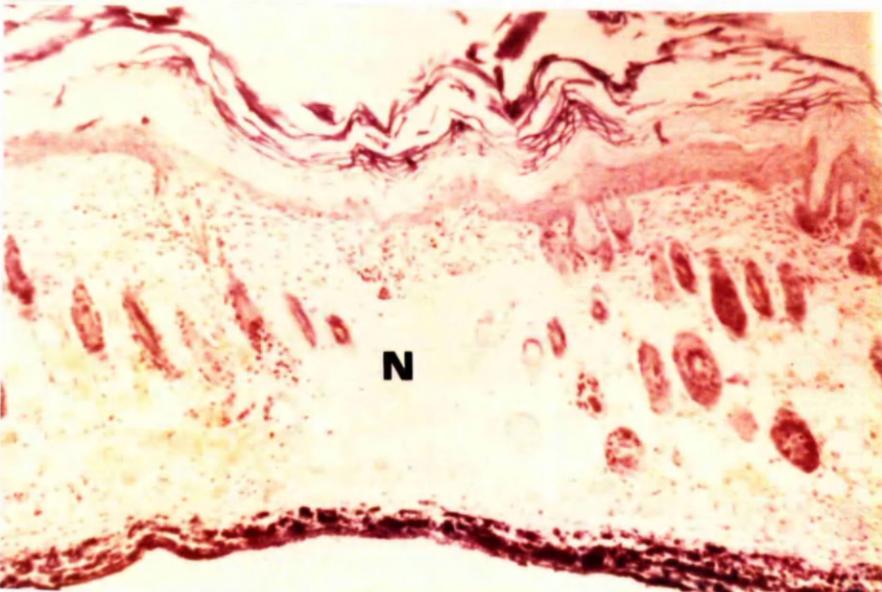
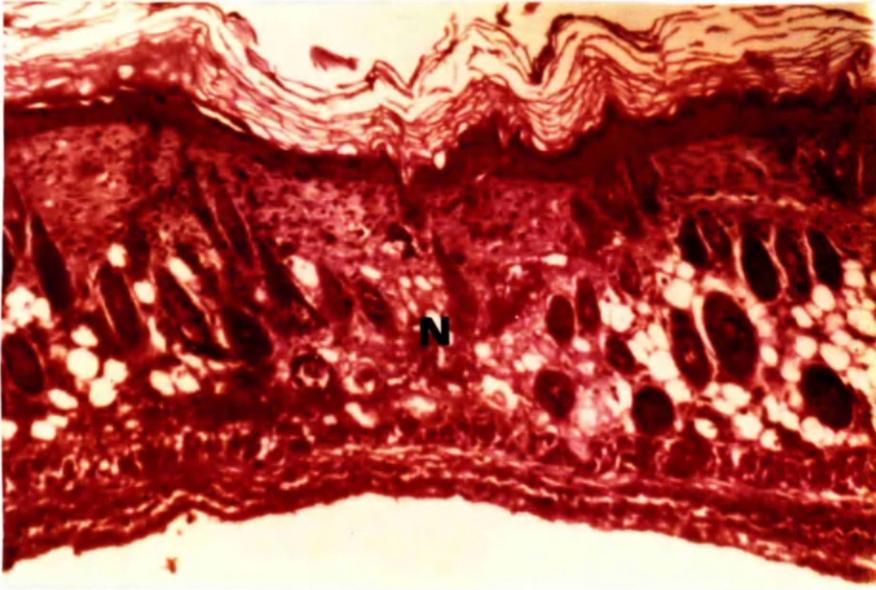


TABLE 24

The effect of adult leucocytes on the mortality of
3-day old mice challenged with 10^6 c.f.u. SM 9

Mice treated with	No. dead 10 days after injection	No. alive 10 days after injection	P (χ^2)
WPEC	14	20	0.1 - 0.2
MEC	38	41	0.1 - 0.2
LEC	28	45	0.01 - 0.05
Hanks BSS	33	28	...

whole peritoneal exudate cells or macrophage-enriched cell suspension and the control group in these experiments. This is difficult to explain since the whole peritoneal exudate contains lymphocytes. These are regarded as only preliminary experiments; further research is required to improve techniques of cell separation and transfer before more definite conclusions can be drawn about the protective effect of different adult cell types. The neonatal mouse model could be developed for such a study of immunological response of the host to experimental staphylococcal infection in addition to further study of the pathogenicity of Staph. aureus.

DISCUSSION

A. Natural Resistance to Staphylococcal Infection

Staphylococcus aureus colonises newborn infants within the first few days of life and thus begins a lasting commensal relationship. For the most part a balance exists between, immune defences of the host, the most important of which in this case is the mechanical barrier of the skin, and the pathogenic potential of Staph. aureus. However, when this balance is upset, for whatever reason, Staph. aureus may cause a great many diseases of varied nature (Table I). The organism produces many biologically active substances which have been the subject of much research. However, only two of these substances, namely staphylococcal enterotoxin and epidermolytic toxin, have been shown to be responsible for specific clinical symptoms. Still a mystery is the involvement of other staphylococcal products in the sequence of events which leads to the more common, and at times severe, pyogenic infections such as furunculosis, abscesses, wound infections and generalised staphylococcal disease.

Man, under normal circumstances, is extremely resistant to staphylococcal infection and this is true also of laboratory animals, a fact which has been a major drawback to progress in staphylococcal research. As mentioned earlier, the staphylococcal disease in which most progress has been made particularly in the understanding of the aetiology is Toxic Epidermal Necrolysis and undoubtedly a most important contribution was the discovery that the symptoms of the disease could be reproduced in the neonatal mouse (see p. 45). Toxic Epidermal Necrolysis

is a disease of infants and children and as argued in the Introduction to this Thesis, man appears to be most susceptible to this and other staphylococcal diseases in infancy.

All of these facts influenced the direction of this investigation towards developing an animal model for experimental staphylococcal infection. For this study a group of staphylococcal strains, including fresh clinical isolates from children, with varied toxin and enzyme patterns was selected.

Strain Wood 46 (SM 1) was included as a classical α -toxinogenic strain, strain BB (SM 15) as a β -toxinogenic strain of animal origin, Staph. epidermidis as a non-toxinogenic strain, strain PS 80 (SM 16) was included as a known virulent human strain which has been widely used in experimental staphylococcal infections in laboratory animals. The other three strains in our study, i.e. SM 9, SM 10 and SM 14, all clinical isolates, had varied toxin/enzyme patterns; of particular note was the fact that SM 9 was virtually non-toxinogenic and produced neither of the two proteases for which we tested. The first objective was to study the response of mice of different ages including neonates to experimental infection by the six strains. The subcutaneous route was chosen, because the most common staphylococcal diseases are associated with the skin and subcutaneous tissues.

I. Age-related susceptibility

This work has established, apparently for the first time, that mice exhibited an age-related susceptibility to experimental staphylococcal

infection when challenged by the subcutaneous route. This was most probably related to the immunological status of neonatal animals in comparison with adults. With few exceptions, which will be discussed later, there has been little work on the virulence of bacterial species in neonatal animals. The greatest contribution to the understanding of immunological development in neonatal animals has come from research in virology. It is well established that mice demonstrate age-related susceptibility to many experimental viral infections (Sigel, 1952; Sawicki, 1961; Marrenikova and Kaptsova, 1965; Hirsch, Zisman and Allison, 1970; Reinarz, Broome and Sagik, 1971 and Hirsch *et. al.*, 1972) and this has led several groups to study the contribution of different immunological cell types to protection during maturation. For this reason, in discussing the possible immunological reasons for the age-dependent susceptibility of neonatal mice to staphylococcal infection I will refer mainly to the work of virologists. While it is true that we cannot draw any direct conclusions about age-related susceptibility to staphylococci from experimental infections caused by viruses, there are certain similarities which bear on the immunological competence of the host against invasion by both types of infecting agent in several instances:-

- (i) The skin is an effective barrier to both, each may take advantage of a break in the skin to set up infection.
- (ii) Both are phagocytosed by macrophages in cutaneous and subcutaneous tissue. If these macrophages subsequently fail to destroy the ingested microbe the infectious process may begin.

2. Immunity and age-related susceptibility

Non-specific resistance is a function of the phagocytic cells, i.e. the macrophages and the polymorphonuclear leucocytes. It has been shown that neonatal mice and rats lack immunocompetence because they lack antigen recognising cells in the form of functional macrophages (Argyris, 1968; Hirsch et. al., 1970) and that functional development of the reticuloendothelial system (RES) is related to the macrophage population (Reade and Casley-Smith, 1965). However, the immunological differences between neonatal and adult macrophages do not appear to be related to differences in phagocytic activity since it has been shown in vivo that phagocytes from newborn and weanling mice are equally able to clear carbon particles from the blood (Hackbarth, Reinarz and Sagik, 1973) and in vitro that macrophages from newborns, weanlings and adults take up labelled virus (Hackbarth et. al., 1973) and sheep RBC (Ishiguru, 1971) with equal facility. Competence seems more likely to be related to differences in bactericidal activity within the macrophage. During the development of the RES of the rat the phagocytic cells of the liver do not develop bactericidal properties (measured against Esch. coli (Lilly) and Salm. typhimurium C5) until the latter part of the suckling period (Karthigasu, Reade and Jenkin, 1965).

With reference to staphylococcal infection of man it is well established from studies of children with chronic granulomatous disease that functional phagocytic cells are of prime importance (Quie et. al., 1967 and Quie and Davis, 1973). These children who suffer from recurrent staphylococcal infection show no impairment of the phagocytic function

of the macrophages but bactericidal activity after phagocytosis is deficient.

The results presented here support the notion of equal phagocytic ability of newborn and weanling mice, as can be seen from the number of staphylococci in the injection site 1 hour after challenge of 21-day old and 3-day old mice. This number was almost the same for both age groups challenged with both strains SM 9 and SM 10 at 10^6 c.f.u. (Fig. 12). The subsequent multiplication of these staphylococci which was markedly different in the two age groups may have been due to differences in bactericidal activities within the macrophages of the neonatal and weanling mice. Certainly it has been shown with some experimental viral infections, that maturation of macrophages with age was important in limiting the spread of infection (Johnson, 1964; Zisman, Hirsch and Allison, 1970).

The preliminary studies of the effect of adult cells indicated that adult macrophages may indeed have protected neonates from the otherwise lethal effects of staphylococci although considerable further work is required to investigate this aspect. In evaluating the role of the monocyte and the neutrophil, one important biological process which must be considered is chemotaxis. I have taken part in a collaborative study of the chemotactic ability of the products of some of our selected strains with colleagues expert in the techniques of measurement of chemotaxis. Adult human blood neutrophils and monocytes were used and results indicated that staphylococci and their products had a direct chemotactic effect for monocytes, and an indirect effect for neutrophils and that Staph. aureus strains also produced factors which possessed inhibitory activity against migration of both cell types (Russell et. al., 1975). Extension of this study to include adult and neonatal

mouse cells may help to elucidate the role of these cells in the response of mice to staphylococci.

The humoral immune response of neonatal mice was not completely competent in producing antibodies to sheep RBC although this varied within strains of mice (Playfair, 1968). It has been suggested that the inability of the neonatal mouse to produce antibodies is due to immature macrophages (Braun and Lasky, 1967 and Argyris, 1968).

In the past there has been much controversy about the protective ability of humoral immunity against infection by Staph. aureus in adult laboratory animals. However, it is now generally accepted that antibodies do contribute a measure of immunity to some types of staphylococcal infection. It has been recently proposed by Easmon and Glynn (1975) that in local infections in mice, circulating α -antitoxin was protective against dermo-necrosis because it became involved in local antibody-antigen interaction, and effected a brisk inflammatory response which proved protective. It seems possible, therefore, particularly in local lesion development, that the immature humoral response may contribute to neonatal susceptibility to staphylococcal infection in the mice studied here. It should prove possible to test this suggestion experimentally by transfer of competent antibody-producing cells from adult mice and/or immune sera.

There is little information on the comparative status of specific cellular immunity (SCI) of neonatal and adult mice or on the maturation of this system. However, it seems likely, since it is known that macrophages and antibodies (cytophilic) are involved that neonatal mice do not have

competent specific cellular immunity.

Because healthy human adults and animals are continuously exposed to staphylococcal antigens due to carriage of staphylococci on the body surface, it has been suggested that a constant level of SCI is responsible for a high natural resistance to Staph. aureus (Gutkowski, Pryjma and Heczko, 1973). Lack of this could be a contributory factor in age-related susceptibility. The preliminary cell transfer experiments indicated that lymphocytes from adult mice possibly protected neonates from challenge with strain SM 9 (Table 24). However, the studies of Gutkowski et. al., and this work are in contrast to the idea that SCI or delayed-type hypersensitivity contributes to enhanced staphylococcal infectivity. In my view much of this work involved extremely complex experimental systems in which many cellular and humoral components probably contributed to the response. For example, one such study involved the use of cotton dust, turpentine, staphylococci and anti-lymphocyte serum from normal and immune mice (Medhurst, Hill and Glynn, 1969). While such experimental systems may be useful they are obviously very complex. The neonatal mouse - a host lacking mature immunological processes - is probably a better and simpler experimental system for the study of the pathogenicity of Staph. aureus and possibly other bacteria. By transfer of adult cells and serum etc. to the neonates, it should be possible to determine the contribution of these to the resistance of the adult mouse and to what extent individual components of the immune system are involved in different phases of the response to staphylococcal challenge. Obviously, care should be taken in generalising about the host factors which determine the outcome of the interaction between the host and the

staphylococcus. As will be discussed later in the following section strains differ in virulence and some strains such as PS 80 (SM 16) and BB (SM 15) were capable of overcoming the host defences of 21-day old mice more efficiently than others.

B. Staphylococcus aureus as a Pathogen

Clearly under certain circumstances Staph. aureus is a potent pathogen. It was pathogenic for neonatal mice, causing severe lesions and death. In this host, we have an opportunity to consider some of the factors which contributed to the pathogenic process by studying their response to characteristically different strains of staphylococci.

I. Differences in virulence

Differences in virulence between strains of Staph. aureus have been demonstrated in mice injected intraperitoneally, intrarenally, intracerebrally, intravenously and subcutaneously. (Elek, 1965; Pereira and Goldberg, 1970; Kimura, 1971; Kondo et. al., 1971; Anderson, 1971; Chesbro, Taylor and Smith, 1973; Easmon, Hamilton and Glynn, 1973). There were obvious differences in virulence between our seven strains of staphylococci on subcutaneous injection both in neonates and older mice. (Table 15)

Strain SM 6 (Staph. epidermidis) was avirulent except at the high dose of 10^8 c.f.u. in 3-day old mice. Although the present study included only a single strain of Staph. epidermidis the results obtained with this strain lend support to the long-accepted belief that coagulase-negative strains can be considered non-pathogenic except in unusual circumstances in which

the organism gains access to a particularly susceptible site such as damaged heart valves. The relatively high virulence of SM 1 (Wood 46) was surprising in view of previous reports that this strain was avirulent in mice particularly in local skin infections (Agarwal, 1967 b and Easmon and Glynn, 1975). The similar virulence of strains SM 9 and SM 10 was also unexpected and was difficult to explain in terms of the known extracellular virulence factors produced by these organisms. This will be discussed more fully in the following sections.

Bovine strains have been reported previously to differ in virulence and, in particular, strain BB has been classed as a strain of high mouse-virulence (Anderson, 1971 and Anderson, 1974). The present results which agree with this confirmed the high virulence SM 15 (BB) for mice in all three age groups. However, the aim of this work was to study the relative mouse virulence of human strains. Highly mouse-virulent strains have been isolated by previous workers (Smith, 1963) who suggested that the ability to clot mouse plasma was an important factor; none of the strains used in this study coagulated mouse plasma.

Two further strains of human origin, SM 14 and SM 16 (PS 80), were also of high virulence. It is noteworthy that β -toxinogenic human isolates (cf SM 14) have previously been associated with mouse virulence (Chesbro *et. al.*, 1973). PS 80 is an unusually virulent strain of Staph. aureus, not only for the mouse but also for man. Clinical evidence of this strain causing boils in otherwise healthy individuals suggests that it possesses the unusual ability to penetrate intact normal human skin (Wilson and Miles, 1975).

Although there is an association of particular phage types with certain lesions (see p. 25), it is interesting to note that two of the very rare reports of experimental staphylococcal infections in man produced no evidence of differences in virulence of Staph. aureus in the human host (Elek and Conen, 1957 and Foster, 1960).

2. Evidence for contribution of specific virulence factors

The factors which contribute to differences in the virulence of Staph. aureus for laboratory animals (and possibly man) have been the subject of the major field of staphylococcal research in the past.

The contribution of these virulence factors to the pathogenesis of Staph. aureus during experimental infections is difficult to assess for reasons which include the difficulty of detecting such factors as toxins in vivo and the multifactorial nature of the infectious process. Despite this, some toxins and other biologically active staphylococcal products have been detected in vivo and the pattern of in-vitro production of biologically active factors has been correlated to some extent with specific effects seen during experimental infection. Some of these were evident in our experiments.

Alpha- and delta-lysins have been shown to be major factors contributing to tissue necrosis both locally in the skin (Takeuchi and Suto, 1974; van der Vijver et. al., 1975) and in deeper tissues (Foster, 1967 and Anderson, 1974). Our study agreed with these findings since only the toxin-producing strains and their cell free culture filtrates were able to produce necrosis in weanling or neonatal mice. In particular, the comparative histological study of the toxinogenic strain SM 10 and the non-toxinogenic strain SM 9 showed the probable

importance of α -lysin in the development of necrosis. Pre-treatment of neonatal mice with mono-specific α -antiserum (kindly donated by Dr. C. Adlam, Wellcome Laboratories, Kent) reduced the necrosis caused by SM 10 as judged by the macroscopic appearance. Unfortunately, because of the small quantity of mono-specific serum available and the practical difficulties in handling damaged (i.e. necrotic) skin tissue from neonatal mice, it was not possible to confirm this histologically. Despite the difference in the lesions produced by these strains, both are equally virulent in terms of number of mice which develop lesions and die. This is evidence against α -toxin as an overriding determinant of virulence.

Protease production by Staph. aureus has also been correlated with virulence for the mouse (Chesbro et. al., 1973) and again our comparative study of SM 9 and SM 10 agreed with this.

Strain Wood 46, the only Protein A-negative Staph. aureus in this study was similar in virulence to SM 9 and SM 10, both Protein A-positive strains. This argues against Protein A being a major determinant of virulence in the neonatal mouse.

Furthermore, leucocidin-producing ability did not seem to be directly related to virulence but, as implied earlier, a more detailed study of the ability of extracellular products of Staph. aureus to affect movement of specific cells of the RES may lead to a better understanding of leucocidal factors and their role in pathogenicity.

Although this study was not directly concerned with the presence or

absence of DOCR (see p. 39) on the cells, the group of staphylococci used included a DOCR-positive strain (SM 16 - PS 80) and a DOCR-negative strain (SM 1 - Wood 46). The greater virulence of strain PS 80 in the neonatal mouse fits in with the idea that DOCR, acting as an aggressin, enhances virulence (Glynn, 1972). It would be interesting to look for DOCR amongst the other three strains of Staph. aureus, particularly strain SM 9, and also to study the effect of purified DOCR on the development of lesion in the neonates.

An important point to stress is that all the strains used in this study of virulence, including those freshly isolated from clinical specimens, were subcultured at least twice on laboratory media before freezing in liquid nitrogen for storage. Since the virulence of Staph. aureus is increased by passage through laboratory animals (Smith, 1958; Beining and Kennedy, 1963; Adlam, Pearce and Smith, 1970 a,b), it is possible that the virulence patterns of our group of seven staphylococci would be different if passaged organisms had been used. Indeed, the virulence for neonatal mice might be further increased. Also, there is some evidence that growth media may be able to enhance the virulence of Staph. aureus (Brock, Turvey and Reiter, 1973). Having established the pattern of results under standard conditions it should prove possible to proceed to an examination of in-vivo grown organisms and organisms grown under various conditions in vitro.

C. Growth of Staph. aureus in vivo

Detailed information about mechanisms of pathogenicity and virulence of bacteria can best be obtained from studies of those bacteria in-vivo because

It is then that the pathogenic processes are in action. Studies in-vitro although helpful may be misleading. The ability to establish local lesions reproducibly in the skin of neonatal mice made it possible to examine in some detail the growth of two strains of Staph. aureus in-vivo. Both strains, i.e. SM 9 and SM 10 were clinical isolates; one, SM 9, was a non-proteolytic, non-toxinogenic strain while the other, SM 10, was proteolytic and toxinogenic, producing predominantly α -lysin. In addition to studying and comparing the growth during lesion development in 3-day old mice, the numbers of organisms in the skin of 21-day old mice was monitored. These weanling mice suffered no observable ill effects on subcutaneous challenge with either strain at a dose of 10^6 c.f.u.

I. The Initial Inoculum

Of the initial inoculum (10^6 c.f.u.) only about 10% was detected at the injection site 1 hour after challenge. This was true for both 3-day old and 21-day old mice challenged with strains SM 9 and SM 10 and we have already discussed the possible significance of this in relation to the comparative populations of phagocytic cells and their immunocompetence in both age groups of mice. The percentage of inoculum detected at 1 hour after challenge was also 10% when strain SM 9 was administered at a dose ten fold higher (10^7 c.f.u.) and a hundred fold lower, i.e. 10^4 c.f.u. This may indicate that for any inoculum of staphylococci, up to at least 10^7 c.f.u. about 10% of the organisms are able to lodge in the skin near to the injection site. This is difficult to explain in terms of actual numbers of organisms but could possibly have been related to the volume of the inoculum.

The outcome of this initial lodgement of staphylococci was dependent on the subsequent ability of the organisms to multiply in the skin. In 21-day old mice challenged with 10^6 c.f.u. of SM 9 and SM 10 there was initial slight multiplication from 24 - 48 hours (a maximum increase in numbers of a hundred fold) but after this time the numbers of staphylococci in the skin fell off rapidly and no lesions were visible. It seems possible, as has already been surmised, that this was a manifestation of non-specific resistance, i.e. the bactericidal capacity of the macrophages and neutrophils in subcutaneous tissues. Was this also true for the 40% of the neonates which survive the infection? I have pointed out previously that by 36 - 48 hours after challenge it was easy to distinguish these neonatal mice which were going to survive. It is important that these mice by 48 hours after challenge are 5 days old and may possibly have undergone a crucial immunological development capable of limiting the multiplication of the injected staphylococci. Certainly, it was established that the onset of resistance, as measured by survival after a challenge of 10^7 c.f.u. SM 9, was fairly narrowly demarcated between 4 and 5 days of age (Table 17). A difference of 24 hours in age meant a considerable difference in the response of neonatal mice to a single challenge dose.

It was equally clear that in 60% of the neonatal mice the staphylococci were able to multiply with great rapidity and in these animals the multiplication rates of the two strains SM 9 and SM 10 were compared.

2. Kinetics of growth in the skin and invasiveness

Both of the strains SM 9 and SM 10 were able to multiply in the skin and underlying tissue to produce severe lesions and death in neonatal mice. SM 9,

the non-toxinogenic strain, multiplied more slowly than SM 10 (Mean generation time 2.29 hours and 1.76 hours respectively). At both the gross and histological levels this strain produced an abscess type of lesion and mice began to die from 36 hours onwards.

The toxinogenic strain SM 10 produced diffuse necrotic lesions and mice began to die from 24 hours onwards. Since strain SM 9 caused similar mortality to strain SM 10 it seems likely that a main contribution of α -lysin is to allow faster multiplication of the organisms in the tissues. It remains to be determined whether it does this by breaking down surrounding tissues and cells, consequently allowing the organisms to multiply faster, or as suggested by van der Vijver and co-workers (1975), it has a growth-promoting effect. These authors found that the number of c.f.u. in skin lesions caused by a mutant strain of Staph. aureus lacking the ability to produce α -lysin was lower than that caused by the wild type. Also in the skin of mice Takeuchi and Suto (1974) observed α -lysin (as well as protease, nuclease and β -lysin) production was associated with multiplication in a suppurative focus. Their diagrammatic representation of the histology of the formation of an abscess and a necrotic lesion in their animals was very similar to the histology observed in this study. They reported the same typical features, i.e. confinement of the staphylococci within the abscess structure by the phagocytes during abscess formation and sub-dermal spread of the organisms, with necrosis of adjacent tissue, in necrotic lesion formation. Anderson (1974) also reported that multiplication of the bovine strain BB in the mammary gland of the mouse was associated with the presence of α -lysin within the gland and the relative failure of strain Mexi to multiply in the same in-vivo situation was associated with the

absence of detectable α -lysin within the gland. Furthermore, Kapral (1974) demonstrated the importance of the production of α -lysin as a contributing factor in intraperitoneal and intrapleural infections of mice and rabbits and suggested that the survival of organisms within abscesses correlated with their ability to produce α -lysin.

From our comparative studies of SM 9 and SM 10 in the 3-day old mice there was also evidence that SM 10 was more invasive as judged by its presence in the liver from 1 hour after challenge and thereafter in increasing numbers (up to 10^4 c.f.u.) until the animals died. Organisms of SM 9 disappeared from the liver by 6 hours after challenge and did not appear again until multiplication had taken place in the skin to a level of about $10^8 - 10^9$ c.f.u. The α -lysin has been connected with invasiveness in mice challenged intraperitoneally (Kimura, 1971). Further evidence from our results was provided by the histology of the lesions (plate 8) where it can be seen that SM 10 was able to spread beneath the dermis and presumably could reach other sites more easily than SM 9 which was confined at least in the early stages of infection (18 - 20 hours) by the phagocytes.

While strain SM 10 multiplied faster in the skin of 3-day old mice than SM 9, an important feature of this process was that both strains reached the same final level in the skin (between 10^{10} and 10^{11} c.f.u.) just before death of the mice. Also at this time the same number of organisms were detected in the liver (around 10^4 c.f.u.) in mice injected with both strains. By virtue of the faster multiplication of SM 10 this occurred earlier with SM 10 (24 - 30 hours) than with SM 9 (36 - 38 hours). The

final numbers of staphylococci detected in the skin and the livers of neonates injected with 10^7 c.f.u. SM 9 was also similar. These findings suggested that if the number of staphylococci in the tissues of mice reached a certain "threshold level" these mice died. Of interest and possible significance here is the fact that 3-day old mice challenged with Staph. epidermidis died if the dose was high enough (10^8 c.f.u.). More extensive in-vivo growth studies in neonates with this strain may confirm the conclusion about a "threshold level" of organisms. There have been previous reports correlating death in adult mice challenged by different routes with the final concentration of staphylococci present in the tissues (Smith et. al., 1960; Gorrill and McNeil, 1963 and Bartell et. al., 1968).

The clinical response and the macroscopic appearance of the mice correlated well with the number of staphylococci recovered from the injection/lesion site. This was true also in the adult mice used for the study of staphylococcal mastitis (Anderson, 1974) who showed that between 10^{10} and 10^{13} c.f.u. were recovered from the mammary glands of mice which were ill. Prevention of death and reduction in severity of the lesions was achieved when Cephaloridine was administered 6 hours after challenge (Figs. 13 and 14) when the number of staphylococci present in the lesion site was around 10^4 c.f.u. (SM 9) and 10^5 c.f.u. (SM 10). However, delay in administration of Cephaloridine until the number of both strains SM 9 and SM 10 was 10^8 c.f.u. did not prevent death and the number of organisms recovered in the antibiotic treated mice just prior to death was still of the order of 10^{10} c.f.u. Further experiments of this type administering different antibiotics at different times during experimental infections should lead to a clearer

understanding of the significance of the "threshold level".

In terms of lesion development, skin specimens taken from mice which had an observable macroscopic lesion rarely contained less than 10^7 c.f.u. and often much larger numbers of staphylococci. If total numbers of staphylococci are as important as these results suggested this is evidence in favour of the clinical procedure of drainage of abscesses which has been a successful treatment since before the advent of antibiotics and still is today.

Although there appeared to be a definite correlation between the number of staphylococci, the clinical state and the eventual death of the animal, the actual cause of death of the mice has not yet been defined. A key question remains: is the "threshold level" of organisms associated with elaboration of lethal amounts of diffusible toxic factors? Further research in the newborn mouse should throw light on this question.

In conclusion, it has been shown that mice exhibit an age-related susceptibility to subcutaneous infection by Staph. aureus which was not found with the limited number of other bacterial species studied. Strains of Staph. aureus vary in virulence in this model. The growth of two characteristically different strains of Staph. aureus was studied in vivo in relation to kinetics of growth and histological features in the skin, the presence of the staphylococci in other organs and the relationship of numbers of staphylococci to the clinical state of the mice. This experimental model we hope will prove a useful tool for further study of the pathogenicity of Staph. aureus and I have listed briefly below the major areas in which I feel it will contribute.

D. Proposed Future Studies in the Neonatal Mouse

- (a) To study the transfer of specific adult cells of varying immunocompetence to the neonatal mice and their effects on the development of lesions and death.
- (b) the effect of specific antibodies on the in-vivo growth rates and lesion development.
- (c) the comparative virulence of mutants of Staph. aureus lacking specific virulence factors.
- (d) the effectiveness of different antibiotics in-vivo.
- (e) the detection of toxins and enzymes in vivo during growth.
- (f) the virulence of specific strains in mice with natural and induced immunological defects.
- (g) the extension of the investigation to include germ-free mice and the effects of exposure to staphylococci in the environment on their susceptibility.

E. Perspectives

The improved standards of hygiene and living conditions and the advent of antibiotic therapy have led to a greatly reduced incidence of serious staphylococcal disease. However, Staph. aureus is still clinically a major pathogen for man, particularly in neonates and in hospitalised patients. Although some strains of Staph. aureus, particularly strain PS 80, apparently show exceptional virulence for man, differences in virulence for humans is very difficult to quantify. Also, it is not justified to monitor changes in virulence of the organism by studying only the incidence and mortality of

staphylococcal infections in man. It must be remembered that the type of hospital patient is always changing as a result of progress in medicine and surgery.

Since the original experiment of Garré in 1885, in which he produced boils by rubbing staphylococci into his arm, very little progress has been made in the understanding of experimental infections of man, for obvious reasons. Intradermal injection techniques required large numbers of organisms to produce small boils. Elek and Conen (1957) had some success in reducing the number of organisms required to cause a large abscess by introducing the inocula on sutures, but lesions produced in this way do not resemble the common lesions of spontaneous infection, i.e. the boil. More recently, Marples and Kligman (1972) have produced acute cellulitis in humans by placing as few as 100 c.f.u. on skin from which the outer layer of the epidermis had been stripped. The severity of the lesion depended on several factors including the need to keep the area moist, and the time between epidermal stripping and application of the organism. The shorter this time, the more severe the lesions presumably because delay meant infiltration of leucocytes in response to the tissue damage, i.e. the stripping process. This is similar to the ability of cotton dust to enhance local infection in the mouse by delaying the onset of the inflammatory response.

Widespread use of antibiotics in the treatment of infection has led to the emergence of plasmid-carrying antibiotic resistant strains. Clinical evidence suggests that plasmid-carrying staphylococci may be less rather than more virulent because the cellular processes required for their

replication are more complex and therefore, reduce their chances of survival (Lacey, 1975). Acquisition of plasmids by staphylococci could, therefore, be important in reducing their virulence for man.

Protection against staphylococcal infection has proved extremely difficult and this may be because the diseases caused by Staph. aureus are very different in nature. It may prove possible to protect against some types of infection, e.g. against bovine mastitis where the infection is localised in the mammary gland.

Recent studies have shown very successful protection of neonates from nursery epidemics of staphylococcal infection by colonising the infants in the first hours of life with a low-virulence strain 502A (Shinefield et. al., 1974). These workers claim that colonisation with this strain has helped as a control measure in at least eight nursery epidemics and this technique will undoubtedly prove important in other situations, although side effects were noted including one fatality amongst the 4,000 neonates treated.

Animal models may not necessarily be applicable to humans but they are necessary as a tool for further research into the pathogenicity of Staph. aureus. Information obtained in using them although perhaps not directly related to human infections should at least contribute to our general understanding of the staphylococcus and its interaction with the host and may serve as a basis for approaches to the problems of infections in man.

SUMMARY

SUMMARY

Several strains of Staphylococcus aureus, all clinical isolates, were tested for haemolytic activity and for the production of extracellular enzymes. From these strains four were chosen for their varied toxin and enzyme patterns. Also included were two reference strains of Staph. aureus, Wood 46 and BB. Each of these six strains was tested for antibiotic sensitivity, production of leucocidin and the presence of protein A on washed cell suspensions. In-vitro growth rates were also measured.

These six characteristically different strains were used in a study of virulence for the mouse. Suspensions of known viability of the strains were prepared, dispensed in small aliquots and frozen in liquid nitrogen. This allowed the same suspension of organisms to be used for animal experiments carried out over a long time. The initial virulence studies were made in three age groups of mice, 3-day old, 10-day old and 21-day old. The response of the mice to subcutaneous challenge with the strains, i.e. death or the development of lesions was recorded. As measured by these criteria the neonatal mice were most susceptible to infection by each strain, although strains varied considerably in virulence. Age-related susceptibility to the four organisms, Esch. coli, P. aeruginosa, Klebsiella sp and Strep. pyogenes, although evident, was less marked than to the staphylococci. Further work is required to determine how widely this phenomenon applies to organisms other than Staph. aureus.

The strains of Staph. aureus varied in virulence as measured by the ability to cause death and to produce lesions in the mice. These lesions were of two types; a diffuse, necrotic type and a localised abscess type. It was noted that the necrotic lesions were produced by the toxinogenic strains and the abscess

lesion was produced by the non-toxinogenic strain, SM 9. Further evidence that toxins (particularly α - and β -lysin) were the cause of the necrosis was obtained by injecting cell-free culture filtrates of each strain into the mice. Filtrates from the toxinogenic strains produced necrosis similar to that observed in a lesion caused by whole organisms. The filtrate from strain SM 9 produced no observable effect. Histological examination of the lesions produced by SM 9 and SM 10, a toxinogenic strain of similar virulence to SM 9, confirmed that necrosis was associated with the toxin-producing strain.

Further comparative growth studies of these two strains were made in 3-day old and 21-day old mice. Numbers of staphylococci present in the injection/lesion site and the liver were counted. Both SM 9 and SM 10 multiplied to a very limited extent in the skin of 21-day old mice but very rapidly in the skin of 3-day old mice. Staphylococci were not recovered from the livers of the weanling mice but were detected in livers of neonatal animals. SM 10 multiplied faster in the skin of the neonates than SM 9 and was present in the liver from the initial stages while SM 9 did not appear in significant numbers until late in the infection. It is suggested that both these properties are associated with the ability of SM 10 to produce α -toxin.

Although the two strains multiplied at different rates, just prior to death the number of staphylococci recovered from the tissues of the neonates was the same for both strains. Also there was good correlation between the clinical state of the mice and the number of organisms in the tissues. Administration of Cephaloridine at different times during the infection confirmed that actual numbers of organisms were an important feature of staphylococcal infection in this model. The possibility of the final number of organisms in the tissues as a direct cause of death is discussed.

Possible future studies involving the neonatal mouse are suggested.

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APPENDICES

Appendix I

Media1. Horse Erythrocyte Agar

Oxoid Blood Agar Base No. 2	90 ml
Sterilised at 15 lb/in ² and cooled to 56°C	
Saline washed horse erythrocytes	10 ml

2. Semi Solid Agar

Oxoid Nutrient Broth No. 2	25 g
Oxoid Blood Agar Base No. 2	10 g
Distilled water to a final volume of	1,000 ml

3. CCY Medium (Gladstone and van Heynigen, 1957 as modified by Woodin, 1959)Yeast Diffusate

Yeast extract (Oxoid)	120 g
Distilled water	85 ml

Dialysed (1.5 cm Visking tubing) against 770 ml distilled water at 4°C for 24 h with constant stirring.

Casamino acid	160 g
Yeast diffusate	1,600 ml
Sodium lactate (70% syrup)	113.6 ml
Sodium glycerophosphate	160 g
Mg SO ₄ .7H ₂ O	0.16 g
Mn SO ₄ .4H ₂ O	0.084 g

Appendix II

Buffers and Diluents1. Phosphate Buffered Saline pH 7.0

$\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$	120 g
NaCl	22.5 g

Dissolve in 3 litres distilled water. Add 11 ml 12N HCl. Adjust pH to 6.95 and add distilled water to a final volume of 5 litres.

2. Bernheimer's Buffer

NaCl	22.5 g
$\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$	120 g

Dissolve in 3 litres distilled water. Add 12N HCl (7 - 9 ml) to adjust pH to 6.95 and add distilled water to a final volume of 5 litres.

3. Bernheimer's Buffer with Albumin

Add 1 mg bovine serum albumin Fraction V (Armour Pharmaceutical Co. Ltd., Eastbourne) for every 1 ml of Bernheimer's buffer.

4. Palitsch Buffer

Solution A - 0.05M sodium tetraborate

$\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$	19.108 g
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Distilled water to 1,000 ml.

Solution B - 0.2M boric acid salt solution

H_2BO_4	12.404 g
NaCl	2.925 g

Distilled water to 1,000 ml.

Mix 1.2 parts A to 8.8 parts B. Final pH should be 7.4.

5. Hank's Balanced Salt Solution

Stock Solution A

1.	NaCl	160 g
	KCl	8 g
	MgSO ₄ · 7H ₂ O	2 g
	MgCl ₂ · 6H ₂ O	2 g
	Distilled water	800 ml
2.	CaCl ₂	2.8 g
	Distilled water	100 ml

Mix 1 and 2 slowly and adjust volume to 1,000 ml with distilled water. Add 2.0 ml chloroform and store in a polythene bottle at 4°C.

Stock Solution B

	Na ₂ HPO ₄ · 12H ₂ O	3.04 g
	KH ₂ PO ₄	1.2 g
	Glucose	20.0 g
	Distilled water	800 ml

When dissolved add 100 ml phenol red solution and adjust to 1,000 ml with distilled water. Add 2 ml chloroform and store as stock solution A.

Phenol Red Indicator Solution (0.4%)

Dissolve 1 g phenol red in 250 ml distilled water.

Sodium Bicarbonate Solution

	NaHCO ₃	1.4 g
	Distilled water	100 ml

Sterilise in a tightly closed screw cap bottle for 10 min. at 115°C (9 lb pressure).

Hank's Solution

1 volume solution A + 1 volume solution B + 18 volumes H_2O (distilled). Sterilise by steaming for 90 min. Immediately before use add 0.5 ml of sterile 1.4% sodium bicarbonate solution to each 20 ml.

6. Gelatin Saline Solution pH 7.6

NaCl	8.5 g
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Gelatin	5 g
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Distilled water to 1,000 ml. Adjust pH to 7.6 with 0.1N NaOH or 0.1N HCl.

7. Bicarbonate Buffer

$NaHCO_3$	3.7 g
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Na_2CO_3	0.6 g
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Distilled water	100 ml
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8. Phosphate Buffer 0.1M pH 7.4

Stock Solution A - 0.2M sodium dihydrogen phosphate

NaH_2PO_4	27.8 g
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Distilled water to	1,000 ml
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Stock Solution B - 0.2M disodium hydrogen phosphate

Na_2HPO_4 or	28.4 g
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$Na_2HPO_4 \cdot 12H_2O$	71.7 g
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Distilled water to	1,000 ml
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19 ml solution A + 81 ml solution B + 100 ml distilled water.

9. Acidic mercuric chloride

$HgCl_2$	15 g
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HCl (conc)	20 ml
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Distilled water	80 ml
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Virulence of strains of *Staphylococcus aureus* for mice of different ages by Sheila McKay and J.P. Arbuthnott (Department of Microbiology, University of Glasgow, Glasgow and Department of Bacteriology, The Royal Infirmary, Glasgow, G4 OSF).

Man is probably most susceptible to acute staphylococcal infection in infancy. With a view to developing an animal model for experimental staphylococcal infection we examined the influence of age on the susceptibility of CD-1 strain mice to *Staphylococcus aureus*.

Six strains of staphylococci of different origin were selected for the study; these had different toxin and enzyme patterns. Standardised suspensions, frozen in small volumes in liquid nitrogen (-196°C) were used as inocula for each animal experiment in which 21-day, 10-day and 3-day old animals were challenged subcutaneously with 10^4 , 10^6 and 10^8 , colony forming units (c.f.u.). In every case the 3-day old animals were most susceptible showing a high mortality rate when challenged with 10^6 and 10^8 c.f.u. In general, at these doses, the animals which survived in each age group developed superficial lesions. Certain strains produced superficial lesions at a dose of 10^4 c.f.u.

Virulence, assessed as the ability to cause either death or lesions, varied considerably among strains, the coagulase negative strain being the least virulent. Interestingly, a non-toxinogenic, non-proteolytic, leucocidin producing strain (SM 9) was comparable in virulence with the toxinogenic strains. The lesions produced by strain SM 9 were of localised abscess type and differed from the more widespread necrotic lesions produced by the toxinogenic strains. A detailed study in neonatal mice challenged with 10^7 c.f.u. of strain SM 9 showed that the percentage mortality decreased markedly at 4 - 5 days of age; by 9 days of age this had decreased to zero per cent.

Proceedings of the Society for General Microbiology (1974), Vol. II, Part 3, p.79.

Comparison of the growth of two strains of Staph. aureus in vivo in the skin of 3-day old and 21-day old mice by Sheila McKay and J.P. Arbuthnott, (Department of Bacteriology, University of Glasgow, Royal Infirmary, Glasgow, G4 OSF).

In a previous study (Sheila McKay and J.P. Arbuthnott, (1973) Proceedings for Society of General Microbiology I, 79) we showed that neonatal CD-1 mice are more susceptible to experimental staphylococcal infection than adult mice. Two of the six strains of staphylococci used in that study, namely SM 9 and SM10 were selected for a further comparative study of growth in vivo; these strains differed in toxin/enzyme patterns but not in virulence for neonatal mice.

Groups of 60 - 100 mice of each age group were challenged subcutaneously with 10^6 colony forming units (c.f.u.) of either SM 9 or SM 10. At various time intervals after challenge the number of viable staphylococci in a piece of skin approximately 4 cm^2 in area containing the injection/lesion site was counted. We found that, for both strains SM 9 and SM 10, in both age groups of mice only 5 - 10% of the inoculum was detectable at 1 hour after injection. In the 21-day old animals the numbers of viable staphylococci increased to approximately 10^6 c.f.u. within 48 hours; thereafter the numbers decreased to a normal control level of 10^3 c.f.u. However, in 3-day old mice both strains multiplied rapidly to a similar level of 10^{11} c.f.u./injection site at which time the mice were moribund. Strain SM 10 was able to multiply approximately 1.75 times faster than SM 9.

The histology of the lesion sites in the neonatal mice at 18 hours after challenge confirmed that the strains produced different lesion types; SM 9 produced a classical abscess type lesion whereas SM 10 produced a necrotic lesion.

It is hoped that this experimental model will provide a basis for further study of the factors affecting staphylococcal pathogenicity.

Proceedings of the Society for General Microbiology (1974), Vol. II, Part 3, p.72.

Effects of staphylococcal products on locomotion of human blood neutrophils and monocytes by R.J. Russell, R.J. McInroy, P.C. Wilkinson, Sheila McKay and Christine MacCartney, (Department of Bacteriology and Immunology, University of Glasgow and Department of Bacteriology, Royal Infirmary, Glasgow).

The effect of staphylococcal products on the locomotion of human leucocytes in Boyden chemotaxis chambers was studied.

Washed staphylococcal cells had little direct effect on leucocyte locomotion. By contrast culture supernatants contained both chemotactic factors and factors which inhibited chemotaxis. Of the 9 strains tested, 8 produced direct chemotactic activity for monocytes up to a dilution of 1 in 1000 whereas none of the supernatants showed direct activity for neutrophils at this dilution. However, staphylococci and their products were chemotactic for neutrophils in the presence of serum complement. Also, when tested at a dilution of 1 in 1000, 7 of the 9 supernatants contained inhibitors of neutrophil chemotaxis, whereas only 3 contained inhibitory activity for monocytes.

Analysis of fractions obtained by preparative isoelectric focusing of a culture supernatant from Staph. aureus (Wood 46) revealed (a) monocyte chemotactic activity was present over a wide pH range, (b) only traces of neutrophil chemotactic activity were detected, (c) strong inhibitory activity for monocyte locomotion was found in distinct peaks at pH 4.1, 4.6, 7.25, 7.4 - 7.5, 8.3 - 8.6, 9.0 and (d) marked inhibition of neutrophil migration was detected at pH 6.1, 6.6, 6.9 - 7.1, 7.5, 8.3 - 8.6, 8.9 - 9.0.

The activities of two highly purified staphylococcal toxins were now examined. The α -toxin inhibited locomotion of both monocytes and neutrophils whereas β -toxin (sphingomyelinase C) had no effect on neutrophils at 10^4 HU/ml but inhibited monocytes at a concentration of 1 HU/ml.

Proceedings of the Society for General Microbiology (1974), Vol. II, Part 3, p.73.