THE ANTIGENIC DETERMINANTS AND REGULATION OF DELAYED HYPERSENSITIVITY TO STAPHYLOCOCCUS AUREUS IN MICE

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ABSTRACT

Delayed hypersensitivity to Staphylococcus aureus in mice is seen only after multiple infections. However, using cyclophosphamide this requirement can be reduced to a single sensitising dose. I have used this model to investigate the antigens involved in the delayed hypersensitivity reaction and one of the control mechanisms involved in regulating this response.

Delayed hypersensitivity to S. aureus could be induced with killed staphylococci at a dose equivalent to 10^8 colony forming units of viable cocci. Induction of a delayed response was possible both with cell walls and peptidoglycan, although the former gave a better response. Previous work has shown that the peptidoglycan contains the major antigenic determinants for delayed hypersensitivity.

Digestion of the cell wall with lysostaphin, modification of the peptidoglycan by (a) chemical methods (b) altered growth conditions and the lack of cross reactivity with cell walls from Streptococcus Group A, all suggested that the pentaglycine cross bridge was of primary importance in the antigenic determinant of peptidoglycan. Soluble fragments obtained from the degradation of cell walls with two specific enzymes, a protease from S. aureus V8 and muramidase from Chaloropsis sp. were able to induce delayed hypersensitivity to S. aureus. A synthetic analogue of the cross bridge, that is, pentaglycine linked to BSA, was able to induce a delayed reaction when challenged with staphylococcal cell walls. An indirect role for teichoic acid in the delayed hypersensitivity reaction was demonstrated with a teichoic acid deficient strain of S. aureus.

The relationship between the antibody response to multiple infections and delayed hypersensitivity was followed using an enzyme linked assay to measure antibody levels. Plasma and plasma fraction transfers showed that both IgM and to a lesser extent IgG were able to suppress delayed hypersensitivity when given before infection. However, plasma absorbed with cell walls or peptidogycan was unable to suppress the delayed reaction.

INTRODUCTION

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1.1. Staphylococcal Skin Infections

The development of a suitable animal model of localised staphylococcal skin infection presents many difficulties. Large doses of above 10^8 staphylococci are necessary to overcome the natural resistance of the skin to infection. Elek and Cohen (1957) and later James and MacLeod (1961) overcame this problem by implanting the staphylococci subcutaneously on cotton sutures. This seemed a better model of the natural production of staphylococcal disease as no more than a few staphylococci were necessary to produce a lesion. The sutures, or other foreign bodies potentiated the infection, possibly acting as a mechanical depot or as an irritant. Noble (1965) made the principle more quantitative by injecting subcutaneously a cotton dust plug from a wide bore needle. This cotton dust plug could be accurately loaded with an appropriate number of staphylococci. The virulence of the bacteria was then assessed by the size of the dermonecrotic lesion at the site of injection.

The skin lesions produced by staphylococcal infection have been associated with several bacterial and host factors. Bacterial factors include, the ability to produce extracellular toxins and enzymes (Jeljaszewicz, 1972) and the presence of a factor which delays the early inflammatory response of the host (Hill, 1968). Humoral immunity, cell mediated (delayed) hypersensitivity, specific and non specific inflammation, all can affect the lesions produced by staphylococcal infections.

1.2. Virulence Factors in S. aureus. Skin Infections.

Agarwal (1967a,b,c) evaluated the virulence of different strains of staphylococci using subcutaneous injections of the bacteria on cotton plugs. Staphylococcal strains such as PS 80, Orbach and 1531 which produced severe lesions, significantly delayed the fluid exudation and emigration of leucocytes to the site of infection. Less virulent strains however, invoked an early inflammatory response, indeed the protective value of an acute inflammatory reaction in bacterial infection had already been described by Miles et al (1957). Thus virulent strains are able to delay the influx of both oedema fluid and leucocytes to the site of infection, providing the invading bacteria with the opportunity to multiply before the host defences can act. Some coagulase negative strains of staphylococci could delay the early inflammatory response without producing dermonecrosis, this led Agarwal (1967b) to propose that virulent strains must be able to both inhibit leucocyte migration and fluid exudation and must also produce the toxins and other factors which were thought to produce dermonecrosis.

Hill (1968) isolated a factor from the walls of virulent staphylococci which had a lesion enhancing effect and also inhibited the accumulation of oedema fluid at the site of infection. This factor was the residue left after deoxycholate extraction of cell walls and consisted mainly of peptidoglycan with some protein. This deoxycholate residue (DOCR) which has also been called an aggressin or impedin acts on the inflammation due to chemical irritants as well as staphylococci, and has its effect by preventing the formation of kinins which are involved in the early stages of acute inflammation. (Easmon, Hamilton and Glynn, 1973).

The delay in the onset of early inflammation was considered by Agarwal not to be of sole importance in virulence, staphylococcal toxins, notably the α-toxin were thought to be responsible for the dermonecrosis. Staphylococci produce at least four haemolysins or cytolytic toxins and a leucocidin (Jeljaszewicz 1972). All strains of staphylococci which give rise to dermonecrosis in the mouse are known to produce either α or β toxins, or both. Mice can be protected from dermonecrosis by previous injection with α toxin producing strains or by the active or passive immunisation with α toxin.

However, Easmon and Glynn (1975a)obtained evidence that the neutralisation of α toxin is of less importance than the factors which produce local inflammation. Previous infections with strains which produced α toxin and therefore led to the production of antibodies against α toxin and with non-toxigenic strains both gave protection against dermonecrosis. They believed that the early inflammation was of paramount importance in determining the outcome of a S. aureus infection. This was supported by the local injection of glycogen at the site of infection which,stimulated an inflammatory response and reduced the final necrosis. Conversely when they suppressed early response with antithymocyte serum, prednisone or by complement depletion with zymosan, necrosis was more severe. This protection is achieved by an antibody dependent hypersensitivity reaction and virulent staphylococci are able to delay the onset of this reaction.

Most pathogenic staphylococci secrete lipase, coagulase and a number of other extracellular enzymes which may function as virulence factors. The possession of a capsule, while an important virulence

factor in intraperitoneal infections confers no increased virulence on staphylococci causing skin infections (Easmon and Glynn 1976).

1.3. Cell Mediated Responses to Staphylococci

The possible role of hypersensitivity in staphylococcal infection was first suggested by Panton and Valentine (1929). They observed that rabbits, given repeated skin infections developed an increased susceptibility to doses of staphylococci one hundred times smaller than that used for infection. Bøe (1945) sensitised rabbits with daily intracutaneous injections of formalin killed staphylococci, so that when challenged with a staphylococcal filtrate vaccine the rabbits developed a delayed hypersensitivity reaction. In such animals, minimal doses of live bacteria produced more severe abscesses than in controls. Bøe was unable to transfer passively the ability to produce a delayed reaction with serum from sensitised rabbits.

Using repeated staphylococcal skin infections to develop delayed hypersensitivity, Johanovsky (1958) demonstrated that the transfer of spleen cells or peritoneal exudate cells from sensitised donor rabbits to normal recipients resulted in the transfer of delayed hypersensitivity. The recipient animals had an increased susceptibility to staphylococcal infection. Johnson, Cluff and Goshi (1961) found that repeated infection in rabbits was associated with the development of delayed hypersensitivity and was unaccompanied by demonstrable serum antibody. These rabbits were more sensitive to staphylococcal skin infections, the lesions of which were maximal at 24 and 48 hours.

In contrast to the findings of Johnson, Cluff and Goshi (1961) and

Johanovsky (1958), Agarwal (1967c) could not produce increased susceptibility in mice with repeated subcutaneous infections. However, when Agarwal transferred lymph node cells from previously infected mice the recipients exhibited a much higher susceptibility and responded to S. aureus injection with severe necrotic lesions. The lymph node cells would not however, transfer the increased susceptibility when given in combination with serum from the previously infected mice. This led Agarwal to suggest that the cell mediated increased susceptibility to staphylococci is not manifested in the presence of antibodies.

Using footpad reactions to measure delayed hypersensitivity in mice, Easmon and Glynn (1975b) showed that repeated infections with S. aureus led to delayed hypersensitivity to crude staphylococcal antigens. Lymph node cells from repeatedly infected mice transferred delayed hypersensitivity to non-infected recipient mice, which responded to staphylococcal challenge with severe necrotic lesions. However, the transfer of delayed hypersensitivity and increased severity of lesions was not seen when the transferred cells were treated with anti-Thy 1 serum. Serum from infected donors protected against dermonecrosis and did not transfer the delayed reaction. As found by Agarwal (1967c) when both serum and cells were transferred into the same animal the effects of the serum suppressed those of the lymph node cells. Easmon and Glynn (1975b) also studied the effect of T lymphocyte depletion on staphylococcal skin infection. Removal of the T cell population by a combination of adult thymectomy and cyclophosphamide gave rise to mice which responded to staphylococcal infection with mild non-necrotic lesions. Nude mice with very few T lymphocytes also produced mild

non-necrotic lesions.

Thus, both the delayed hypersensitivity and necrosis are attributed to cell mediated immune responses because of their timing which is maximal at 24-48 hours, their histology, a mononuclear cell infiltrate and because both responses can be transferred by T cells from immune donors.

Cell mediated (delayed) hypersensitivity is typically associated with intracellular bacteria such as mycobacteria, brucellae and listeriae. However, as shown already S. aureus which is predominantly an extracellular bacterium can induce delayed hypersensitivity. The first interest in the kinetics of the induction of delayed hypersensitivity to S. aureus was expressed by Taubler (1968). He used a mouse footpad model to demonstrate the induction of delayed hypersensitivity by eight weekly injections of 10^8 viable staphylococci. When challenged with a soluble preparation of disrupted staphylococci, mice sensitised in this manner responded with significantly higher footpad swellings at 24 and 48 hours than normal mice.

Taubler and Mudd (1968) demonstrated delayed hypersensitivity in mice using the in vitro inhibition of macrophage migration by a crude staphylococcal antigen. This cell migration inhibition was specific in that macrophages from S. aureus sensitised mice were not inhibited from migrating by an unrelated antigen.

Subcutaneous staphylococcal injections on cotton dust plugs (Noble 1965) were used to induce delayed hypersensitivity in a study by Easmon and Glynn (1975b). They reduced the number of weekly infections from eight to four and the number of bacteria from 10^8 to 10^5 colony

forming units when injected on cotton dust plugs. The footpad reactions were suppressed by antithymocyte serum and characterised histologically as delayed hypersensitivity responses by a mononuclear infiltration at the site of challenge.

It has been demonstrated (Johnson, Cluff and Goshi, 1961; Tribble and Bolen 1978) that non-viable S. aureus will only induce delayed hypersensitivity when used in conjunction with Freunds complete adjuvant. Induction of delayed hypersensitivity has been accomplished with several components of staphylococcal cell walls. Heczko, Grov and Oeding (1973) sensitised mice with multiple injections of protein A in Freunds complete adjuvant, the use of the incomplete adjuvant gave rise only to short lived Arthus reactions. Single injections of peptidoglycan in both Freunds complete and incomplete adjuvant have been used by Dziarski (1978) to induce delayed hypersensitivity in guinea pigs.

In the classical model of cell mediated immunity in Listeria infections (Mackaness 1970), specifically activated lymphocytes induce increased bactericidal activities in macrophages. This seems in contrast to the work already described on staphylococcal skin lesions where delayed hypersensitivity is accompanied by increased susceptibility to infection. This was supported by the work of Lenhart and Mudd (1971) who found that the staphylococcidal ability of rabbit peritoneal macrophages was not increased in repeatedly infected animals. Pryjma, Zembala, Grov and Heczko (1975) obtained further evidence for this by demonstrating that the bactericidal activity of peritoneal exudate cells was inversely related to the delayed type skin reactivity of the donor, that is animals with strong delayed hypersensitivity reactions showed depressed

staphylococcidal activity. The increased susceptibility to infection, which accompanies delayed hypersensitivity could therefore be a reflection of this decreased bactericidal activity.

In contrast, Shayegani, DeCourcy and Mudd (1973) took macrophages from mice which had received repeated infections with S. aureus and observed an increased killing ability by these cells after further exposure to staphylococcal antigen. Macrophages not exposed to further antigen did not show any increased killing ability, indeed they were less effective than normal macrophages. Baughn and Bonventre (1975) studied the production of lymphokines by cultures of lymphocytes taken from mice exhibiting delayed hypersensitivity. Lymphokines were only produced when the culture fluid contained staphylococcal antigens. While these lymphokines could not activate macrophages to increase intracellular killing, they were able to enhance phagocytosis, thus rapidly clearing staphylococci from the extracellular fluid. This led Baughn and Bonventre to propose that cell mediated immunity to S. aureus is manifested at the level of phagocytosis and is not associated with increased intracellular killing.

Despite receiving much attention the relationship between delayed hypersensitivity and cell mediated immunity remains ambiguous. Some consider that hypersensitivity and immunity are merely coincidental events. The opposite view is that hypersensitivity and immunity are different expressions of the same process, namely cell mediated immunity. The complex reactions and interactions amongst the various sets and subsets of cells involved are as yet uncertain. However it is possible that these reactions will provide information leading to a better understanding

of this relationship. (Youmans, 1975; Lefford, 1975; Salvin and Neta 1975).

1.4. The Use of Cyclophosphamide to Study the Regulation of Cell Mediated Hypersensitivity.

The regulatory mechanisms involved in controlling the immune reaction have, during recent years attracted much attention, despite this the interactions of the immune system remain poorly understood and still await a unifying theory. Humoral and cell mediated responses have been reported to be regulated by B lymphocytes, T lymphocytes, macrophages, antibody and immune complexes (Asherson and Zembala, 1976).

Much of the work surrounding the regulation of delayed hypersensitivity has involved the use of the selective immunosuppressant drug, cyclophosphamide. Cyclophosphamide is an alkylating agent which is powerfully cytotoxic towards rapidly dividing cells (Dewys, Goldin and Mantel, 1970). It is itself inactive, but is converted by hepatic microsomal enzymes into a series of active metabolities which are incorporated into the DNA double helix, causing the incorrect translation of the DNA resulting in cell death (Berenbaum, Cope and Double, 1973).

Turk, Parker and Poulter (1972) studied the contact hypersensitivity reaction in guinea pigs and observed that the intensity of the reaction to 2,4 dinitrofluorobenzene was increased and prolonged by treatment with cyclophosphamide three days prior to sensitisation. They suggested that a B cell response which normally regulates contact sensitivity was blocked by treatment with cyclophosphamide. B cells were directly

implicated, in the suppression of delayed hypersensitivity to ovalbumin in guinea pigs (Turk, Parker and Poulter, 1972). Cyclophosphamide treatment removed the suppression of delayed hypersensitivity to ovalbumin, passively transferred spleen cells from ovalbumin-sensitised animals were able to restore the suppression. However, when spleen cells depleted of B lymphocytes by passage through a rabbit anti-guinea pig gamma globulin coated column were passively administered there was no suppression. Turk and Parker (1973) postulated that cyclophosphamide removes the B cells which normally modulate the T cell reaction.

One possible mechanism of B suppressor cell action is through feedback inhibition by antibody. Askanase, Hayden and Gershon (1975) however, augmented delayed hypersensitivity to sheep red blood cells with small doses of cyclophosphamide which do not influence antibody production. This led to the suggestion that a cyclophosphamide sensitive T cell was involved in the regulation of delayed hypersensitivity. Polak and Turk (1974) substantiated this by demonstrating a subset of T cells which divide rapidly and are sensitive to cyclophosphamide. Antigen specific T suppressor cells regulate immunity at various levels, they can affect both the generation of immune cells (Moorhead 1976) and the functional expression of immune cells (Zembala et al, 1975) that is, they can act by afferent or efferent suppression.

Cyclophosphamide has been used to study the regulation of the cell mediated reactions following infection with both S. aureus (Easmon and Glynn 1977) and Listeria monocytogenes (Kerckhaert, Hofhuis and Willers 1977) and following the injection of tularaemia vaccine in guinea pigs

(Ascher, Parker and Turk, 1977).

Easmon and Glynn (1975a) demonstrated that immune serum from twice infected mice protected non-infected mice from the dermonecrotic effects of S. aureus challenge. However, lymph node cells from twice infected mice had the opposite effect and recipients responded to staphylococcal infection with increased dermonecrosis. When immune serum and lymph node cells were transferred together, the effects of the serum overode those of the cells. Thus, there appears to be a serum dependent suppression of the cell mediated dermonecrosis.

Cyclophosphamide was used as a selective immunosuppressant by Easmon and Glynn (1977) to study further the regulatory mechanisms involved. Mice received intraperitoneal injections of cyclophosphamide (200 mg/kg) two days before a single subcutaneous S. aureus infection, this enhanced the delayed footpad responses to levels otherwise seen only after at least four weekly infections. A minimum dose of 100 mg/kg body weight was necessary, which is greater than those small doses shown by Askenase et al (1975) to enhance cell mediated immunity without affecting antibody synthesis.

In addition to being a potent immunosuppressant cyclophosphamide has anti-inflammatory properties. Because of Agarwal's (1967c) proposal that late necrosis is inversely related to early inflammation, it was necessary for Easmon and Glynn to demonstrate that the enhancement of delayed hypersensitivity by cyclophosphamide was not due to any reduction in inflammation but was immunologically mediated. They used cobra venom factor as an anti-inflammatory agent and glycogen to stimulate inflammation at the site of skin infection. However, when used separately,

or in combination these two agents had no effect on the outcome of the response and it is therefore unlikely that the anti-inflammatory effect of cyclophosphamide effects the enhancement of delayed hypersensitivity to S. aureus.

Thus in normal mice there are regulatory mechanisms which prevent the effector cells of cell mediated immunity and delayed hypersensitivity displaying their harmful responses to S. aureus infection. During the course of multiple infection, (or by the action of cyclophosphamide) these suppressor systems are overidden.

Immune serum, when given before infection to cyclophosphamide treated mice suppressed the induction of delayed hypersensitivity as measured by footpad tests two weeks later. However, the serum had no effect when given before antigen challenge. This differs from the system observed by Mackaness et al (1974) where immune serum suppressed delayed hypersensitivity to sheep red blood cells both at the time of induction and challenge.

In contrast to immune serum, spleen cells from twice infected mice could not suppress delayed hypersensitivity when given at the time of induction. However, when cell transfers were performed twenty four hours before footpad challenge the delayed response was significantly reduced. It therefore appears that there is dual control of delayed hypersensitivity to S. aureus with immune serum preventing the induction of delayed hypersensitivity and immune cells suppressing its expression.

In an attempt to categorise the cells responsible for suppressing delayed hypersensitivity Easmon and Glynn (1979) passed the cells through nylon wool columns to remove lymphocytes and treated them

with carbonyl iron to remove phagocytic cells. Only the cells removed by the nylon wool columns were able to suppress delayed hypersensitivity. These lymphocytes were treated with anti-Thy 1, 2 plus complement and also antiheavy chain serum plus complement. The suppressor cells were unaffected by the former but were lysed by the latter treatment and were therefore classified as B-lymphocytes.

1.5. Chemical Composition of Staphylococcal Cell Wall Antigens.

Although extracellular and ribonucleic acid associated antigens do occur in staphylococci, this discussion is limited to the chemistry of the cell wall antigens lipoteichoic acid, teichoic acid, peptidoglycan and protein A.

Lipoteichoic acid

Lipoteichoic acid, as the name implies is a complex of teichoic acid and lipid. It is derived from the cell membrane but can function as a cell surface component, and thus presents an analogy with the lipopolysaccharide of gram-negative bacteria. The lipoteichoic acids of staphylococci possess a component of glycerol teichoic acid which is covalently linked to a glycolipid (Knox and Wicken 1973).

Teichoic Acids

Teichoic acids are water soluble polymers of glycerol or ribitol phosphate, a sugar and D-alanine held together by phosphodiester linkages. In the cell wall teichoic acid is covalently bound to peptidoglycan by a phosphodiester bond to the 6-hydroxyl group of a muramic acid residue. The teichoic acids comprise between 20-50% of the weight of the wall. In S. aureus (Figure 1.) the polymer is of the ribitol type (unlike lipoteichoic acid which is of the glycerol type) with each ribitol unit linked

by phosphodiester bonds at the primary hydroxyl group. N-acetyl glucosamine is linked to the 4-hydroxyl group of each D-ribitol residue and D-alanine is esterified to the 2-hydroxyl groups.

Peptidoglycan

Peptidoglycan (murein or mucopeptide) is a cell wall polymer common to gram positive, gram negative bacteria, rickettsiae and blue green algae. It is composed of a polysaccharide or glycan backbone which is cross linked by short peptides. It constitutes about 50% of the weight of the cell wall. The whole structure forms a network, which completely surrounds the cell providing resistance to the internal osmotic pressure of the cytoplasm and giving the bacteria its characteristic shape. The polysaccharide backbone consists of β 1,4-linked N-acetyl glucosamine residues in which each alternative residue is substituted at the 3-OH group by D-lactic acid and is then called muramic acid.

The peptide moiety is usually made up of a tetrapeptide or pentapeptide of the sequence L-Ala, D-Glu, L diaminoacid - D-Ala (D-Ala), whereby the L-Ala residue is linked to the carboxyl group of muramic acid by a peptide bond. These tetrapeptide/pentapeptide units are cross linked between the C-terminal D-alanine of one peptide to the distal amino group of the diaminoacid at position 3 of another peptide. The cross-linking is performed either by a single aminoacid or by a short peptide.

The aminoacid sequence of the peptidoglycan of S. aureus strain Copenhagen was elucidated by Ghuysen et al (1965) (Figure 2). The tetrapeptide unit consists of L-alanine bound to muramic acid, followed by D-glutamate, L-lysine and D-alanine. The α carboxyl group of the glutamate is amidated and the γ carboxyl group of D-glutamate is linked

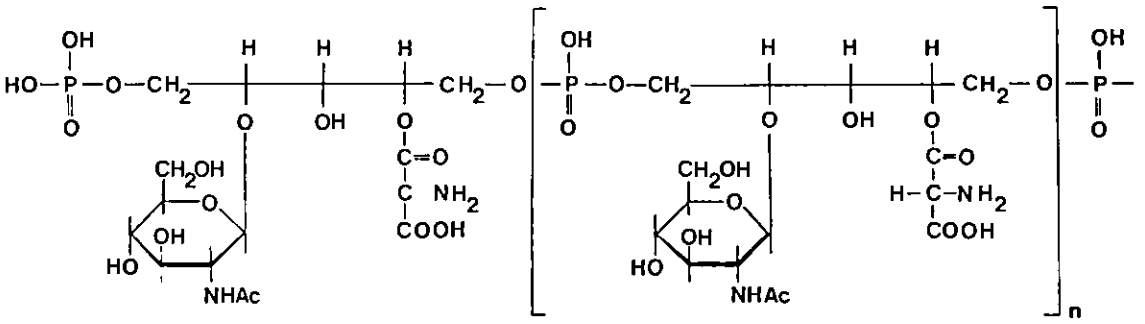


Figure 1.

Repeating unit of a ribitol teichoic acid from Staphylococcus aureus.

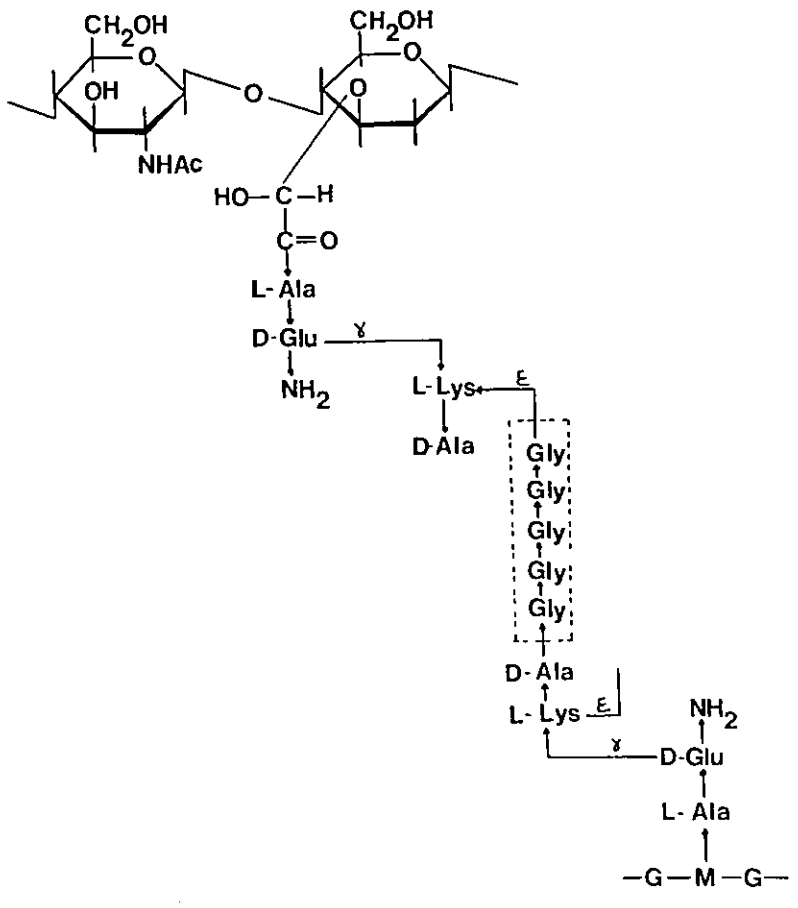


Figure 2.

Fragment of the primary structure of the peptidoglycan of Staphylococcus aureus. G, N-acetylglucosamine; M, N-acetylmuramic acid.

to the α amino group of lysine. The interpeptide bridge is composed by 5 glycine residues which extend from the C-terminal of D-alanine of one peptide to the ε-amino group of L-lysine of another peptide on another polysaccharide chain. In S. aureus a high percentage of the peptide units are cross linked which leads to polymers of considerable size, containing on average fifteen repeating units. Other staphylococcal strains differ in the amino acid sequence of their interpeptide bridges. In S. epidermidis, for example a single glycine residue is replaced by L-serine. A few coagulase negative staphylococci have an interpeptide bridge consisting of tetraglycyl-L-alanine, where an L-alanine residue is bound to the ε amino group of the tetrapeptide lysine.

Protein A

Most strains of S. aureus contain protein A. It can be either cell bound or extracellular. Cell bound protein A is found predominantly on the cell wall where it is covalently linked to the peptidogycan. In the strain S. aureus Cowan I protein A comprises 6% (w/w) of the cell wall, it has been calculated that every 325th peptidoglycan monomer is linked to a protein A molecule of molecular weight 42,000. (Sjoquist 1973). Protein A is best known for its peculiar property of reacting with the Fc part of IgG.

1.6. Antigens Involved in Delayed Hypersensitivity.

Although delayed hypersensitivity to S. aureus had been demonstrated using crude fractionated antigens many years previously, it was not until 1971 that Kowalski and Berman (1971) tried to purify and define the antigens involved. They studied the skin reactions in sensitised and non-sensitised mice to intradermal injections of whole cells, cell walls, insol-

uble peptidoglycan, teichoic acid and soluble cell wall fragments. In non-sensitised mice, all the antigens except teichoic acid gave rise to an acute inflammatory response, this might have been due to an activation of the alternative pathway of complement by surface components of the bacteria. Delayed hypersensitivity which was measured by a protracted indurated response was present only in sensitised animals and was not elicited by teichoic acid nor by soluble peptidoglycan fragments. However, insoluble peptidoglycan and peptidoglycan linked to teichoic acid were able to evoke hypersensitivity reactions which remained erythematous and indurated for at least 30 hours.

Kowalski and Berman postulated that it was the peptidoglycan portion of the cell wall that was responsible both for the delayed hypersensitivity and the acute inflammatory lesions. However, they used the protracted erythema and induration as the sole criteria of delayed hypersensitivity and neglected to perform the classical tests of passive transfer with lymphoid cells but not serum together with an histological examination showing an infiltration with mononuclear cells.

Using the in vitro assay of macrophage migration inhibition from capillary tubes Targowski and Berman (1975) extended the work of Kowalski and Berman. Their results agreed with those obtained from the in vivo experiments. Cell walls and peptidoglycan-teichoic acid complexes were able to inhibit the migration of peritoneal exudate cells from mice sensitised with S. aureus. As seen in the in vivo study peptidoglycan and teichoic acid were ineffective antigens.

Other studies have shown delayed hypersensitivity to staphylococcal protein A. Heczko, Grov and Oeding (1973) elicited delayed hypersensit-

ivity with protein A in guinea pigs sensitised with protein A in Freunds complete adjuvant. Using the inhibition of macrophage migration test Helgeland, Naess and Grov (1975) demonstrated that protein A suppresses the migration of macrophages from guinea pigs sensitised with protein A.

Helgeland, Naess and Grov also reported the inhibition of specifically macrophages by peptidoglycan, however they suggested that this inhibition might have been due to the intrinsic toxicity of the peptidoglycan and not the production of any migration inhibition factor. This toxic effect of peptidoglycan on macrophages was investigated by Bultman et al (1975) who suggested that the immunologically specific inhibition of macrophages was indistinguishable from the inherent toxicity of the peptidoglycan.

Easmon and Glynn (1978) used the mouse footpad swelling technique to investigate the delayed hypersensitivity elicited by various cell wall components. Cyclophosphamide pretreatment of mice was used in this study to overcome the need for multiple infections to induce delayed hypersensitivity. The antigens used to stimulate the delayed response by Easmon and Glynn were cell walls, trypsinised cell walls, peptidoglycan, teichoic acid, and protein A. Neither teichoic acid nor protein A were able to stimulate delayed hypersensitivity. However, in agreement with the results of Kowalski and Berman (1971) both cell walls and peptidoglycan could elicit a delayed reaction in S. aureus sensitised mice.

A different approach has been adopted by Tribble and Bolen (1978) who followed the footpad responses of mice receiving one to eight injections of S. aureus, by challenging with several antigens. They demonstrated a biphasic response, the first peak of delayed hypersensit-

ivity after three injections was primarily dependent upon protein antigens associated with the bacterial membrane. After seven injections, there was a second higher peak, which was dependent upon cell walls, protein A and membrane components (Bolen and Tribble 1979). These results were confirmed using the in vitro methods of spleen cell stimulation and macrophage migration inhibition (Tribble and Bolen 1979).

Recently, Bolen and Tribble (1981) isolated a membrane protein which was responsible for the early delayed hypersensitivity response i.e. the first peak in the biphasic response. The active immunogen called purified membrane protein was classified as a glycoprotein of molecular weight 15,600 Daltons. However, this purified protein did contain some cell wall components which, when removed resulted in a decrease in the delayed hypersensitivity response elicited by this antigen. Thus despite the purification of the glyco protein which was thought to be responsible for the early phase of delayed hypersensitivity Bolen and Tribble were unable to rule out the involvement of cell wall fractions.

1.7. The Immunochemistry of Peptidoglycan.

The antigenic specificity of staphylococcal peptidoglycan for the humoral response has been widely studied. Examination of the methods used in such studies might provide information useful in the investigation of the specificity of the delayed hypersensitivity reaction.

The cross reactions between solubilised peptidoglycan from Staphylococcus aureus and serum from rabbits immunised with Group A variant streptococci first led Karakawa et al (1968) to suggest that

antibodies are raised against the tetrapeptide units of peptidoglycan and, to a lesser extent the polysaccharide backbone. These two parts of peptidoglycan are identical in staphylococci and Group A variant streptococci, Figure 3. The immunogenicity of the polysaccharide backbone was confirmed by Rolicka and Park (1969) who went on to show that N-acetylglucosamine rather than muramic acid was the immunodominant sugar. A similar situation had already been described in streptococcal peptidoglycan by Karakawa.

Helgeland, Grov and Schleifer (1973) demonstrated by an indirect haemagglutination technique that the reaction between S. aureus peptidoglycan and anti-staphylococcal serum was more complex than originally thought. The peptide subunit was shown to contain the major antigenic determinants, with both the tetrapeptide and pentapeptide L-Ala - D-Glu - L-Lys - D-Ala - (D-Ala), having strong affinities for S. aureus antiserum. They also isolated antibodies against the C-terminal of the pentaglycine bridges.

Schleifer and Seidl (1974) immunised rabbits with synthetic pentapeptide Gly - L-Ala - L-Ala - D-Ala - D-Ala linked to human serum albumin. The antibodies raised cross reacted on Ouchterlony agar gel diffusion with sonically disrupted peptidoglycan from Staphylococcus Sp. strain 24. By inhibiting the precipitin reactions with various structural analogues of the peptide unit they were able to show that the immunodominant site is the C-terminal D-Alanyl - D-Alanine. A similar study concerning the antigenicity of the pentaglycine cross bridge was performed by Seidl and Scheifer (1977). They immunised mice with an albumin pentaglycine conjugate which possessed free C-terminal residues then,

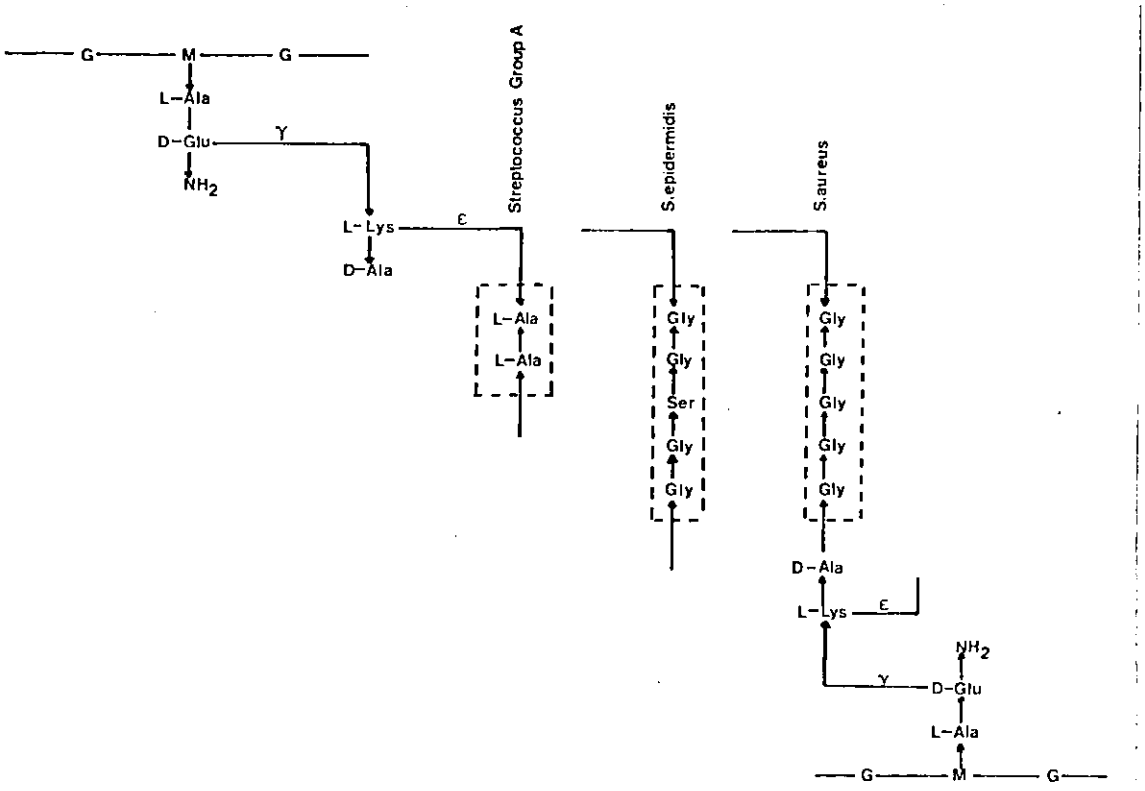


Figure 3.

Fragments of the primary structures of the peptidoglycans from Streptococcus Group A, Staphylococcus epidermidis and Staphylococcus aureus.

using a latex agglutination method they demonstrated cross reactions between antibodies to the synthetic immunogen and staphylococcal peptidoglycan. However, Seidl and Schleifer did point out that serum raised against peptidoglycan mostly contains antibodies with specificities for the polysaccharide chain and the peptide sub unit, with only a small fraction of the antibodies directed against the interpeptide bridge.

The presence of antibodies against polyglycyl peptides in anti-staphylococcal sera was also demonstrated by Ranu (1975). He sensitised tanned sheep red cells with BSA linked to polyglycyl peptides showing either a free aminoterminal end or a free carboxyl terminus and found antibodies reacting with both termini. The presence of these antibodies is somewhat difficult to explain as there appear to be no free amino or carboxyl groups in the pentaglycine cross bridge. However, Wise and Park (1965), Tipper and Strominger (1965) and Matsuhashi et al (1965) all proposed that the pentaglycine cross bridge with a free amino terminal is present during the synthesis of peptidoglycan. Carboxy termini are less abundant and are thought only to arise by the action of autolytic enzymes.

1.8. Aims of This Study.

The role of the delayed hypersensitivity and/or cell mediated immunity in the pathogenesis of staphylococcal infection has received intermittant attention since the first reports of Panton and Valentine, in 1929. Despite this, some 50 years later there is still no complete understanding of the mechanism involved in delayed hypersensitivity and the part played by this in staphylococcal infections.

This study has set out to fill some of the remaining gaps in our knowledge of this reaction; they are:-

1. To characterise the antigens involved in delayed hypersensitivity to S. aureus.

2. To investigate the interactions between humoral immunity and delayed hypersensitivity to S. aureus.

MATERIALS and METHODS

MATERIALS AND METHODS

2.1. Mice

Inbred female CBA mice were used in all experiments, these had previously been shown to produce a good delayed hypersensitivity response to staphylococci. (Easmon and Glynn, 1975b). All mice weighed between 20 and 25g.

2.2. Bacteria and Culture Conditions

(a) Bacteria

The majority of S. aureus infections were with the non-encapsulated laboratory strain 8530. Occasionally the protein A deficient strain Wood 46 and the teichoic acid deficient strain 52A5 were used. The latter was a gift from Dr B.J. Wilkinson, Illinois State University, U.S.A. S. epidermidis NCTC 5955/ATCC 155 and Streptococcus Grp. A Strain T27 were gifts from The Central Public Health Laboratory, Colindale.

(b) Growth Conditions

When staphylococci were required for infection they were grown overnight in Nutrient broth (Oxoid) at 37^{o}C and diluted as necessary with 0.9% saline. When required for the isolation of antigens they were grown on an orbital shaker for 7 hrs at 37^{o}C in 250 ml Erlenmeyer flasks containing 100 ml of nutrient broth. Growth was harvested by centrifugation at 500 g for 20 minutes.

The streptococci were grown by similar methods but using Todd Hewitt Broth (Oxoid).

(c) Modification of Peptidoglycan by Altered Growth Conditions

S. aureus strain 8530 were grown on an orbital shaker for 7 hours

at 37°C in 250 ml. Erlenmeyer flasks containing 100 ml of yeast extract glucose broth supplemented with 0.3% NaCl and 3% glycine (Hammers, Schleifer and Kandler 1972).

2.3. Cyclophosphamide

Mice were given intraperitoneal injection of cyclophosphamide (Endoxana, Ward Blenkinsopp) 200 mg/kg body weight, dissolved in water for injection.

2.4. Sensitisation of Mice

(a) Sensitisation with S. aureus

Two days after the cyclophosphamide, mice were given a subcutaneous injection of staphylococci suspended on a small cotton dust plug as described by Noble (1965).

(b) Sensitisation with soluble fractions and synthetic analogues in Freunds Adjuvants.

Mice were pretreated with cyclophosphamide as before. The soluble fraction or the synthetic analogue was dissolved in saline and emulsified with an equal volume of Freunds Adjuvant (Difco Labs., Detroit, U.S.A.). 0.2 ml of the emulsion was then injected intradermally into the backs of shaven mice.

2.5. Measurement of Delayed Hypersensitivity

Twelve days after sensitisation, delayed hypersensitivity was assessed by the footpad swelling technique. Both the hind footpad thicknesses were measured carefully with an engineer's micrometer, then 20 µl of antigen suspended in 0.9% saline was injected into the right hand footpad and the same volume of saline injected into the left

hind footpad. The change in footpad size was measured at 4 hours, 24 hours, and 48 hours. All measurements were made in triplicate and care was taken not to squash the swollen footpad. The net footpad swelling was taken as, the swelling in the right hand footpad minus the swelling in the left hind footpad. All experiments used groups of between 4 and 8 mice.

2.6. Production of antigens

(a) Cell walls

S. aureus cell walls were prepared from a frozen block of S. aureus strain V8 which was supplied by The Centre of Applied Microbiological Research, Porton. S. epidermidis NCTC 5955, S. aureus 52A5 and Streptococcus Group A strain T27 were grown as previously described.

The bacteria were mixed with saline to give a cream-like suspension, added to with an equal volume of No. 11 Ballotini glass beads and then disintegrated in a Braun homogeniser, cooled with carbon dioxide. Disruption was continued until no whole gram positive cells could be distinguished on a gram stain. The crude cell walls were recovered by decantation and by washing the beads with saline. The cell walls were washed three times in 1M NaCl and 3 times in 1M phosphate buffer (pH 7.0). Removal of whole cells was performed by 3 cycles of differential centrifugation at 2,000 rpm for 5 minutes and 11,000 rpm for 10 minutes. All washings and centrifugations were carried out at $4^{o}C$. Autolytic enzymes were then destroyed by steaming in buffer for 25 minutes. Finally, the cell walls were treated with ribonuclease (B.D.H. Ltd)

200 µg/ml for 2 hrs. followed by trypsin (B.D.H. Ltd) 500 µg/ml for 2 hrs then washed in distilled water and freeze dried.

The cell wall preparations were observed by electron microscopy after staining with phosphotungstic acid.

(b) Peptidoglycan

Peptidoglycan was prepared by several previously reported methods. (i) Extraction with 1% deoxycholate solution for 4 hours at 2°C. (Hill 1967).

(ii) Extraction with 0.1M NaOH for 1 hour at 100°C (Archibald,Baddiley and Heptinstall 1969).

(iii) Extraction with 0.5M NaOH for 4 hours at room temperature (Archibald,Coapes and Stafford 1969).

(iv) Extraction with 0.1M NaOH for 16 hours at room temperature (Hughes and Tanner (1966).

(v) Extraction with 10% TCA for 36 hours at 2°C. (Schleifer 1975).

(vi) Extraction with 10% TCA for 16 hours at 60°C. (Schleifer 1975).

After preparation, the peptidoglycan residue was washed and freeze dried. The teichoic acid remaining after extraction was estimated by determining the total phosphate content.

(c) Lysotaphin digested cell walls

Cell walls were hydrolysed using the lytic enzyme lysostaphin. Five hundred milligrammes of cell walls were incubated with 500 µg of lysostaphin (Becton, Dickinson Ltd) at 37°C in 0.1M potassium phosphate

buffer (pH 7.4). Aliquots were removed at 15 minute intervals and the hydrolysis terminated by placing in a boiling water bath for 4 minutes. The insoluble cell wall material was centrifuged down, washed with distilled water and freeze dried. Hydrolysis was continued for 105 minutes.

The reaction was monitored by:-

(1) Reduction in optical density at 620 nm

(2) Measurement of increasing reducing power in the supernatant (Ghuysen and Strominger 1965) see Section 2.11.

(3) Measurement of release of free amino groups into the supernatant (Ghuysen and Strominger 1965). see Section 2.11.

The insoluble cell wall preparations from each aliquot were used as antigens.

(d) Solubilised cell wall fragments

S. aureus V8 protease enzyme (Houmard and Drapeau 1972). One hundred milligrammes of cell walls were incubated at 37°C with 1 mg (560 units) of the S. aureus V8 protease enzyme (Miles Biochemicals) in tris buffer (pH 7.8) for 6 hours. The suspension was centrifuged and the insoluble fraction washed in distilled water, both the soluble and insoluble fractions were then freeze dried. The soluble fraction was either used as a crude antigen or purified by gel filtration. The insoluble cell walls were weighed before and after enzyme digestion.

Chaloropsis B enzyme (Tipper, Strominger and Ghuysen 1964). One hundred milligrammes of cell walls were incubated at 37°C with 500 units of Chaloropsis B enzyme (Miles Biochemicals) in 10 mls sodium acetate buffer (pH 4.7) for 18 hours. The insoluble and soluble extracts were treated as for the protease enzyme extracts.

Gel Chromatography of soluble cell wall digests.

Gel filtration of the two enzyme digests was carried out on a Sephadex G-25 fine column (1 x 25 cm). Distilled water was used as eluent and the column operated at 4°C. The fractions were monitored by measuring the optical density at 280 nm.

Analytical Procedures

The purified fractions obtained from the protease digestion were analysed by paper chromatography for aminoacids and aminosugars. One mg sample of each fraction was hydrolysed in 200 µl HCl Aristar grade (B.D.H. Ltd) at 100°C in sealed, evacuated pyrex tubes for 24 hours. After hydrolysis, the samples were redissolved in distilled water and subjected to descending paper chromatography on Whatman No. 4 chromatography paper using N-butanol, acetic acid, water (60:20:20) as solvent. The papers were dried and sprayed with or dipped in ammoniacal Ag NO_3 or ninhydrin. Muramic acid, N-acetyloglucosamine, ribitol, alanine, glycine, glutamic acid and lysine were used as standards.

Polyacrylamide Gel Electrophoresis

The purity of the active fraction from the protease digest was checked using polyacrylamide Gel Electrophoresis. 10% S.D.S. polyacrylamide gels were run on a multiphor flatbed apparatus and stained with 1% coomassie blue.

2.7. Chemical Modification of Peptidoglycan with Acetoacetic Anhydride

S. aureus peptidoglycan was acetoacetylated with acetoacetic anhydride (diketene) (Sigma Chemical Co.Ltd) according to the method described by Marzotto (1968). Ten milligrammes peptidoglycan was

suspended in 5 ml. borate buffer (pH 8.5) and reacted with 0.5 ml aceto-acetic anhydride for 6 hours, at room temperature with periodical shaking. The pH was maintained using 2M NaOH. The modified product was dialysed against distilled water for 24 hours at room temperature to remove any remaining soluble reactants and products. The aceto-acetylated peptidoglycan was washed three times in distilled water and freeze dried (Figure 4).

2.7.1. Selective Unblocking of Acetoacetylated Hydroxyl Groups

10 mg of the modified peptid oglycan was stirred continuously in 50 ml of 0.2M carbonate-bicarbonate buffer (pH 9.5) for 24 hours at room temperature. Buffer and reactants were removed by dialysis against distilled water and the product was washed and freeze dried.

2.7.2. Total unblocking of the Acetoacetylated Groups

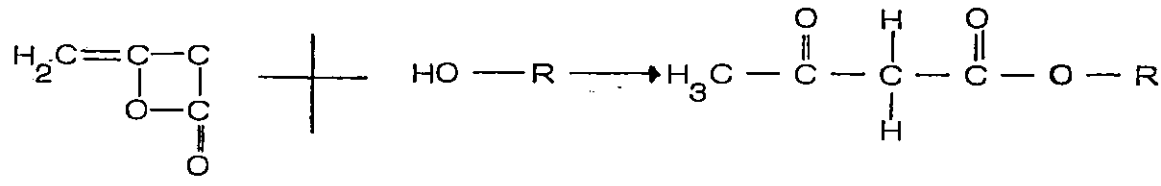
20 mg of acetoacetylated peptidoglycan was continually stirred in 100 mls of 10% aqueous hydroxylamine hydrochloride (pH 7.0) at room temperature for 24 hours. Again the product was dialysed washed and freeze dried.

2.8. Peptide-Protein Conjugation Using Carbodiimide

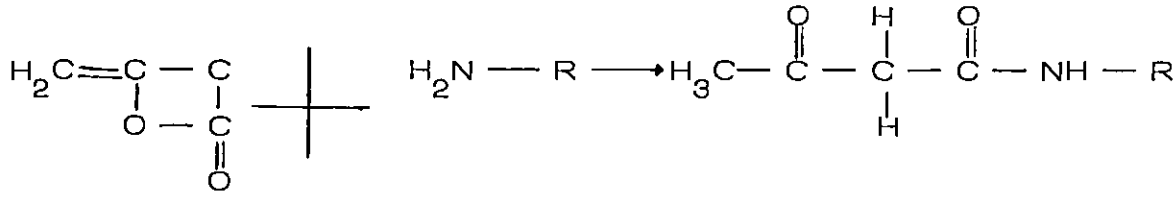
Pentaglycine (Sigma Chemical Co.Ltd) was linked to BSA (Sigma Chemical Co.Ltd) using the bifunctional reagent 1 cyclo-3-(2-morpholino-ethyl)-carbodiimide metho-p-toluene sulphonate (Aldrich Chemical Co. Ltd), by a modification of the method used by Goodfriend, Levine and Fasman (1966). 10 mg BSA and 10mg pentaglycine were dissolved together in 2 ml distilled water. 50 mg of the carbodiimide dissolved in 0.25 ml of distilled water was added and the reaction allowed to proceed

The Acetoacetylation Reactions

Figure 4.

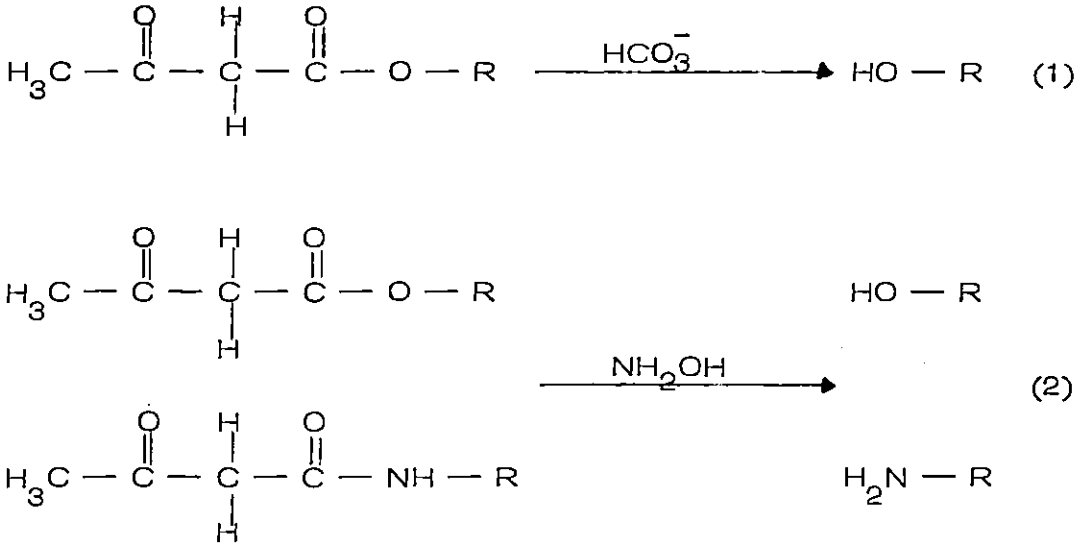


ACETOACETIC ANHYDRIDE | HYDROXYL RESIDUE | ACETOACETYLATED PRODUCT



ACETOACETIC ANHYDRIDE | AMINO RESIDUE | ACETOACETYLATED PRODUCT

The Selective Unblocking of the Acetoacetylated Products



(1) The unblocking of acetoacetylated hydroxyl residues by treatment with bicarbonate.

(2) The unblocking of acetoacetylated hydroxyl and amino residues by treatment with hydroxylamine.

for 30 minutes. The product was passed down a Sephadex-G-25 fine column and eluted with NH_4HCO_3. The non-retarded fractions absorbing at 280 nm were pooled and freeze dried.

2.9. The Measurement of Antistaphylococcal Antibody by an Enzyme Linked Immunosorbant Assay (ELISA).

One hundred microlitres of antigen (S. aureus strain Wood 46 at an optical density of 1.0) in carbonate-bicarbonate buffer (pH 9.6).

An enzyme linked immunosorbant assay was set up to measure serum antibodies to S. aureus. The assays were performed in flatbed polvinyl chloride microtitre trays (Dynateck Laboratories, Inc.). S. aureus Wood 46 was used as antigen. This strain is deficient in protein A and overcomes the problem of non specific binding of S. aureus to immunoglobulins. A conjugate of horseradish peroxidase coupled to rabbit immunoglobulins was used in each assay. It is important in the ELISA technique that there is an excess of antigen, dilutions of known positive and negative sera were therefore tested against increasing antigen concentrations (Figure 5). A plateau was reached at concentrations of antigen above an optical density of 1.0. The concentrations of S.aureus Wood 46 used in each assay was then kept within the plateau range. Preliminary experiments using several dilutions of serum and conjugate were performed to find their optimal concentrations for the assay.

Plates were coated with 100 µl of antigen in carbonate-bicarbonate buffer (pH 9.6) and incubated at room temperature for 30 minutes. The plates were washed three times with phosphate buffered saline containing 0.05% Tween at this time and before the addition of each of the subsequent

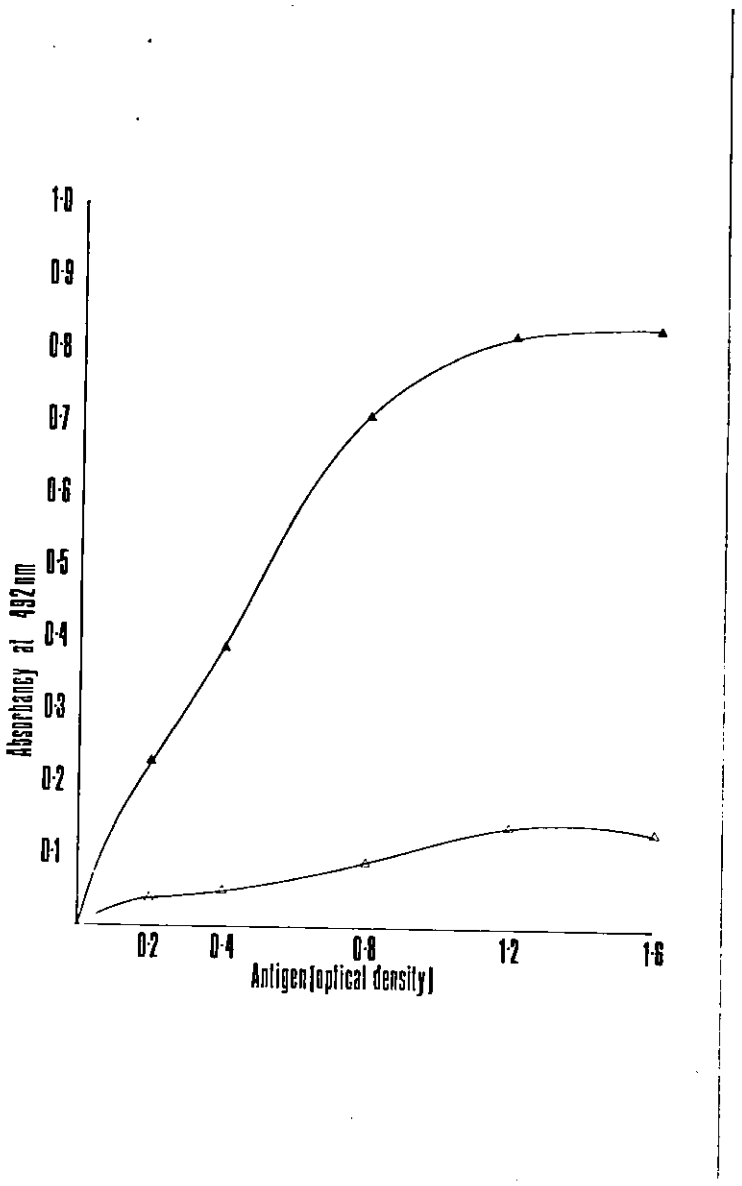


Figure 5.

The measurement of antistaphylococcal antibody by an enzyme linked assay, the effect of antigen concentration.

▲ positive serum

△ negative serum

reagents. Two hundred microlitres of the test sera, diluted 1:100 in PBS-Tween was added to each well of the microtitre tray. Trays were incubated for one hour at 37°C in a moist atmosphere. After washing, antimouse immunoglobulins conjugated to horseradish peroxidase (Dako immunoglobulins, Mercia Brocades Ltd) were added at a dilution of 1:100. The conjugated antibody was incubated in the trays for two hours at 37°C in a moist atmosphere. After washing, 100 µl of the substrate o-phenylenediamine (Sigma Chemical Co.Ltd) 0.4 mg/ml dissolved in phosphate citrate buffer pH 5.0 was added to each well. Colour development was terminated by the addition of 50 µl of 2.5M H_2SO_4. The optical density of each well was read at 492 nm. A negative control, using the pooled sera from several normal mice was tested in each tray. No standardisation of the assay was required as all sera were measured on the same day.

2.10. Plasma for Measurement of Antibody Levels

Mice were bled by cutting off the tips of their tails and allowing the blood to be drawn in heparinised capillary tubes. These tubes were plugged, centrifuged, and the plasma removed and stored at minus 70°C.

2.11. Plasma Transfers

Mice were bled directly from the heart into heparinised 1 ml syringes, the blood was pooled and mixed with heparin at a concentration of 10 units/ml. Plasma was separated by centrifugation and stored at minus 70°C. The immune plasma or plasma fractions were injected into recipient mice via the tail veins 1 hour before sensitisation.

(a) Gel Chromatography of Immune Plasma

Plasma from mice given four infections was applied to a Sephacryl G-200 (Pharmacia Fine Chemicals) column (80 x 1.6 cm). The column was run at room temperature and eluted with PBS, the fractions monitored by measuring the optical density at 280 nm. The fractions under each of the 3 peaks were pooled and concentrated down to the original volume with an Amicon Filter (membrane pore size in excess of 50,000M.W).

(b) Absorption with Cell Walls and Peptidoglycan

Plasma was mixed with cell walls or peptidoglycan (1 mg/ml) for 2 hours at room temperature. The immune complexes were removed by centrifugation and millipore filtration (pore size 0.4 μm).

2.12 Total Phosphate Determination

Phosphate was determined by a modification of the method used by Ames and Dubin (1960). The sample (less than 50 μl) was mixed in a 1 x 7.5 cm pyrex test tube with 0.02 ml of 10% Mg $(NO_3)_2$ in ethanol; the mixture was evaporated to dryness in a bunsen flame and further heated in a strong flame until the brown fumes disappeared. The tube was allowed to cool, 0.3 mls of 0.5M HCl added and heated in a boiling water bath for 15 minutes to hydrolyse pyrophosphate. After cooling, 0.7 ml of ascorbic-molybdate mixture was added and incubated at 45°C for 20 minutes, the optical density was then read at 820 nm against a blank containing water. The ascorbic-molybdate mixture which was made up daily, contained 1 part of 10% ascorbic acid to 6 parts 0.42% ammonium molybdate in 2M H_2SO_4.

2.12.1. Measurement of Free Amino Groups

Free amino groups were measured by reaction with dinitrofluoro-benzene (DNFB), using the method of Ghuysen Tipper and Strominger (1965). The sample (25 µl) was mixed with 100 µl of 1% sodium borate, $Na_2B_4O_7.10\ H_2O$. 10 µl of 10 mM DNFB in ethanol was added and each sample immediately placed in a water bath at 60°C for 30 minutes. After incubation, 500 µl of 2M HCl was added and the absorbancy read at 420 nm against a blank containing water.

2.12.2. Measurement of Reducing Power

Reducing groups were measured by the Ghuysen, Tipper and Strominger (1965) modification of the original Park and Johnson method. The sample (containing reducing power equivalent to 1-5 n moles of N-acetylglucosamine)was diluted to 100 µl in a 1 ml tube. 100 µl of a fresh mixture of 0.05% potassium ferricyanide and a carbonate-cyanide solution 1:1 was added. The mixture was heated for 15 minutes in a boiling water bath, cooled and 250 µl of ferric iron solution added. This was allowed to stand for 15 minutes, before the absorbancy was read at 690 nm against a blank containing water.

Stock Solutions.

Carbonate-Cyanide Solution:	6.3g sodium carbonate and 0.65g potassium cyanide inllitre of water.
Ferric Iron Solutions:	1.5g Ferric ammonium sulphate and 1g of sodium dodecyl sulphate in 1 litre of 0.15M Sulphuric acid.

RESULTS

In this study cyclophosphamide was given two days before sensitisation to enhance delayed hypersensitivity to S. aureus to levels normally only seen after multiple infections. This method of potentiating delayed hypersensitivity was first applied to S. aureus by Easmon and Glynn (1977). In addition, they performed spleen cell transfers to confirm that this was indeed a cell mediated delayed hypersensitivity response.

The results are presented as footpad swellings measured 4, 24 and 48 hours after the injection of challenge antigen.

3.1.1. Induction of delayed hypersensitivity to S. aureus.

Preliminary experiments were performed to determine whether delayed hypersensitivity to S. aureus could be induced with killed bacteria. The induction of cell mediated responses is usually dependent upon the presence of viable organisms. However, Tribble and Bolen (1978) demonstrated it was possible to induce hypersensitivity to S. aureus with formalin and heat killed S. aureus in Freunds Complete Adjuvant.

Two days after the intraperitoneal administration of cyclophosphamide (200 mg/kg body weight), mice were sensitised with S. aureus. One group of mice was given subcutaneous infections of 10^5 cfu of viable staphylococci and another group given 10^8 heat killed cfu of S. aureus. The response to footpad challenge with 20 µg S. aureus cell walls is shown in Table 1. There was little difference in the 4 hour responses.between the sensitised and non-sensitised control mice. However, by 24 and 48 hours a delayed response had developed in both

TABLE 1.

The footpad swellings in mice sensitised with 10^8 heat killed and 10^5 viable S. aureus and challenged with 20 µg S. aureus cell walls.

Sensitising Agent	Footpad swellings mm(± SD) 4 hours	Sig'(P)*	24 hours	Sig'(P)*	48 hours	Sig'(P)*
10^8 heat killed S. aureus	0.52(0.15)	NS	0.76(0.18)	<0.005	0.60(0.18)	<0.001
10^5 viable S. aureus	0.54(0.29)	NS	0.78(0.44)	<0.02	0.58(0.30)	<0.01
Control	0.70(0.04)		0.28(0.12)		0.11(0.10)	

*Student's T test comparing the mean footpad swellings in the sensitised and non-sensitised control mice.

Each group contained 6 mice.

NS = Not significant

groups of sensitised mice and statistically significant differences in footpad swellings between these and the control mice were measured. Cyclophosphamide treated mice were injected subcutaneously with 10^5, 10^7, $5x10^7$ and 10^8 heat killed or with 10^5 viable S. aureus. The footpad responses on challenge with 20µg S. aureus cell walls are shown in Table 2. Sensitising doses of $5x10^7$ or more heat killed S. aureus induced delayed hypersensitivity reactions, with significantly greater footpad swellings at 24 and 48 hours than in the controls. Delayed reactions induced by 10^7 and 10^5 heat killed bacteria, were not significantly greater than that seen in the control mice. Formalin killed S. aureus consistantly induced greater footpad swellings than an equivalent number of heat killed staphylococci but the differences were not statistically significant (Table 3).

3.1.2. The induction of delayed hypersensitivity with cell walls and peptidoglycan from S. aureus.

The last series of experiments demonstrated a requirement for 10^8 heat killed S. aureus to induce a good delayed hypersensitivity response. Attempts were then made to induce delayed hypersensitivity in cyclophosphamide treated mice with subcutaneous injections of cell walls and peptidoglycan on cotton dust plugs. The sensitising doses of 0.0625 mg, 0.125 mg and 0.25 mg cell walls and peptidoglycan were based on the dry weight of 10^8 S. aureus. Figure 6 shows the 24 hour footpad swellings when these mice were challenged with 20µg of cell walls. Both the peptidoglycan and the cell walls induced significant 24 hour footpad swellings, however dose for dose cell walls induced greater footpad swelling than peptidoglycan.

TABLE 2.

The footpad swellings in mice sensitised with increasing doses of heat killed S. aureus and challenged with 20 μg S. aureus cell walls.

Sensitising Agent (S. aureus)		Footpad swellings mm (±SD) 4 hours	Sig'(P)*	24 hours	Sig'(P)*	48 hours	Sig'(P)*
10^8	heat killed	0.64(0.04)	NS	0.82(0.16)	<0.005	0.63(0.10)	<0.002
$5x10^7$	heat killed	0.62(0.09)	NS	0.71(0.17)	<0.025	0.60(0.11)	<0.01
10^7	heat killed	0.58(0.09)	NS	0.66(0.17)	NS	0.48(0.13)	<0.02
10^5	heat killed	0.72(0.14)	NS	0.51(0.29)	NS	0.26(0.31)	NS
10^5	viable	0.59(0.16)	NS	0.90(0.25)	<0,025	0.68(0.13)	<0.01
control		0.62(0.21)		0.35(0.19)		0.17(0.14)	

*Student's T test comparing the mean footpad swellings in the sensitised and non-sensitised control mice. Groups of 6 mice.

NS = Not significant

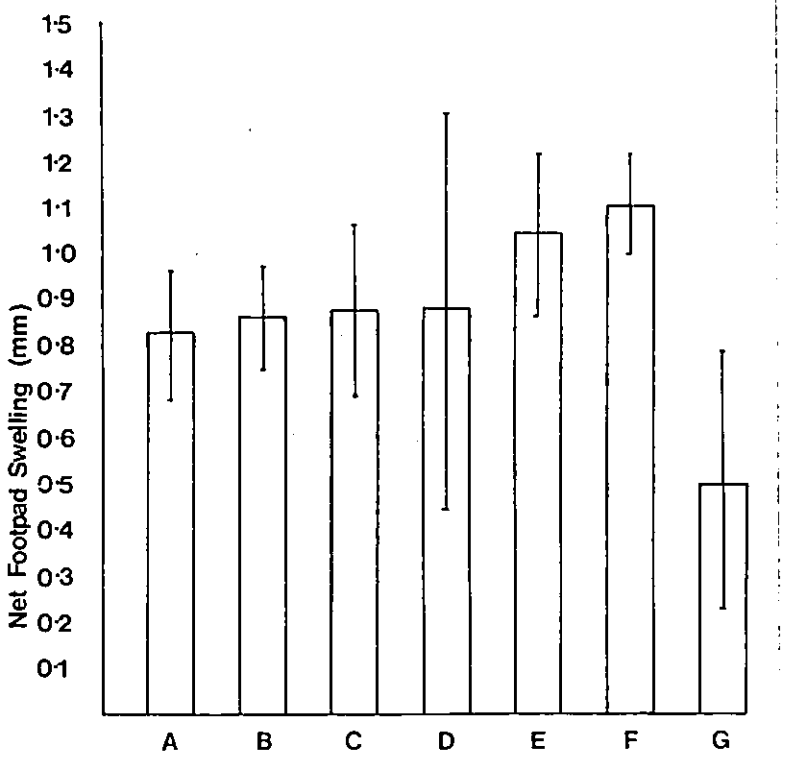


Figure 6.

The 24 hour footpad swelling in mice sensitised with cell walls or peptidoglycan from S. aureus and challenged with 20µg S. aureus cell walls.

Sensitising doses		Significance (P)*
A	0.0625 mg peptidoglycan	NS
B	0.125 mg peptidoglycan	<0.05
C	0.250 mg peptidoglycan	<0.05
D.	0.0625 mg cell walls	NS
E	0.125 mg cell walls	<0.02
F	0.250 mg cell walls	<0.01
G	control	

Standard deviations shown by bars on histogram. *Student's t test.

TABLE 3.

The 24 hour footpad swelling in mice sensitised with formalin or heat killed S. aureus and challenged with 20 µg S. aureus cell walls.

Sensitising Agent (S. aureus)	24 hour footpad swelling (±SD)	Significance (P)*
10^8 heat killed	0.61(0.22)	<0.002
10^8 formalin killed	0.74(0.14)	<0.001
control	0.17(0.10)	

*Student's t test comparing the mean footpad swellings in sensitised and non sensitised control mice. Each group contained 5 mice.

3.2. Use of lysostaphin to split the peptidoglycan structure

In an attempt to identify the antigenic determinants for the delayed hypersensitivity reaction lysostaphin, a staphylolytic enzyme mixture was used to degrade the peptidoglycan in cell walls. Lysostaphin is a combination of three enzymes, endo-N-acetyl glucosaminidase, N-acetyl-muramyl-L-alanine amidase and endopeptidase (glycinase), the sites of action of these enzymes are shown in Figure 7. Cell walls treated with lysostaphin for up to 90 minutes were then used to elicit a delayed hypersensitivity reaction in S. aureus sensitised mice. The degradation of the cell walls was monitored by the release of free amino groups and the increase in the reducing power of the supernatant and by the fall in optical density of the cell wall suspension. The release of free amino groups measures the rates of action of the amidase and endopeptidase enzymes, therefore reflecting the breakdown of the peptide part of the peptidoglycan. The release of reducing power measures the rate of action of the glucosaminidase enzyme and therefore shows the degradation of the polysaccharide chain of peptidoglycan.

The effect of the lysostaphin on cell walls is represented in Figures 8 and 9. After 60 minutes treatment the optical density measured at 620 nm had fallen to 25% of its original value and thereafter remained constant. The release of the cell wall constituents over the period of lysis is shown in Figure 9. There was a rapid release of amino groups, especially after 60 minutes. This was accompanied by a much slower release of reducing power, indicating that lysis was primarily associated with the loss of integrity of the peptide part of the peptidoglycan.

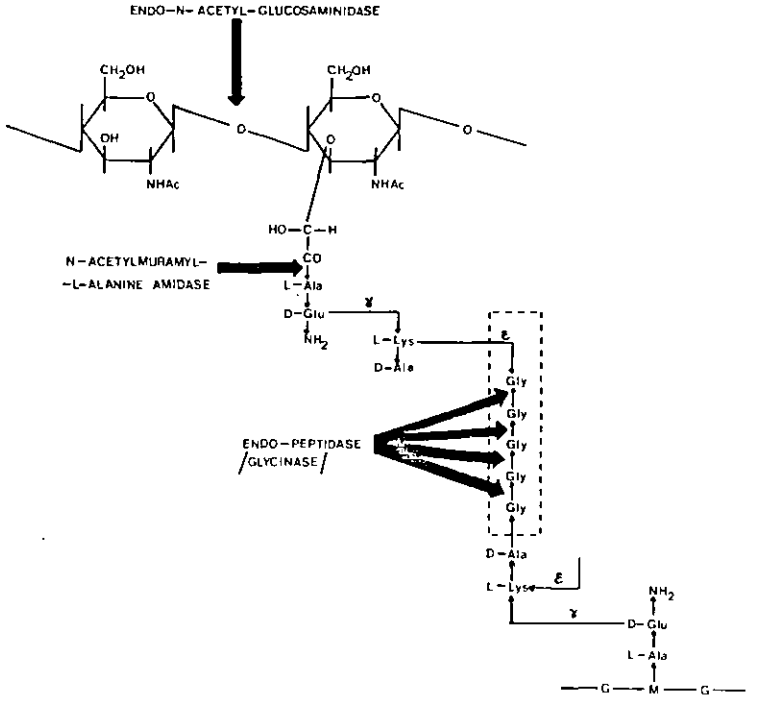


Figure 15.

Action of three enzymatic activities of lysostaphin: endo-N-acetyl-glucosaminidase, N-acetyl-muramyl-L-alanine amidase, and endo-peptidase (glycinase) on staphylococcal peptidoglycan.

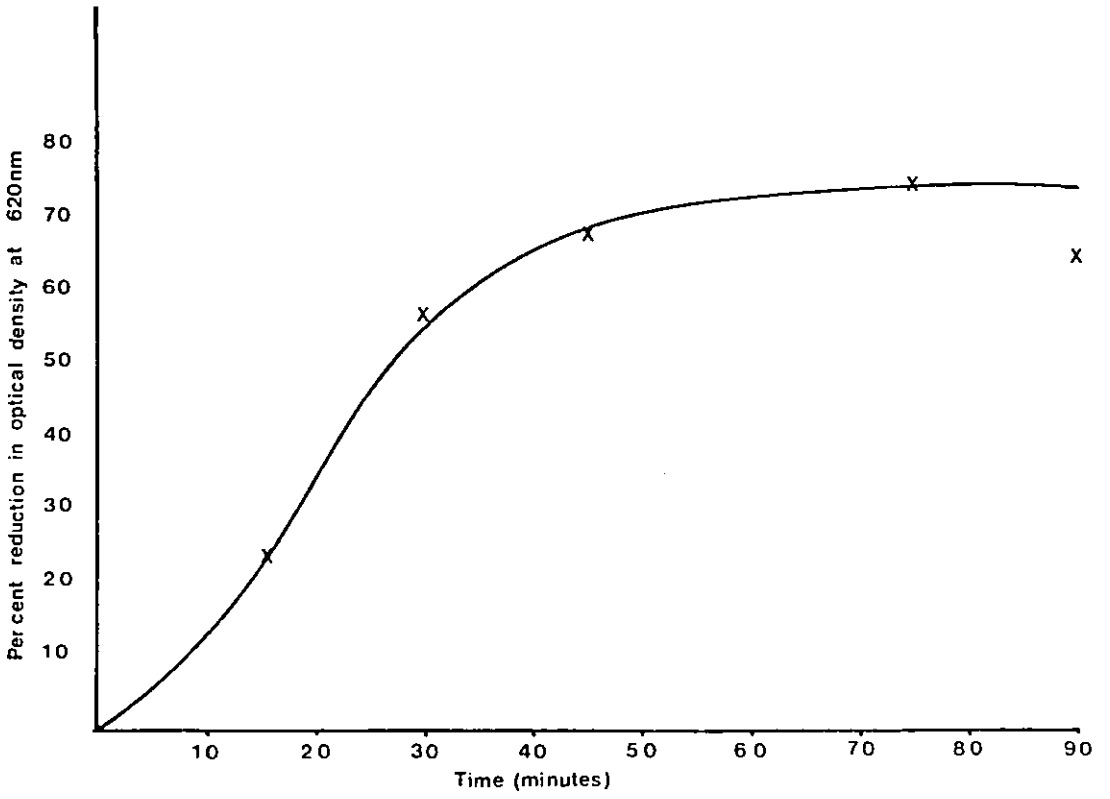


Figure 8.

Lysis of S. aureus cell walls by lysostaphin, measured by the reduction in optical density of the cell wall suspension at 620 nm.

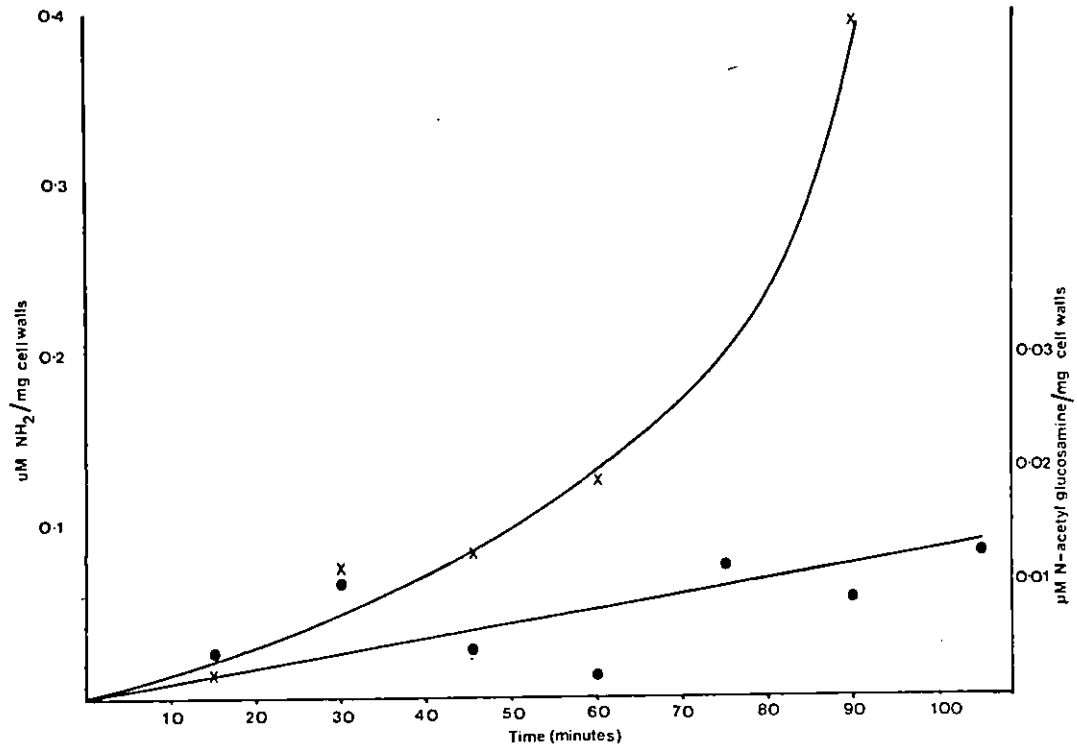


Figure 9.

Release of cell wall constituents by lysostaphin. (X) free amino groups, (●) reducing power.

Table 4 shows the 24 hour footpad swellings obtained when 20 µg of the lysed cell walls were used as challenge antigens in S. aureus sensitised mice. Both the untreated cell walls and the cell walls lysed for 60 minutes gave significantly greater 24 hour footpad swellings in the sensitised mice than in the non sensitised controls. However, the cell walls which had been subjected to 90 minutes of lysostaphin digestion elicited no delayed hypersensitivity reaction, with 24 hour footpad barely above the control levels. It is of interest to note that the ability of the cell walls to stimulate a delayed hypersensitivity reaction was lost after 60 minutes of lysis, at which time the release of free amino groups was maximal and the release of reducing power still low. This suggests that the antigenic determinant for the delayed hypersensitivity reaction involves the peptide part of the peptidoglycan.

3.3. Modification of peptidoglycan by growth in a glycine enriched medium and its effect on the ability of peptidoglycan to elicit delayed hypersensitivity.

Supplementation of the growth medium with high concentrations of glycine can inhibit bacterial growth and alter the structure of the peptidoglycan (Hammes, Schleifer and Kandler 1972). The effect of such modifications on the immunogenicity of S. aureus cell walls was examined.

3.3.1. The effect on S. aureus of growth in a medium supplemented with 3% glycine.

The growth of S. aureus in 3% glycine enriched, yeast extract glucose broth is shown in Figure 10. Clearly the glycine enrichment of

TABLE 4.

The 24 hour footpad swelling in mice sensitised with S. aureus and challenged with 20 µg of lysostaphin treated cell walls.

Period of lysostaphin treatment (minutes)	Sensitisation of mice	24 hr footpad swelling(±SD)	Significance (P)*
0	+	0.79(0.20)	<0.025
control	−	0.46(0.13)	
60 minutes	+	0.94(0.21)	<0.02
control	−	0.41(0.07)	
90 minutes	+	0.58(0.02)	NS
control	−	0.48(0.06)	

*Student's t test comparing the mean footpad swellings in the sensitised and non-sensitised control mice. Each group contained 4 mice.

NS = Not significant.

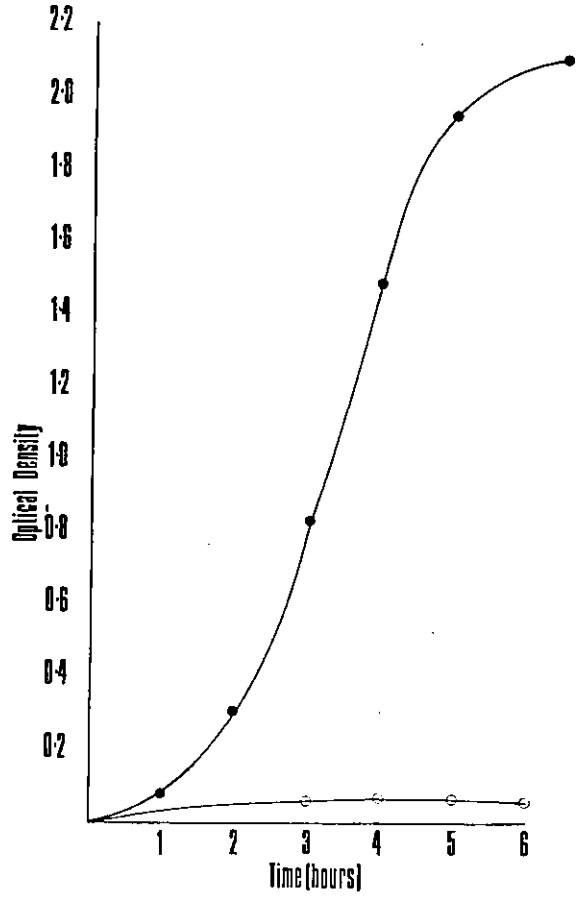


Figure 10.

Inhibition of staphylococcal growth by medium supplemented with 3% glycine.

● Growth in yeast extract glucose broth

o Growth in yeast extract glucose broth plus 3% glycine

the medium has inhibited the staphylococcal growth.

The staphylococci cultured in the supplemented medium exhibited many changes from the original strain. The bacteria were gram negative, did not form any of the usual clusters, were variable in size and gave a negative result with the slide coagulase test. Subcultured onto blood agar the bacteria reverted to being both gram and coagulase positive. When subcultured onto glycine enriched agar the colonies were smaller and less pigmented than on nutrient agar.

It seems that growth in a 3% glycine enriched medium induces a phenotypic change which produces a variant of S. aureus similar to the L-forms isolated from cultures containing penicillin.

3.3.2. Delayed hypersensitivity stimulated by cell walls from S. aureus grown in a glycine enriched medium.

Cell walls from S. aureus grown in a glycine enriched medium were compared with cell walls from S. aureus grown in the usual medium for their ability to elicit a delayed hypersensitivity response. As expected, challenge with 20 µg of normal cell walls gave rise to significant delayed reactions at 24 and 48 hours (Table 5). Challenge with 20 µg of cell walls from staphylococci grown in the glycine enriched medium showed a different picture. There was a high 4 hour response in both the sensitised and non-sensitised mice. This was significantly greater than the 4 hour response seen when normal cell walls were used for challenge. However, the delayed footpad swellings at 24 and 48 hours in the sensitised mice were not significantly greater than the controls (for a full statistical analysis of this experiment see Appendix). It is possible that the high early inflammatory response obtained with the modified cell

TABLE 5.

The footpad swellings elicited in S. aureus sensitised mice by 20 µg cell walls from S. aureus grown in a glycine enriched medium.

Challenge Antigen	Sensitisation of mice	Footpad swellings mm(±SD) 4 hours	Sig'(P)*	24 hours	Sig'(P)*	48 hours	Sig'(P)*
Cell walls from normal S. aureus	+	[a]0.43(0.07)	NS	0.61(0.10)	<0.002	0.51(0.11)	<0.002
Cell walls from normal S. aureus	-	[b]0.40(0.07)		0.27(0.12)		0.09(0.11)	
Cell walls from S. aureus grown in 3% glycine	+	[a]0.71(0.19)	NS	0.57(0.21)	NS	0.34(0.14)	NS
Cell walls from S. aureus grown in 3% glycine	-	[b]0.65(0.16)	---	0.39(0.07)	---	0.14(0.12)	---

*Student's t test comparing the mean footpad swellings in the sensitised and non-sensitised control mice. Sensitised mice were in groups of 6, control mice were in groups of 4.

NS = Not significant

a = Student's t test comparing these two mean footpad swellings $P < 0.05$

b = Student's t test comparing these two mean footpad swellings $P < 0.02$

walls persisted up till 24 hours and therefore masked a delayed hypersensitivity reaction. Thus it seems that although the modified cell walls can stimulate inflammatory reactions it is not certain if they can elicit a delayed hypersensitivity reaction.

3.4. The chemical modification of peptidoglycan by acetoacetic anhydride

Acetoacetic anhydride is a powerful reagent which will specifically acetoacetylate free hydroxyl and amino residues. This series of experiments studied the effect of the chemical modification of peptidoglycan on its ability to stimulate a delayed response in S. aureus sensitised mice. The modified groups were then selectively removed, first from the hydroxyl residues, then from the amino groups and the activity of the partially and completely restored peptidoglycan was again measured.

3.4.1. Delayed hypersensitivity elicited by the acetoacetylated peptidoglycan

Cyclophosphamide treated mice were sensitised with S. aureus. Twelve days later footpad tests were performed, challenging mice with 20 µg each of peptidoglycan and acetoacetylated peptidoglycan. Peptidoglycan elicited a significant delayed reaction at 24 and 48 hours (Table 6). However, the modified peptidoglycan did not stimulate any delayed response. Thus the acetylation of the free amino and hydroxyl groups of peptidoglycan has resulted in the loss of the ability to elicit delayed hypersensitivity to S. aureus.

3.4.2. Partial and complete removal of the acetoacetyl modifications and its effect on the ability to elicit delayed hypersensitivity.

Cyclophosphamide treated S. aureus sensitised mice were footpad

TABLE 6.

The footpad swellings in mice sensitised with S. aureus and challenged with 20 µg of acetoacetylated peptidoglycan from S. aureus.

Challenge Antigen	Sensitisation of mice	Footpad swellings mm (±SD) 4 hours	Sig'(P)*	24 hours	Sig'(P)*	48 hours	Sig'(P)*
normal peptidoglycan	+	0.66(0.09)	NS	1.23(0.46)	<0.02	0.82(0.07)	<0.02
normal peptidoglycan	-	0.76(0.14)		0.49(0.13)		0.23(0.23)	
acetoacetylated p'g	+	0.72(0.11	NS	0.56(0.14)	NS	0.39(0.15)	NS
acetoacetylated p'g	-	0.83(0.18)		0.45(0.16)		0.25(0.17)	

* Student's t test comparing the mean footpad swellings in the sensitised and non-sensitised control mice. Each group contained 4 mice.

NS = Not significant

tested with:

(a) 20 µg peptidoglycan

(b) 20 µg acetoacetylated peptidoglycan

(c) 20 µg acetoacetylated peptidoglycan with the modifications removed from the hydroxyl residues by treatment with Na_2CO_3

(d) 20 µg acetoacetylated peptidoglycan with the modifications removed from both the hydroxyl and amino residues by treatment with hydroxylamine.

The footpad responses are shown in Table 7. As expected, the peptidoglycan elicited delayed reactions at 24 and 48 hours. The delayed hypersensitivity elicited by the modified peptidoglycan was significantly less than that elicited by the normal peptidoglycan. The modified peptidoglycan did however elicit a significant delayed response at 24 and 48 hours. This was probably due to the incomplete modification of peptidoglycan by the acetoacetic anhydride. The acetoacetylated peptidoglycan treated with Na_2CO_3 stimulated only poor delayed reactions at 24 and 48 hours, which were not significantly greater than the control values. However, the hydroxylamine treated acetoacetylated peptidoglycan stimulated a full delayed hypersensitivity reaction. Therefore, while removal of acetoacetyl groups from the hydroxyl residues did not return any activity to the peptidoglycan, removal of all the modifications completely restores the ability to stimulate delayed hypersensitivity. The antigenic determinant for delayed hypersensitivity is therefore not associated with the hydroxyl residues of the polysaccharide chain of peptidoglycan, but it could be associated with the amino groups found in the peptide part of peptidoglycan.

TABLE 7.

The footpad swellings in mice sensitised with S. aureus and challenged with 20µg peptidoglycan, 20 µg acetoacetylated peptidoglycan, 20 µg sodium carbonate treated peptidoglycan and 20 µg hydroxylamine treated peptidoglycan.

Challenge Antigen	Sensitisation of mice	4 hours	Sig'(P)*	24 hours	Sig'(P)*	48 hours	Sig'(P)*
peptidoglycan	+	0.50(0.24)	NS	[a]1.34(0.23)	<0.001	1.04(0.34)	<0.002
peptidoglycan	−	0.41(0.17		0.39(0.06)		0.10(0.07)	
acetoacetylated p'g'	+	0.35(0.10)	NS	[a]0.82(0.21)	<0.01	0.55(0.18)	<0.01
acetoacetylated p'g'	−	0.65(0.12)		0.31(0.11)		0.06(0.07)	
Na_2CO_3 treated aceto-acetylated p'g'	+	0.54(0.19)	NS	0.64(0.31)	NS	0.18(0.20)	NS
Na_2CO_3 treated aceto-acetylated p'g'	−	0.84(0.20)		0.49(0.15)		0.05(0.09)	
hydroxylamine treated acetoacetylated p'g'	+	0.57(0.17)	NS	1.23(0.40)	<0.01	1.03(0.03)	<0.001
hydroxylamine treated acetoacetylated p'g'	−	0.75(0.20)		0.32(0.15)		0.06(0.06)	

*Student's t test comparing the mean footpad swellings in sensitised and non-sensitised mice. Each group contained 4 mice.

NS = Not significant

a = Student's t test comparing these two mean footpad swellings $P<0.02$.

3.5. Cross reactions between S. aureus and Streptococcus Group A and S. aureus and Staphylococcus epidermidis.

Peptidoglycans from staphylococci and streptococci are structurally very similar (Figure 3) and antiserum raised against one will cross react with cell walls from the other. Cross reactions in delayed hypersensitivity were observed and related to the structures of the peptidoglycans to locate the antigenic determinants for this reaction.

3.5.1. Staphylococcus aureus and Streptococcus Group A strain T 27.

Mice sensitised with S. aureus and pretreated with cyclophosphamide were footpad tested with 20 µg of cell walls from either S. aureus or Streptococcus Group A strain T 27. As expected, mice challenged with S. aureus cell walls gave rise to significant delayed reactions (Table 8). However, challenge with the streptococcal cell walls produced no significant swelling at 24 hours.

3.5.2. S. aureus and S. epidermidis NCTC 5955.

Two groups of cyclophosphamide treated mice were injected with 10^5 cfu S. aureus on a cotton dust plug. Twelve days later footpad tests were performed using 20 µg cell walls from either S. aureus or S. epidermidis as challenge antigens. Similarly, two groups of S. epidermidis sensitised mice were challenged with 20 µg S. aureus and S. epidermidis cell walls. Control experiments were performed by challenging non-sensitised mice with the two types of cell walls. The footpad swellings are shown in Table 9. The highest levels of delayed hypersensitivity were obtained when both sensitisation and challenge were with bacteria and walls of the same species. Cross reactions did occur between the two staphylococcal species and delayed responses were achieved when

TABLE 8.

The footpad swelling in mice sensitised with S. aureus and challenged with 20 µg staphylococcal and streptococcal cell walls.

Challenge Antigen	Sensit-isation of mice	24 hr.footpad swellings(±SD)	Significance (P)*
S. aureus cell walls	+	0.82(0.11)	<0.0005
S. aureus cell walls	−	0.30(0.04)	
Strep' Grp.A cell walls	+	0.31(0.13)	NS
Strep' Grp.A cell walls	−	0.35(0.21)	

*Student's t test comparing the mean footpad swellings in the sensitised and non-sensitised mice. Sensitised mice were in groups of 6, control mice were in groups of 4.

NS = Not significant.

TABLE 9.

The footpad swellings in mice sensitised with S. aureus and S. epidermidis and challenged with 20 µg of the homologous or heterologous cell walls.

Challenge Antigen	Sensitisation of mice	Footpad swelling mm (±SD) 4 hours	Sig'(P)*	24 hours	Sig'(P)*	48 hours	Sig'(P)*
S. aureus	S. aureus	0.56(0.09)	NS	0.90(0.21)	<0.01	0.50(0.31)	NS
S. aureus	S. epidermidis	0.61(0.08)	NS	0.77(0.11)	NS	0.32(0.17)	NS
S. aureus	------	0.64(0.09)		0.41(0.11)		0.10(0.12)	
S. epidermidis	S. epidermidis	0.64(0.09)	NS	0.89(0.22)	<0.02	0.40(0.19)	NS
S. epidermidis	S. aureus	0.47(0.08)	NS	0.71(0.17)	NS	0.36(0.23)	NS
S. epidermidis	------	0.72(0.08)		0.55(0.08)		0.19(0.14)	

*Student's t test comparing the mean footpad swelling in the sensitised and non-sensitised mice. Each group contained 4 mice.

NS = Not significant.

mice sensitised with one of the bacteria were challenged with the heterologous cell walls. However, the swellings in the sensitised mice were not significantly greater than that observed in the non-sensitised control mice.

The structure of the peptidoglycans of S. aureus and Streptococcus Group A are, apart from their interpeptide bridges, identical. The lack of cross reactivity between these two bacteria must indicate that the interpeptide bridge is important in the antigenic determinant for delayed hypersensitivity. The peptidoglycans of S. aureus and S. epidermidis only differ in that the pentaglycine interpeptide bridge of S. aureus is replaced in S. epidermidis by four glycines and a single alanine residue (Schleifer, 1973) on this basis therefore, the partial cross reactivity is not unexpected.

3.6. The use of soluble cell wall fractions to induce and elicit delayed hypersensitivity.

It is possible using specific cell wall lytic enzymes to obtain soluble fragments of the cell wall, I have used two such enzymes, a muramidase from Chaloropsis sp. and a specific protease from S. aureus V8. These fragments have then been used in an attempt to both induce and elicit delayed hypersensitivity, to determine whether the intact three dimensional structure of the cell wall is necessary for this reaction and to find which parts of the cell wall are of importance.

3.6.1. The induction of delayed hypersensitivity with soluble cell wall fragments produced by muramidase from Chaloropsis sp.

Cyclophosphamide treated mice were sensitised with the crude soluble extract produced by the action of the Chaloropsis sp. muramidase

on S. aureus cell walls. The extract (0.1 ml) was administered intradermally in FCA, at a concentration of 2 mg/ml. Twelve days later footpad tests were carried out using both cell walls and the soluble extract as challenge antigens (Table 10). The soluble extract induced a delayed hypersensitivity reaction which was elicited both by the soluble extract itself and by S. aureus cell walls. Where the extract was used to elicit delayed hypersensitivity there was a rapid fall off in swelling between 24 and 48 hours. This could be due to the soluble antigen being rapidly removed from the site of injection.

3.6.2. The stimulation of delayed hypersensitivity to S. aureus by the soluble extracts produced by Chaloropsis sp. muramidase and S. aureus V8 protease.

The crude soluble extracts produced by Chaloropsis sp. muramidase and S. aureus V8 protease were used to stimulate a delayed hypersensitivity response in cyclophosphamide treated S. aureus sensitised mice. Table 11 shows that the muramidase extract was able to elicit significant delayed swelling at 24 hours, however, this swelling did not follow the classical delayed hypersensitivity pattern and had subsided by 48 hours. Again, this was probably due to the prompt removal of antigen from the site of injection. The solubilised cell walls produced by the protease enzyme elicited no significant delayed swelling, there was however, some early inflammatory response.

3.6.3. The purification of the soluble cell wall extract produced by Chaloropsis sp. muramidase.

The soluble extract (30 mg) dissolved in 1 ml distilled water was applied to a Sephadex G-25 column. Elution was performed at 4^{o}C with

TABLE 10.

The footpad swellings in mice sensitised with the soluble extract produced from S. aureus cell walls by Chaloropsis sp. muramidase.

Challenge Antigen	Sensitisation of mice	Footpad swellings mm(±SD) 4 hours	Sig'(P)*	24 hours	Sig'(P)*	48 hours	Sig'(P)*
S. aureus cell walls	+	0.39(0.10)	NS	1.01(0.32)	<0.02	0.94(0.12)	<0.001
S. aureus cell walls	-	0.52(0.13)		0.29(0.04)		0.12(0.07)	
soluble extract	+	0.40(0.06)	NS	0.80(0.14)	<0.02	0.38(0.13)	<0.025
soluble extract	-	0.29(0.11)		0.16(0.25)		0.06(0.10)	

*Student's t test comparing the mean footpad swellings in the sensitised and non-sensitised mice. Each group contained 4 mice.

NS = Not significant.

TABLE 11.

The footpad swellings in mice sensitised with S. aureus and challenged with 20 µg of the soluble extracts produced from S. aureus cell walls by muramidase from Chaloropsis sp. and protease from S. aureus V8.

Challenge Antigen	Sensitisation of mice	Footpad swellings mm(±SD) 4 hours	Sig'(P)*	24 hours	Sig'(P)*	48 hours	Sig'(P)*
muramidase extract	+	0.21(0.19)	NS	0.66(0.10)	<0.01	0.18(0.05)	NS
muramidase extract	-	0.20(0.09)		0.21(0.20)		0.09(0.07)	
protease extract	+	0.28(0.14)	NS	0.07(0.09)	NS	0.00	NS
protease extract	-	0.27(0.09)		0.00		0.05(0.10)	

*Student's t test comparing the mean footpad swellings in the sensitised and non-sensitised mice. Sensitised mice were in groups of 6, non-sensitised control mice were in groups of 4.

NS = Not significant

distilled water and the soluble extract separated into three fractions (Figure 11), each fraction was collected and freeze dried.

3.6.3.1. The delayed hypersensitivity elicited by the purified fractions.

Each of the three fractions produced by the gel filtration of the muramidase extract was used in an attempt to stimulate delayed hypersensitivity in cyclophosphamide treated, S. aureus sensitised mice. Table 12 shows that only fraction 1 could elicit a delayed response, stimulating significantly greater 24 hour footpad swellings in the sensitised mice than in the controls. Again as with previous experiments using the crude soluble extract as challenge antigen, the swelling at 48 hours was not significantly greater in the sensitised mice than in the controls. In this respect these reactions are more like the Jones-Mote reaction, where protein antigens in FIA are used to induce delayed hypersensitivity than classical tuberculin delayed hypersensitivity reactions.

3.6.4. The purification and analysis of the extract produced from cell walls by the S. aureus V8 protease enzyme.

Lysis with the protease enzyme solubilised 70% of the cell wall material. The soluble products were subsequently separated by Sephadex Gel chromatography into two fractions (Figure 12) and then analysed by paper chromatography. The higher molecular weight fraction was shown to contain the amino acids glycine, lysine, alanine and glutamic acid, some small quantities of sugars were also detected. The lower molecular fraction contained only sugars, no amino acids were found. The purity of the first fraction was checked using polyacrylamide gel electrophoresis, this showed a single protein band when stained with Coomassie blue. Preliminary experiments indicated that immunological

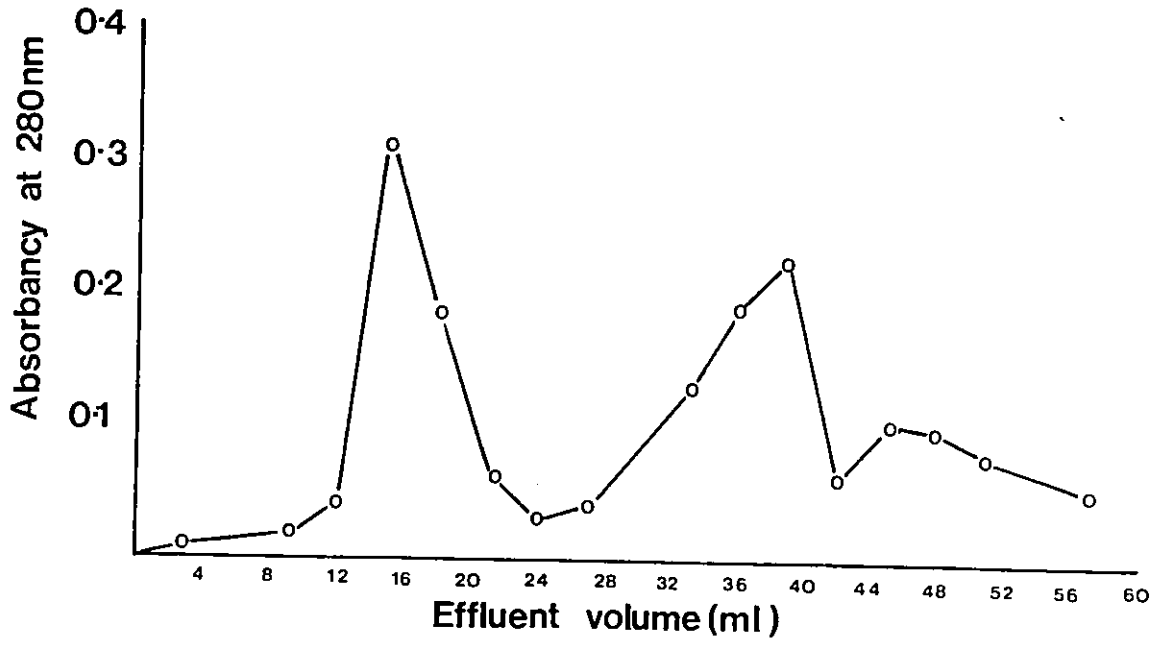


Figure 11.

Gel filtration of the soluble extract produced from cell walls by Chaloropsis sp. muramidase.

TABLE 12.

The footpad swellings in mice sensitised with S. aureus and challenged with 20 µg of the fractionated soluble extracts produced from S. aureus cell walls by the Chaloropsis sp. muramidase.

Challenge Antigen	Sensitisation of mice	Footpad swellings mm(±SD) 4 hours	Sig'(P)*	24 hours	Sig'(P)*	48 hours	Sig'(P)*
Fraction 1	+	0.35(0.14)	NS	0.40(0.13)	<0.005	0.22(0.11)	NS
Fraction 1	-	0.30(0.04)		0.11(0.06)		0.07(0.08)	
Fraction 2	+	0.21(0.10)	NS	0.18(0.07)	NS	0.09(0.03)	NS
Fraction 2	-	0.20(0.05)		0.15(0.08)		0.08(0.06)	
Fraction 3	+	0.17(0.12)	NS	0.19(0.13)	NS	0.05(0.05)	NS
Fraction 3	-	0.20(0.12)		0.21(0.16)		0.09(0.15)	

*Student's t test comparing the mean footpad swellings in the sensitised and non-sensitised control mice.

Each group contained 4 mice.

NS = Not significant

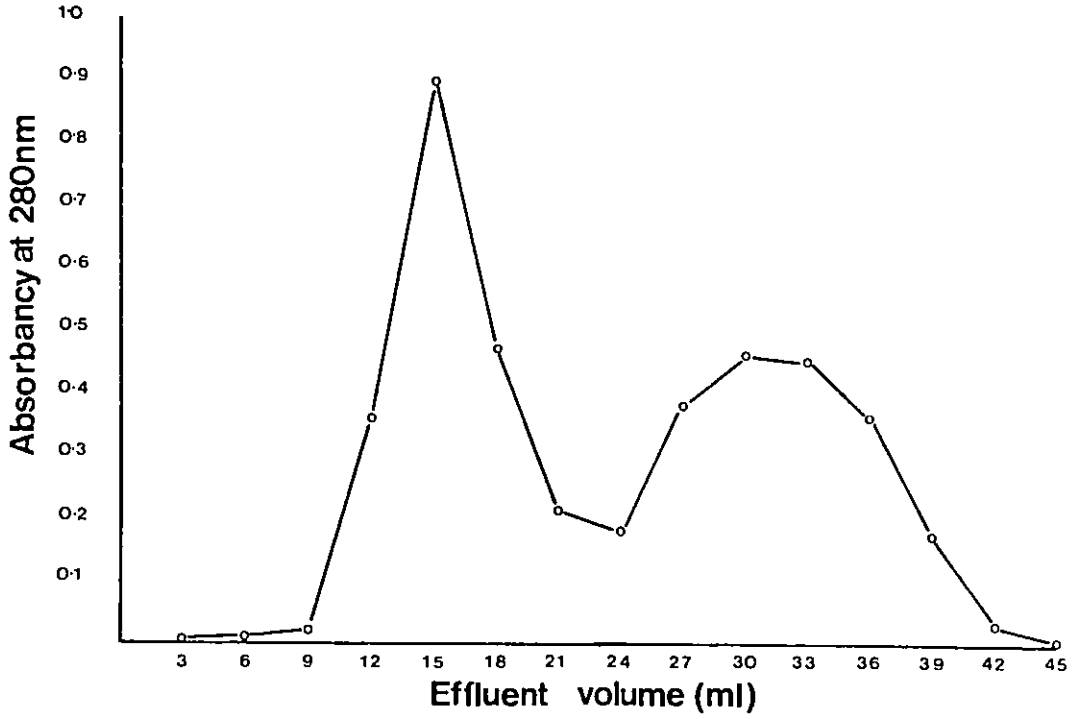


Figure 12.

Gel filtration of the soluble extract produced from S. aureus cell walls by S. aureus V8 protease enzyme.

activity was restricted to the high molecular weight fraction and this was therefore used in all subsequent experiments. This reaction in Freunds Complete Adjuvant was used to induce delayed hypersensitivity in cyclophosphamide treated mice (Table 13). Challenge with 20 µg cell walls elicited significant delayed swellings at 24 and 48 hours. Cell walls however, did not elicit any delayed swellings in mice sensitised with FCA alone, thus showing that there was no cross reactivity with the mycobacteria present in the adjuvant, nor was there any enhancement of pre-existing immunity by the adjuvant.

3.6.5. The induction of delayed hypersensitivity with the active fraction from the soluble cell wall extracts produced by the Chaloropsis sp. muramidase and S. aureus V8 protease.

Mice pretreated with cyclophosphamide were given intracutaneous injections of 0.2 ml of the active fractions from either the muramidase produced extract or the protease produced extract in Freunds Complete or Incomplete adjuvant. All emulsions were at a final concentration of 2 mg/ml. Twelve days after sensitisation, 20 µg staphylococcal cell walls were used to stimulate a delayed hypersensitivity response. In each case delayed reactions were induced and the 24 hour footpad swellings were significantly greater than in the control group of mice. Both extracts produced greater swelling when used in conjunction with FCA than with the incomplete adjuvant (Table 14).

3.7. The induction of delayed hypersensitivity to S. aureus with synthetic analogues of the pentaglycine cross bridge.

Previous experiments have indicated that the pentaglycine cross bridge is the major antigenic determinant for the delayed hypersensitivity

TABLE 13.

The footpad swellings in mice sensitised with the soluble extract produced from S. aureus cell walls by the protease enzyme and challenged with 20 µg S. aureus cell walls.

Sensitising Agent	Footpad swellings mm (± SD)					
	4 hours	Sig'(P)*	24 hours	Sig'(P)*	48 hours	Sig'(P)*
extract/FCA	0.62(0.20)	NS	0.81(0.13)	<0.005	0.71(0.18)	<0.002
FCA alone	0.61(0.11)	NS	0.37(0.07)	NS	0.20(0.10)	NS
Control	0.69(0.21)		0.43(0.14)		0.23(0.04)	

*Student's t test comparing the mean footpad swellings in the sensitised and non-sensitised mice.

Sensitised mice were in groups of 6, control mice were in a group of 4.

NS = Not significant

\+ = challenge with 20 µg cell walls.

TABLE 14.

The footpad swelling in mice sensitised with soluble cell wall extracts in Freunds Adjuvant and challenged with 20 μg S. aureus cell walls.

Sensitising Agent	24 hr. footpad swelling (±SD)	Significance(P)*
muramidase extract/FCA	0.84(0.06)	<0.001
muramidase extract/FIA	0.49(0.09)	<0.01
protease extract/FCA	0.66(0.15)	<0.002
protease extract/FIA	0.52(0.05)	<0.002
Control	0.19(0.10)	

*Students t test comparing the mean footpad swellings in the sensitised and non-sensitised control mice. Each group contained 4 mice.

reaction to S. aureus. These experiments aim to sensitise mice with synthetic analogues of the cross bridge and then to stimulate delayed hypersensitivity with S. aureus cell walls.

3.7.1. The induction of delayed hypersensitivity with pentaglycine and polyglycine.

Cyclophosphamide treated mice were injected intradermally with 0.2 ml of either polyglycine or pentaglycine in FCA at a concentration of 2 mg/ml. Twelve days later footpad tests were carried out using 20 µg S. aureus cell walls as a challenge antigen. No delayed swellings were elicited at 24 or 48 hours (Table 15). It is probable that these two antigens were too small to elicit an immunological reaction. To overcome this pentaglycine was coupled to a larger carrier protein.

3.7.2. The induction of delayed hypersensitivity with pentaglycine coupled to BSA.

Cyclophosphamide treated mice were injected intracutaneously with 0.5 ml of pentaglycine coupled to BSA in FCA, at a concentration of 1 mg/ml. Footpad tests were performed twelve days later using 20 µg of BSA-5Gly and 20 µg of S. aureus cell walls as challenge antigens.

No evidence of delayed reaction was seen when BSA-5Gly was used as challenge antigen, however the cell walls did give rise to a delayed hypersensitivity reaction, with significantly higher 24 and 48 hour footpad swellings in the sensitised mice than in the controls (Table 16). There was a possibility that the induction of the delayed hypersensitivity was due to a cross reaction of cell walls with BSA. Therefore, the experiment was repeated using BSA/FCA and BSA-5Gly/FCA to sensitise mice. In this experiment sensitisation was with 0.2 mls of

TABLE 15.

The footpad swellings in mice sensitised with polyglycine and pentaglycine and challenged with 20 µg *S. aureus* cell walls.

Sensitation	Footpad swellings mm (±SD)					
	4 hours	Sig'(P)	24 hours	Sig'(P)*	48 hours	Sig'(P)*
polyglycine/FCA	0.56(0.17)	NS	0.30(0.14)	NS	0.10(0.10)	NS
pentaglycine/FCA	0.48(0.22)	NS	0.30(0.13)	NS	0.15(0.19)	NS
	0.45(0.16)		0.22(0.06)		0.05(0.10)	

*Student's t test comparing the mean footpad swellings in the sensitised and non-sensitised. Sensitised groups contained 6 mice, the control group contained 4 mice.

NS = Not significant

TABLE 16.

The footpad swellings in mice sensitised with pentaglycine coupled to BSA and challenged with *S. aureus* cell walls, BSA – pentaglycine and BSA.

Challenge Antigen	Sensitisation	Footpad swellings mm (± SD) 4 hours	Sig'(P)*	24 hours	Sig'(P)*	48 hours	Sig'(P)*
cell walls	+	0.82(0.13)	NS	0.92(0.25)	<0.02	0.51(0.36)	<0.05
cell walls	–	0.58(0.19)		0.42(0.08)		0.10(0.11)	
BSA-5Gly	+	0.24(0.15)	NS	0.09(0.09	NS	0.08(0.08)	NS
BSA-5Gly	–	0.15(0.09)		0.05(0.06)		0.06(0.11)	
BSA	+	0.10(0.12)	NS	0.02(0.02)	NS	0.07(0.08)	NS
BSA	–	0.05(0.05)		0.01(0.02)		0.04(0.07)	

*Student's t test comparing the mean footpad swellings in the sensitised and non-sensitised control mice.

Each group contained 4 mice.

NS = Not significant

2 mg/ml. The 24 hour footpad swellings elicited by challenge with 20 µg S. aureus cell walls are shown in Table 17. Significant footpad swellings were obtained only in mice sensitised with BSA-5Gly, the BSA sensitised mice did not produce significant 24 hour footpad swellings.

3.8. The extraction of peptidoglycan from cell walls.

Cell wall polymers including teichoic acid are covalently attached to the cell wall peptidoglycan and thus require harsh extraction procedures to remove them. Numerous methods have been reported which purify peptidoglycan for immunobiological studies. This series of experiments compares the efficiency of several of the published purification methods with the ability of the resulting peptidoglycan to stimulate a delayed hypersensitivity response. Organic phosphate was measured before and after extraction to estimate the efficiency of extraction (phosphate is only found in teichoic acid and not in peptidoglycan). Twenty microgrammes of each preparation was then used to stimulate delayed hypersensitivity in cyclophosphamide treated mice (Table 18).

The extraction methods used differed widely in their ability to remove teichoic acid from cell walls. One per cent sodium deoxycholate was only able to remove 29% of the cell wall phosphate (teichoic acid). The sodium hydroxide extractions removed between 29 and 75% of the teichoic acid, this extraction being dependent upon temperature and time, but not on the concentration of the sodium hydroxide. Similarly, the trichloracetic acid extraction was dependent upon temperature, only 35% of the teichoic acid was removed at 2^{o}C whereas 76% was removed at 60^{o}C.

Apart from the 'peptidoglycan' extracted with sodium hydroxide at

TABLE 17.

The footpad swelling in mice sensitised with BSA-pentaglycine and BSA and challenged with S. aureus cell walls.

Sensitisation	24 hour footpad swelling(±SD)	Significance(P)*
BSA-5Gly/FCA	0.44(0.10)	< 0.01
BSA/FCA	0.27(0.06)	NS
Control	0.19(0.06)	

*Student's t test comparing the mean footpad swellings in the sensitised and non-sensitised control mice. Each group contained 4 mice.

NS = Not significant

TABLE 18.

Delayed Hypersensitivity stimulated in S. aureus sensitised mice by peptidoglycans, isolated by different methods.

Preparation	% Teichoic Acid removed	DH Response
Cell walls		+++
DOC	29	+++
0.1M NaOH, 1hr 22°C	29	+++
10% TCA, 36hrs 2°C	35	+++
0.1M NaOH, 1hr 100°C	54	NIL
0.5M NaOH, 4 hrs 22°C	61	+
10% TCA, 16 hrs 60°C	76	+
0.1M NaOH, 4 hrs 22°C	75	+

100°C all the preparations were able to elicit a delayed hypersensitivity response in S. aureus sensitised mice. However, the magnitude of the delayed response stimulated by the other 'peptidoglycans' varied inversely with the quantity of teichoic acid remaining. Thus there seems to be some involvement of teichoic in the delayed hypersensitivity reaction to S. aureus. However, Easmon and Glynn (1978) failed to find any delayed hypersensitivity to teichoic acid in S. aureus sensitised mice. It is possible that teichoic acid has an indirect role in promoting the reaction to peptidoglycan. This can be further investigated using a mutant strain of S. aureus which is known to be deficient in teichoic acid.

3.8.1. The delayed hypersensitivity stimulated by cell walls from a teichoic acid deficient strain of S. aureus.

Cell walls from the teichoic acid deficient strain, S. aureus 52A5 were used to stimulate delayed hypersensitivity in mice treated with cyclophosphamide and sensitised with S. aureus 8530. Routine phage typing was used to check that the 52A5 strain had not reverted to its parent H strain, 52A5 was untypable at routine test dilution or 100 times routine test dilution. 20µg of cell walls from 52A5 elicited significant delayed swellings at both 24 and 48 hours. However, this response was far lower than the delayed response stimulated by the normal S. aureus V8 cell walls. (Table 19). This result is in agreement with the previous experiment, in that teichoic acid seems to play an indirect role in the potentiation of the delayed hypersensitivity stimulated by peptidoglycan.

TABLE 19.

The footpad swellings in mice sensitised with S. aureus and challenged with 20 µg of cell walls from teichoic acid deficient and normal strains of S. aureus.

Challenge Antigen	Sensitisation	Footpad swellings mm (±SD) 4 hours	Sig'(P)*	24 hours	Sig'(P)*	48 hours'(P)*
S. aureus V8 cell walls	+	0.49(0.17)	NS	1.13(0.17)	<0.005	0.91(0.13) <0.001
S. aureus V8 cell walls	-	0.43(0.19)		0.30(0.14)		0.23(0.06)
S. aureus 52A5 cell walls	+	0.57(0.14)	NS	0.74(0.13)	<0.005	0.52(0.11) <0.01
S. aureus 52A5 cell walls	-	0.59(0.11)		0.28(0.08)		0.27(0.10)

*Student's t test comparing the mean footpad swellings in the sensitised and non-sensitised control mice.

Sensitised mice were in groups of 6. Control groups contained 4 mice.

NS = Not significant.

3.9. The relationship between delayed hypersensitivity and the antibody response to S. aureus.

Delayed hypersensitivity to S. aureus in mice is usually seen only after at least four subcutaneous infections. That is, it is only after four infections that mice respond positively to footpad challenge with cell walls. It is known that immune serum suppresses delayed hypersensitivity if given before induction (Easmon and Glynn 1977). However, little is known about the class of antibody involved or what these antibodies are directed against. Also, there has been no work published on the relationship between the induction of delayed hypersensitivity to S. aureus and the corresponding humoral response.

3.9.1. The antibody response to doses of S. aureus required to induce delayed hypersensitivity.

The antibody levels were followed in mice during the sensitisation for delayed hypersensitivity by:-

(a) 4 weekly subcutaneous infections

(b) pretreatment with cyclophosphamide followed by a single subcutaneous infection.

The sera obtained from individual mice obtained during the course of the experiment were stored and the antibody levels measured on the same day using an enzyme linked immunosorbant assay (ELISA). Figure 13 shows that even before infection, the mice possessed antibodies against S. aureus, presumably due to the so called natural immunity induced by this organism. In the mice given multiple infections, no increase in the level of antibody was noticable until after the second infection. A gradual increase in antibody levels took place reaching a plateau at four weeks. As expected, the cyclophosphamide pretreated

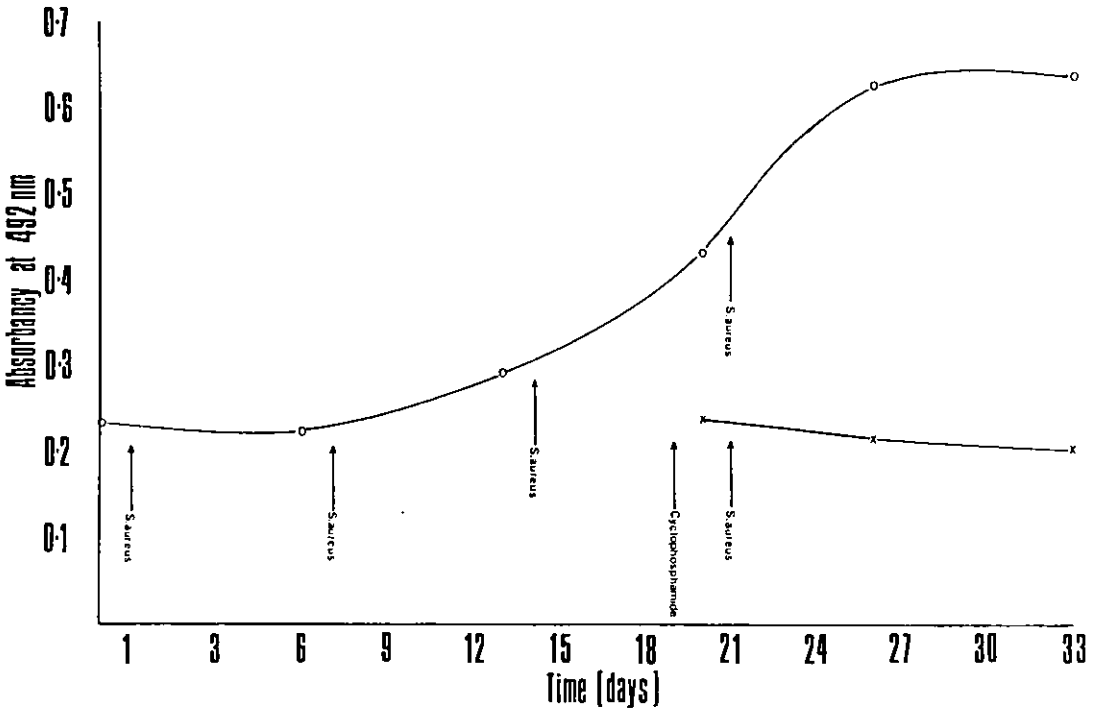


Figure 13.

The antibody response to multiple S. aureus subcutaneous infections and to a single infection after cyclophosphamide treatment.

mice displayed no increase in antibody levels.

Both sets of mice were tested for delayed hypersensitivity 12 days after the last infection. Both the 4 times infected and the cyclophosphamide pretreated mice responded to footpad challenge with cell walls giving high 24 hour swellings.

3.9.2. Suppression of delayed hypersensitivity with factors from immune plasma.

One hour prior to sensitisation with S. aureus cyclophosphamide treated mice were given intravenous injections (0.2 mls) of:-

(a) Immune plasma from four times infected mice

(b) IgM extracted from the plasma of four times infected mice

(c) IgG extracted from the plasma of four times infected mice

(d) The remaining serum proteins from the plasma of four times infected mice.

(e) Immune plasma which had been absorbed with peptidoglycan

(f) Immune plasma which had been absorbed with cell walls

Twelve days after sensitisation, the mice were footpad tested for delayed hypersensitivity with S. aureus cell walls.

As previously shown by Easmon and Glynn (1977) the immune plasma (Figure 14) significantly suppressed the delayed hypersensitivity response. Both the IgM and to a lesser extent the IgG also suppressed the delayed reaction. The remaining proteins in the plasma had no effect on the reaction. Plasma absorbed with either cell walls or peptidoglycan given prior to sensitisation caused no subsequent suppression of the delayed hypersensitivity response. Thus, the humoral factors which suppress the induction of delayed hypersensitivity are IgM and IgG, although the

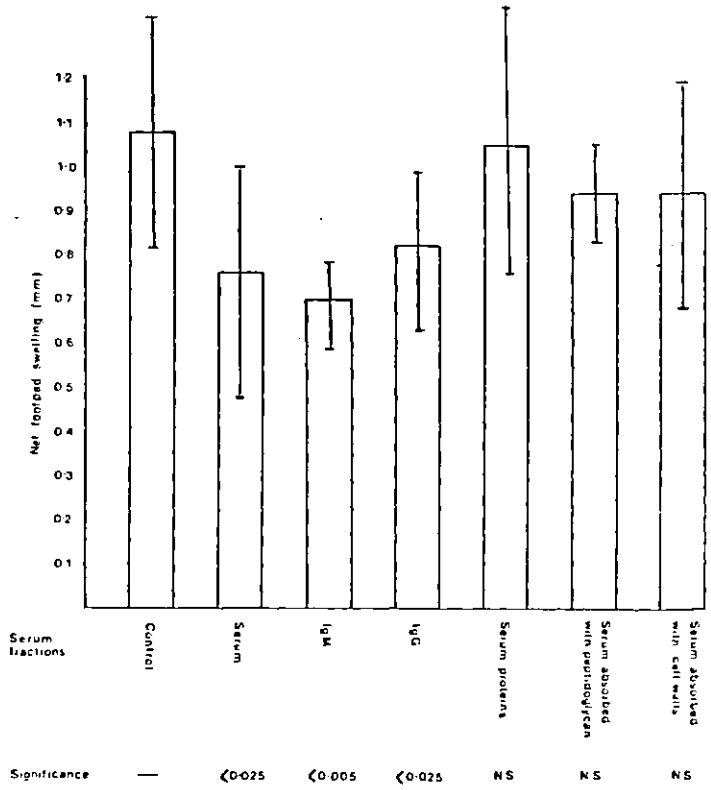


Figure 14.

The 24 hour footpad swellings in mice given immune plasma fractions prior to infection with S. aureus.

Bars on histogram show standard deviations.

Each group contained 8 mice.

former is more effective and these antibodies are directed against a component of the staphylococcal cell wall, possibly the peptidoglycan.

DISCUSSION

DISCUSSION

4.1. Induction of delayed hypersensitivity to S. aureus.

Delayed hypersensitivity to S. aureus in mice can be induced by multiple infections of viable staphylococci (Taubler, 1968; Taubler and Mudd, 1968 and Easmon and Glynn, 1975). However, the need for multiple infections can be removed by treatment with cyclophosphamide two days prior to a single subcutaneous infection. The levels of delayed hypersensitivity in these mice are then similar to those seen in mice given four weekly infections (Easmon and Glynn, 1977). I have used this cyclophosphamide model to study the antigens involved in the delayed hypersensitivity reaction to S. aureus.

Previous studies on the induction of delayed hypersensitivity to S. aureus have all required infection with viable bacteria (Taubler, 1968; Easmon and Glynn, 1975) or, the injection of killed bacteria with Freunds complete adjuvant (Tribble and Bolen, 1978). Using cyclophosphamide to dispense with the requirement for multiple infections, I have induced delayed hypersensitivity with 10^8 heat killed S. aureus (Table 1) to the same levels as seen with 10^5 viable staphylococci. It was not possible however to induce delayed hypersensitivity with 10^5 heat killed bacteria. The subcutaneous injections of both viable and killed S. aureus were performed on cotton dust plugs, using the method described by Noble (1965). Agarwal (1967a) studied the effect of cotton dust on subcutaneous infections with S. aureus. He demonstrated that cotton dust allows bacterial growth to take place at the site of infection and that within 24 hours 10^5 staphylococci will multiply to 10^8. Thus it seems that sensitisation of mice is not dependent upon viable bacteria or the presence of

Freunds adjuvant, but it is dependent upon the quantity of antigen and it appears that 10^8 S. aureus are necessary. The induction of cell mediated hypersensitivity reactions are often described as being dependent upon viable bacteria but, there are examples of induction with killed organisms. Indeed several mycobacterial species are immunogenic when killed, although larger doses are usually required for sensitisation than when viable organisms are used (Rook, 1980).

In view of the ability of killed staphylococci to induce delayed hypersensitivity, attempts were made to sensitise mice with cell walls and peptidoglycan from S. aureus. Kowalski and Berman (1971) and Easmon and Glynn (1978) demonstrated that peptidoglycan contains the antigenic determinants for delayed hypersensitivity to S. aureus and this stimulates greater footpad swelling in S. aureus sensitised mice than cell walls. However, weight for weight cell walls always induced the greater footpad response (Figure 6). Thus there seems to be a paradox, with peptidoglycan containing the antigenic determinants and eliciting the better footpad swelling in S. aureus sensitised mice, but cell walls inducing higher levels of delayed hypersensitivity than peptidoglycan. An explanation for this is provided by the nature of the lesions formed after the subcutaneous injections of cell walls and peptidoglycan. Peptidoglycan forms a severe necrotic lesions which is maximal at 24 hours and thereafter heals with the rapid disintegration of the peptidoglycan. Cell walls however, form a chronic granulomatous lesion in which the cell wall material is remarkably persistant (Abdulla and Schwab, 1966). Thus cell walls provide a more prolonged sensitisation and promote the infiltration of many more cells into the granuloma. This ability of cell walls to induce delayed hyper-

sensitivity may be analysed by experiments in which the lesions formed by the cell walls are excised from the animal. There might also be an indirect role for teichoic acid in slowing the degradation of the cell walls by the muramidase type enzymes of the host. This situation is known to occur with streptococcal cell walls (Lahav et al, 1974) and will be discussed in detail later.

Therefore, it seems that at least 10^8 S. aureus are necessary for the induction of delayed hypersensitivity in cyclophosphamide treated mice. When infections with 10^5 viable S. aureus are given to induce delayed hypersensitivity it is evident that these must multiply to 10^8 before inducing delayed hypersensitivity. This is supported by the work of Easmon (1981) where the local injection of antibiotics into the area of the lesion could prevent the induction of delayed hypersensitivity by 10^5 viable bacteria, only if given immediately after infection.

4.2. The antigenic determinants involved in the delayed hypersensitivity reaction to S. aureus.

Several staphylococcal antigens have been implicated in the delayed hypersensitivity reaction to S. aureus. However, both Kowalski and Berman (1971) and Easmon and Glynn (1978) have given conclusive evidence that peptidoglycan is the only cell wall antigen which stimulates delayed hypersensitivity in S. aureus sensitised mice. S. aureus peptidoglycan consists of 3 parts; a polysaccharide backbone cross linked by tetrapeptide units and pentaglycine bridges (Figure 2). All three of these have determinants which stimulate antibody formation, with the terminal D-alanine of the tetrapeptide unit being the immunodominant factor. (Helgeland,

Grov and Schleifer, 1973).

I have attempted to elucidate which, if any, of these determinants is involved in the delayed hypersensitivity reaction. The methods I have used include; the enzymatic degradation, the chemical and biological modification and cross reactions of the peptidoglycan and finally the use of synthetic analogues. Lysostaphin was first isolated from the culture filtrate of S. epidermidis NRRL B-2628 by Schindler and Schuhardt (1965), who described it as an antibiotic which lyses S. aureus. However, its main use has been in the classification of staphylococcal species (Heczko, Grov and Pulverer, 1975) and in the study of the biological activity of peptidoglycan (Schleifer 1975). Lysostaphin is in fact a combination of three enzymes, endo-N-acetyl glucosaminidase, N-acetyl-muramyl-L-alanine amidase and endopeptidase (glycinase), the sites of action of which are shown in Figure 15.

Tipper and Strominger (1971) investigated the actions of lysostaphin and demonstrated that the endopeptidase action on the pentaglycine cross bridge, preceeds the actions of the N-acetyl muramyl-L-alanine amidase and the endo-N-acetyl-glucosaminidase. The endo-N-acetyl glucosaminidase is only able to hydrolyse the polysaccharide chain after it has been stripped of its peptide by the action of the other two enzymes. The results I have obtained are in agreement with this theory of lysostaphin action. There is a rapid hydrolysis of the peptide signified by the release of free amino groups while the release of reducing power which measures the hydrolysis of the glycan chain is extremely slow (Figures 8 and 9).

I have investigated the effect on the cell walls of treatment with lysostaphin and compared the ability of the treated cell walls with that of

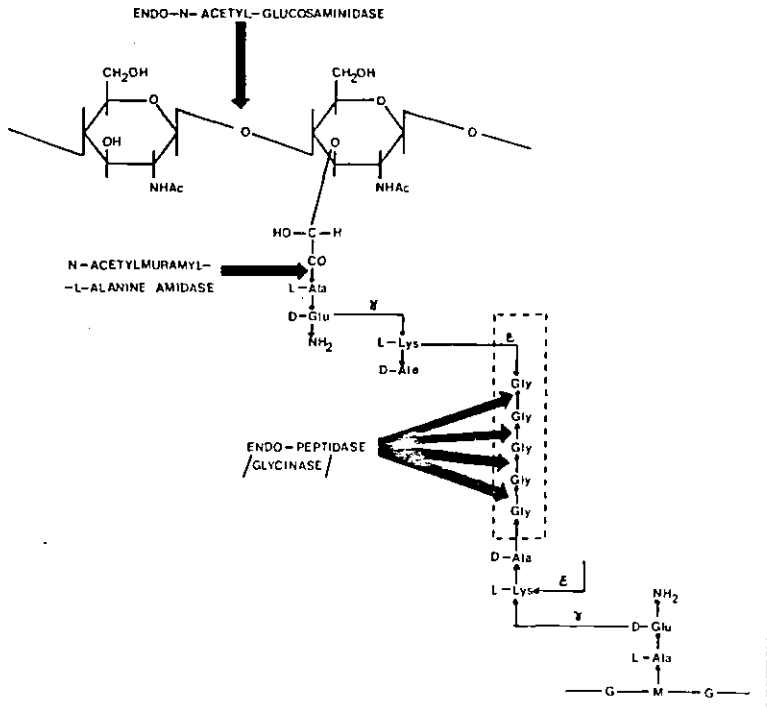


Figure 15.

Action of three enzymatic activities of lysostaphin: endo-N-acetyl-glucosaminidase, N-acetyl-muramyl-L-alanine amidase, and endo-peptidase (glycinase) on staphylococcal peptidoglycan.

normal cell walls to elicit a delayed hypersensitivity reaction in S. aureus sensitised mice.

After 90 minutes of lysostaphin treatment, the cell walls lost the ability to elicit a delayed response (Table 4). This occurs after the reduction in optical density has reached its maximum, at the time when the release of free amino groups is maximal but there is still only a slow release of reducing power. (Figures 8 and 9).

Therefore, there is a coincidence between the loss of the ability of the treated cell walls to elicit a delayed response and the maximal release of free amino groups. I take this as an indication that the antigenic determinants of the peptidoglycan are contained within the peptide part and not the polysaccharide chain (Figure 2) of this macromolecule.

The use of lysostaphin in the isolation of cell wall antigens should be borne in mind by future workers, it is pointless as some have done (Tribble and Bolen, 1978) to prepare antigens with treatments including lysostaphin action and then claim that the peptidoglycan fractions possess no immunobiological activity.

One possible method for gaining further insight into which parts of the cell wall peptidoglycan are important in the antigenic determinants for the delayed hypersensitivity reaction is to alter its normal structure and then observe this effect on its immunogenicity. There are several ways of doing this. One is by the addition to the growth medium of sub-inhibitory concentrations of penicillin or other cell wall inhibiting antibiotics such as vancomycin, both of which lead to the accumulation of

cell wall precursors. Penicillin acts by binding to several proteins which affect the cross linking of the peptide sub units. In contrast, vancomycin acts by inhibiting the transfer of muramic and pentapeptide units across the cell membrane. Another method is to alter the nutrients in the medium limiting certain factors necessary for growth, or to use a quite unbalanced growth medium. I have chosen a method of growing the S. aureus in a 3% glycine enriched medium (Hammes, Schleifer and Kandler, 1972). This has the effect of inhibiting growth, giving rise to irregular sized cocci and leads to the accumulation of uridine-N-acetyl muramic acid and other cell wall precursors. The glycine is incorporated into the peptidoglycan at positions 1, 4 and 5 of the peptide sub unit, which then becomes a poor substrate for the carboxy peptidase II and transpeptidase enzymes of peptidoglycan synthesis. This results in a higher percentage of uncrosslinked peptidoglycan. The effect of a glycine enriched growth medium on the morphology of S. aureus has already been described in the results. Presumably, they appear gram negative as a result of the loss of integrity of the cell walls and slide coagulase negative because the coagulase enzyme can no longer bind to cell walls. The 3% glycine therefore induces a phenotypic alteration in the cell wall peptidoglycan which is only maintained in this growth medium.

When used as challenge antigen in S. aureus sensitised mice these modified cells did not elicit a significant delayed response (Table 5). This means that replacement of some of the alanine groups in the tetra-peptide unit by glycine and the resultant decrease in cross bridging by pentaglycine has removed the ability of peptidoglycan to elicit a delayed

hypersensitivity response in S. aureus sensitised mice. The increase in the 4 hour footpad swelling is a totally different problem, it is probably an antibody mediated Arthus reaction. But why is it higher with the cell walls from S. aureus, grown in the glycine enriched medium? Dziarski and Kwarecki (1975) have shown that Arthus reactions do occur in S. aureus sensitised mice and are produced by the cell wall antigens protein A and peptidoglycan but not teichoic acid. Also, there is the constant presence of antibodies against peptidoglycan (due to the indigenous bacteria of the respiratory and gastrointestinal tracts) which will allow for the formation of immune complexes. Perhaps the increased early reaction stimulated by the altered cell walls is due to the loss in integrity of the peptidoglycan which therefore becomes more susceptible to the lytic enzymes of the host, thus allowing quicker release of soluble antigens which will undoubtedly lead to a more pronounced Arthus reaction. Peptidoglycan from S. aureus activates both the classical and alternative pathways of the complement system (Pryjma et al, 1975) and possesses its own intrinsic toxicity (Dziarski and Kwarecki, 1975) both of which can explain the early inflammatory response, but not the increased reaction promoted by the altered cell walls from the staphylococci grown in 3% glycine.

It has been known for some time that extensive substitution of protein antigens can destroy their serological specificity. In studying the factors which govern the immune response to Salmonella flagellin Parish (1971) induced tolerance to this normally highly immunogenic molecule by chemical modification with diketene (acetoacetic anhydride). This powerful reagent will acetoacetylate free amino and hydroxyl groups

and can therefore be used to gain some understanding of the role of these groups in the antigenic specificity of the molecule.

I have used a similar method to study the immunogenicity of S. aureus peptidoglycan for the delayed hypersensitivity reaction. The acetoacetic anhydride will modify the free amino and hydroxyl groups of the peptidoglycan as shown in Figure 4. This modified peptidoglycan was no longer able to elicit a delayed response in S. aureus sensitised mice Table 6. A result which considering the large amount of substitutions is not unexpected. The modified groups were then sequentially removed, first from the hydroxyl groups by treatment with sodium carbonate and then from the amino groups by treatment with hydroxylamine Table 7. The removal of the modifications from the hydroxyl groups had no effect on the inability of the modified peptidoglycan to elicit delayed hypersensitivity. However, removal of the modifications from the amino groups restored the activity of the peptidoglycan. This must mean that the polysaccharide backbone, where the hydroxyl groups are found is not involved in the antigenic determinants for delayed hypersensitivity, but the antigenic determinant involves or is closely related to (i.e. in the 3 dimensional structure) a free amino group. There are two possibilities for this. One is the free amino group on the glutamic acid residue at position three in the tetrapeptide unit, the other is the N terminal part of the interpeptide bridge. It is known that peptidoglycans are not completely cross linked and in S. aureus at least 15 per cent of the cross bridges show free unsubstituted amino groups (Seidl and Schleifer, 1978). Indeed, antibodies with specificity against this N terminal of the pentaglycine bridge are known to occur in antistaphylococcal antisera

(Ranu, 1975). Thus the polysaccharide chain can be eliminated from having any involvement in the antigenic specificity of the delayed hypersensitivity reaction to S. aureus. This is in contrast to the humoral response where antibodies are directed against the N-acetyl glucosamine of the glycan chain (Karakawa et al, 1968; Rolicka and Park, 1969). It is however, possible to associate the determinant with either or both of two free amino groups on the peptide part of the peptidoglycan. Again, this is in contrast to the humoral response in which the immunodominant aminoacids are the C-terminal alanine of the tetrapeptide or the C-terminal alanine of the uncross-linked pentapeptide (Helgeland, Grov and Schleifer, 1973) and only a very small amount of the antibodies are directed specifically against the pentaglycine interpeptide bridge.

Many workers have reported cross reactions between peptidoglycan of one strain of staphylococcus and antibodies raised against a heterologous strain, or indeed against other gram positive bacteria, most notably streptococci (Karakawa et al, 1968). The basis for this cross reactivity is that the polysaccharide backbones and the tetrapeptide units of most staphylococcal and streptococcal strains are identical. The interpeptide bridges however, are normally a characteristic of each strain. By way of an aside, these cross reactions between peptidoglycans of several species of bacteria are believed to form part of the so called natural immunity whereby antibodies raised against the indigenous bacteria of the gastrointestinal and respiratory tracts protect against invading pathogens. The cross reactions between different species and strains led to the proposal that the immunodominant group of S. aureus peptidoglycan is the tetrapeptide unit, a theory which has been since proved correct with the

use of synthetic analogues. (Schleifer and Seidl, 1974).

The polysaccharide chain and the interpeptide bridge play minor roles in the humoral antigenicity of peptidoglycan, although recently Schopfer et al (1980) have indicated that in patients with recurrent staphylococcal infections and hyperimmunoglobulinemia E it is the penta-glycine cross bridge which binds the IgE (Schopfer et al, 1980).

I have used the approach of studying the cross reactions, in the delayed hypersensitivity reaction in an attempt to discover the important antigenic determinants. When used as a challenge antigen in S. aureus sensitised mice, Streptococcus Group A strain T 27 cell walls were unable to elicit delayed response (Table 8). However, partial cross reaction did occur between S. epidermidis and S. aureus (Table 9). The structures of the peptidoglycan of these three bacteria are shown in Figure 3, they all possess exactly the same polysaccharide backbone and tetrapeptide unit. The Streptococcus Group A has a cross bridge of two alanine residues, S. epidermidis a cross bridge of four glycines linked in the middle by a single serine residue, while S. aureus has a pentaglycine cross bridge. The teichoic acid types of these bacteria differ and S. epidermidis and Streptococcus Group A contain no protein A.

The inability of the streptococcal cell walls to cross react can only indicate that neither the polysaccharide chain nor the tetrapeptide unit are involved in the antigenic specificity for delayed hypersensitivity i.e. in direct contrast to the humoral response. The partial cross reaction of S. epidermidis and S. aureus is expected since there is only a minor difference in one amino acid of the peptidoglycan. Also, antibodies raised the synthetic immunogen pentaglycine conjugated to albumin will cross

react both with S. aureus and S. epidermidis peptidoglycan (Seidl and Schleifer, 1977). There is the additional possibility that a considerable proportion of the S. epidermidis cross bridge is made of pentaglycine and does not contain any serine, a situation which has already been demonstrated in S. epidermidis strain Texas 26, where 20% of the cross bridges are pentaglycine. (Tipper, 1968). The result also rules out the direct involvement of teichoic acid, as the different types should not give rise to any cross reactions. Taken together, these experiments have indicated that the antigenic determinants involved in delayed hypersensitivity to S. aureus are associated with the pentaglycine cross bridge.

This lack of cross reactivity in delayed hypersensitivity reactions between closely related peptidoglycans is distinctly beneficial. Delayed hypersensitivity is normally deleterious and obviously it would be extremely disadvantageous and reactions would become more widespread if they were induced and elicited by heterologous bacteria.

It is possible that the immunogenicity of staphylococcal peptidoglycan relies on an intact three dimensional structure to hold the antigenic determinants in the correct configuration for lymphocyte stimulation. I have used solubilised cell wall extracts to show that the three dimensional conformation is not necessary for immunogenicity and to gain some further insight into the actual antigenic determinants for delayed hypersensitivity to S. aureus. The two enzymes used for solubilisation of the cell walls were:-

(1) Muramidase from Chaloropsis sp., this differs from egg white lysozyme in that it has both β-1, 4-N-acetyl muramidase and β-1, 4-N, 6-O-diacetyl muramidase activity, while egg-white

lysozyme only has the former activity and is therefore not active against cell walls from S. aureus.

(2) Protease from S. aureus V8, this enzyme specifically cleaves peptide bonds on the carboxyl terminal side of glutamic acid.

The results indicate that while the soluble extracts produced by the two enzymes can both induce delayed hypersensitivity in both Freunds complete and incomplete adjuvants (Table 14), only the extract produced by the muramidase could elicit delayed hypersensitivity in S. aureus sensitised mice (Table 11). The composition of the soluble extract produced by Chaloropsis sp. muramidase has been well documented (Ghuysen and Strominger, 1965). It is known that the first fraction produced after gel filtration on Sephadex G-25, is a mixture of fragments of a glycopeptide-teichoic acid complex and a glycopeptide complex (Figure 11). The second fraction is a mixture of N-acetyl glucosamine and free amino acids. As expected, fraction I is the active constituent of the solubilised cell walls (Table 12).

The solubilisation of cell walls by the protease enzyme has not been previously documented. The enzyme should split the tetrapeptide unit giving rise to a polypeptide of units of the pentaglycine bridge attached to lysine and alanine residues and to the polysaccharide chain with alanine and glutamine attached. However, paper chromatography of the two fractions shows that while fraction I (Figure 12), the active fraction contains all 4 amino acids in high concentration and some sugars, Fraction II contains higher concentrations of sugars with only traces of aminoacids. This can only be explained by either solubilisation occurring

before the enzyme has broken all the glutamate lysine bonds or by the two main products remaining together on gel filtration, or both. Nevertheless, solubilisation with this enzyme has resulted in the loss of ability to elicit a delayed hypersensitivity response in S. aureus sensitised mice. I can only suspect that this is due to the splitting of the antigenic determinant from teichoic acid which then cannot perform its indirect role in delayed hypersensitivity. This fraction can still induce delayed hypersensitivity, probably because the Freunds adjuvant takes over the role of the teichoic acid in maintaining the antigen at the site of injection.

As well as further establishing the role of teichoic acid, in the delayed hypersensitivity reaction, these experiments add weight to the previous findings that the antigenic determinant is contained in the peptide part of the peptidoglycan molecule and also indicate that the intact three dimensional conformation of peptidoglycan, which is destroyed by these enzymes is not necessary for T-lymphocyte stimulation.

The experiments which I have already discussed, have all indirectly indicated that the antigenic determinant for delayed hypersensitivity is contained in the peptide part of peptidoglycan and is almost certainly the pentaglycine bridge. There is, however, only one possible method of directly implicating the pentaglycine bridge as the antigenic determinant and that is by the use of synthetic analogues. A similar approach was used to evaluate the immunogenicity of peptidoglycan for the humoral response (Helgeland et al, 1973; Schleifer & Seidl, 1974; Seidl and Schleifer 1977; Ranu, 1975). I decided to use the synthetic analogues in FCA

to induce delayed hypersensitivity and then to challenge with S. aureus cell walls, rather than to sensitise mice with S. aureus and challenge with the analogues. The main reason for this was my experience with enzymatically solubilised cell walls, where one fraction would induce but not elicit delayed hypersensitivity to S. aureus probably due to the effects of adjuvant which was used in combination with the sensitising antigen.

It was however, impossible to induce delayed hypersensitivity with pentaglycine or polyglycine (Table 15). This is quite understandable as pentaglycine must be near to the minimum size required for immunogenicity. Schecter et al (1970) overcame this problem by covalently coupling the small peptide to larger protein carriers. In order to increase the immunogenicity I coupled pentaglycine to bovine serum albumen, using the bifunctional reagent carbodiimide. This reagent is assumed to activate carboxyl groups and subsequently couples of these to free amino groups with the formation of a peptide bond. This coupling results in BSA attached to pentaglycine peptides by both the amino and carboxyl groups. Thus, there are two synthetic analogues of the pentaglycine bridge, one with a free amino terminal and another with a free carboxyl terminal.

It is known that in S. aureus, about 20% of the interpeptide bridges are uncross-linked and possess free amino groups (Schleifer and Kandler, 1972). These are the determinants for the very small amount of antibodies that are directed against the pentaglycine bridge in antiserum raised against peptidoglycan. (Seidl and Schleifer, 1977). Occasionally,

however, antibodies with specificity against the carboxyl terminus are found. These free carboxyl groups on the pentaglycine are thought only to arise through the action of autolytic enzymes (Ranu, 1975).

When challenged with S. aureus cell walls, mice sensitised with BSA-pentaglycine did give rise to a delayed hypersensitivity reaction (Tables 16 and 17), thus providing direct proof that the pentaglycine bridge is involved in the antigenic determinant for delayed hypersensitivity. However, it is not possible to say whether the N-terminal, the C-terminal or neither of these is involved in binding to the lymphocyte.

The ability of hapten-carrier complexes, to induce and elicit delayed hypersensitivity presents a problem since Benacerraf and Gell (1959) first demonstrated a requirement for both homologous haptens and carriers in sensitisation and challenge. Clearly, this is not the situation when S. aureus cell walls are used to challenge mice sensitised with synthetic analogues. However, the rules of carrier specificity are not absolute, which has been demonstrated by Alkan et al (1976) and Janeway et al (1975), where guinea pigs were sensitised with 2, 4 dinitrophenol-mycobacteria and subsequently produced a delayed hypersensitivity response when challenged with a variety of DNP-proteins. Also, Schechter et al (1970) using short peptides, coupled to protein carriers demonstrated that antibodies only show carrier specificity when the hapten attached is less than a tetrapeptide.

4.3. Preparation of peptidoglycans

Teichoic acids are covalently bound to peptidoglycan and require rather harsh extraction methods to remove them. Many different

extraction procedures have been used, each having its own disadvantage. Moreover, little is known about how these extraction methods affect the biological activity of the peptidoglycan. I have used three methods of extraction; trichlor acetic acid, dilute sodium hydroxide and deoxycholate. There are several other methods available, for example formamide at 150°C or N N-dimethylhydrazine but I considered these either too dangerous or, as with the latter, too much of the peptidoglycan material is dissolved. Even the extraction procedures I have used are far from ideal. Trichloracetic acid is usually used between 2-4°C. However, this gives an incomplete removal of teichoic acid. For a better extraction, a higher temperature is necessary and this can result in partial degradation of the peptidoglycan. A similar situation exists with dilute sodium hydroxide, at high temperatures this dissolves the peptidoglycan due to the liability of the interpeptide pentaglycine bridges (Archibald, Coapes and Stafford, 1969). Extraction with deoxycholate is very mild but, does not completely remove the teichoic acid (Hill, 1967).

I have compared the efficiency of the extraction procedures with the ability of the resulting 'peptidoglycans' to elicit delayed hypersensitivity in S. aureus cyclophosphamide treated mice. There appears to be a general inverse relationship between the amount of teichoic acid remaining and the magnitude of the delayed response (Table 18). All these responses except one were of the delayed type, with increased footpad swellings at 24 and 48 hours. It was only the magnitude of the response that was changed. There was one exception to this, peptidoglycan prepared by sodium hydroxide at 100°C could not elicit any reaction at 24 hours. This is quite understandable since this treatment splits the pentaglycine bridge,

and as I have already indicated, this is the antigenic determinant for delayed hypersensitivity to S. aureus.

The involvement in delayed hypersensitivity of teichoic acid is much more complicated and can only be postulated. It has already been demonstrated by Kowalski and Berman (1971) and Easmon and Glynn (1978) that teichoic acid is not directly involved in the antigenic determinant for delayed hypersensitivity. It may however, play an indirect role, either purely by confering greater size on the antigenic particle, thus making it more immunogenic and less likely to be quickly removed from the site of injection (Katsura et al, 1977). Or it could function in a manner similar to that proposed by Lahav et al (1974) for streptococcal teichoic acids, preventing the action of the cell wall lytic enzymes and thus allowing retention at the site of injection.

In an attempt to clarify the role of teichoic acid, I have used the mutant strain S. aureus 52A5 which is deficient in teichoic acid. This mutant is deficient in an enzyme phospho-Glc N Ac translocase which is involved in the synthesis of the linkage unit between teichoic acid and peptidoglycan (Bracha, Davidson and Mirelman, 1978). Shaw (1971) found that the walls of 52A5 had a phosphate content of 10% of that of the parent strain, also muramic acid and glucosamine were in equimolar concentrations, rather than there being the usual excess of glucosamine. No ribitol residues could be isolated from the cell walls of 52A5. The mutant is not susceptible to the phage 52A at a routine test dilution or at 100 times the routine test dilution, because this phage normally binds to the teichoic acid. The phosphate present in the cell walls is thought to represent muramic acid linked to phosphate.

When used as a challenge antigen in S. aureus sensitised mice, these cell walls gave rise to a similar response as the peptidoglycans with a substantial proportion of their teichoic acid removed i.e. delayed hypersensitivity reaction was observed (Table 19) but at a considerably lower level than with normal cell walls. Thus while teichoic acid does not contain any of the relevant antigenic determinants for delayed hypersensitivity it does seem to have an indirect role in potentiating the delayed response.

4.4. The regulation of delayed hypersensitivity to S. aureus.

Delayed hypersensitivity to S. aureus in mice is normally only seen after at least four subcutaneous infections. However, this need for repeated infections can be removed by prior injection with cyclophosphamide, whereupon the levels of delayed hypersensitivity elicited by staphylococcal cell walls are of the same order as with mice given multiple infections. The cyclophosphamide removes factors which suppress the appearance of delayed hypersensitivity in once infected mice, between one and four infections there must be an alteration in the regulatory mechanisms allowing the cells mediating delayed hypersensitivity to overcome its suppression after a single infection. Easmon and Glynn (1977) have investigated the suppressor factors involved in delayed hypersensitivity to S. aureus and have found evidence for two mechanisms of regulation. The first, humoral regulation was only observed when immune serum or plasma was given immediately before infection i.e. it suppressed the induction of delayed hypersensitivity. The second, a cellular regulatory system had its effect on the expression of delayed hypersensitivity. The cells involved in this suppression were most active

when taken from twice infected mice and were probably B lymphocytes (Easmon and Glynn, 1979).

I have followed the antibody levels using an ELISA assay during the induction of delayed hypersensitivity by multiple infections of S. aureus. Considering these results together with the induction of delayed hypersensitivity by multiple infections (Figure 13), there seems to be a concomitant increase in antibody levels and delayed hypersensitivity, with high levels of antibody and delayed hypersensitivity existing together at the same time. However, this is in contrast to much of the work described on the relationship between humoral and cellular immunity. Parish (1971b) has shown an inverse relationship to exist between antibody production and delayed hypersensitivity in that the chemical modification of a strong antibody producer Salmonella flagellin enhances, the delayed hypersensitivity response and decreases the magnitude of antibody production. Also, delayed hypersensitivity usually preceeds the appearance of detectable antibody and there is a depression of delayed hypersensitivity just before the appearance of circulating antibodies to protein antigens (Neta and Salvin, 1976). Clearly, these situations do not exist in delayed hypersensitivity to S. aureus but one cannot ideally compare infection with a viable multiplying bacteria to injection of a soluble protein or a sheep red blood cell.

The levels of antibody (Figure 13) do not start to rise until after the second infection, which presents an interesting analogy with the findings of Easmon and Glynn (1975), that mice given two subcutaneous infections are protected from dermonecrosis when given a subsequent infection in the same way as passive transfer of immune serum protects

against dermonecrosis. Thus high levels of antibody exist side by side with delayed hypersensitivity so that the harmful dermonecrotic effects of the latter are not expressed.

As I have already described, immune serum or plasma does suppress delayed hypersensitivity when given before infection, the results (Figure 14) have indicated that this suppression is mainly due to IgM and to a lesser extent IgG and these antibodies are directed against a determinant in the cell walls of S. aureus, possibly the peptidoglycan. If this is the case, then the most likely part of the peptidoglycan against which the antibodies are determined is the carboxy terminal alanine of the tetrapeptide unit which is the immunodominant part of peptidoglycan (Helgeland, Grov and Schleifer, 1973) and this could be verified by raising antibodies against a synthetic analogue of this unit. However, there is another possibility, it has recently become clear that immune responses can be regulated by cells and antibodies that are specific for idiotypic determinants, i.e. the specific antigen combining sites of antibody and T cell receptors. These idiotypes can be themselves immunogenic in the same species or even in the same individual, which leads to a network of idiotype, anti-idiotype, anti-(anti-idiotype) etc. Jerne (1973) postulated that interactions amongst this network are of central importance in the regulation of the immune response. This presents us with the possibility that serum suppression of delayed hypersensitivity is brought about by anti-idiotypic antibodies. In this particular case, the determinant for the anti-idiotypic antibodies could be the specific T cell receptor mediating delayed hypersensitivity. One piece of evidence in favour of such a response is the finding that serum

from both twice and four times infected mice suppress delayed hypersensitivity equally as well but, there are large differences in the quantities of antibody in the two serums, thus only small quantities of antibody are necessary to suppress delayed hypersensitivity. A similar finding was made by Easmon (1980) who demonstrated that the quantity of passively transferred serum necessary to suppress delayed hypersensitivity could be reduced to as little as 0.075 ml. It is known, that quite small amounts of anti-idiotypic antibodies can inhibit an idiotypic response (Eichman, 1974). It would be difficult to explain away the small quantities of antibody needed for suppression using the sheep red blood cell model of Mackaness (1974) who suggested that antibody either combines with antigen to block the antigen from stimulating T-cells or, immune complexes switch off the T-cell responses leading to a delayed hypersensitivity reaction.

Asherson and Zembala (1976) suggested that one of the functions of immune regulatory systems is in the control of potentially harmful responses. This certainly appears to be the case in S. aureus infections, where the induction of the harmful delayed hypersensitivity is prevented by antibodies. Its elicitation is prevented by the B-cell suppressor system and when both these systems are over ridden as in mice given multiple infections, then high levels of antibody prevent the harmful effect of delayed hypersensitivity, namely dermonecrosis, from being expressed.

CONCLUSIONS

5. CONCLUSIONS

Delayed hypersensitivity can be induced in cyclophosphamide treated mice with heat killed S. aureus at a dose equivalent to 10^8 colony forming units. Previous work has shown that the normal dose of 10^5 viable S. aureus used to induce delayed hypersensitivity will grow to 10^8 bacteria within 24 hours, suggesting that the quantity of antigen associated with 10^8 S. aureus is necessary to induce delayed hypersensitivity. Delayed hypersensitivity can be induced with both cell walls and peptidoglycan from S. aureus using a dose equivalent to the dry weight of 10^8 S. aureus.

The antigenic determinant for delayed hypersensitivity is known to be associated with the cell wall peptidoglycan. The enzymatic degradation of the cell wall by lysostaphin, the modification of the peptidoglycan by altered growth conditions and by chemical methods, plus cross reaction studies with similar peptidoglycans from Streptococcus Group A and S. epidermidis all implicate the pentaglycine in the antigenic determinant. The need for the intact three dimensional structure of the peptidoglycan is dispelled using enzymatically solubilised cell walls to induce and elicit delayed hypersensitivity. Final proof that the antigenic determinant involved in the delayed hypersensitvity reaction is the pentaglycine bridge is obtained using synthetic analogues to induce delayed hypersensitivity. However, it is not known whether the amino terminal, the carboxyl terminal of the pentaglycine bridge or neither of these binds to the T-lymphocyte.

The preparation of peptidoglycan by the removal of teichoic acid

from cell walls is never completely satisfactory, with none of the methods used leading to a pure peptidoglycan and one method producing a peptidoglycan which is unable to elicit a delayed hyper sensitivity. It should be borne in mind by future workers that during its preparation some of the peptidoglycan will be destroyed and it will also be invariably contaminated with teichoic acid. Teichoic acid does seem to play an indirect role in the stimulation of delayed hypersensitivity by slowing down the breakdown of peptidoglycan by lytic enzymes.

The humoral suppression of the induction of delayed hypersensitivity appears to be mediated by IgM and to a lesser extent IgG. These antibodies are specific for an unknown determinant in the cell wall peptidoglycan. In four times infected mice, high levels of delayed hypersensitivity and humoral immunity exist side by side, with the high antibody levels protecting mice from the harmful dermonecrotic effects of delayed hypersensitivity.

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APPENDIX

Example of analysis of data

Taken from Table, 5. The footpad swelling elicited in S. aureus sensitised mice by 20 µg cell walls from S. aureus grown in a glycine enriched medium.

1. Measurement of footpad swelling using an engineers micrometer.

Time		0 Hrs						4 hrs					
Mouse		R			L			R			L		
G	1.	2.33	2.36	2.34	2.32	2.33	2.43	3.21	3.19	3.14	2.26	2.27	2.27
r	2.	2.28	2.29	2.29	2.34	2.34	2.36	2.90	2.88	2.87	2.54	2.60	2.63
o	3.	2.30	2.29	2.31	2.26	2.29	2.31	3.22	3.18	3.18	2.42	2.44	2.42
u	4.	2.23	2.24	2.25	2.33	2.33	2.36	3.13	3.13	3.12	2.40	2.40	2.40
p	5.	2.28	2.28	2.29	2.23	2.23	2.25	2.91	2.91	2.96	2.23	2.25	2.23
(A)	6.	2.32	2.39	2.38	2.31	2.31	2.30	3.16	3.14	3.20	2.40	2.35	2.32
G	1.	2.32	2.30	2.32	2.32	2.34	2.39	2.76	2.83	2.84	2.52	2.50	2.49
r	2.	2.29	2.30	2.29	2.26	2.25	2.25	2.75	2.77	2.76	2.35	2.37	2.39
o	3.	2.31	2.29	2.31	2.28	2.28	2.29	2.70	2.71	2.75	2.28	2.29	2.29
u	4.	2.32	2.32	2.30	2.25	2.27	2.25	2.98	2.92	2.92	2.31	2.35	2.40
p (B)	5.	2.33	2.25	2.27	2.25	2.31	2.29	2.70	2.80	2.82	2.43	2.43	2.40
G	1.	2.43	2.42	2.42	2.43	2.41	2.40	2.85	2.84	2.90	2.53	2.51	2.52
r	2.	2.42	2.41	2.42	2.34	2.33	2.35	3.08	3.04	2.98	2.57	2.60	2.56
p	3.	2.34	2.35	2.40	2.28	2.26	2.25	2.93	2.95	2.95	2.45	2.43	2.44
(C)	4.	2.26	2.23	2.25	2.34	2.31	2.34	2.81	2.80	2.80	2.47	2.50	2.49
G	1.	2.24	2.23	2.20	2.38	2.39	2.40	2.95	2.96	2.98	2.31	2.33	2.28
r	2.	2.40	2.42	2.38	2.42	2.43	2.38	3.02	3.01	3.05	2.43	2.44	2.45
p	3.	2.32	2.29	2.33	2.26	2.24	2.28	3.07	3.07	3.09	2.29	2.31	2.31
(D)	4.	2.37	2.39	2.38	2.38	2.38	2.37	3.04	3.01	3.01	2.32	2.34	2.36

	Sensitisation	Challenge
Group A --	S. aureus	Cell walls from S. aureus grown in 3% glycine enriched media
Group B --	S. aureus	Cell walls from normal S. aureus
Group C --	---	Cell walls from normal S. aureus
Group D --	---	Cell walls from S. aureus grown in 3% glycine enriched media

Time	24 hrs						48 hrs					
Mouse	R			L			R			L		
1.	2.88	2.89	2.84	2.31	2.29	2.30	2.93	2.88	2.85	2.35	2.32	2.30
2.	2.72	2.73	2.76	2.51	2.54	2.47	2.46	2.51	2.45	2.42	2.38	2.38
3.	2.77	2.77	2.78	2.33	2.31	2.32	2.70	2.71	2.72	2.37	2.35	2.30
4.	3.02	3.01	3.03	2.60	2.60	2.63	2.60	2.60	2.63	2.26	2.32	2.35
5.	2.86	2.90	2.92	2.32	2.32	2.30	2.68	2.68	2.70	2.30	2.30	2.37
6.	3.21	3.19	3.19	2.33	2.35	2.39	2.73	2.75	2.73	2.42	2.42	2.44
1.	2.96	2.93	2.94	2.43	2.40	2.44	3.10	3.04	3.06	2.44	2.44	2.46
2.	3.17	3.18	3.17	2.38	2.38	2.39	2.97	2.98	2.98	2.38	2.39	2.41
3.	2.90	2.95	2.91	2.43	2.41	2.42	2.75	2.74	2.75	2.34	2.39	2.37
4.	3.00	2.98	3.00	2.34	2.35	2.34	2.82	2.83	2.83	2.33	2.34	2.33
5.	3.05	3.01	3.02	2.38	2.35	2.37	2.90	2.90	2.90	2.35	2.36	2.35
1.	2.80	2.85	2.81	2.42	2.39	2.42	2.71	2.69	2.72	2.46	2.49	2.48
2.	2.66	2.71	2.66	2.45	2.40	2.46	2.52	2.52	2.51	2.43	2.41	2.47
3.	2.72	2.71	2.72	2.45	2.47	2.46	2.55	2.53	2.48	2.46	2.44	2.41
4.	2.72	2.70	2.69	2.43	2.43	2.47	2.52	2.50	2.48	2.48	2.44	2.46
1.	2.67	2.65	2.65	2.36	2.36	2.36	2.46	2.44	2.44	2.34	2.35	2.29
2.	2.89	2.83	2.82	2.46	2.43	2.44	2.64	2.64	2.65	2.48	2.51	2.51
3.	2.68	2.69	2.70	2.33	2.30	2.35	2.41	2.42	2.44	2.36	2.36	2.35
4.	2.78	2.76	2.73	2.40	2.39	2.41	2.43	2.42	2.45	2.31	2.32	2.33

2. Calculation of net footpad swelling

Net footpad swelling = swelling in right hind footpad minus swelling in left hind footpad

Time		4 hours		24 hours		48 hours	
Mouse			Mean ±SD		Mean ±SD		Mean ±SD
G	1.	0.90)		0.56)		0.56)	
r	2.	0.35)		0.28)		0.15)	
o	3.	0.75)	0.71±0.19	0.45)	0.57±0.21	0.36)	0.34±0.14
u	4.	0.82)		0.82)		0.40)	
p	5.	0.66)		0.54)		0.33)	
(A)	6.	0.76)		0.79)		0.25)	
G	1.	0.45)		0.56)		0.66)	
r	2.	0.35)	0.43±0.07	0.76)	0.61±0.10	0.54)	0.51±0.11
p	3.	0.43)		0.49)		0.37)	
(B)	4.	0.54)		0.60)		0.45)	
	5.	0.37)		0.66)		0.55)	
G	1.	0.33)		0.40)		0.22)	
r	2.	0.37)	0.40±0.07	0.16)	0.27±0.12	0.)	0.09±0.11
p	3.	0.50)		0.16)		0.)	
(C)	4.	0.39)		0.34)		0.12)	
G	1.	0.82)		0.47)		0.29)	
r	2.	0.60)	0.65±0.16	0.42)	0.39±0.07	0.15)	0.14±0.12
p	3.	0.73)		0.32)		0.01)	
(D)	4.	0.46)		0.36)		0.11)	

Test of Significance

The student's t test was used to assess the differences between the footpad swellings of the sensitised and the control mice.

The test statistic t is given by the formula

$$t = \frac{X_1 - X_2}{\sigma}$$

where $X_1 - X_2$ = the difference in means

σ = the standard error of the difference in means

$$\sigma = \sqrt{\frac{X_1^2 - (X_1^2)/N_1 + X_2^2 - (X_2^2)/N_2}{(N_1 - 1) + (N_2 - 1)} \times \frac{1}{N_1} + \frac{1}{N_2}}$$

Testing for significant difference between the 24 hour swellings in Groups B and C.

X_B	X_C	$\sum X_B^2$	$(\sum X_B)^2$	$\sum X_C^2$	$(\sum X_C)^2$
0.56	0.40	1.9269	9.4249	0.3268	1.1236
0.76	0.16				
0.49	0.16				
0.60	0.34				
0.66					

$$\sigma = \sqrt{1.9269 - \frac{9.4249}{5} + \frac{0.3268 - 1.1236}{4} \times \left[\frac{1}{5} + \frac{1}{4}\right]}$$

$$\sigma = \sqrt{\frac{0.04192 + 0.0459}{7} \times 9}$$

$$\sigma = 0.075137$$

$$t = \frac{X_B - X_C}{} = \frac{0.34}{0.075137}$$

$$= 4.5251$$

The computed value of t is greater than the value given in the statistical table for P = 0.01 (3.499) where there are seven degrees of freedom. This is taken as proof that a statistical difference exists between the mean footpad swellings at 24 hours in groups B and C

Similarly testing for a significant difference between the 24 hour swellings in groups A and D. t = 1.6687

This value of t is less than the tabulated value for P= 0.1 (1.895) where there are 8 degrees of freedom. Thus it is unlikely that there is a significant difference between the mean footpad swellings at 24 hours in the two groups.