CONSTRUCTION AND CHARACTERISATION OF AUXOTROPHIC MUTANTS OF SALMONELLA TYPHIMURIUM AS LIVE VACCINES IN THE MURINE MODEL OF TYPHOID FEVER.

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ABSTRACT

This thesis describes the construction of auxtrophic mutants of Salmonella typhimurium and their assessment as live vaccines in the mouse typhoid model.

Stable transposon generated auxotrophic mutations in the aromatic and purine biosynthetic pathways were introduced into mouse virulent strains of S. typhimurium. These mutations, in aroA, purA, purE or aroA purA resulted in requirements for aromatic compounds, adenosine, inosine, or aromatics plus adenosine respectively. Auxotrophic mutants had limited capacities to replicate in vivo in mice with genetically determined susceptibility (BALB/c) or resistance (A/J) to S. typhimurium infection. purE mutants were less attenuated than either aroA, purA or aroA purA mutants.

Following intravenous infection of BALB/c mice, both aroA and purA mutants persisted for several weeks in livers and spleens. aroA mutants persisted at higher levels than purA mutants but the duration of persistence of the former was shorter. The aroA purA double mutants established a persistent low level infection, which lasted at least 10 weeks.

The ability of these mutants to protect mice against challenge with virulent strains of S. typhimurium was

assessed after both intravenous and oral immunization. The aroA mutants gave good protection when given by either route. purA mutants were poor vaccines when given intravenously and ineffective when given orally. aroA purA double mutants were ineffective vaccines by either route.

When given orally, aroA mutants are capable of invading and infecting the mononuclear phagocyte system of infected mice. Neither purA nor aroA purA double mutants were able to do this.

The immune responses were followed in BALB/c mice immunized intravenously to analyse the differences in efficacy of the different mutants. Sera from immunized mice were analysed for salmonella antibodies using an ELISA technique. High titres developed after intravenous infections with all mutants, although aroA immunization did induce higher titres. When compared for their ability to activate macrophages, aroA mutants were more effective than purA mutants.

The data presented show that different auxotrophic mutants have widely varying degrees of attenuation, vaccine efficacy, and ability to induce humoral and cell-mediated immune responses. The consequences of these findings in relation to the design of live vaccines for use in man are discussed.

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ABBREVIATIONS

ALS	Adenosine monophosphate.
AMP	Anti-lymphocyte serum.
cfu	Colony forming units.
conc	Concentrated.
cy	Cyclophosphamide.
DHB	Dihydroxybenzoic acid.
FCS	Foetal calf serum.
GMP	Guanosine monophosphate.
IFN	Interferon.
IL-2	Interleukin-2.
IMP	Inosine monophosphate.
LPS	Lipopolysaccharide.
MAF	Macrophage activating factor.
ml	Mililitre.
mm	Milimetre.
mM	Milimolar.
n	Number.
NMS	Normal mouse serum.
NRS	Normal rabbit serum.
PABA	Para-amino benzoic acid.
pfu	Plaque forming units.
SD	Standard deviation.
se	Standard error.
SMA	Spent medium antigen.

UDP Uridine monophosphate.

ug Microgram.

ul Microlitre.

w/v Weight per volume.

v/v Volume per volume.

INTRODUCTION

1.1 Typhoid Fever: pathogenesis and control.

1.1.1 Pathogenesis and epidemiology.

Typhoid Fever is an acute febrile disease of man caused by *Salmonella typhi*. A milder form called paratyphoid is caused by serotypes of *S. paratyphi*. *S. typhi* is transmitted via the faeco-oral route. After surviving the normal host barriers, such as stomach acid and normal gut flora, the bacteria enter the body via the Peyer's patches in the ileum. Here, they enter and survive and multiply within cells of the macrophage line. They spread to the lymphatics and mesenteric nodes and, after transport around the body, they accumulate and multiply in the organs of the mononuclearphagocyte system (MPS), namely the spleen, liver, lymph nodes and bone marrow. *S. typhi* can then be isolated from the blood.

There is no direct animal model for *S. typhi* infections since it only causes a serious illness in man. In chimpanzees *S. typhi* can cause a mild infection after oral infection (Edsall *et al*, 1960; Gaines *et al*, 1968) but the costs make these experiments prohibitive. Volunteer studies carried out in the 1960s, gave much experimental data on both the pathogenesis of typhoid fever and on vaccine efficacy (Hornick *et al*, 1970; Woodward, 1980). In these studies, volunteers drank various doses of *S. typhi* (Quailes strain) in 30ml milk. These studies had the advantage that

the vaccination and exposure histories of the volunteers were known and dose regimes could be controlled. The symptoms in volunteers who developed typhoid were essentially identical to those in naturally acquired disease. The chance of developing clinical illness depended on the dose given. No volunteers (0/14) who received 10^3 S. typhi developed typhoid. 10^5 gave an incidence of 28% (32/116), 10^7 gave 50% (16/32) and a dose of 10^9 S. typhi gave clinical typhoid to 95% (40/42). The incubation period was longer in the low dose groups although there was a wide range of incubation periods in all dose groups. In the field, transmission is by ingestion of contaminated food or water. The critical dose in natural infections is thought to be lower than that seen in the volunteer studies (Blaser and Newman, 1982). The dose received varies according to the route of transmission. Water-borne typhoid probably results from a dose of about 10^5 whilst S. typhi can grow to high density in food, resulting in a much higher dose (Hornick et al. 1970).

WHO figures suggest there are about 1 million cases of typhoid each year mostly in developing countries. The majority of cases are school-age children or young adults. Typhoid is rare in young children: the reason for this is unclear. The apparent resistance of the older age groups has been attributed to increased immunity following subclinical infection (Ashcroft et al, 1967). This view has been

recently strenghtened by Murphy *et al* (1987) who showed that 90% of adult volunteers tested in Santiago had peripheral blood lymphocytes which proliferated when stimulated *in vitro* with *S. typhi* antigens. These authors suggest that there are seven subclinical cases of typhoid in Santiago for every one diagnosed.

1.1.2 Parenteral vaccination against typhoid fever.

The first attempts to vaccinate against typhoid were in the late 19th century when Wright (Wright and Semple, 1897; Wright, 1900,1902) tested crude killed *S. typhi* preparations in India and the Boer War. A major field trial was set up during the First World War in which 95% of all British troops in France were vaccinated. The results were encouraging with a lower incidence of typhoid in British forces compared with other forces in similar conditions. Vaccine preparations used in the first half of this century were crude. One improvement, suggested by Felix (1938; 1941), was that vaccines should be inactivated by 75% alcohol, since this preserves Vi antigen, a capsular polysaccharide antigen found on most strains of S. typhi. It was later established that acetone treatment is better for preservation of Vi antigen (Landy, 1953).

In the late 1950s and early 1960s, a series of properly controlled field trials were set up by the WHO to determine the efficacy of parenteral inactivated typhoid vaccines.

Most of these trials were centred in endemic areas, and were conducted with school-age children and young adults. The results of the first field trial, in Yugoslavia, which was designed to compare alcohol and heat/phenol inactivated preparations, (Yugoslav Typhoid Commision, 1957; 1962) suggested that the heat/phenol inactivated vaccine was better. The next set of trials were to test and compare the efficacy of dried heat-phenol inactivated (L) and acetone inactivated (K) vaccine preparations. Trials were set up in British Guiana (Typhoid Panel, UK Department of Technical Co-operation, 1964; Ashcroft et al, 1967), Poland (Polish Typhoid Commitee, 1965) and USSR (Hejfec, 1965; Hejfec et al, 1966). These suggested that the K vaccine gave a higher degree and longer lasting levels of protection than the L vaccine.

Absenteeism resulted in 10% of the vaccinees in the Polish and Guiana trials receiving only one dose. Those in the British Guiana trial who received only one dose were solidly protected throughout the whole 7 year observation period (Ashcroft et al, 1967), whilst those in the Russian trials were protected for only 10 months (Hejfec et al, 1968; 1969). A trial was set up in Tonga (Tapa and Cvjetanovic, 1975) to try to resolve the conflicting results with vaccinees getting one or two doses of vaccine K. Two doses gave only 40% protection over a 7 year follow up period (compared to 79-93% in other trials) and one dose gave no

protection. It has been suggested that the low vaccine efficacy seen in this trial was because typhoid is food-borne in Tonga. This would result in high infective doses which may overwhelm the effects of vaccination.

The field trials were all conducted in areas where typhoid is endemic. It is probable that many of the vaccinees had already had subclinical exposure to S. typhi and that the vaccination was acting as a booster. Volunteer challenge studies were carried out at the University of Maryland to test the efficacy of vaccine in volunteers with no previous exposure to S. typhi (Hornick et al, 1970; Woodward, 1980). Volunteers received 3 doses of the L or K vaccines and were challenged orally with varying doses of S. typhi (Quailes Strain) from 3 months to 1 year later. The levels of protection were low. In those challenged with 10^5 S. typhi (approximately 1 ID_{25}) protection was around 70% in both the L and K vaccinated groups. Volunteers challenged with 10^7 or 10^9 S. typhi showed no significant levels of protection. These results support the theory that the parenteral inactivated vaccine is effective against low dose water-borne typhoid (such as in British Guiana) but ineffective against high dose, food-borne typhoid (such as in Tonga).

1.1.3 Immune responses during clinical typhoid and vaccination.

Since Widal's (1896) first reports of agglutination, immune responses to typhoid have been measured by titres of serum antibodies to surface antigens. The main antibodies tested are against the lipopolysaccharide (LPS) O antigen, the Vi capsular antigen and flagella protein H antigens. The validity of this as a measure of protective immunity is doubtful. In the field-trials, no correlation was seen between anti-O and anti-Vi titres and protection, although it was suggested that anti-H titres may indicate levels of protection (Benenson, 1964). However, in the volunteer studies (Hornick et al, 1970) there was no correlation between post-vaccination antibody titres and protection.

Antibody titres to Vi polysaccharide were found not to correlate with immunity (Hornick et al, 1970). Robbins and co-workers advocate the use of purified capsular polysaccharide as a parenteral vaccine. Recent field trials in South Africa (Klugman et al 1987) and Nepal (Acharya et al, 1987) show that a Vi polysaccharide vaccine induced approximately 70% protection. These levels are similar to those given by traditional whole cell vaccines and again are probably due to boosting of existing immunity. The vaccine did have the advantage of not giving the unpleasant side effects attributed to the endotoxin component of the whole cell vaccine (Klugman et al, 1987). It should be noted that

the posession of Vi antigen is not essential for virulence since both Hornick et al (1970) and Hone et al (1988) have reported the incidence of typhoid caused by non-Vi producing strains.

Since the portal of entry of typhoid is the gut, one would expect that immunity at the gut mucosa might prevent infection. Parenteral killed vaccines are unable to induce mucosal immunity, and inactivated typhoid vaccines given orally are ineffective in humans (Hornick et al, 1970; Du Pont et al, 1971; Chuttani et al, 1971; 1972; 1973). S. typhi specific IgA has been isolated from the stools (Cancelleri and Fara, 1985), and IgA secreting cells from the blood (Kantele, Arvilommi and Jokinen, 1986) of volunteers vaccinated with a live vaccine, strain Ty21a.

It is assumed that in order to control typhoid, as with other intracellular infections, a cell mediated immune response is required. Unfortunately, few human studies have been performed to supply supporting data. There have been reports of leukocyte migration inhibition responses in typhoid patients (Kumar et al, 1974; Balakvishna Sama et al, 1977; Nath et al, 1977) and reports of lymphocyte proliferative responses from blood lymphocytes of typhoid patients (Mogensen, 1979; Murphy et al 1987). No leukocyte migration inhibition responses were seen in vaccinees who received a parenteral inactivated vaccine (Nath et al,

1977), although blood lymphocytes from vaccinees do give a significant proliferative response (J. Rhodes personal communication).

1.1.4 Conclusions from parenteral vaccination: the need for a new vaccine.

The observations described above highlight both the effectiveness of parenteral killed typhoid vaccines and their limitations. The main criticisms of parenteral typhoid vaccination are:

1. It fails to stimulate a cell mediated immune response.
2. It fails to stimulate mucosal immunity.
3. It is ineffective against high dose challenge.
4. There are many unpleasant, often severe, side-effects.

It is clear that a new vaccine is desirable. Ideally this vaccine must:

1. Be capable of inducing protective humoral, mucosal and cellular immune responses.
2. Have no side-effects.
3. Be effective when delivered orally.
4. Be effective against high dose infections.

Work to develop such a vaccine has been underway for over 20 years. Most work has been carried out with the mouse model

and the results have suggested that live vaccines are more effective (Collins, 1974). Live vaccine candidates have been developed and will be discussed in detail later.

1.2 The Mouse Model of Typhoid Fever.

1.2.1 The need for an animal model.

Human volunteer studies are impractical: they present risks for the volunteers and the information they generate is limited. A parallel animal model is required for more detailed studies on pathogenesis and immunity. Fortunately, certain species of salmonella, such as S. typhimurium and S. enteritidis, cause a disease in mice which closely resembles typhoid.

Many factors affect the course and outcome of salmonella infections in mice. They include the route of infection used, the dose and virulence of the infecting organism, and the susceptibility of the mouse to infection. At least three factors influence the susceptibility of the mouse to infection: genetically determined susceptibility, acquired immunity and secondary impairment of immunity. The following section will describe in detail pathogenesis and immunity in this model, drawing comparisons with the human disease where appropriate.

1.2.2 Routes of infection.

The natural route of infection in both human and murine typhoid is oral. Early work with the mouse model suggested that the bacteria entered the blood stream directly from the gut. Collins (1970) found that S. enteritidis could be detected in the livers and spleens of orally infected mice before detection in mesenteric lymph nodes. However, more detailed studies did not confirm this view. Carter and Collins (1974) followed the course of oral infection by S. enteritidis in CD1 and B6D2 mice. The bacteria invaded primarily via the Peyer's patches of the terminal ileum and thence into the distal mesenteric nodes, from which they were cultured in the first 24 hours. No early bacteremia was found. Infection was possible when bacteria were injected directly into the caecum or the rectum but since the bacteria drained into different lymph nodes to those following an oral infection, it was concluded that these were not natural routes of infection. Carter and Collins (1975) used ^{3}H-thymidine incorporation to follow cellular proliferation in response to infection in the Peyer's patches and spleens of B6D2 mice. In orally infected mice, Peyer's patches responded before spleens, suggesting that they were infected first. When mice were infected intravenously (i.v.), Peyer's patches and spleens responded together. This is further evidence to support the theory that the infection passes through the gut lymphoid system before systemic spread.

Oral salmonella infection is influenced by factors other than the virulence of the bacteria and host immunity. The stomach acid presents a natural barrier to infection, as does the normal gut flora. In gnotobiotic mice (Collins and Carter, 1978) or mice whose gut flora has been disrupted by antibiotic treatment (Bohnhoff and Miller, 1962; Que and Hentges, 1985) much lower oral doses are lethal.

Experimental models have been established which use parenteral infection to by-pass these factors. The course of the infection can be altered depending on whether the oral, subcutaneous (s.c.), intraperitoneal (i.p.) or i.v. routes are used. Subcutaneous infection mimics the oral route in so far as the infection passes through draining lymph nodes, albeit different ones (Carter and Collins, 1974; Collins, Auclair and Mackaness, 1977). The i.p. and i.v. routes bypass lymph node involvement. In the i.p. model the bacteria are free to multiply extracellularly within the peritoneal cavity. This creates a large infectious pool, which continually seeds the rest of the body. In the i.v. model the majority of the inoculum is found in livers and spleens within a few minutes of injection. There has been great debate over the relative merits of the various parenteral routes in studing immunity to mouse typhoid. The results obtained by the i.v., or s.c. model are probably closer than the i.p. model to the oral route (Collins, 1971;

1974; 1979). The i.v. model has been used in this study when parenteral infection was required.

1.2.3 Factors determining the virulence of salmonella.

The factors which confer virulence on salmonella are multiple and mostly unknown. Different strains of salmonella are also highly host specific; for example S. pullorum is avirulent for mice but highly virulent for chickens, whilst S. typhi is only virulent in man. The factors which determine this host specificity are unknown.

Salmonella can actively invade eukaryotic cells (Finley, Gumbiner and Falkow, 1988), but the importance of this in pathogenesis is not known. Genetic approaches have now identified genes in enteric pathogenes such as Shigella spp (Sansonetti, Kopeco and Formal, 1982ab) and Yersinia spp (Isberg and Falkow, 1985; Miller and Falkow, 1988) which are required for invasion. A mutant of S. enteritidis has been isolated which is unable to invade Hep2 cells (Miller, personal communication). However, when tested for virulence in BALB/c mice its oral LD50 is similar to that of its parent. Futher "invasion minus" mutants of S. choleraesuis have been identified (Finley et al in press) and are currently being tested for mouse virulence.

Virulent salmonella can resist the bactericidal activity of macrophages and survive and grow within them (Mitsuhashi,

Sato and Tanaka, 1961; Ushiba, 1965; Lissner, Swanson and O'Brien, 1983; Fields et al, 1986; Harrington and Hormaeche, 1987). Once inside the cells they are resistant to the bactericidal actions of antibody and complement (Rous and Jones, 1916). Fields et al (1986) have described a number of mutants of S. typhimurium with reduced ability to survive within cultured macrophages. Many of these mutants have reduced virulence. It has been suggested that flagella may play a role in intracellular survival (Carsiotis et al, 1984; Weinstein et al, 1984) but this view has been disputed (Benjamin et al, 1986ab).

Many bacterial pathogens contain plasmids which encode potential virulence determinants. These include shigella, yersinia and salmonella. Many salmonella contain large molecular weight plasmids. Recent genetic studies have shown a clear role for these plasmids in virulence. Strains cured of their plasmids, or with transposons inserted in the plasmid, have reduced virulence in mice and chickens. The role of the plasmid in virulence is still unclear since it has been variously ascribed to adhesion and invasion (Jones et al, 1982), serum resistance (Vanderbosch, Rabert and Jones, 1987), or resistance to inflamatory macrophages (Heffernan et al, 1987; Popoff, personal communication). Interestingly, S. typhi does not possess such a plasmid. Transfer of a plasmid from S. typhimurium to S. typhi does

not bestow mouse virulence on S. typhi (Popoff personal communication).

The lipopolysaccharide (LPS) in the cell wall is important in virulence. When the structure of the LPS is altered, such that the O-polysaccharide side chains are lost, the strain changes from smooth to rough. In many strains this change is associated with loss of virulence (Nakano and Saito, 1969; Roantree, 1971). Some exceptions do exist, Fields et al (1986) reported a rough variant of a virulent S. typhimurium which is virulent for BALB/c mice, and Hormaeche (personal communication) has a rough strain of S. choleraesuis which is highly virulent. When this rough strain is administered i.v. to BALB/c mice, it is first killed to a greater extent than a smooth strain but then the survivors grow rapidly, killing the mouse. These observations suggest that the smooth LPS is important for S. choleraesuis to resist initial killing seen in the first few hours after infection, but plays a negligible role in intracellular survival and growth.

The type of O-antigen present on the bacteria plays a role in virulence in the i.p. infection (Makela, Valtonen and Valtonen, 1973). Salmonella strains were constructed to be isogenic except for their O-antigens. A virulent S. typhimurium (0-1, 4, 5, 12) was constructed to be 0-1, 9, 12 and a virulent S. enteritidis (0-1, 9, 12) was

constructed to be 0-1, 4, 5, 12. In the i.p. model, strains posessing 0-1, 4, 5, 12 were always more virulent than those with 0-1, 9, 12. Similarly, 0-6, 7 strains were less virulent than either 0-4, 5 or 0-9 strains (Makela et al, 1973). No differences were seen between the virulence of 0-4, 5 and 0-9 strains when they were given i.v. (Hormaeche personal communication). The differences in virulence in the i.p. model can be explained by the different abilities of the O-antigens to activate the alternative complement pathway. 0-6,7 LPS consumes complement faster than 0-9, which is, in turn, faster than 0-4,5 LPS (Grossman and Leive, 1984). This is more important in the i.p. than the i.v. model because the resident peritoneal macrophages phagocytose bacteria poorly unless they are opsonised. Therefore, 0-6,7 strains which bind more complement than 0-9 or 0-4, 5 strains are more readily phagocytosed and killed by resident peritoneal macrophages. This reduces the effective infecting dose and so appears to reduce virulence (Saxen, Reima and Makela, 1987).

1.2.4 Genetic control of resistance to salmonella infections.

Early workers with the mouse model noted that different mouse strains had different degrees of susceptibility or resistance to salmonella infections (Webster, 1924; 1933ab; Gowen, 1948; 1960). Studies with inbred mouse strains have allowed identification of several genes which control

resistance to salmonella. These will be discussed in detail below.

ity.

Inbred mouse strains fall into two distinct groups when their s.c. or i.v. LD^{50}'s for S. typhimurium C5 are measured (Plant and Glynn, 1974; 1976; 1977; 1979; Hormaeche, 1979a). Some mouse strains are highly sensitive, while others are highly resistant. No intermediate stages are found, although the ity^r phenotype can be overcome by other genetically determined susceptibilities discussed below. Experiments with F1 hybrids and backcrosses suggested that resistance was coded by a single dominant gene or cluster of closely linked genes, designated ity (Plant and Glynn, 1976; 1977), which mapped to chromosome 1 (Plant and Glynn, 1979). ity also controls innate resistance to L. donovani (under the name lsh) (Plant et al, 1982), M. tuberculosis BCG (bcg) (Gros, Skamene and Forget, 1981; Forget et al, 1981) and M. lepraemurium (Brown, Glynn and Plant, 1982). Not all intracellular pathogens are controlled by ity, since L. monocytogenes (Stevenson, Kongshavn and Skamene, 1981; Gervais Stevenson and Skamene, 1984) and Yersinia enterocolitica (Hancock, Schaedler and MacDonald, 1986; 1988), for example, have different genetic control systems.

ity acts in the early part of the salmonella infection by controlling the net rate of increase of salmonellae in

livers and spleens of infected mice (Hormaeche, 1979ab) and its effects are evident within the first 24 hours of infection (Swanson and O'Brien, 1983). The effect of ity is mediated solely by macrophages. Its expression is sensitive to silica treatment (O'Brien, Scher and Formal, 1979), which affects macrophage function, but is resistant to radiation (Hormaeche et al, 1983) suggesting non-dividing, resident macrophages are involved. Expression of ity is independent of delayed type sensitivity (DTH) (Hormaeche, 1979b) or T-cells (O'Brien and Metcalf, 1982). ity can be expressed in vitro by cultured peritoneal macrophages (Lissner et al, 1983) and liver kupffer cells (Harrington and Hormaeche, 1986). There is still some debate over the exact mode of action of ity, with claims that it is due to either bacteriostatic or bacteriocidal activities of macrophages. In vivo evidence obtained by following the survival of non-replicating, temperature sensitive strains (Hormaeche, Pettifor and Brock, 1981), or by following in vivo growth by segregation of a non-replicating phage in mice of different ity types (Homaeche, 1980), has suggested a bacteriostatic mechanism. lsh has been demonstrated in vitro with Leishmania infected kupffer cells (Crocker, Blackwell and Bradley, 1984). This report also suggested a bacteriostatic mechanism. Neither the ability of macrophages to phagocytose (Groschel, Paas and Rosenberg, 1970; Swanson and O'Brien, 1983; Gros et al, 1983) nor the ability of C. parvum to activate them (Briles et al, 1986) is affected by ity, although ity^r macrophages

are reported to have higher levels of expression of I-A antigens than *ity*^{s} (Zwilling, Vespa and Massie, 1987).

Genetics of resistance in C3H lineage mice.

C3H/HeJ mice carry the mutant allele *Lps*^{d} which makes them hyporesponsive to endotoxin. C3H lineage mice are *ity*^{r} and, except for C3H/HeJ, are endotoxin responsive. C3H/HeJ mice are susceptible to s.c. or i.p. infection with *S. typhimurium* (Robson and Vas, 1972) when compared to most other C3H strains, and this difference has been attributed to the *Lps*^{d} allele (O'Brien *et al*, 1980). The role of *Lps*^{d} in susceptibility was disputed by Eisenstein and co-workers (Eisenstein *et al*, 1982) who found that whilst C3H/HeJ mice were LPS hyporesponsive and salmonella susceptible, C3H/HeNCrlBr were LPS responsive but still salmonella sensitive. Other strains showed intermediate resistance. It is probable that the factors determining susceptibility in C3H lineage mice are multiple. (C3H/HeJ x C3H/FeJ) F1 mice were both salmonella resistant and LPS responsive, suggesting that each strain had a separate defect which was complemented in the F1 hybrid. Further mating experiments confirmed the multifactorial nature of susceptibility in C3H mice (O'Brien and Rosenstreich, 1983; O'Brien *et al*, 1985).

xid.

CBA mice carry the resistant, *ity*^{r}, allele. CBA/N mice carry an X-linked gene, *xid*, which confers a defiency or absence

of B-cell function (Scher, 1980). Mice homologous for xid are susceptible to S. typhimurium infections (O'Brien et al, 1979), apparently because they are unable to make an adequate antibody response later in the infection (O'Brien, Scher and Metcalf, 1981).

Control by MHC linked genes.

Hormaeche et al (1985) described late emerging resistance in ity^s mice of the B10 lineage. Using H2 congenic B10 mice they showed that this resistance is linked to H2 and maps to the I-E subregion. Mice with a resistant haplotype are more able to clear infection with less virulent strains from livers and spleens than those with a sensitive haplotype.

Other genes.

C57L and DBA/2J mice, although both ity^r, ultimately succumb late in the infection. This is genetically controlled and is thought to be encoded by a single recessive gene which has not yet been mapped but is not linked to ity, lps or xid (O'Brien, Taylor and Rosenstreich, 1984).

Genetic control in other animals.

Genetic control similar to ity has recently been found in chickens (Barrow, personal communication). Conclusive evidence of genetic control of resistance to typhoid in man is yet to be produced. However, some studies (De Vries et al 1979; Naylor, 1983) suggest it may exist.

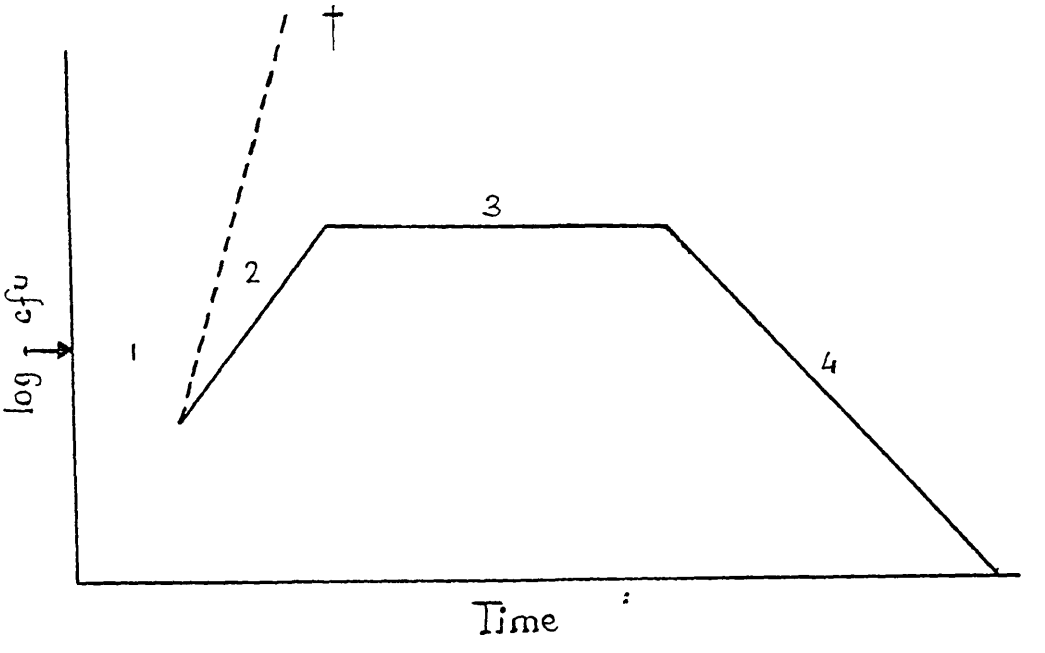


Fig. 1.1 Schematic representation of the four phases of an i.v. salmonella infection in livers and spleens. Phase 1 represents the initial reduction of the innoculum. Phase 2 is exponential growth of the challange. Phase 3 is control of bacterial growth and plateau. Phase 4 shows clearance of the infection from the MPS.

1.2.5 The course of an i.v. infection in the mouse model.

The course of a salmonella infection in mice can be divided into distinct phases. The exact course followed, and its outcome, depends on the combination of mouse strain, bacterial strain and route of infection used. Since the i.v. route of infection has been used for much of the work described in this thesis the following description will concentrate on this model. The infection has 4 phases which are represented in Fig. 1.1. The description covers only the infection in livers and spleens. Lymph nodes throughout the body are also seeded during i.v.infection (Collins, 1969ab). The i.p., s.c. and oral routes differ only in the early stages of the infection. Once established in livers and spleens, the course of the infection is essentially the same.

Phase 1. An infecting dose, administered i.v., is rapidly removed from the blood. The majority of the challenge is found in livers and spleens within the first 30 minutes of infection. Once entrapped in livers and spleens, the numbers of viable bacteria are reduced over the next 6 hours (Akeda et al, 1981). Resident, fixed macrophages are responsible for this killing since the process is resistant to X-irradiation, which affects polymorphonuclear leukocytes (PMNL) and monocytes, but sensitive to carrageenan, a macrophage inhibitor (Akeda et al, 1981). A similar situation exists with listeria (Mitsuyama et al, 1978;

Newborg and North, 1980) and tuberculosis (North, 1974) infections. The initial blood clearance is enhanced by opsonic antibody (Biozzi et al, 1961; Akeda et al, 1981; Mackaness, Blanden and Collins, 1966) as is the intracellular killing (Akeda et al, 1981).

Phase 2. Some of the bacteria entrapped in liver and spleen may survive the initial period of intracellular killing and then grow exponentially within the macrophages of the liver and spleen. The outcome of the infection depends on whether the mouse is able to control the growth of the bacteria before it reaches lethal levels (always between 10^8 and 10^9/organ, Meynell and Meynell, 1958; Hobson, 1957c). If a susceptible mouse is challenged with a virulent strain, or a resistant mouse with a large dose of a virulent strain, the mouse is overcome by the infection and dies. This is portrayed in Fig. 1.1 as a broken line. Should the challenge organism be less virulent or a low dose of a virulent strain given to a resistant mouse, the bacteria still grow exponentially but more slowly (Hormaeche, 1979ab; Hormaeche et al, 1981).

Phase 3. The third phase of the infection begins at around days 4 or 5. At this time, the mouse is able to mount a cellular immune response which can control the growth of the bacteria (Zinkernagel, 1976). This is followed by a plateau where bacterial growth is kept in check. Control of growth

appears to require cell division since it is sensitive to irradiation (Newborg and North, 1980; Hormaeche et al, 1983; Heffernan et al 1987). It is apparently dependent on an influx of macrophages, which radiation shielding experiments suggest are derived from the bone marrow (Hormaeche, personal communication). The exact mechanisms of the initial macrophage activation in salmonella infections remain unclear. Macrophage activation, as measured by suppression of growth, appears to be a local event since two salmonella strains within the same mouse grow and are controlled independently of each other (Maskell et al, 1987a). The induction of plateau where bacterial growth is controlled appears to be independent of T-cells. In nude mice this has been shown for salmonella (O'Brien and Metcalf, 1982) and listeria (Newborg and North, 1980). The nude mouse model has the disadvantage that there appears to be a high base line level of macrophage activation in uninfected mice (Newborg and North, 1980). More recently, work with severe combined immunodeficency (SCID) mice, which lack all functional lymphocytes, has shown that macrophage activation by listeria, as judged by control of growth and expression of I-A antigens, is independent of T-cells (Bancroft et al, 1986). In the salmonella model, Maskell et al (1987a) showed that T-cells are not required for supression of growth. Here, mice were depleted of T-cells by adult thymectomy and in vivo treatment with monoclonal antibodies against T-cells. Growth of virulent S. typhimurium C5 was controlled

equally well in normal and T-cell depleted (B10xA/J) F1 mice.

If activation of macrophages in salmonella and listeria infections is independent of T-cells, we are left with the question of what provides the activating signals. Bancroft et al (1986) have proposed that natural killer (NK) cells are the source of interferon (IFN)-gamma which activates macrophage in SCID mice. It has recently been reported that C3H lineage mice, immunised with an aroA S. typhimurium mutant, SL3235, have a burst of NK activity between the second and fourth day after infection (Schafer and Eisenstein, 1988). This is the period immediately before macrophage activation as assessed by resistance to salmonella and listeria challenge (Killar and Eisenstein, 1985) and tumor cell killing (Schafer, Nancy and Eisenstein, 1988). It is possible that NK cells are the source of macrophage activating lymphokines: this area needs further examination.

Once plateau has been attained, the bacterial levels may remain constant, often indefinitely (Hobson, 1957a; Maskell and Hormaeche, 1985), or the mouse may clear the infection from its MPS. Maintenance of plateau probably depends on the presence of activated macrophages. This is supported by the observations that during plateau, mice are immune to a number of heterologus intracellular pathogens, such as

listeria (Zinkernagel, 1976), and also immune to salmonella of different serotypes (Collins, Mackaness and Blanden, 1966). A similar situation is seen in mice infected with brucella and mycobacteria (Mackaness, 1964). Activated macrophages alone do not appear unable to maintain plateau completely. Work with nude mice shows that whilst they are capable of controlling both salmonella and listeria infections the levels of bacteria in livers and spleens still slowly increase and the mice eventually succumb to the infection (O'Brien and Metcalf, 1982; Newborg and North, 1980). There is presumably some involvement of T-cells in this period since most mouse strains are capable of mounting a delayed type hypersensitivity response to salmonella antigens and salmonella specific T-cells can be detected (Attridge and Kotlarski, 1985ab; Paul et al, 1985) but the role has not yet been elucidated.

Phase 4. The fourth phase is clearance of the infection from livers and spleens. Mice appear capable of completely eliminating only the most attenuated of salmonella. It is thought that T-cells play a role in clearance although this has still to be proven. Circumstantial evidence is that T-cell depleted mice have impaired ability to clear an avirulent rough mutant 11XR (Davies and Kotlarski, 1976) and the observation of H2 control of clearance in H2 congenic B10 mice (Hormaeche et al, 1985) may suggest macrophage, T-cell interactions. Following clearance the

level of activation of the macrophages decreases. It is assumed that a population of memory T-cells remains which will serve to re-activate macrophages in a second infection. This will be discussed in detail in the next section.

1.3 Acquired immunity in the mouse model.

1.3.1 General.

Immunity to typhoid may result from either survival of a virulent infection or vaccination. Vaccines may be either killed bacteria, or bacterial fractions, or they may be living bacteria. Recovery from a clinical infection results in both humoral and cell mediated immune responses. Live vaccination mimics a virulent infection (indeed early vaccination experiments were with sub-lethal doses of virulent bacteria) and results in both humoral and cell mediated immunity. Killed vaccines, however, whilst inducing high levels of antibody, are incapable of inducing a cell mediated response. There has been much debate over the relative merits and contribution of each arm of the immune system to immunity to typhoid. Much of the controversy over the relative importance of cell mediated immunity (live vaccines) or humoral immunity (killed vaccines) can be attributed to the various experimental methods used when studying immunity in the mouse model. The route of infection, the mouse strain and the criteria used to assay

protection, all influence both the result of immunisation experiments and their interpretation.

Whatever the route of infection used, both humoral and cell mediated immunity play a role in immunity to salmonella. However, the i.p. and s.c. routes of infection emphasise the role of antibody, whilst the i.v. route emphasises the role of cellular immunity. This will be discussed in detail below. The natural oral route is obviously the model most relevant to human typhoid. Since this route is affected by the non-specific factors discussed earlier, and many mouse strains are difficult to infect orally, many workers have chosen to use parenteral routes.

The mouse strain used is important. Genetically resistant mice are easier to immunise with killed vaccines than genetically susceptible mice (Robson and Vas, 1972). This has been highlighted by Eisenstein and co-workers (Eisenstein, Killar and Sultzer, 1984) who found that resistant C3H/HeNCrlBR mice were easily vaccinated with killed endotoxin-protein vaccines, whilst susceptible C3HeB/FeJ mice were not. Much of the work in this thesis is with susceptible (ity^{s}) BALB/c mice, which have proved very difficult to immunise effectively with killed vaccines (Robson amd Vas, 1970).

Many vaccination experiments rely solely on levels of survival following a single dose of challenge as their criterion for protection. This tells us nothing about how the mouse controls the challenge. Collins and co-workers advocated following the growth of the challenge in the MPS of the mice (Collins, 1974). Using these techniques, they showed that mice, which appear protected by survival data, may have undergone a severe illness which would probably have resulted in a case of clinical typhoid if extrapolated to man.

These varying experimental methods gave rise to claims in support of the importance of humoral or cell mediated immunity. The following sections will discuss the evidence for both arms of the immune response and show how, in fact, both play a role in immunity.

1.3.2 Humoral Immunity.

It has long been recognised that live vaccines are more effective than killed vaccines at stimulating a protective immune response. Whilst some workers proposed that this was due to a cellular immune response (Hobson, 1957b; Mitsuhahi *et al*, 1958; Saito *et al*, 1962), others claimed it was because live vaccines could stimulate a humoral response which was in some way superior to killed vaccines (Jenkin and Rowley, 1962).

Jenkin, Rowley and Auzins (1964) reported that immunity could be passively transfered with serum. However, in this report protection was measured only by mortality (60% survived in treated, 20% in untreated) and the challenge was i.p.. Rowley, Turner and Jenkin (1964) reported antibodies associated with the membranes of peritoneal macrophages could adoptively transfer immunity to an i.p. challenge. These cytophilic antibodies were suggested as the mediators of cellular immunity. It was further suggested (Jenkin and Rowley, 1965) that a heat labile antigen was present in live, but not dead, vaccines. Antibodies to this antigen were proposed as being important for protection. All of these studies used the i.p. route of infection and relied solely on mortality data.

Later studies showed that the difference in efficacy between live and killed vaccines was not due to antigenic differences. Mackaness, Blanden and Collins (1966) compared the ability of sera from mice immunised with either live or killed vaccines to passively transfer immunity. Both sera were able to increase the rate of initial blood clearance after i.v. challenge, but were unable to affect the subsequent multiplication in liver and spleen, even though the donors vaccinated with the live vaccine were capable of doing this. Blanden, Mackaness and Collins (1966) found that sera from mice immunised with heat killed or live S. enteritidis were equally able to enhance phagocytosis by

macrophages *in vitro*. Transfer of immunity by immune serum could only be shown against i.p. challenge.

Kenny and Herzberg (1968) reported bactericidal and haemagglutinating antibodies within 4 days of administration of either heat killed or live vaccines. They found that killed vaccines were effective against both i.p. and s.c. challenge. Collins carefully examined the role of antibody in the various routes of challenge by pre-opsonising *S. enteritidis* before challenging normal (Collins, 1969a) or immunised (Collins, 1969b) mice. In the i.v. challenge, he found that antibody had an effect in the first phase of the infection, by enhancing the blood clearance and killing of the salmonella over the first hour. As previously shown by Mackaness *et al* (1966), subsequent growth of the surviving salmonella was not affected by the preopsonization. Antibody played a greater role in the i.p. and s.c. routes. Its effect was to slow the rates of dissemination of the challenge from the peritoneal cavity, or site of injection, to the liver and spleen. However, antibody was incapable of stopping this spread and, once the bacteria reached the livers and spleens, they grew rapidly. The delay gave the mice time to mount a cellular immune response, which could control the growth of the challenge. This view was supported by Herzberg, Nash and Hino (1972) and by Akeda *et al* (1981).

In human typhoid, vaccination is parenteral and challenge is usually oral with a modest dose (Hornick et al, 1970; Blaser and Newman, 1982). Collins and Carter (Collins, 1970; 1972a; 1973; Collins and Carter, 1972) assessed the efficacy of parenteral vaccination against oral challenge. Mice vaccinated with killed S. enteritidis were challenged orally with S. enteritidis. No protection was seen against challenge with virulent S. enteritidis 5694, but some protection was evident against challenge with the less virulent strain Se795 (Collins, 1972a). As with parenteral challenge, immunisation only delayed spread of the infection, giving the mouse time to mount a de-novo cellular immune response. It is probable that this is similar to the situation in human typhoid, where parenteral vaccination is probably only effective against low dose oral challenge (Hornick et al, 1970).

Despite the elegant studies of Collins, claims are still made for a crucial role for antibody in protection and the use of killed vaccine is still advocated (Svenson, Nurminen and Lindberg, 1979; Nakoneczna and Hsu, 1983; Hsu, Nakoneczna and Guo, 1985; Killon and Morrison, 1986). These studies generally use the i.p. model and mortality as a basis of protection.

Attempts have been made to immunise mice orally with killed or avirulent preparations to stimulate immunity at the

intestinal mucosa (Collins and Carter, 1972; Waldman, Grunspan and Ganguly, 1972; Moser et al, 1980). These studies showed that large oral doses of killed bacteria gave only very low levels of protection. Moser et al (1980) found that levels of intestinal anti-salmonella antibody correlated with protection.

1.3.3 Cell Mediated Immunity.

Collins and co-workers (Collins, 1974; 1979) found that immunisation with live bacteria elicited a strong antibacterial response, which immediately controlled the growth of a superinfecting challenge. This was attributed to a cell-mediated immune response. Immunity of this kind was not seen in mice immunised with killed vaccines.

Early work with cell-mediated immunity was performed using pathogens other than salmonella, especially listeria, BCG and brucella. Mackaness and his co-workers found that once this antibacterial response had been stimulated it was non-specific in its effects. They found that, for example, a brucella immune mouse was also immune to mycobacteria, salmonella and listeria (Mackaness, 1969). The induction of this response required live organisms which were able to establish a persistent infection. Avirulent strains of salmonella (Collins et al, 1966; Collins, 1968a) or mycobacteria (Collins, 1971b) which were cleared rapidly from the mice did not induce a protective response. The

level of immunity was correlated with the level of carriage of the immunising bacteria: a high level of carriage gave a high level of immunity (Collins et al, 1966; Collins, 1968a). Killed vaccines were unable to induce an antibacterial response unless given in Freunds complete adjuvent (CFA). When given in CFA, even strains which were ineffective as live vaccines, such as S. pullorum were effective (Collins, 1972b; 1973).

Adoptive transfer studies were carried out with listeria infected mice to confirm the cellular nature of the immunity (Miki and Mackaness, 1964). Resistance was not transferable with serum but was transferable with live spleen cells. Urea extracts of spleen cells were ineffective, thus ruling out a role for cytophilic antibody. The transfer of resistance was ablated with anti-lymphocyte serum (ALS). It was proposed that lymphocytes were the cells responsible for the specificity and that macrophages were the effector cells. It was later established that the lymphocytes are T-cells (Lane and Unanue, 1972; Blanden and Langman, 1972; North, 1973ab; 1975).

Collins and Mackaness (1968) found that infection of mice by live salmonella induced a delayed type hypersensitivity (DTH) response. They claimed that a DTH response was a necessary part of protective immunity. This idea was further supported by the observations that avirulent salmonella

which did not protect did not induce DTH either. Killed vaccines produced an Arthus response rather than a DTH response (Collins and Mackaness, 1968).

It has since been shown that DTH is not the ideal marker for protective immunity. Hormaeche et al (1981) found that whilst BALB/c mice infected i.v. with the semi-virulent S. typhimurium strain M525 did not display a DTH response until day 14, on day 8 they were immune to a superinfecting challenge by virulent S. typhimurium C5R. Attridge and Kortlarski (1985b) used local adoptive transfer to study DTH in mice immunised with avirulent S. enteritidis 11XR. They found that DTH could be transfered locally to the footpad by non-adherent cells of the $lyt1^{+}$ 2^{-} phenotype. DTH was class II restricted at the I-A subregion of H2. Protection could not be transferred with DTH or any T-cell fraction. Immunisation with salmonella aroA mutants may induce a DTH response. Eisenstein and co-workers (Eisenstein, Killar, Stocker and Sultzer, 1984; Killar and Eisenstein, 1985) found that i.v. immunised salmonella susceptible C3H/HeJ and C3HeB/FeJ, and resistant C3H/HeNCrlBr and CD1 mice were highly immune to virulent challenge. When tested for DTH, however, only the C3H/HeNCrlBr and CD1 mice showed any DTH response (Killar and Eisenstein, 1985; 1986). Induction of DTH by aroA mutant SL3235 in C3H/HeNCrlBr mice was dose dependent, and lower doses, although protective, did not induce DTH.

Divergence of DTH and protection have been reported in other intracellular infections. Orme and Collins (1984) found that distinct subsets of activated T-cells mediated protection and DTH. In L. major infections Liew, Howard and Hale (1984) found that i.v. immunisation with irradiated promastigotes lead to protection, with no DTH, while s.c. immunisation gave DTH but also caused exacerbation of the challenge infection.

Little work has been done to show the role of T-cells in immunity to salmonella infections. Attridge and Kotlarski (1985a) followed lymphokine release by cultured lymphocytes from mice immunised with the attenuated S. typhimurium strain 11XR. $Lyt1^{+}2^{-}$ cells were found to release interleukin 2 (IL2) and macrophage activating factor (MAF) when cultured in vitro with 11XR antigens. The cells were I-A restricted in the same way as the DTH response. Paul et al (1985) isolated T-cells from mice immunised with a spent medium antigen (SMA) commonly used to elicit DTH (Plant and Glynn, 1974). These cells proliferated in response to SMA and could be maintained continuously if IL2 was supplied. BALB/c mice were given 10^{6} cells i.v. and were challenged i.p. 3 hours later with small doses (30 or 60 bacteria) of virulent S. typhimurium. Some slight protection was seen as judged by mortality. Killar and Eisenstein (1985) and Maskell et al (1987a) attempted to adoptively transfer immunity with T-cells but both were unsucessful.

Listeria infections (unlike most salmonella, mycobacteria and brucella) are cleared from the MPS resulting in sterile immunity. The mice can, however, respond rapidly to a challenge and are highly immune. North (North, 1975; North and Deissler, 1975) examined the T-cell responses involved. Two populations of cells were found; a short lived population of dividing cells, which was present during the period of infection and rapidly disappeared upon clearance, and a small population of non-dividing cells which remained for several months. The short-lived cells, which were thought to be responsible for activating the macrophages, required constant antigenic stimulation, whilst the long-lived population provided memory. The long-lived population could transfer DTH and low levels of immunity, and were found to be responsible for re-activating a new population of short-lived cells.

Work with the listeria and mycobacteria models have given more evidence for the role of different T-cell subsets in immunity to intracellular bacterial infections (Kaufman, 1987). Listeria specific $L3T4^-$ $lyt2^+$ and $L3T4^+$ $lyt2^-$ cells have been identified. In adoptive transfer studies both are required for good levels of protection and DTH (Kaufmann, Simon and Hahn, 1979; Kaufmann, Hug, Vath and Muller, 1985). T-cell clones have been established of each phenotype (Kaufmann and Hahn, 1982; Kaufmann, 1984). The $L3T4^+$ clones show behaviour typical for T-helper cells. Like the

lyt1^{+} 2^{-} salmonella specific cells, they are I-A restricted and produce both IL2 and IFN-gamma in culture. Similar properties have been found for M. tuberculosis L3T4^{+} cells (Kaufmann and Flesch, 1986) and human CD4^{+} M. leprae specific cells (Emmrich and Kaufmann, 1986). The Lyt2^{+} clones required IL2 but when cultured in the presence of IL2 would produce both it and IFN-gamma. They were found to have an antigen specific ability to lyse listeria infected macrophages in class I restricted manner (Kaufmann, Hug and DeLibero, 1986). When investigated further, it was found that the L3T4^{+} cells also had listeria specific cytolytic activity. This was class II restricted, and required that the target macrophages were first treated with IFN-gamma, probably to allow expression of class II antigens.

While T-cells are the mediators of cellular immunity, the macrophage is the effector. In both the primary and secondary responses, when stimulated the resident macrophages of the MPS divide (North, 1969). More importantly, the stimulation also causes an influx of mononuclear phagocytes from the blood and bone marrow (North, 1970) which soon constitute the majority of cells within the lesion. These recruited cells can make an increased H_2O_2 during listeria infections: the resident liver kupffer cells cannot (Lepay et al, 1985). Although attempts to transfer immunity to salmonella with immune T-cells have been unsuccessful, it has been possible to

transfer immunity with macrophages. Killar and Eisenstiein (1985) found that immunity could be transfered by adherent spleen cells from mice immunised with an aroA S. typhimurium. Successful transfer was only possible during the second week of the infection. At this point, the mice show maximal resistance to listeria (Killar and Eisenstein, 1984) and their peritoneal exudate shows maximal ability to kill cultured tumor cells (Schafer et al 1988). Maskell et al (1987a) found that immunity could be transfered from infected mice by spleen cell suspensions. T-cell depletion by monoclonal antibody and complement did not affect the ability to transfer protection, whilst carbonyl iron treatment did, again suggesting that macrophages were the protecting cells. Although, as noted earlier, the initial macrophage activation events appear to be T-cell independent, the evidence suggests that T-cells are important later in the infection and for recall of memory.

1.4 The development of live vaccines.

1.4.1 The requirements for a live vaccine.

In the mouse model, vaccination with sub-lethal doses of virulent organisms induces high levels of immunity. However, the vaccinating strain often kills some of the mice, and survivors were always chronically infected. Therefore, such strains would be unacceptable for use as live vaccines in

humans. Attempts have been made to design attenuated vaccines for use in man. They should be safe, even in an immunocompromised host, non-reverting, and immunogenically identical to the virulent organism (Stocker, Hoiseth and Smith, 1983). Development work has centred on the mouse model but some putative vaccine candidates have been constructed in S. typhi and tested, with varying degrees of success, in man.

1.4.2 Early live vaccines tested in the mouse model.

Early live vaccines tested in the mouse model ranged from low doses of virulent organisms discussed above, to naturally attenuated strains, such as S. gallinarum (Collins et al, 1966) and S. typhimurium M525 (Hormaeche, 1981), or rough mutants (Kenny and Hertzberg, 1968). Such strains were often excellent vaccines in the experimental model but are not suitable candidates to apply to S. typhi and human use. More recently, attempts have been made to attenuate salmonella using more precise genetic methods. Approaches taken have been either to alter virulence determinants or to change the ability of the salmonella to survive in vivo. Examples of each approach are described below.

1.4.3 Temperature sensitive mutants.

Mutants unable to grow at the temperature of the host, have been isolated for pathogens such as L. monocytogenes (Gervais et al, 1986) and salmonella. Fahey and Cooper

(1970ab) developed a temperature sensitive S. enteritidis. When given orally or parenterally it induced good levels of long-lasting immunity. A similar protective mutant of S. enteritidis has been reported by Ohta et al (1987). Hormaeche et al (1981b) isolated a temperature sensitive mutant of S. typhimurium C5. This strain grew slowly in livers and spleens of i.v. infected mice, but did not kill them. This strain would protect salmonella resistant and susceptible strains of mice against subsequent i.v. challenge. Non-specific immunity against listeria was seen during the vaccinating infection (Nauciel et al, 1985). Morris-Hooke (personal communication) has construted a temperature sensitive S. typhi mutant. As yet, no human trials are proposed.

1.4.4 Plasmid cured and other attenuated strains.

As discussed earlier, the large plasmid present in some salmonella strains encodes virulence determinants. Attenuated strains which are cured of the plasmid have been successfully used as live vaccines in the mouse model (Nakamura et al, 1985; Gulig and Curtiss, 1987). Since S. typhi does not possess such a plasmid, this approach cannot be extended to human vaccines. Some of the attenuated "macrophage survival" mutants isolated by Fields et al (1986) have been found to be effective vaccines (Heffron personal communication). It is possible that analogous S. typhi mutants could be constructed.

1.4.5 Streptomycin dependent mutants.

The first live S. typhi vaccines tested in man were attenuated by streptomycin dependence. These strains have ribosomes which require the presence of streptomycin for efficient protein synthesis. Two streptomycin dependent mutants of S. typhi were developed independently by Reitman (1967) and Mel et al (1974). The Mel strain, derived from S. typhi Ty2, was fed to 1104 yugoslavian adults and 622 children without adverse side effects. The Reitman strain, 27V, was derived from S. typhi 19V and required streptomycin at 10ug/ml to grow. In mice, it was attenuated unless streptomycin was present. The strain was tested extensively for safety and immunogenicity in human volunteers (Levine et al 1976). When given orally with one gram of streptomycin, and bicarbonate, the vaccine gave no side effects. Challenge studies showed good levels of protection if the vaccine was a fresh culture. When lyophilised bacteria were used, however, no protection was seen. Since wide-scale use of a vaccine depends on lyophilised preparations, it was decided that streptomycin dependent mutants were unacceptable. They had, however, shown that a live attenuated vaccine is effective in man against typhoid.

1.4.6 galE mutants.

Rough salmonella strains, which do not possess O-antigens, are generally attenuated. The galE gene encodes the enzyme uridine diphosphate (UDP) galactose-4-epimerase. This

converts UDP glucose to UDP galactose. Since UDP galactose is incorporated into smooth LPS, galE mutants are rough. If exogenous galactose is supplied, this can be converted into UDP-galactose via galactose-1-phosphate, so allowing synthesis of smooth LPS. Galactose is, however, toxic to the cells since galactose-1- phosphate accumulates and causes cell lysis within a few hours (Fukasawa and Nikaido, 1961). This toxicity can be relieved by supplying glucose with the galactose.

Germanier (Germanier, 1970; 1972; Germanier and Furer, 1971) investigated the attenuation and the protective capacity of a S. typhimurium galE mutant, G30D. It was found to be attenuated when given to mice by the i.p., i.v., s.c., and oral routes. When mice were immunised i.p., G30D established a limited infection in livers and spleens, which was cleared by day 38. When administered orally, it was capable of colonizing the liver and spleen for 14 days.

Immunisation by all routes gave good levels of protection against i.p. challenge. Immunisation orally induced long-lasting immunity against both i.v. and oral challenge. During the immunising infection, immunity was non-specific, with protection against both S. typhimurium and S. enteritidis, but by 66 days it was specific. Oral immunisation with G30D induced serum antibodies, intestinal IgA and DTH to salmonella antigens (Moser et al, 1980).

Morris, Wray and Sojka, (1976) suggested that immunity to galE mutants was dependent on B-cells. They treated CBA mice with either cyclophosphamide (cy) or anti-lymphocyte serum (ALS) before immunisation with G30D and/or challenge with a virulent S. typhimurium. Cy treated mice were unable to control the immunising dose of G30D and died. ALS treated mice had no T-cell responses as judged by proliferation to PHA, but also had depressed B-cell responses as measured by proliferation to LPS. ALS treated, vaccinated mice made no antibody response to G30D. When challenged with virulent S. typhimurium all unimmunised groups died. Normal, vaccinated mice controlled the superinfecting infection effectively. ALS treated, vaccinated mice, were given an injection of anti G30D serum 24 hours before challenge, to make up for the lack of B-cell function. When challenged, they could control growth of the challenge effectively, but less well than the normal vaccinated group. These results suggest that cy treatment was affecting macrophage function and that it was this, rather than a lack of T or B-cells which rendered mice susceptible and non-vaccinatable.

galE lesions do not attenuate all salmonella serotypes. Nnalue and Stocker (1987) isolated galE mutants of S. choleraesuis (0-6, 7). They were found to be as virulent as their parents, possibly because the amounts of galactose in 0-6, 7 LPS are much less than in S. typhimurium.

1.4.7 galE S. typhi vaccines used in humans: Ty21a.

The success of galE S. typhimurium suggested that galE S. typhi would be effective in humans. Germanier used chemical and ultraviolet mutagenesis to isolate a galE mutant of S. typhi Ty2. This strain, known as Ty21a, (Germanier and Furer, 1975) lacks UDP-galactose epimerase activity and also has reduced galactose sensitivity due to further lesions in the Leloir pathway enzymes. It is avirulent for mice with an LD_{50} greater than 10^8 if given in saline or mucin. It shows protective capacity in the mouse model (Germanier and Furer, 1983)

Ty21a was tested for safety in human volunteers (Gilman et al, 1977). One hundred and fifty five men took between three and five doses containing between 3 and $10x10^{10}$ organisms, after bicarbonate. There were no adverse side-effects. Some of the volunteers were then challenged with virulent S. typhi Quailes strain. A significant level of protection against disease and stool carriage was seen in vaccinees, provided the vaccinating dose of Ty21a had been grown in medium (Brain Heat Infusion) supplemented with 0.1% galactose. No protection was given by Ty21a grown in BHI with no added galactose.

The results in volunteers led to field trials in Egypt and South America. In the Egyptian trial, 16,486 6-7 year old school children received three doses of vaccine, containing

$3x10^9$ live Ty21a per dose. Over the next three years, only one case of typhoid was diagnosed in children who had received 2 or more doses, compared with 22 cases in the placebo group of 14,557 children (Wahdan *et al*, 1982). This trial was followed by a large trial in Santiago, Chile. This area has the highest recorded incidence of typhoid in the world. In this trial, 109,000 children were given three doses of Ty21a in enteric coated capsules (Levine *et al*, 1987). Protection was 67% over a three year period. This is much lower than the 96% protection seen in the Egyptian trial. However, differences in vaccine formulation, population genetics and the higher incidence of typhoid in Santiago may all have contributed to this difference. Efficacy of 67% is still as good as that shown by parenteral vaccination (Ashcroft *ef al*, 1967) with none of the associated side-effects.

Apart from its *galE* lesion, Ty21a has several other phenotypic differences from its Ty2 parent: it neither possesses the Vi antigen, nor does it produce hydrogen sulphide. It also has several amino acid auxotrophies different from Ty2. There has been a recent report from the Santiago trial, showing that galactose fermenting revertants of Ty21a could be isolated from stools of vaccinees using MacConkey medium. These gal^+ isolates were still attenuated in mice (Silva *et al*, 1987).

Hone *et al*, (1987) have recently described the construction of a genetically defined *galE* mutant of Ty2. This mutant was further modified so that it did not make Vi antigen by insterting a *via* mutation (Hone *et al*, 1988). This strain was serum sensitive and attenuated in the mouse model but when tested in human volunteers two of four people who ingested $7x10^8$ bacteria became ill and developed a typhoid like disease. They recovered after antibiotic treatment. This is further evidence to suggest that the *galE* lesion in Ty21a is not the major reason for attenuation.

1.4.8 *Attenuation by auxotrophic requirements.*

For a bacterial pathogen to grow *in vivo*, there must be a sufficient supply of all nutrients that it requires. Should the pathogen require a nutrient not available *in vivo*, it will be unable to multiply and will be attenuated. This has been observed several times in the past with reports of attenuation of *Yersinia pestis* (Burrows and Bacon, 1954), *Bacillus anthracis* (Ivanovics, Maraji and Dobozy, 1968) and *S. typhi* (Bacon, Burrows and Yates, 1950; 1951ab) by auxotrophy.

Burrows *et al* (1950; 1951ab) produced a bank of *S. typhi* mutants by chemical mutagenesis. When tested for virulence in the i.p. mouse model, some mutants were attenuated. Many of the attenuated isolates had auxotrophic requirements,

especially for para-aminobenzoic acid (PABA), histidine, aspartic acid, methionine, and purines and pyrimidines.

Recent advances in bacterial genetics and genetic manipulation have allowed the construction of mutant strains with genetically defined lesions. Prompted by the work of Bacon *et al*, most attention has been paid to the aromatic and purine biosynthetic pathways.

1.4.9 *Aromatic compound requiring mutants.*

Bacteria, plants and fungi possess a biosynthetic pathways to produce aromatic compounds. This pathway is the only means by which they can synthesise aromatic compounds such as p-aminobenzoic acid (PABA) (and so folate and nucleotides), dihydroxy benzoic acid (DHB) (and so the siderophore enterochelin), and the aromatic amino acids tyrosine , tryphophan and phenyl-alanine. Mammals do not possess such a pathway, and so have to take in required aromatic compounds in their diet.

The aromatic biosynthetic pathways have been studied in detail. The pathway found in *E. coli* and *Salmonella* spp is shown in Fig. 1.2. The enzyme functions of the pathway to chorismate are highly conserved. In *E. coli* and salmonella, every step of the pathway is cataylised by a different enzyme which are encoded by individual genes. In fungi, such as *Aspergillus nidulans* (Charles *et al*, 1986) and

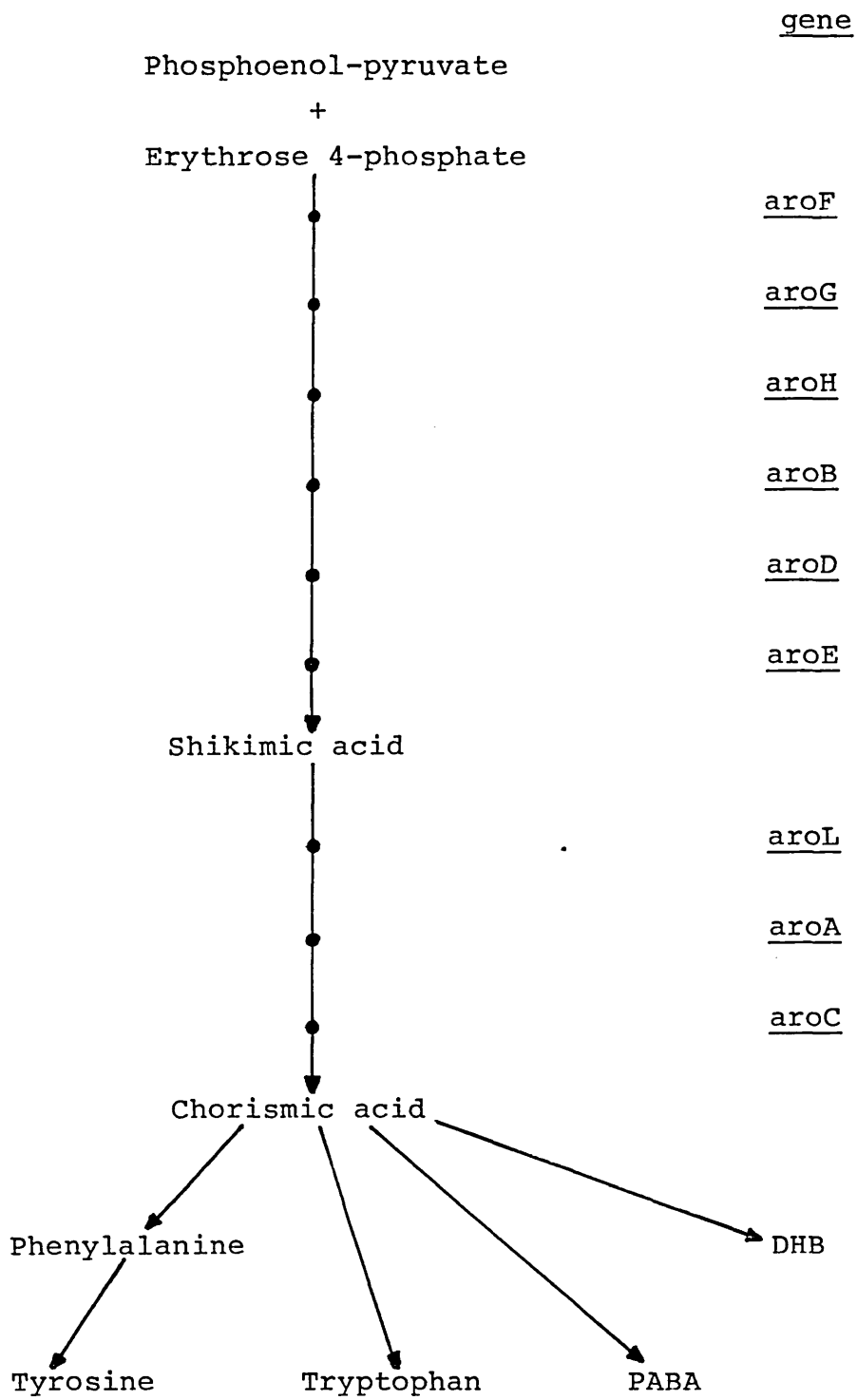


Fig. 1.2 The aromatic biosynthetic pathway in salmonella.

Saccharomyces cerevisiae (Duncan, Edwards and Coggins, 1987) there exist multi-enzymic complexes, cataylising several reactions. The aroA genes have been cloned and sequenced from E. coli (Duncan, Lewendon and Coggins, 1984), S. typhimurium (Stalker, Hiatt and Comai, 1985), B. pertussis (Maskell, Morrissey and Dougan, 1988), S. cerevisiae (Duncan et al, 1987) and A. nidulans (Charles et al, 1986). The amino acid sequences show a large degree of homology with several blocks of sequence with 100% homology. The aromatic biosynthetic pathway is obviously of key importance since it is so highly conserved throughout such a wide range of organisms. In light of the observation of Bacon et al (1951), that PABA requiring S. typhi were attenuated, Hoiseth and Stocker (1981), used Tn10 mutagenesis to construct mutants of mouse virulent S. typhimurium, with non-reverting deletions in the aroA gene of the aromatic biosynthetic pathway. aroA derivatives of several mouse virulent strains were found to be highly attenuated when administered to BALB/c mice. Whereas <10 cfu (colony forming units) of the parental strains administered i.p. were lethal, no ill effects were seen in mice infected i.p. with 10^6 cfu of the aroA derivatives. The aroA derivatives were found to be effective as live vaccines (Hoiseth and Stocker, 1981). Thirty days after i.p. immunisation with $2x10^5$ cfu of an aroA mutant (SL3261), BALB/c mice were highly immune to an i.p. challenge with $5x10^5$ cfu of its virulent parent (SL1344). Similarly, mice

immunised i.p. with $2x10^4$ cfu SL3261, or orally with $2x10^8$ cfu SL3261 were immune to an oral challenge with $3x10^7$ cfu SL1344.

Eisenstein and co workers have studied the behaviour of SL3235, a S. typhimurium aroA mutant, in C3H lineage mice. They were highly attenuated in both genetically resistant and susceptible C3H mouse strains (Eisenstein et al, 1984; Killar and Eisenstein, 1985). When C3H/HeJ or C3H/HeNCrlBr mice were immunised i.p. with SL3235, the bacteria established a limited infection in the livers and spleens. Mice immunised with SL3235 were highly immune to an i.p. challenge with a virulent S. typhimurium. Significant levels of protection were achieved within three days of immunisation and were still evident 7 months later. Non-specific immunity to listeria challenge was evident between days 6 and 21 of the immunising infection (Killar and Eisenstein, 1985). All protection was assessed by mortality following i.p. challenge. Immunisation with SL3235 induced a cellular immune response (Killar and Eisenstein, 1984; 1985; 1986). DTH was evident in some mouse strains. T-cells were primed as judged by PETLEs, one and three months following immunisation. Immunity, however, could only be transfered with macrophage fractions.

Initial experiments with aroA mutants indicated the potential value of aroA mutants as oral vaccines. We have

shown (Maskell et al, 1987b) that SL3261 given orally was able to invade and establish a persistent infection in the MPS of BALB/c mice. SL3261 persisted in livers, spleens, mesenteric lymph nodes and Peyer's patches for between 5 and 6 weeks. Although persistence was low level (between 10^3 and 10^4 cfu per organ), mice were highly protected against oral challenge with 10^{10} virulent SL1344 57 days (Maskell et al, 1987a) and 70 days (Dougan et al, 1988) later.

Salmonellosis is an economically important disease in both cows and sheep. In bovine salmonellosis, the most important serotypes are S. typhimurium and S. dublin. aroA mutants derived from both serotypes have proven to be excellent vaccines in calves (Smith et al, 1984abc; Robertson et al, 1983) when given either intramuscularly or orally. Some serotype cross protection was also evident. An aroA mutant of a sheep virulent phage type of S. typhimurium has also been shown to be an effective vaccine against salmonellosis in sheep (Mukkur et al, 1987).

1.4.10 Purine dependent mutants.

The attenuation and vaccine efficacy of S. typhimurium aroA mutants suggested the use of S. typhi aroA mutants in man. However, should a single attenuating mutation revert to wild type, the vaccine strain would be fully virulent. Therefore, to ensure safety if this unlikely event occurred, it was

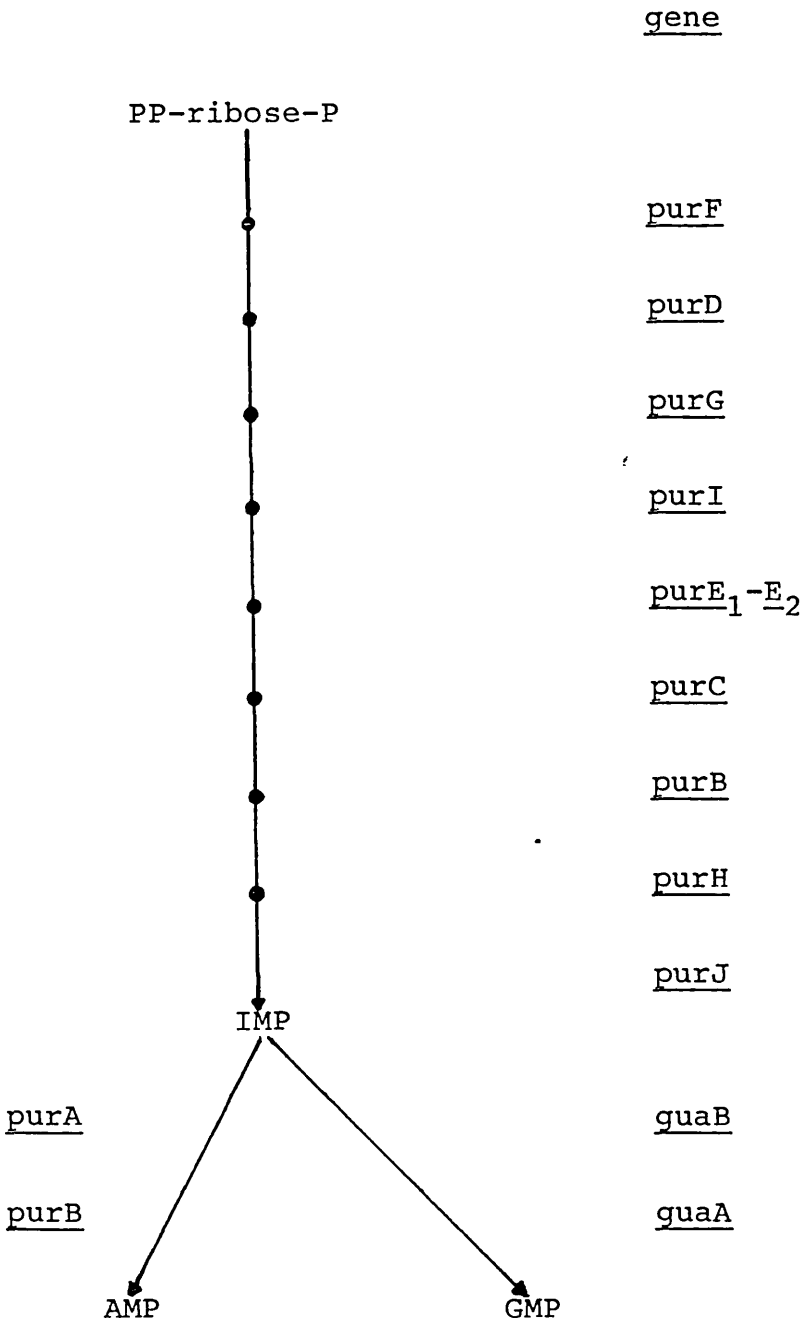


Fig 1.3 Main features of de-novo purine biosynthesis in salmonella.

proposed S. typhi vaccines should contain a second attenuating lesion (Stocker, 1988).

Prompted by the work of Bacon et al (1950; 1951ab), purine dependence was selected as the second attenuating mutation, and S. typhi mutants with deletions in aroA and various purine biosynthetic genes, were constructed (Stocker, 1988; Dougan, personal communication). The de novo purine biosynthetic pathway is shown in Fig 1.3. It consists of a single pathway to inosine monophosphate (IMP), at which point it splits into pathways to adenosine monophosphate (AMP) and guanosine monophosphate (GMP).

Attenuation by purine dependence has been assessed in the mouse model. McFarland and Stocker (1987) constructed purine dependent mutants of S. typhimurium and S. dublin. Mutants with lesions in the pathway to IMP (purF, purC, purG or purJGD operon) had partially reduced virulence. Similar partial attenuation was seen in a purHD S. choleraesuis mutant (Nnalue and Stocker, 1987). Mutants between IMP and GMP (guaA, guaB) were less virulent than the common pathway mutants (McFarland and Stocker, 1987). Mutants in the pathway to AMP (purA, purB) were highly attenuated.

BALB/c mice immunised with a S. typhimurium purA mutant were protected against virulent challenge 30 and 58 days later.

Challenge was with $2.5x10^3$ given i.p. with mortality from a group of three mice the only criterion for protection.

1.4.11 Auxotrophic S.typhi vaccine candidates.

Edwards (PhD thesis, University of Stanford, 1985) constructed two derivatives of S. typhi as candidate typhoid vaccines. The strains, one posessing and one without Vi antigen, contained deletions in both aroA and purA. These strains were tested for safety in human volunteers with no side effects (Levine et al, 1987). Volunteers received one dose of 10^8, 10^9 or 10^{10} cfu of either strain, or two doses of the Vi^+ strain four days apart. Positive stool cultures were obtained from most volunteers who had received 10^9 cfu or more, but no positive blood cultures were obtained. The immune responses were followed in vaccinees. Few of the 37 volunteers showed a significant rise in serum antibodies. Low levels of T-cell priming , as measured by proliferation in response to S. typhi antigens, was observed in 16/18 of the volunteers who received 10^9 cfu or more. Cell mediated immunity, measured by an ADCC type test (Tagliabue et al, 1984) was claimed to be evident in all 37 vacinees.

Similar S. typhi aroA purA mutants have been constructed by G. Dougan at Wellcome Research Laboratories (personal communication). One strain has been found to be safe in human volunteers but no immune response was seen.

1.5 Aims of and reasons for the work.

The work described in this thesis was commenced to complement attempts to construct a rationally attenuated live *S. typhi* vaccine. Since the behaviour of *aroA* *S. typhimurium* in the mouse typhoid model was well characterised *aroA* was proposed as the prime attenuating lesion (Dougan *et al*, 1987; Stocker, 1988). As discussed above, a *pur* mutation was proposed as a second attenuating lesion. No information existed on the behaviour of defined *pur* or *aroA* *pur* mutants in the mouse typhoid model.

The aims of the project were:

1. To use transposon Tn*10* mutagenesis to construct mutants of mouse virulent *S. typhimurium* with lesions in *aroA*, *purA*, *purE*, and combinations thereof.

2. To determine the effects (if any) of the mutations on the *in vitro* characteristics of the strains.

3. To examine the behaviour of the strains in the mouse typhoid model following infection by parental and oral routes determining:

   i. degree of attenuation.

   ii. *in vivo* behaviour and persistence.

   iii. ability to vaccinate.

4. To examine the immune responses to vaccination with each vaccine type.

MATERIALS AND METHODS

2.1 Bacterial strains, bacteriophage and media.

The strains of S. typhimurium used are shown in table 1.1. Derivatives of the avirulent S. typhimurium strain LT2, with transposon Tn10 inserted into either the aroA, purA or purE genes, were obtained from J. Roth, Salmonella Genetic Stock Centre, Calgary. The mouse virulent strains SL1344 and HWSH were used both as parents of the auxotrophic mutants and for challenge experiments. They were obtained from B.A.D. Stocker, Stanford University, and H. Williams Smith, Houghton Poultry Research Centre, respectively. The virulent strain C5, occasionaly used as virulent challenge, was obtained from C.E Hormaeche, University of Cambridge. All auxotrophic derivatives were constructed during this study except SL3261, an aroA derivative of SL1344 (Hoiseth and Stocker, 1981), which was obtained from B.A.D. Stocker. The virulent strain of Listeria monocytogenes LUGI 23 was obtained from C.E. Hormaeche.

High frequency transducing mutants P22 HT105 int (Schmieger, 1972) and P1 vir were obtained from T. Foster, Moyne Institute, Trinity College, Dublin. Phage were propagated in soft agar overlays and were titrated by spotting 2ul of 100-fold dilutions onto a soft agar overlay containing 100ul of an overnight culture of the bacterial host. Plaques were counted after overnight incubation at 37^{o}C.

Bacteria were grown in L-broth or L-agar (Davis, Botstein and Roth, 1980). L-agar was solidified with 1.5% Bacto agar (Difco). Soft agar for phage growth contained 0.8% agar. Minimal media (Davis, et al, 1980) contained M9 salts, glucose (1% w/v) and Ca/Mg salts. It was made with double distilled, deionized water and solidified with 2% Noble Agar (Difco). To allow growth of aroA mutants, minimal medium was supplemented with the aromatic amino acids tyrosine, tryptophan and phenylalanine at a concentration of 40ul/ml and PABA and DHB at 10ul/ml. purA and purE mutants required adenosine at 40ug/ml. SL1344 and its derivatives required histidine at 40ug/ml. All supplements were purchased from Sigma and were disolved in double distilled deionized water and sterilised by 0.22um filtration. Tyrosine was sterilised by steaming. Adenosine was solublised by adding one or two drops of conc HCL. Bochner medium (Bochner et al, 1980) was made up as described by Davis et al (1980) except that fusaric acid was at 2ug/ml instead of 6ug/ml.

TABLE 2.1

Strains of S. typhimurium Used in This Study.

Strain	Phenotype
LT2 *aroA* 554::Tn*10*	aro^- tet^R
LT2 *purA* 874::Tn*10*	pur^- tet^R
LT2 *purE* 884::Tn*10*	pur^- tet^R
HWSH	Prototroph
SL1344	his^-
C5	Prototroph
HWSH *aroA*	aro^-
HWSH *purA*	pur^-
HWSH *aroA purA*	aro^- pur^-
HWSH *purE*	pur^-
SL3261	aro^- his^-
SL1344 *purA*	pur^- his^-
SL3261 *purA*	aro^- pur^- his^-

2.2 Construction of auxotrophic mutants of mouse virulent S. typhimurium strains.

Auxotrophic deletion mutants were created in mouse virulent strains using transposon Tn10 mutagenesis as described by Davis et al (1980). This is outlined in Figure 2.1 and comprised two steps. Firstly, a Tn10 inactivated gene was transduced from an avirulent LT2 derivative into the mouse virulent strain using P22. Second, a tetracyline sensitive auxotrophic mutant, due to imprecise excision of the transposon, was selected using Bochner medium.

2.2.1 P22 transductions.

A high titre P22 lysate was prepared from the LT2::Tn10 strains using the plate method. Ten-fold dilutions of phage were prepared and 10ul was mixed with 100ul of an overnight culture. After incubation at 37^{o}C for 1 hour, 3ml of soft agar was added and the mixture poured over a fresh L-agar plate containing tetracycline (50ug/ml). Following overnight incubation at 37^{o}C the soft agar overlay on the plate of the highest phage dilution showing complete lysis was scraped into a centrifuge tube with 3ml T-2 buffer plus a few drops of chloroform. After vigorous vortexing, the lysate was clarified by centrifugation (10,000xg 10 minutes). The lysates were titrated on the strains on which they were grown and generally contained between 10^{9} and 10^{11} plaque forming

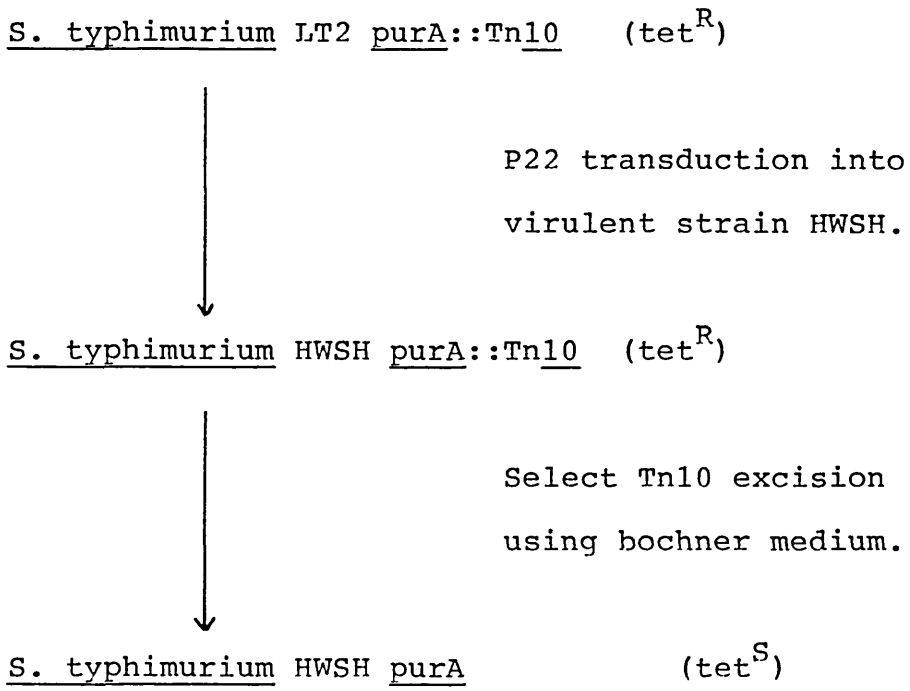


Fig. 2.1 Method for selection of Tn10 generated deletions in the purA gene of virulent S. typhimurium HWSH. The Tn10 inactivated purA gene is transduced from S. typhimurium LT2 into HWSH using phage P22, selecting for the tetracyclin resistance encoded by Tn10. Tetracyclin sensitive deletion mutants, caused by imprecise excision of Tn10, were then selected on Bochner medium.

units (pfu) per/ml. Lysates were stored at 4°C with a few drops of chloroform until needed.

2.2.2 Transduction of virulent strains.

Virulent parental strains were stored in 0.5ml aliquots over liquid nitrogen as described below. One aliquot was used to inoculate 10ml of L-broth. Transductions were always carried out with late log cultures grown at 37°C with shaking. One hundred microlitres of culture was mixed with 10ul of neat, 10^{-1} or 10^{-2} dilutions of P22 lysate. After incubation for 30 minutes at 37°C, the mixture was spread over an L-agar plate containing tetracycline (50ug/ml) and EGTA (5mM) and incubated overnight at 37°C. Transductants were picked as tet^R colonies and were passaged on the same medium until free of contaminating phage. They were then checked for the correct auxotrophic characteristics using minimal agar supplemented as appropriate.

2.2.3 Selection of mutants with deletions induced by imprecise excision of Tn10.

Like many transposons, Tn10 can excise at high frequency (Kleckner, Roth and Botstein, 1977). Excision results in loss of antibiotic sensitivity. Excision can be one of two forms: precise or imprecise. When a transposon excises precisely, it jumps out leaving the ends of the gene in which it was inserted free to reform the wild type gene. Imprecise excision results in part of the surrounding host

DNA being removed with the transposon. Therefore, when the ends of the gene re-join there is a deletion or a rearrangement resulting in loss of gene function. Excision of Tn10 occurs at fairly high frequency (approximately 10^{-6}) and can be selected for on Bochner medium which selects for tet^S phenotype. Bochner medium contains autoclaved chlorotetracycline. This induces the tet^R genes without having any antibiotic effects. The membrane changes induced by the tet^R genes make the cell sensitive to the antibiotic fusaric acid. This is helped by zinc ions. Bochner medium contains low amounts of fusaric acid acid zinc.

Cultures were grown overnight in L-broth containing 50ug/ml Tetracycline. Cultures were diluted 10^{-1}, 10^{-2}, 10^{-3} and 100ul of each dilution was spread over a Bochner plate and incubated overnight at $37^{o}C$. Tet^S colonies were identified as those growing faster than the surrounding background. Colonies were picked and twice re-passaged on Bochner plates before testing auxotrophy and tetraclycline resistance.

2.3 In vitro characterisation of S. typhimuriun strains.

2.3.1 Slide agglutinations and phage sensitivity.

Strains were tested for their serological characteristics by slide agglutination with O-4, O-5, O-6, 7 and O-9, and, Hi, H2, and Hd specific diagnostic sera provided by Wellcome

Diagnostic Ltd, Dartford, Kent. Only suspensions made in PBS from fresh plates or liquid cultures were tested.

Sensitivity to smooth LPS specific P22 and rough LPS specific P1 phages by titrating phage lysates (10^{10} pfu/ml) on overlays containing the test strain. A sensitive strain gave single plaques on the 10^{-8} dilutions whilst a resistant strain gave no plaques on the neat dilution. SL1344 and its derivatives were more resistant to P22 since they contain a lysogenic P22 like phage (Stocker, personal communication).

2.3 Lipopolysaccharide characterisation.

LPS was purified using the hot phenol/water method of Westphal, Luderitz and Bister (1952). Overnight L-broth cultures (10 ml) wre harvested by centrifugation (10,000×g), the pellet washed with distilled water and suspended in 3ml of ditilled water. This was heated to 65°C in a water bath and 3ml of 90% phenol (BRL, Ultra Pure) at 65°C was added. The mixture was vortexed and then incubated at 65°C for 30 minutes with vortexing every 7-8 minutes. The mixture was cooled in ice for 10 minutes and then the aqueous and phenol phases separated by centrifugation (10,000×g) at 0°C. The aqueous phase was removed and dialysed against distilled water, at 4°C for 24 hours with three changes of water, to remove any remaining phenol. LPS was precipitated by adding 1/10 volume 3M sodium acetate and 10 volumes of analar ethanol at -20°C. Precipated LPS was pelleted by centrifugation (10,000×g) washed with

cold ethanol and dried in a speed vac at 37°C. The pellet was re-dissolved in 400ul of double distilled, deionized water. 10-20ul was used per track on SDS Polyacrylamide gel electrophoresis (PAGE).

2.3.4 SDS PAGE

Whole cell lysates were separated on polyacrylamide gels under reducing conditions (Lamelli, 1970). Bacterial cultures were diluted in L- broth to give an A_{600} of unity. One ml of this was then pelleted with an Eppendorf microfuge and dried by inversion. The bacterial pellet was re-suspended in 100ul of PAGE final sample buffer. The sample was boiled for 5 minutes and 20ul was added per track. Samples could be stored at -20°C until needed. Molecular weight standards were purchased from Sigma. They were dissolved in final samples buffer to give a final concentration so that 20ul contained 1ug of each protein constituent. They were stored in 50ul aliquots at -20°C until required, at which time they were boiled for 5 minutes before loading on the gel. LPS samples were mixed with an equal volume of final sample buffer and boiled for 5 minutes before loading.

2.3.5 Silver Stain.

LPS was separated on 14% acrylamide gels containing 7M urea. LPS was visualised by silver Staining using the method of Tsai and Frach (1982). Gels were fixed overnight in 40% EtOH

5% acetic acid. The LPS was then oxidised by 0.7% periodic acid (BDH Analar) in fixing solution and the gel was then washed in double distilled deionized water (3x15 minutes). The LPS was stained for 10 minutes with ammonial silver nitrate. This was made just before use as follows: 4ml of .880 ammonia was diluted in 56ml of 0.1M NaOH, 20ml of 20% $AgNO_3$ in water was added with stirring by a magnetic stirrer. A brown precipitate appeared, and then rapidly disappeared. The stain was made up to 300ml with water and poured over the gel. After staining the gel was again washed in water (3x15 minutes) and then the stain developed by adding 300ml developer (0.5ml 37% formaldehyde, 100mg citric acid/litre). When LPS bands were visible the reaction was stopped by extensive washing in distilled water. The gel was photographed immediately.

2.4 Mouse experiments.

2.4.1 Mice.

BALB/cJ mice were bred at Wellcome Research Laboratories from breeders purchased from OLAC (1976) UK Ltd (Blackthorn Bicester, Oxon). A/J mice were bred in the Department of Pathology, University of Cambridge from breeders purchased from Jackson Laboratories (Bar Harbor Me). Male and female mice of 7-10 weeks age were used. In any one experiment, all animals were age and sex matched.

2.4.2 Preparation of inocula.

Wild type and auxotrophic salmonella were grown overnight at 37°C as 250ml L broth cultures without shaking. L. monocytogenes was grown overnight at 37°C in 100ml L broth with shaking (200rpm). Bacteria were snap frozen and stored over liquid nitrogen, in 0.5ml aliquots.

For infection of mice, a vial was thawed in a 37°C water bath and diluted as appropriate in PBS. When inocula were prepared for oral infections, or to determine LD_{50} values for attenuated strains, more bacteria were required than were present in a frozen vial. To prepare inocula with the high cell densities required, 250ml overnight L-broth cultures were harvested by centrifugation (10,000 x g) and re-suspended in PBS to give a concentration of 10^9 - 10^{11} cfu/ml. Where large groups of mice were infected, bacteria from several litres of culture were pooled. For lymph node priming experiments, where large doses were given s.c., bacteria were grown and concentrated as above and then the concentrated suspension was stored in 0.5ml aliquots over liquid nitrogen.

The number of bacteria administered was confirmed by triplicate or quadruplicate pour plates of an appropriate dilution.

2.4.3 Infection of mice.

Mice were infected i.v with 0.2ml via the lateral tail vein. Subcutaneous injections were 0.1 or 0.2ml given in the groin or at the base of the tail. Oral infection was with a gavage tube: a blunt 2 inch 18g needle with a smoothed 2mm wide metal sheath over the end. Mice were lightly anesthetised with anaesthetic grade ether and held by the skin between the eyes. They were allowed to swallow the needle which passed into the stomach. 0.2ml of inoculum was administred from a 1ml tuberculin syringe attached to the needle. This method had been found to be the most reproducible method for oral infection.

2.4.4 LD50 determinations

The i.v. and oral LD_{50}'s for virulent strains was obtained by infecting groups of 5 mice with serial ten fold dilutions in PBS. For attenuated strains, 8-10 mice were used per dose. Deaths were recorded over the following four weeks and the LD_{50}'s was calculated using the method of Reed and Muench (1937).

2.4.5 Growth curves.

Large groups of mice were infected. At various times, three to five mice were killed and their livers and spleens were removed aseptically and placed in a stomacher 80 bag (Seward Medical Ltd). When mice had been infected orally, mesenteric lymph nodes and the most distal Peyers patch were also

removed. The organs were homogenised for 1-2 minutes in 10ml of sterile distilled water using a Colworth Stomacher 80.

Viable counts were performed on these homogenates using the pour plate method. Normally, 1ml of homogenate was added to 25ml agar but for more sensitive counting up to half (5/10ml) of an organ could be included in one plate. When counts of >1000 cfu per organ were expected, counts were performed using a Coleworth droplet. This technique allows serial dilutions to be made in agar (at 50^{o}C). One millilitre of agar is taken up into a plastic straw and dispensed in 0.1ml amounts. Dilutions are made by dispensing 0.2ml of organ homogenate into 1.8 ml of agar. After mixing, 0.2ml is dispensed into the next tube, and a further 0.5 ml is plated as five 0.1 ml spots onto a petri dish. Further dilution steps can be made as appropriate and upto 7 dilutions can be spotted onto one 9 cm petri-dish.

Counts were generally performed in L-agar. However, when studying the growth of virulent wild type challenge in mice immunised 28 days earlier, it was necessary to distinguish between the challenge and residual immunising bacteria. This was done by using minimal medium for all counts. This did not allow growth of auxotrophic strains but did not affect the counts of wild type strains. When following the growth of L. monocytogenes in salmonella infected mice, polymixin B (20 ug/ml, Sigma) was added to the L-agar to inhibit growth

of the salmonella (MIC < 1 ug/ml). Listeria (MIC > 50ug/ml) counts were not affected. Viable counts are expressed as geometric mean ± 1 SD of counts per organ. When counts on pour plates showed no colonies the organ was assigned the highest theoretical count for the organ (ie, 10 for a 1ml pour plate, 2 for a 5ml plate etc) to calculate the geometric mean.

2.4.6 Serum samples.

Mice, anaesthetised with ether, were bled from the axilla or by cardiac puncture without recovery. Small amounts (<0.5ml) were removed with recovery from the tail vein. Blood was allowed to clot at room temperature and then stored overnight at 4°C. Sera were clarified in an Eppendorf Microfuge and stored at -20°C.

2.5 Measurement of immune responses.

2.5.1 Detection of antibodies to salmonella.

Salmonella antibodies were detected using an ELISA. Plates were coated with heat killed (HK) S. typhimurium, diluted test sera were added followed by a horseraddish peroxidase conjugate.

To prepare HK S. typhimurium, one litre of HWSH was grown overnight in L-broth in a rotary shaker(250rpm) at 37°C and harvested by centrifugation. The pellet was suspended in PBS

to give a suspension with an an absorbance of 100 at 600 nm (approximately 10^{11}cfu/ml) and heat killed by incubation at 65°C for 2 hours. Sterility was checked by adding 100ul of the heat killed suspension to 100ml of L-broth and looking for no bacterial growth after overnight incubation at 37°C. The suspension was stored in 2ml aliquots at -20°C until use, when it was diluted as appropriate in PBS.

A high titre reference serum was required for the ELISA tests. A suspension of HK SL1344 at 10mg/ml was provided by D. Maskell, Wellcome Research Labs. This was diluted in PBS to give a suspension at 400ug/ml and immulsified with 3 parts Freud's incomplete adjuvant (Difco). Ten mice were injected s.c. in the groin with 100ul (10mg) and boosted s.c. with a further 100ul on days 30 and 45. On day 55, the mice were exsanguinated by cardiac puncture. Sera were tested for anti-salmonella antibodies by ELISA. Sera with 50% maximal binding titres of less than 10,000 were discarded. The other sera (7/10) were pooled and stored in 1ml aliquots at -20°C until use.

Flat bottomed ELISA plates (Linbro, Nunc) were coated with heat killed HWSH the day before use by adding 100ml of a suspension with an absorbance at 600 nm of 0.25 to each well. This was incubated at 37°C for 3 hours and then overnight at 4°C. The coated plate was removed from the fridge and the wells washed three times with PBS Tween 20

(0.05% v/v) using a Nunc immunowash-8. Wells were blocked for 3 hours at 37°C with 100ul of blocking buffer (2% Bovine serum albumin: Sigma, Fraction V, in PBS). All sera and conjugates were diluted in blocking buffer and incubations were at 37°C. After washing 50ul of 2-fold dilutions of test sera was added. The plate was incubated for one hour. Each plate contained a positive reference serum, the high titre anti SL1344 serum diluted in 2 fold steps starting at 1/1000 and a normal mouse serum diluted as the test samples. After washing 50ul of a 1/1000 dilution of rabbit anti-mouse HRPO conjugate (Biorad) was added and incubated for 1 hour. After a final wash, 100ul of substrate was added. The substrate used was 10.8mg o-phenylenediamine hydrochloride (Sigma) dissolved in 1ml methonol. This was diluted 1:100 by adding 100ul to 9.9ml PBS and 5ul 30% H_2O_2. After 30 minutes incubation, the reaction was stopped with 20ul of 2.5M H_2SO_4 and the A_{490} measured with a Titreteck ELISA reader directly into a microcomputer.

The titres of individual sera were calculated as arbitrary units by method of Manclark, Meade and Burnstyn, (1986) using a computer programme provided by D. Burnstyn, NIH, USA. Results are shown as geometric mean of four sera $\pm$ 2se.

2.5.2 Lymph node proliferation assay.

The inguinal and periaortic lymph nodes were removed from mice immunized s.c. at the base of the tail and single cell suspensions in PBS made by gently grinding between two frosted glass slides. The resulting single cell suspension was washed twice in PBS and the cells re-suspended at a concentration of 4 x 10^6 cells/ml in Clicks EHAA (Eagles High Amino Acid) medium (GIBCO) supplemented with 0.5% fresh normal mouse serum. One hundred microlitres of this suspension was added to microtitre wells (96-well, Costar) with antigen at the indicated concentration in a further 100ul medium. The cultures were then incubated for 3 or 4 days before pulsing for 18 hours with tritiated thymidine. Cultures were harvested on an automatic cell harvesting device and counted.

2.5.3 Staining of adherent spleen cells for Ia and surface immunoglobulin.

Spleen cell suspensions were made by gently grinding between two frosted glass slides. Red cells were removed by distilled water lysis and the suspension washed three times in PBS. Cell viability was determined by trypan blue exclusion and was always over 90%. Cells were suspended in medium (Clicks EHAA containing 10% FCS) at the desired concentration.

Spleen cells were suspended at $2x10^7$/ml in and 0.5 ml was added to each well of a chamber slide (Flow). Cells were allowed to adhere for 4 hours at 37°C. Non-adherent cells were removed by vigorous washing with PBS, and the adherent cells fixed over night with 2% paraformaldehyde.

Monoclonal antibodies, MKD6 specific for $I\text{-}A^d$ (Kappler et al, 1981), Y-P3, specific for the public determinant present on I-A molecules and raised in BALB/c mice (Janeway et al, 1984) and a polyclonal rabbit serum against mouse immunoglobulin were provided by J. Tite, Wellcome Res Labs. FITC-avidin was purchased from Vector.

Cells washed once with staining buffer (PBS containing 1% FCS and 1% NRS) and 0.5 ml antibody in staining buffer added. After incubation for 20 minutes at room temperature, the cells were washed three times with staining buffer and 0.5 ml FITC-avidin (diluted 1:50 in staining buffer) was added for a further 20 minutes. The cells were washed three times with staining buffer and observed for fluorescence.

2.5.4 ^{51}Cr-release assay.

YAC-1 lymphoma (NK sensitive) and P815 mastocytoma (NK resistant) cell lines were provided by J. Tite. Target cells to be radiolabeled ($2x10^6$-$5x10^6$ cells) were suspended in 0.5ml serum free medium containing 100 uCi of sodium

chromate solution (Amersham), and incubated at 37°C for 1 h. The labeled cells were washed 3 times in medium containing 10% FCS before suspension in the same medium for the ^{51}Cr-release assay. The ^{51}Cr-release-assay was performed in round-bottomed 96-well plates (Linbro, Flow Labs). Effector cells were mixed with target cells as indicated in the text in final volume of 200 ul. The microtiter plate was then centrifuged at 750 x g for 4 min. before incubation at 37°C in 5% CO_2 in air for the time indicated. At this time 100ul was removed from each well and counted. Spontaneous release was measured as the ^{51}Cr release from the target cells in medium alone, maximum release was measured as the release from target cells in 1% Triton X-100 solution. Specific ^{51}Cr release was calculated according to the formula:

$$\% \text{ specific release} = \frac{\text{Experimental release - spontaneous release}}{\text{Maximum release - spontaneous release}} \times 100$$

RESULTS

3.0 CONSTRUCTION ISOLATION AND CHARACTERISATION OF MUTANTS OF MOUSE VIRULENT S. TYPHIMURIUM WITH DELETIONS IN GENES OF THE AROMATIC AND PURINE BIOSYNTHETIC PATHWAYS.

3.1 Construction and isolation of the mutants.

The derivatives of S. typhimurium LT2 with Tn10 inserted in aroA, purA or purE, were checked on minimal medium and found to show the correct auxotrophic phenotypes. Transducing lysates were prepared as described in materials and methods, and were used to transduce the virulent strains SL1344 and HWSH with selection for tet^R. This procedure always yielded a high frequency of transductants. Because Tn10 can excise precisely at high frequency to regenerate an intact gene, transductants harbouring Tn10 are unsuitable for use as live vaccines. Therefore, Bochner medium was used to select tet^S isolates, containing deletions generated by the imprecise excision of Tn10. Such isolates were still auxotrophic. Bochner selection for tet^S colonies was not clear-cut, since tet^S colonies grew out of a slower growing tet^R background. The growth of this background could be further slowed by using Bochner medium with no added glucose, or as modified by Maloy (1981). Between 20-50 putative tet^S colonies were picked and passaged twice on the same medium. Isolates which still grew were screened for tetracycline sensitivity and for auxotrophic phenotype. In general only about 25% of the

isolates first picked from Bochner medium were both tet^S and auxotrophic.

To construct double mutants, with lesions in both aroA and purA, either HWSH aroA or SL3261 were retransduced with the phage lysate prepared using LT2 purA::Tn10. Transductants, dependent on both aromatic compounds and adenosine, were selected and passed through the Bochner selection procedure as described above. SL3261 is already partially resistant to fusaric acid (Hoiseth and Stocker, 1981), therefore SL3261 purA::Tn10 was plated on Bochner medium containing 6ug/ml fusaric acid.

3.2 In vitro characterisation of the auxotrophic mutants.

It is important that live vaccines neither revert to virulence nor have major antigenic differences from their virulent parents. The stability of the auxotrophic character was checked before commencing immunization experiments. The LPS and protein profiles of the auxotrophic mutants were also characterised in vitro, to ensure no differences existed between the mutants and their parents.

3.2.1 Stability of lesions causing auxotrophic character.

Tet^S isolates were checked for the stability of their auxotrophic characteristics. Cultures were grown overnight at 37^oC in 100 ml L-broth in an orbital shaker and harvested

by centrifugation. After washing with PBS, they were suspended in PBS at a concentration of at least 10^{11} cfu/ml. One millilitre of this suspension was spread over a 14 cm minimal medium plate. After 3-4 days incubation at 37°C the plate was checked for prototrophic revertants. On no occasion were such revertants isolated. It was concluded that in all cases the reversion frequency was greater than 10^{-11}, although the nature of Tn10 generated lesions should result in reversion rates even lower than this.

3.2.2 Characterization of the LPS of auxotrophic mutants.

LPS is thought to be a major virulence determinant in salmonella infections. The LPS of the auxotrophic isolates was checked by agglutination with S. typhimurium specific antisera, sensitivity to phages P22 (smooth LPS specific) and P1 (rough specific), and by silver staining of purified LPS separated on 14% PAGE gels containing 7M urea . All the isolates selected showed correct agglutination profiles for S.typhimurium: O-4,5 positive, O-9, 6,7 negative. All were P1 resistant and all, except one HWSH aroA purA isolate, were P22 sensitive. When purified LPS was analysed by silver staining (fig. 3.1) all showed a smooth profile except the P22 resistant HWSH aroA purA isolate. Only the smooth isolates were further characterized in the mouse model.

3.2.3 Analysis of protein profile of mutants.

The protein profiles of the isolates were examined using SDS PAGE as described in materials and methods. Fig 3.2 shows lysates of each of the mutant isolates separated on a 10% polyacrylamide gel. There are no obvious differences seen when profiles of the mutant strains are compared with those of their parents. Flagella protein H antigens were checked by slide agglutination. All mutants were agglutinated by sera specific for phase 1 Hi and polyvalent phase 2 (specific for S. typhimurium), but not by phase 1 Hd (S. typhi).

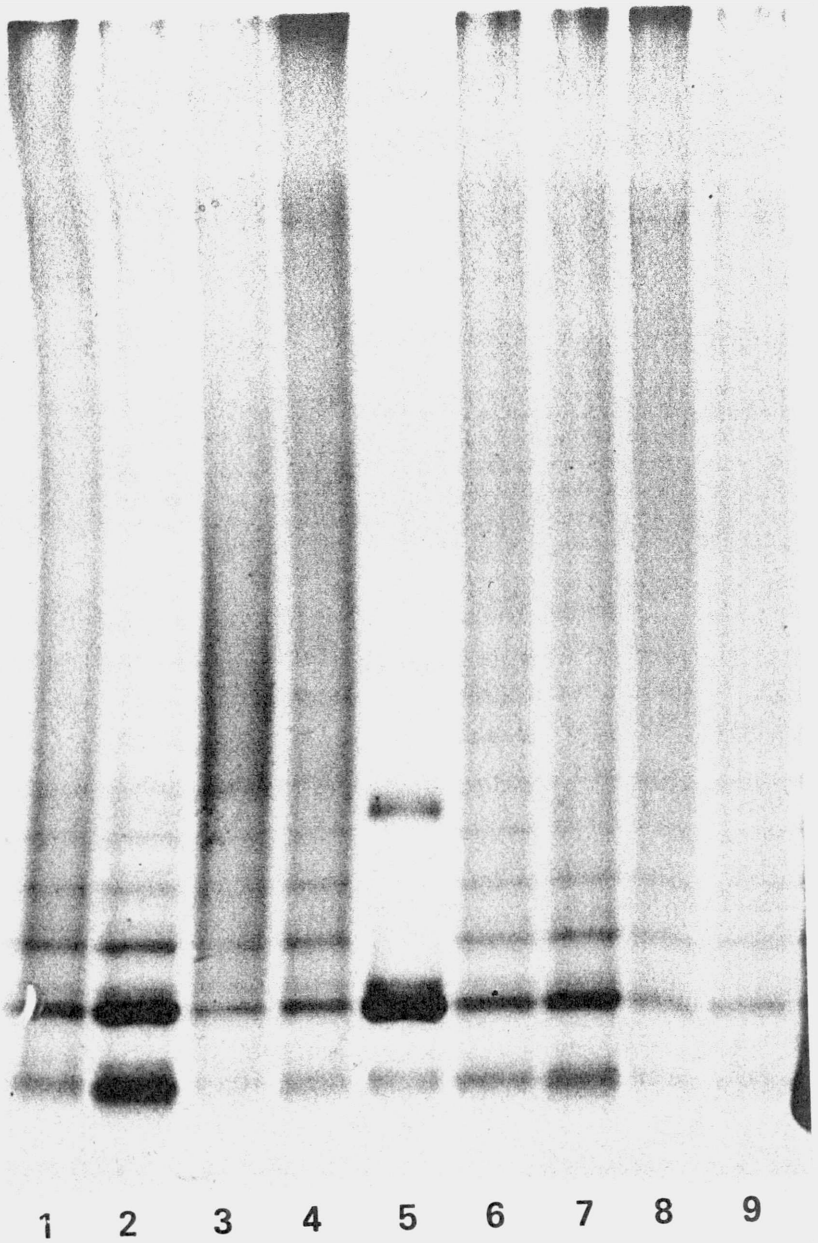


Fig. 3.1 LPS profiles of S. typhimurium strains HWSH, SL1344, and their auxotrophic derivatives. LPS was visualised by silver staining after separation a 14% acrylamide gel.

1: HWSH; 2: HWSH aroA; 3: HWSH purA; 4: HWSH aroA purA; 5: HWSH aroA purA (rough); 6: SL1344; 7: SL3261; 8: SL1344 purA; 9: SL3261 purA.

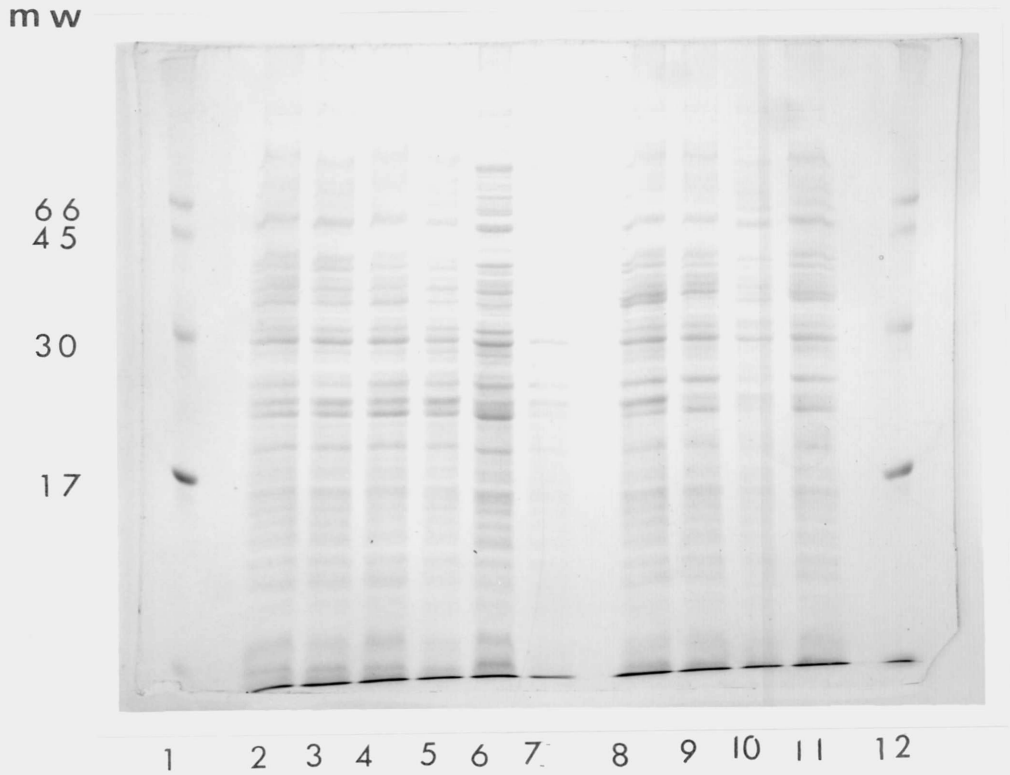


Fig. 3.2 Protein profiles of S. typhimurium strains HWSH, SL1344, and their auxotrophic derivatives. Whole cell lysates were separated on a 10% acrylamide gel and the protein stained with Comassie Blue.

1: Molecular weight standards; 2: HWSH; 3: HWSH aroA; 4: HWSH purA; 5: HWSH aroA purA; 6: HWSH aroA purA (rough); 7: HWSH purE; 8: SL1344 9: SL3261; 10: SL1344 purA; 11: SL3261 purA 12: Molecular weight standards.

3.3 Characterization of the attenuation conferred by auxotrophy and behaviour of the mutants in vivo following infection of inbred mice.

3.3.1 LD_{50} following i.v. innoculation.

The degree of attenuation conferrred by the auxotrophic requirements was first assessed by determining the LD_{50} value for each mutant after i.v infection of BALB/c mice (table 3.1). The two parental strains were virulent, with LD_{50} values being reproducably less than 10 cfu. The LD_{50}'s for the auxotrophic mutants show that they are all greatly attenuated when compared with their parents. The *aroA* mutants had LD_{50}'s of over 10^7 cfu, which is in good agreement with published data on *aroA* mutants (Hoiseth and Stocker, 1981). The mutants with lesions in *purA* or *aroA purA* were further attenuated, with LD50s between 10^8 - 10^9 cfu. Any mouse which died after i.v injection of the high doses of *aroA purA* mutants that are required for lethality did so within 24 hours of injection, suggesting that death was due to toxic causes rather than infection. Mice infected with the first dose of *aroA* mutants above the LD_{50} died between 1-2 weeks after injection, suggesting that death was due to infection.

HWSH *purE* mutant was considerably less attenuated than any of the other mutants tested, although the lethal dose varied from mouse to mouse. For example, some mice would die after

injection of as few as 100 organisms while, in the same experiment, mice injected with 10^4-10^5 survived.

The LD_{50}'s were determined after infecting salmonella resistant (*ity*^{r}) A/J mice i.v. (table 3.2). It can be seen that they are not greatly different from those obtained in BALB\c mice.

3.3.2 *LD_{50} following oral innoculation.*

The oral LD_{50} values were determined for parental and auxotrophic strains in BALB\c mice. The parental strains consistently had LD_{50} values of between 10^5 and 10^6 cfu for HWSH and between 10^6 and 10^7 cfu for SL1344. Neither aromatic nor purine mutants could kill the mice when given orally, even with doses as high as 10^{10} cfu. When orally infected mice were autopsied at the end of LD_{50} experiments *aroA*, *purA* and *aroA purA* infected mice showed no visible signs of infection. Mice infected with HWSH *purE* showed gross splenomegaly and large hepatic and splenic abscesses, although they had shown no outward signs of distress.

Table 3.1.

Log LD_{50} values obtained by infecting BALB/c mice i.v. with two highly virulent *S. typhimurium* strains and their auxotrophic derivatives. All LD_{50}'s were calculated 28 days after infection except HWSH *purE*, which was calculated after 56 days.

S. typhimurium strain	log LD_{50}
HWSH	<1
HWSH *aroA*	7.4
HWSH *purA*	8.6
HWSH *aroA purA*	8.9
HWSH *purE*	3.8
SL1344	<1
SL3261	7.1
SL1344 *purA*	7.7
SL3261 *purA*	8.7

Table 3.2

LD_{50} of *S. typhimurium* HWSH and auxotrophic derivatives following i.v. infection of A/J mice. LD_{50}s were calculated after 28 days.

Strain	Log LD_{50}
HWSH	4.5
HWSH *aroA*	7.6
HWSH *purA*	8.7
HWSH *aroA purA*	8.9

3.3.3 In vivo growth and persistence in BALB/c mice following i.v. infection.

The ability of the virulent and attenuated strains to grow and persist in the livers and spleens of BALB/c mice after i.v. inoculation was determined.

Virulent parental strains. Parental strains grew rapidly following i.v. administration of 10^2 - 10^3 cfu, reaching levels of 10^8 - 10^9 cfu per organ by Days 5 - 6, resulting in death of all the mice infected (data not shown).

aroA derivatives. The aroA mutant of HWSH grew much less efficiently than its parent. Its in vivo growth curve (fig. 3.3) is similar to those found for aroA mutants of S. typhimurium C5 (Hormaeche personal communication), S. dublin aroA (Our unpublished observations) and of SL3235 given i.p. (Killar and Eisenstin, 1985). The in vivo behaviour of SL3261 has been previously characterised in detail (Maskell, PhD thesis, University of Cambridge, 1985; Maskell, et al, 1987b; O'Callaghan et al, 1988). Approximately one week after i.v. infection with HWSH aroA or SL3261, marked splenomegaly was observed (Table 3.3a). The splenomegaly peaked at Day 14 and resolved as the infection cleared.

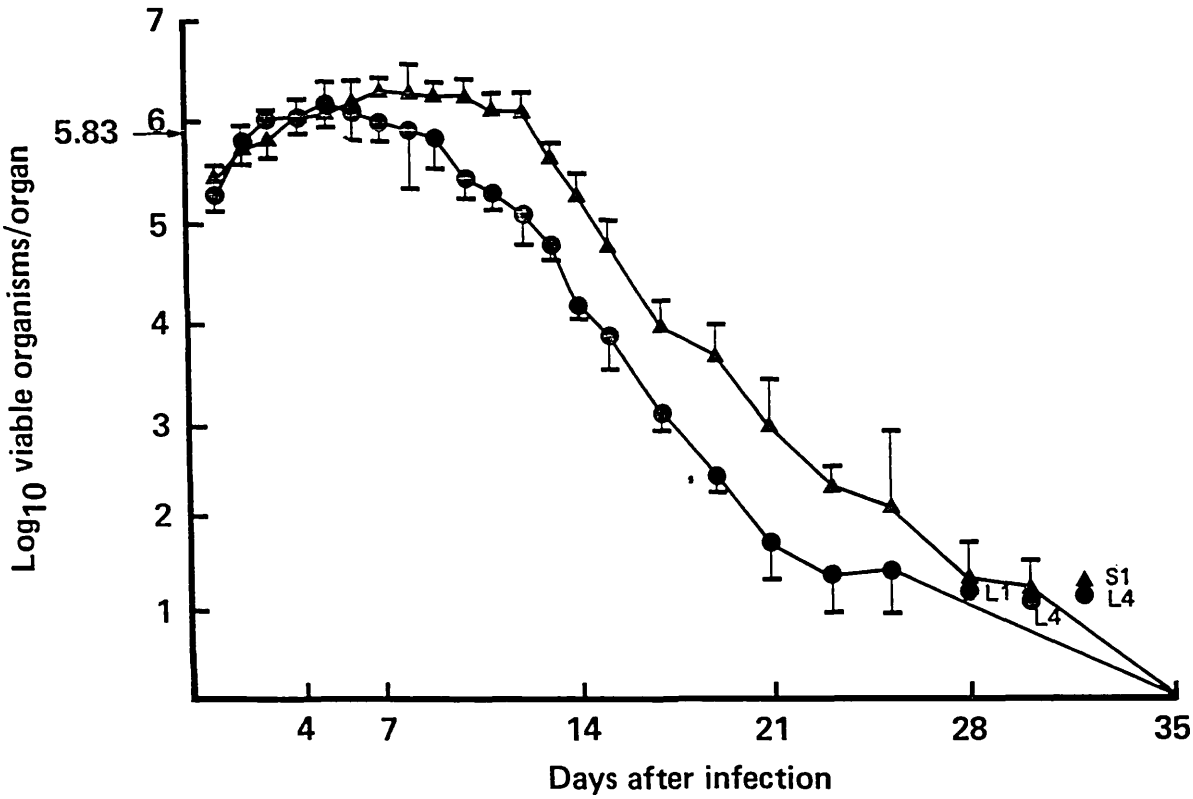


Fig. 3.3 Growth and persistence of HWSH aroA in livers (●) and spleens (▲) of BALB/c mice infected i.v. with 6.3 x10^5 cfu. Points represent geometric mean ± 2 se of 4 mice.

Table 3.3a

Spleen weights of mice immunized i.v. with S. typhimurium aroA mutants.

Days after infection	Mean spleen weight (grams) ±2se after immunization with: SL3261	HWSH aroA
1	-	0.17 ± 0.08
4	0.28 ± 0.01	0.28 ± 0.02
7	0.41 ± 0.006	0.57 ± 0.05
14	0.98 ± 0.13	1.05 ± 0.1
21	0.61 ± 0.17	0.38 ± 0.11
28	0.34 ± 0.05	0.28 ± 0.05
35	0.23 ± 0.02	-
42	0.26 ± 0.02	-

Table 3.3b

Spleen weights of mice immunized i.v. with S. typhimurium purA mutants.

Days after infection	Mean spleen weight (grams) ±2se after immunization with: 1344 purA	HWSH purA
1	0.15 ± 0.02	0.17 ± 0.02
4	0.16 ± 0.02	0.14 ± 0.01
7	0.23 ± 0.02	0.155 ± 0.03
14	0.29 ± 0.025	0.15 ± 0.01
21	0.38 ± 0.05	0.25 ± 0.025
28	0.28 ± 0.04	0.24 ± 0.03
35	0.22 ± 0.03	0.18 ± 0.02
49	0.22 ± 0.02	0.20 ± 0.02

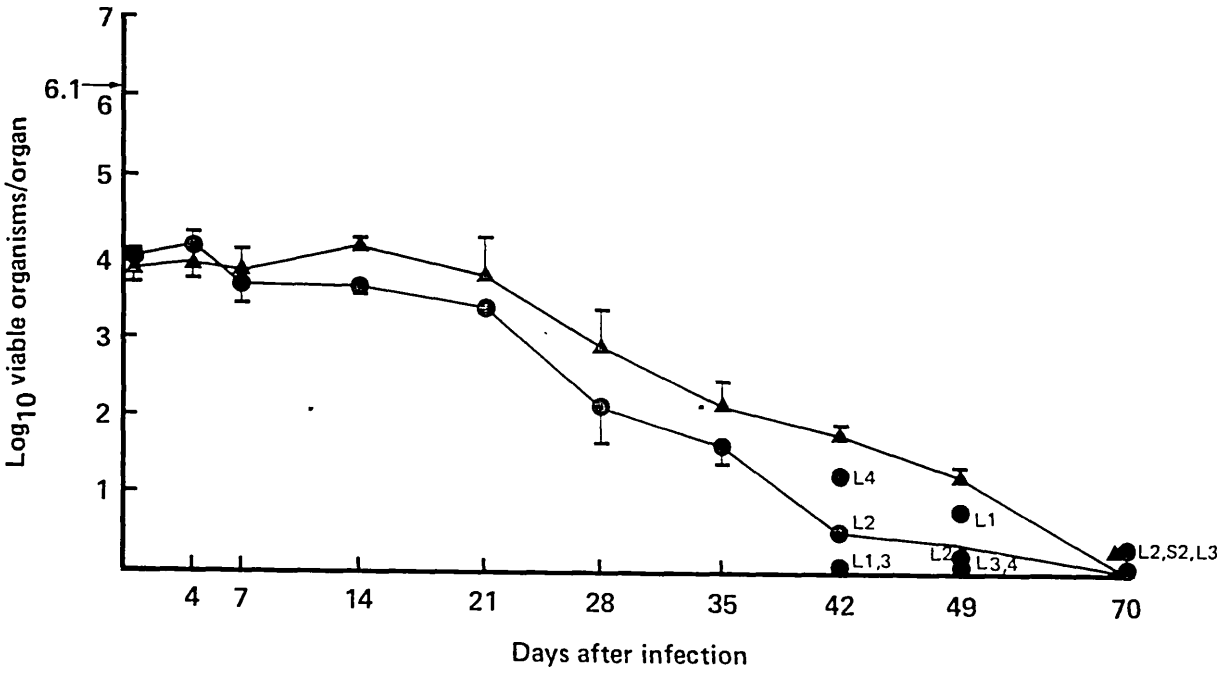


Fig. 3.4a Growth and persistence of HWSH purA in livers (●) and spleens (▲) of BALB/c mice infected i.v. with 1.3 x10^6 cfu. Points represent geometric mean ± 2 se of 4 mice.

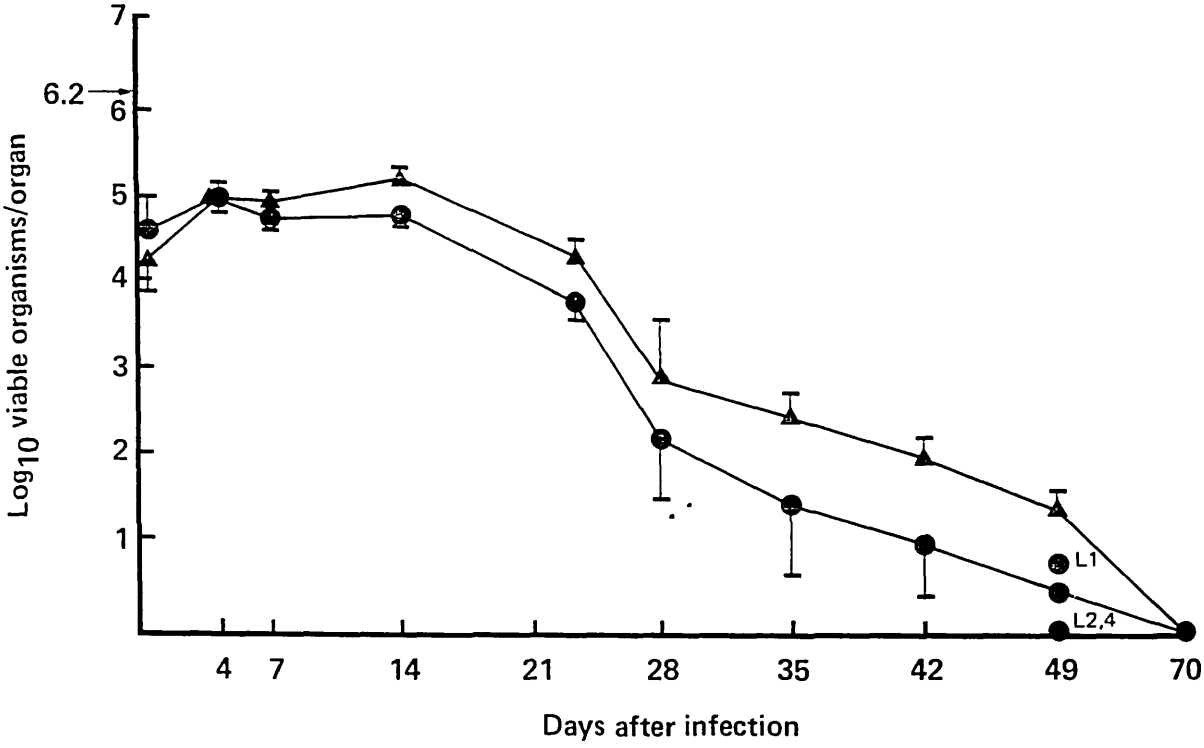


Fig. 3.4b Growth and persistence of SL1344 purA in livers (●) and spleens (▲) of BALB/c mice infected i.v. with 1.6×10^6 cfu. Points represent geometric mean ± 2 se of 4 mice.

purA derivatives. The purA derivatives of HWSH and SL1344 grew poorly in vivo (fig 3.4). One hundred BALB/c mice were infected i.v. with 1.6 x 10^6 cfu SL1344 purA or 1.3 x 10^6 cfu HWSH purA. Viable counts showed an initial drop for both strains of almost 2 logs over the first 24 hours, as opposed to 1 log for the aroA mutants. After this, both strains exhibited a similar pattern of persistence although SL1344 purA, but not HWSH purA, showed a small increase in viable counts in livers and spleens over the first few days. Both strains were then cleared from livers and spleens but at a slower rate than the aroA derivatives. SL1344 purA and HWSH purA induced minimal changes in spleen weight (Table 3.3).

aroA purA double mutants. aroA purA derivatives were less able to survive in vivo than either aroA or purA mutants. One hundred BALB/c mice were infected i.v. with 2 x 10^5 cfu HWSH aroA purA (fig 3.5a). Viable counts again showed more than 2 logs clearance of the inoculum over the first day. Bacterial counts continued to decline over the first seven days of infection. Following this, levels of persistence of between 5 x 10^2 and 1 x 10^3 cfu per organ were detected for 4 weeks, followed by a slow clearance over the next 6 weeks. After the sixth week of infection, individual mice had no detectable organisms in either liver or spleen, but never simultaneously in both organs. Ten weeks after infection, low numbers of bacteria were still detectable in livers or spleens. At no

stage in the infection was splenomegaly detected (data not shown).

Twenty five mice were infected i.v. with $1x10^6$ cfu SL3261 purA (fig 3. 5b). There was again a decrease in viable counts of 2 logs over the first 24 hours followed by low level persistence. The actual levels of persistence were slightly higher than in the HWSH aroA purA infection, but this may be due to the larger infecting dose used.

HWSH purE One hundred BALB/c mice were injected i.v. with 3.3×10^2 cfu HWSH purE (fig. 3.6). Viable counts on day 1 showed an initial decrease of approximately 1 log. The bacteria then grew rapidly in livers and spleens until day 14 after which, in most cases, the counts declined. Ten percent of the mice infected died during the experiment and, in many cases, large abscesses were observed in livers and spleens. These abscesses contained large numbers of bacteria. One hundred and nineteen days after infection, HWSH purE still persisted in livers and spleens at around 1×10^3 cfu. Minimal medium plates supplemented with liver or spleen homogenate from uninfected mice supported growth of HWSH purE.

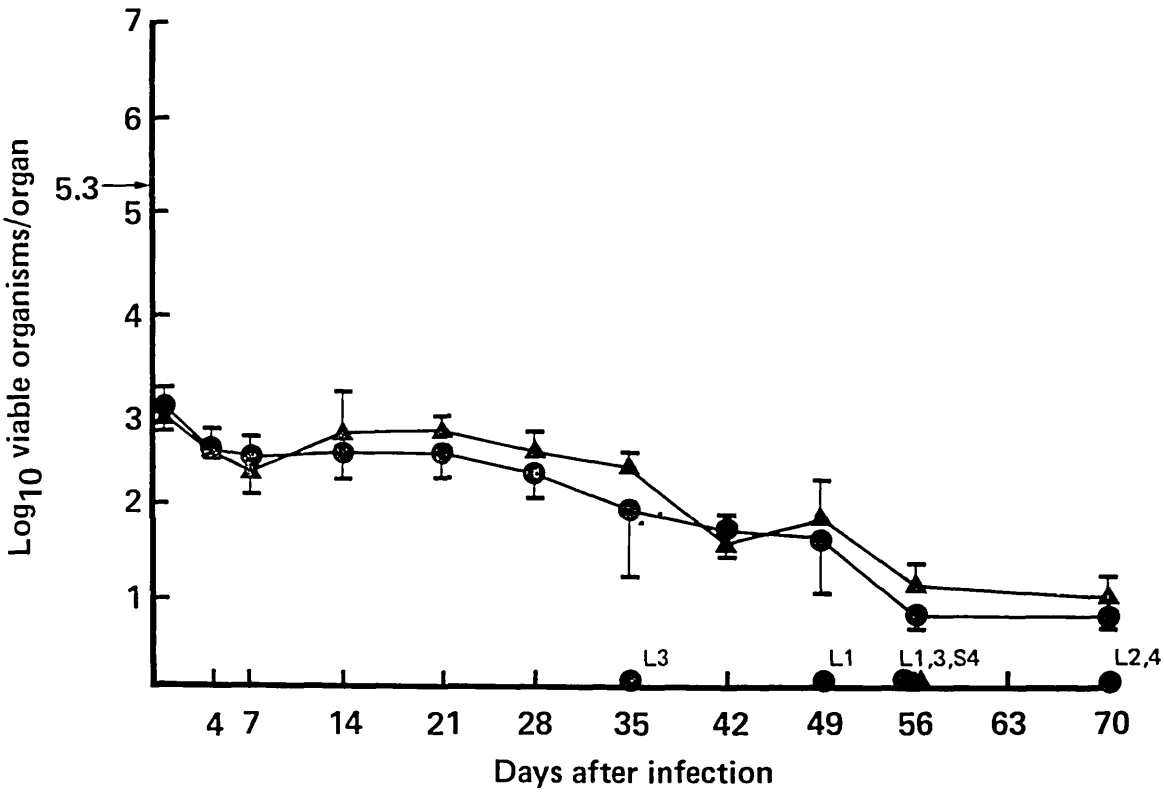


Fig. 3.5a Growth and persistence of HWSH aroA purA in livers (●) and spleens (▲) of BALB/c mice infected i.v. with 2 x10^5 cfu. Points represent geometric mean ± 2 se of 4 mice.

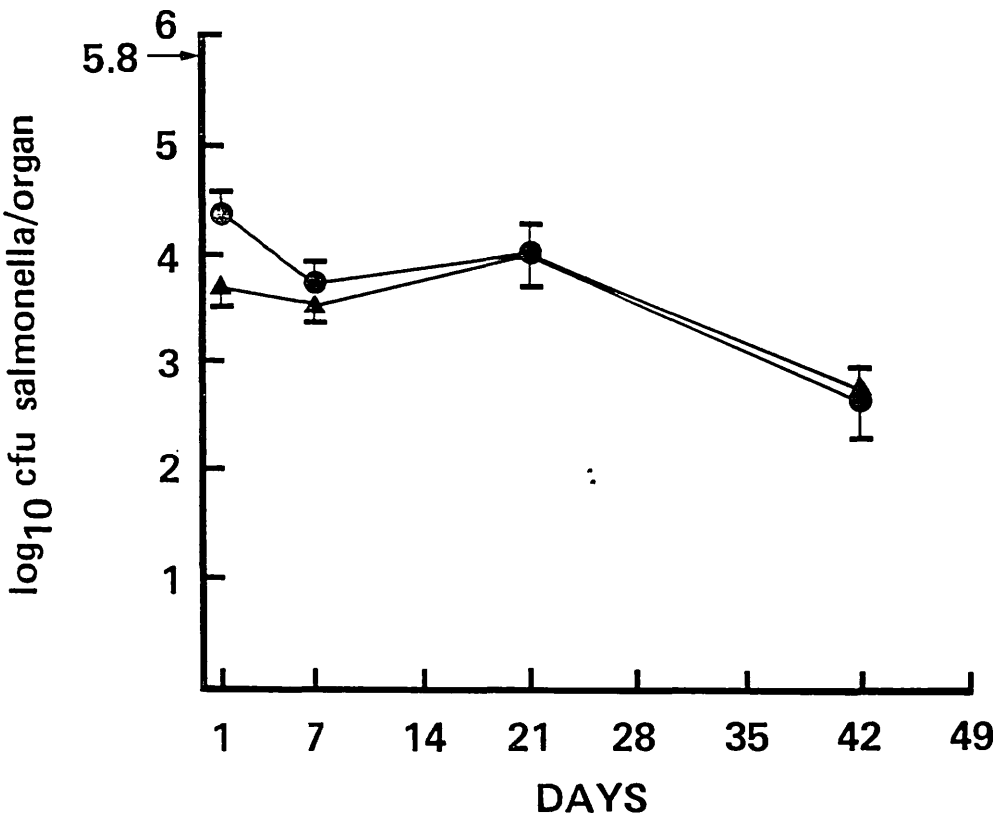


Fig. 3.5b Growth and persistence of SL3261 purA in livers (●) and spleens (▲) of BALB/c mice infected i.v. with x10 cfu. Points represent geometric mean ± 2 se of 4 mice.

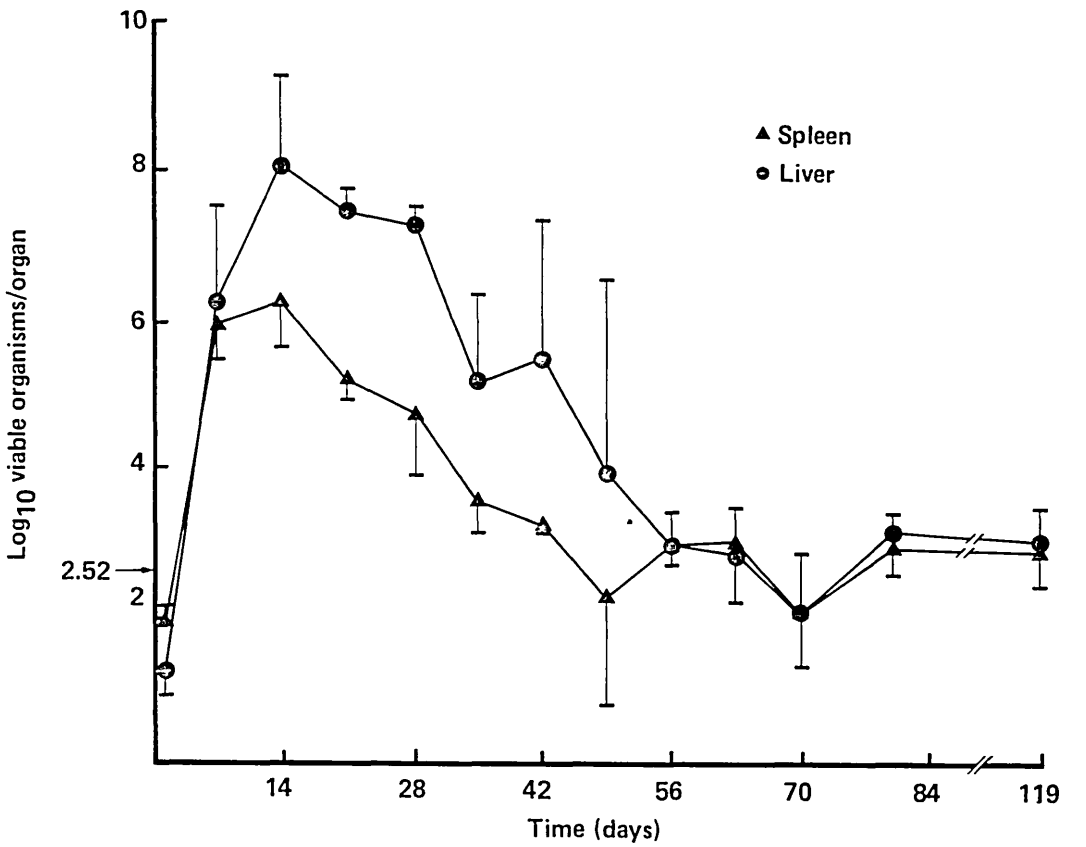


Fig. 3.6 Growth and persistence of HWSH purE in livers (o) and spleens (▲) of BALB/c mice infected i.v. with 3.3 x10^2 cfu. Points represent geometric mean ± 2 se of 4 mice.

4.0 VACCINATION WITH AUXOTROPHIC MUTANTS.

4.1 Ability of attenuated mutants to induce protective immunity to i.v. challenge in i.v. immunised BALB/c mice.

The ability of the aroA, purA and aroA purA mutants to act as live vaccines was assessed. Inbred mice were vaccinated i.v. or orally and then challenged with a virulent strain by the same route, either 28, 70 or 91 days after immunisation. Protection was assessed in two ways. First, by determining the LD_{50} of the virulent strain in immunised and control mice. Second, by comparing the growth of the challenge strain in livers and spleens of immunised and control mice.

4.1.1 Efficacy of immunisation with aroA mutants.

4.1.1.1 Immunity to challenge with virulent parents.

HWSH aroA immunised: HWSH challenge. Mice were immunized i.v. with $8x10^5$ cfu HWSH aroA. LD_{50} determinations at day 28 (table 4.1) showed good protection. By day 70, the level of protection had waned slightly but was still good. On day 70, 40 mice were challenged i.v. with $2x10^3$ cfu HWSH (fig 4.1) In the control mice, this strain grew rapidly, killing all mice by day 5. In the immunized mice, day 1 counts of

Table 4.1

Efficacy of i.v. immunisation with $8x10^5$ cfu *S. typhimurium* HWSH *aroA* in protecting BALB/c mice against i.v. challenge with *S. typhimurium* HWSH.

Immunising strain.	Log LD_{50} of HWSH at day	
	28	70
HWSH *aroA*	5.1	4.2
Unimmunised	<1	<1

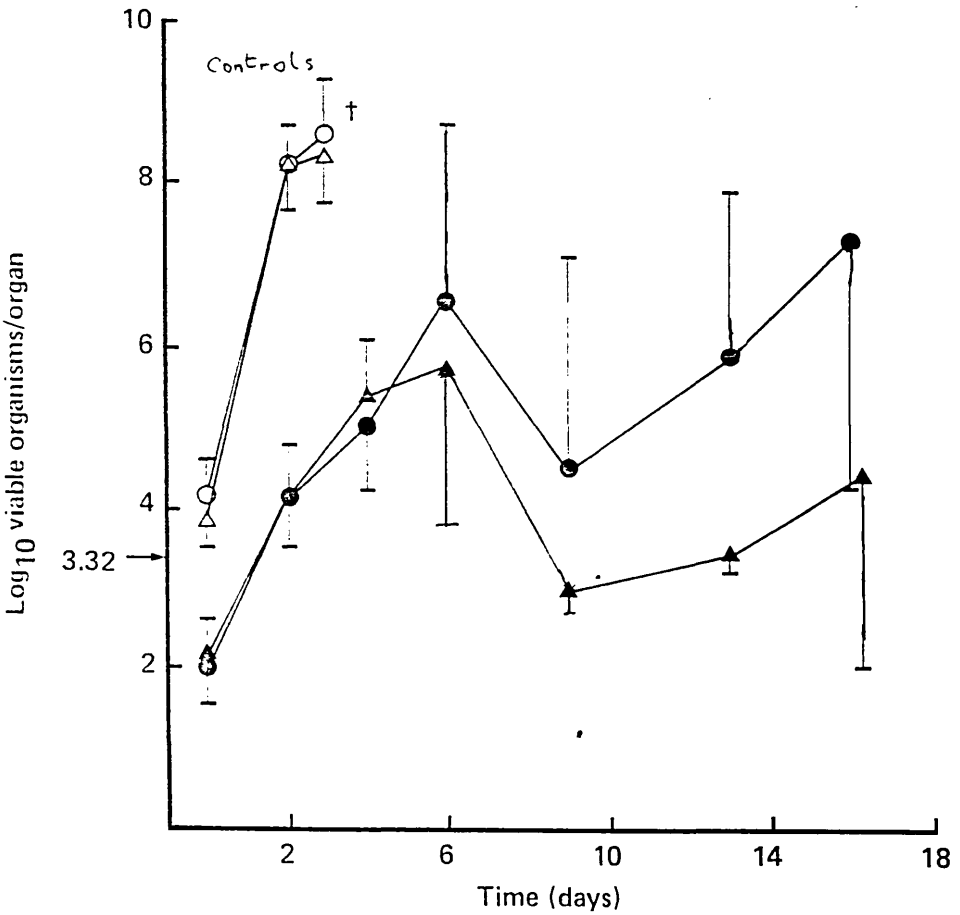


Figure 4.1 The ability of HWSH aroA to immunise BALB/c mice against its virulent parent. Mice were immunised i.v with HWSH aroA and challenged i.v. with HWSH 70 days later. Counts are shown as geometric mean ± 2se in livers (●) and spleens (▲).

HWSH were two logs lower than in controls. The challenge grew rapidly until day 5. Between days 5 and 7, growth was controlled in the majority of mice resulting in a chronic carriage of levels between 10^3 and 10^4 in livers and spleens. Five mice were unable to control the infection and died between days 6 and 8. Although after day 7 the majority of organs carried between 10^3 and 10^4 cfu, many livers (and occasionly spleens) had very high counts. These high counts were always associated with large abscesses which, if dissected out and counted separately, were found to contain almost all the bacteria present in the organ. The surrounding tissue generally appeared healthy. Mice with these abscesses, even one with liver abscesses which occupied two whole lobes and contained over 10^{10} HWSH, appeared well. When these abscesses occured, mean counts were high, and standard errors were large.

SL3261 immunised: SL1344 challenge. Mice immunized i.v. with $8x10^5$ cfu of the aroA mutant SL3261 were highly protected against SL1344 challenge 28 and 91 days later (table 4.2). When a small group (9) of mice, immunised with SL3261 28 days earlier, was challenged i.v. with 10^4 cfu SL1344 this challenge was rapidly eliminated (fig 4.2). Twenty-four hours after challenge, no detectable SL1344 (<10 cfu) were found in livers and only a few (10 cfu) in spleens. On day 4, more sensitive detection methods (5ml pour plates, ie 1/2 organ) showed very low numbers in

Table 4.2.

Efficacy of i.v. immunisation with $8x10^5$ SL3261 in protecting BALB/c mice against i.v. challenge S. typhimurium SL1344.

Immunising strain.	LD_{50} of SL1344 at day	
	28	91
SL3261	6.4	5.8
Unimmunised	<1	0.85

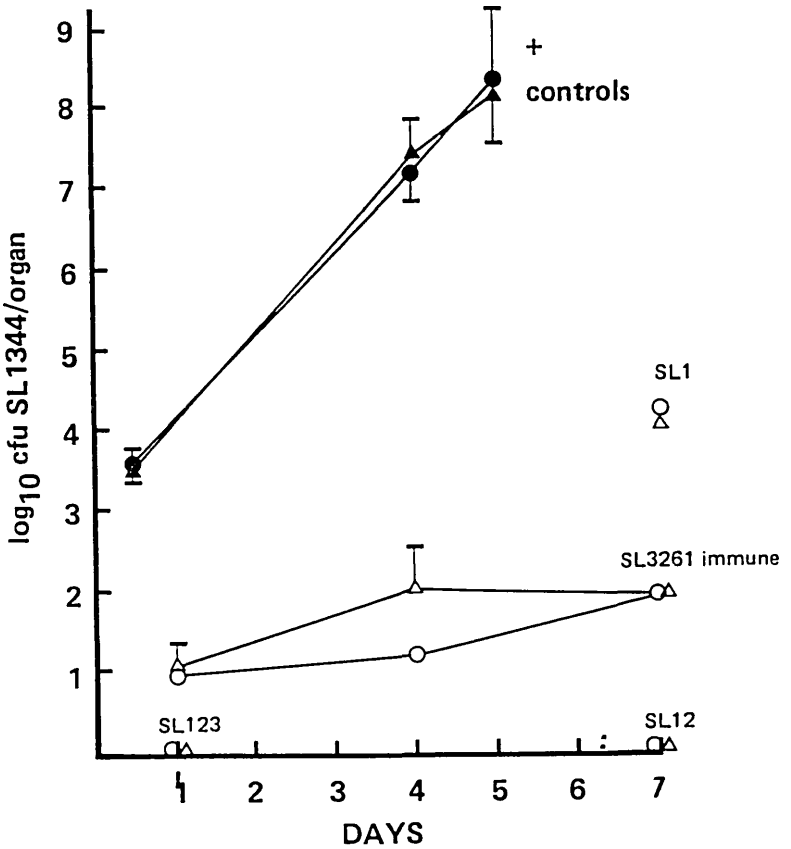


Figure 4.2 The ability of SL3261 to immunise BALB/c mice against its virulent parent. Mice were immunised i.v. with SL3261 and challenged i.v. with SL1344 28 days later. Counts are shown as geometric mean ± 2se in livers (●) and spleens (▲).

immunized mice when organs from control mice contained around 10^7 cfu. All controls were dead on or by day 5. On day 7, 2/3 SL3261 immune mice had no detectable SL1344 (<2 cfu) whilst the third had abscesses in the liver and spleen and counts of 10^4 cfu.

4.1.1.2 Comparison of the efficacy of immunisation with HWSH aroA and SL3261 against challenge with S. typhimurium C5.

The vaccination results obtained when challenging with the parent strains suggest that SL3261 is a better vaccine than HWSH aroA. These results are not directly comparable since HWSH appears to be more virulent than SL1344 when their growth rates in vivo and oral LD_{50}'s in BALB/c mice are compared. In order to compare SL3261 and HWSH aroA directly, two groups of mice were immunized i.v. with 1×10^6 and 2×10^6 cfu respectivly and challenged i.v. with S. typhimurium C5 28 and 70 days later.

Day 28 challenge. The LD_{50} values of the day 28 challenge (table 4.3) showed that both SL3261 and HWSH aroA immunised mice were highly protected. However, when the growth of the i.v. challenge was followed (fig 4.3), some differences were evident in the ability of SL3261 and HWSH aroA immunized mice to control the growth of the C5 challenge. Day 1 counts in the control mice showed levels of C5 about 1/2 log lower than the challenge dose. The challenge grew rapidly killing all remaining mice by day 7. In both immunised

Table 4.3.

Comparison of the efficacy of i.v. immunisation with HWSH *aroA* or SL3261 in protecting BALB/c mice against i.v. challenge with *S. typhimurium* C5.

Immunising strain.	LD_{50} of C5 at day	
	28	70
HWSH *aroA*	6.2	3.3
SL3261	6.6	5.5
Unimmunised	<1	<1

Fig. 4.3/4.4 Comparison of the efficacy of i.v. immunisation with HWSH aroA and SL3261 in protecting BALB/c mice against i.v. challenge with S. typhimurium C5.

Points represent geometric mean ± 2se cfu C5 in livers (●) and spleens (▲) of mice challenged 28 or 70 days after immunisation.

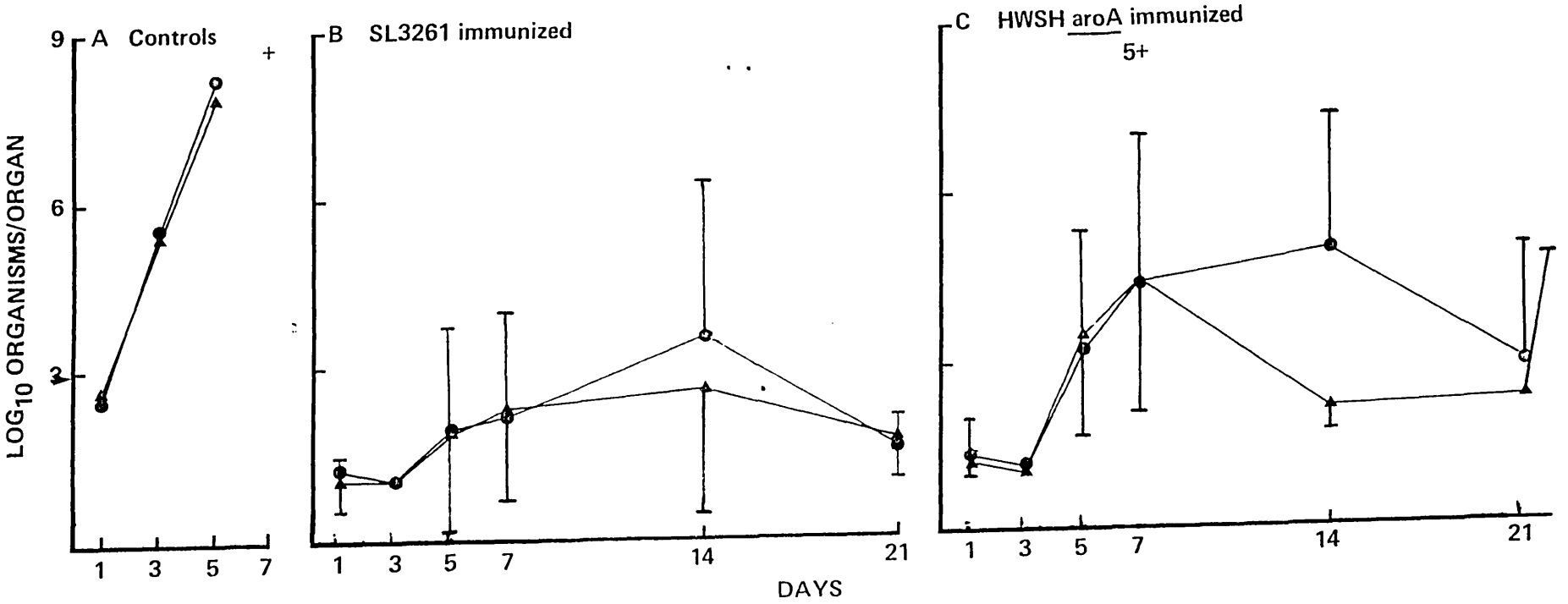


Fig. 4.3 Day 28 challenge

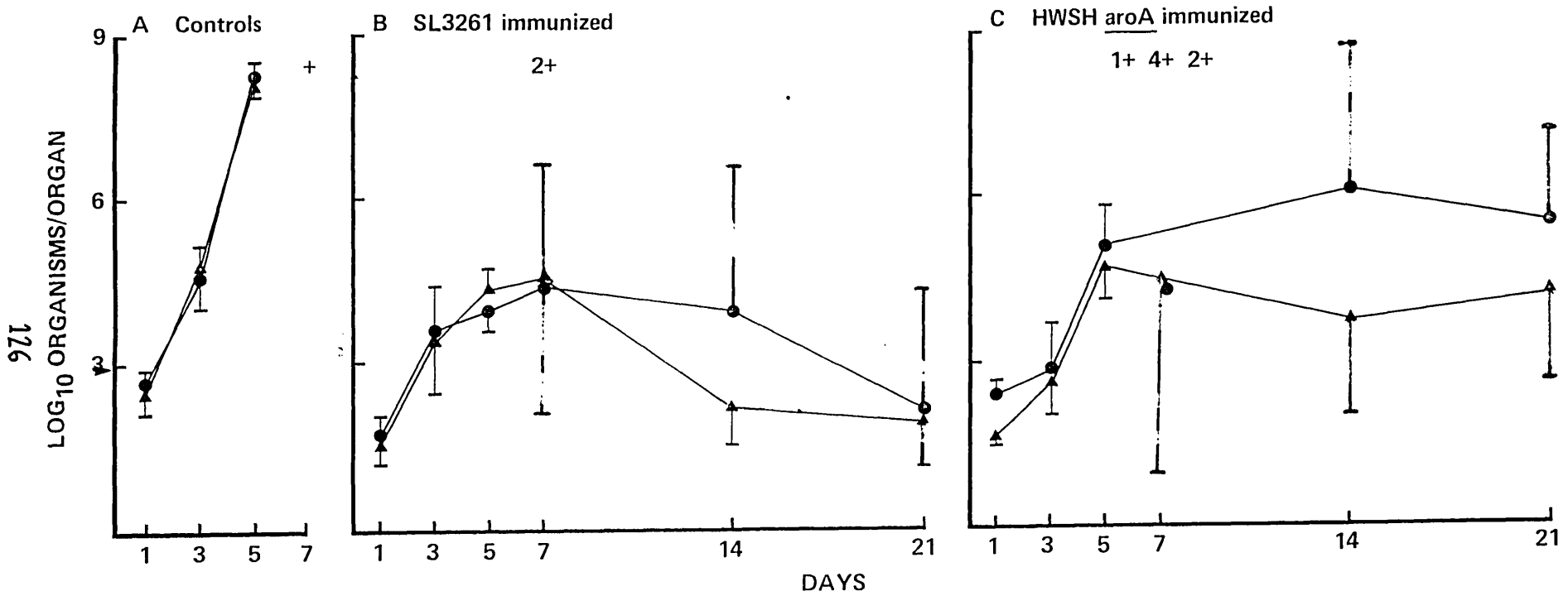


Fig. 4.4 Day 70 challenge

groups reduction of the C5 challenge over the first 24 hours was greater than in controls. SL3261 immune mice were better able to clear the challenge than HWSH aroA immune mice. No C5 (<10 cfu) was detectable in the mice sampled on day 3. On day 5, some mice were clear in both the SL3261 and HWSH aroA groups, whilst others carried between 10^4 and 10^6 cfu. Some of the HWSH immunised mice looked sick. On day 7, carriage levels ranged between <10 and 10^8 cfu with lower levels in SL3261 immune mice. Five of the HWSH aroA immune mice died on day 7. On and after day 7, when high numbers of C5 were found in individual organs, this was always associated with abscesses similar to those seen with HWSH challenge. Abscesses were more common in HWSH aroA immunised mice than in SL3261 immunised. In organs that did not develop abscesses, the C5 challenge was controlled but not cleared. Instead, a persistent infection followed, with carriage levels between 10^2 and 10^3 cfu. Occasionaly a mouse was clear of challenge (<10 cfu detectable); here it is most likely that the challenge was completely inactivated during the first day of the infection, rather than later when a chronic infection was underway.

Day 70 challenge. Mice were challenged i.v. with C5, 70 days after immunisation. The LD_{50} determinations in both vaccinated groups showed good levels of protection (table 4.3), but it appeared that SL3261 immunisation was more effective than HWSH aroA immunisation. This was confirmed

when the growth of an i.v. challenge of 10^3 cfu C5 was followed (fig 4.4). There was again an increased clearence of the challenge over the first 24 hours, although it was not as great as at day 28. The surviving bacteria grew rapidly in both SL3261 and HWSH aroA immunised mice. The majority of mice were able to control the growth of the challenge. However, six HWSH aroA immune mice died between days 6 and 8, and two SL3261 mice died on day 7. On day 7, mice were found to have varying degrees of C5 carriage, ranging from moribond mice with 10^7-10^8 cfu, to mice with only 10^2 cfu. On days 14 and 21 most mice in both groups had a chronic infection persisting at around 10^3 cfu in livers and spleens. Again, occasional abscesses increased mean counts and gave large standard errors. In the SL3261 group some mice were found to have clear (<10 cfu) organs. This is again probably due to clearance in the early stage after the infection rather than clearance of a chronic infection. Again, abscesses were more common in the HWSH aroA immune group than the SL3261 immune group.

4.1.2 Efficacy of immunisation with purA mutants.

4.1.2.1 Immunity to challenge with virulent parents.

HWSH purA immunised: HWSH challenge. No data is available to show whether HWSH purA immunises effectively against challenge with virulent HWSH. Initially, a HWSH purA mutant was isolated and characterised in great detail. It was

attenuated, and capable of causing limited infection in livers and spleens of BALB/c mice, which persisted for 28 days. It was found to be an ineffective vaccine. After SL1344 purA was constructed and characterised, it was apparent that the behaviour of this strain was very different. To ensure that this was a true observation attempts, were made to restore virulence to HWSH purA by complementing the purA lesion. This was done by transducing HWSH purA with a P22 lysate grown on HWSH, and selecting purine independent isolates on minimal medium. Several isolates were obtained, but none of these showed mouse virulence. It was assumed that the HWSH purA isolate was in fact LT2 purA. A new HWSH purA mutant was constructed. To ensure that it was a mutant of HWSH, a nalidixic acid resistant mutant of HWSH was used as the parent. HWSH was made resistant to nalidixic acid by plating 10^{10} cfu onto L agar containing 50ug/ml nalidixic acid selecting a spontaneously resistant isolate. This isolate showed the same virulence as HWSH and was used as the parent of the HWSH purA mutant described in this thesis.

SL1344 purA immunised: SL1344 challenge. Twenty mice immunised i.v. with 1.5×10^6 cfu SL1344 purA were challenged i.v. with SL1344 28 days later. The LD_{50} value of 4.5 (as opposed to <1 in controls) showed that the mice were protected although the level of protection was not as high

as in mice immunised with a similar dose of SL3261 (see table 4.2).

4.1.2.2 Comparison of the efficacy of immunisation with HWSH purA and SL1344 purA against S. typhimurium C5 challenge.

Fifty mice were immunised i.v. with either $1.3x10^6$ cfu HWSH purA or $1.6x10^6$ cfu SL1344 purA. They were challenged i.v. with C5 either 28 or 70 days later, and the LD_{50} calculated (Table 4.4). At day 28, SL1344 purA immune mice showed good levels of protection against C5 challenge, as they had done against SL1344 challenge. Again, this was less than the level of protection induced by SL3261 immunisation. The mice immunised with HWSH purA were poorly protected against C5 challenge. When mice were challenged at day 70, the protection induced by SL1344 purA had waned considerably and HWSH purA immunised mice were still not protected.

Table 4.4

Comparison of the efficacy of i.v. immunisation with HWSH *purA* or SL1344 in protecting BALB/c mice against i.v. challenge with *S. typhimurium* C5.

Immunising strain.	LD_{50} of C5 at day	
	28	70
HWSH *purA*	2	1.5
SL1344 *purA*	5	2.4
Unimmunised	<1	<1

4.1.3 Efficacy of immunisation with aroA purA mutants.

4.1.3.1 Efficacy of immunisation with HWSH aroA purA against HWSH challenge.

Twenty mice were immunised i.v. with $3x10^5$ cfu HWSH aroA purA. They were challenged i.v. with HWSH 28 days later and the LD_{50} calculated to be log 1.3. This showed no protection against the challenge when compared with the LD_{50} of <1 in controls. Seventy days after immunisation, mice were challenged i.v. with $1.4x10^3$ cfu HWSH and the growth of the challenge followed (fig 4.5). The growth of the challenge was identical in both immunised and control mice.

4.1.3.2 Efficacy of immunisation with SL3261 purA against challenge with S. typhimurium C5.

Twenty mice, immunised i.v. with SL3261 purA were challenged with C5 28 days later. The LD_{50} of 2.8 in immunised mice, compared with <1 in controls, showed only minimal levels of protection.

4.2 Ability of auxotrophic derivatives of SL1344 given i.v. to protect salmonella resistant A/J mice against i.v. challenge with SL1344.

Groups of 25 A/J mice were immunised i.v. with SL3261 (8×10^5 cfu), SL1344 purA (1.8×10^6 cfu) or SL3261 purA (7.2×10^5 cfu). Seventy days after immunisation, they were

challenged i.v. with SL1344. The LD_{50} of SL1344 in unimmunised mice was 3.8 whilst in all vaccinated groups the LD_{50} was 6.7.

4.3 The ability of auxotrophic mutants given orally to protect BALB/c mice against oral challenge.

Mice were infected orally with 10^9-10^{10} of the auxotrophic mutants and then challenged orally with the parental virulent strains 4 weeks later (Table 4.5). Both aroA strains were excellent vaccines giving challenge LD_{50} values 5-10,000 fold higher than controls. Unlike the aroA derivatives neither the purA nor the aroA purA derivatives induced any protection orally against an oral challenge.

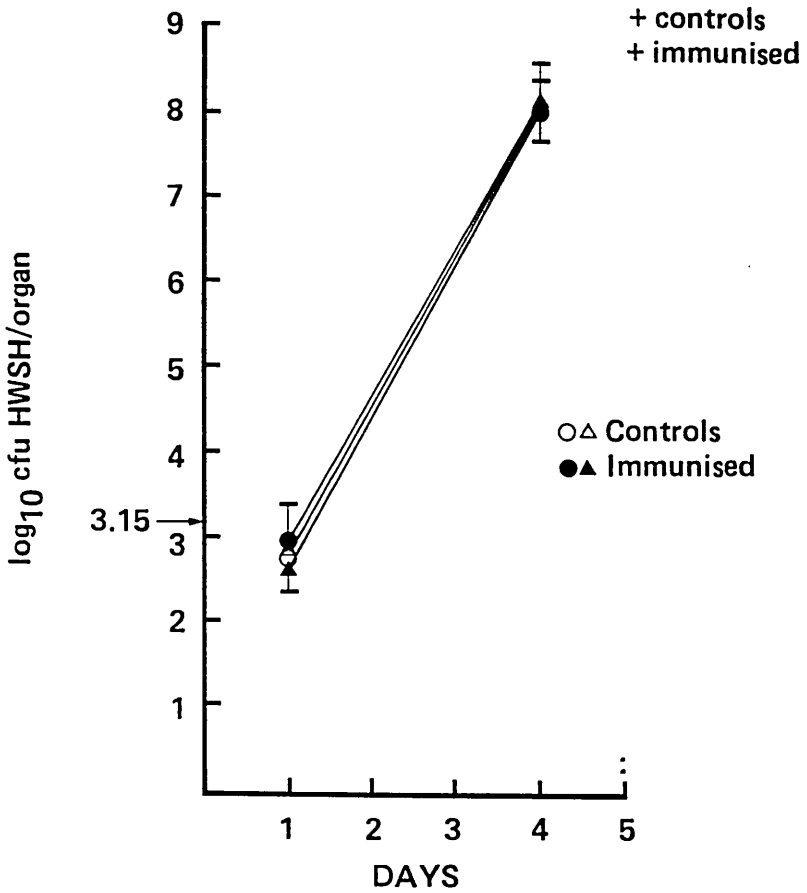


Figure 4.5 The ability of HWSH aroA purA to immunise BALB/c mice against its virulent parent. Mice were immunised i.v. with HWSH aroA puA, then challenged i.v. with HWSH 70 days later. Counts are shown as geometric mean ± 2se per organ in livers (●) and spleens (▲).

Table 4.5

The ability of auxotrophic *S. typhimurium* strains given orally to protect BALB/c mice against an oral challenge with virulent *S. typhimurium* 28 days after immunisation.

	Challenge (Log LD_{50} values oral)
Immunizing Strain	HWSH
HWSH *aroA*	8.82
HWSH *purAaroA*	<5.22
Unimmunized	5.32
	SL1344
SL3261	10.3
SL1344 *purA*	6.68
SL3261 *purA*	5.58
Unimmunized	6.2

5.0 ANALYSIS OF SERUM IgG AND IgM LEVELS FOLLOWING I.V. IMMUNISATION USING ELISA.

5.1 Antibody levels after immunisation with HWSH derived strains.

The IgG responses in mice immunised with the HWSH derived mutants are shown in Fig 5.1. Immunisation with HWSH *aroA* induced an IgG response which was greater and more rapid than that following immunisation with either HWSH *purA* or HWSH *aroA purA*. Levels of IgG increased during the first 35 days after immunisation. Unfortunately, no sera were taken after day 35 (when the mice cleared the infection: Fig 3.3). Immunisation with HWSH *purA* and HWSH *aroA purA* induced lower, but still significant levels of IgG.

The IgM levels are shown in Fig 5.2. Unfortunately, the assay system does not allow quantitative comparison of IgG with IgM. The levels of IgM in HWSH *aroA* immune sera increased in a similar way to IgG levels. As with the IgG response, the IgM response in the HWSH *purA* and HWSH *aroA purA* sera was much smaller than that seen in HWSH *aroA* immunised mice.

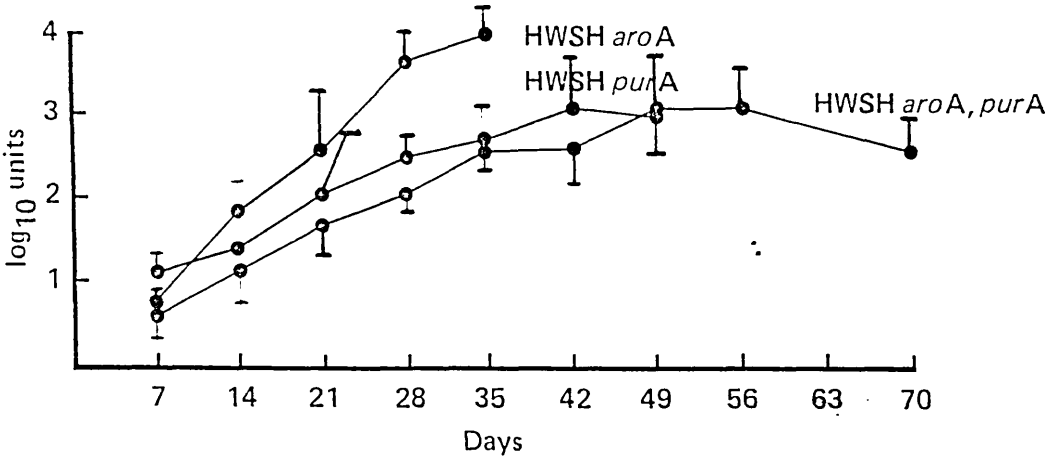


Fig. 5.1 Salmonella specific IgG levels in sera from mice immunised i.v. with HWSH derived mutants. Levels are expressed in arbitrary units with each point representing the geometric mean ± 2se of 4 mice.

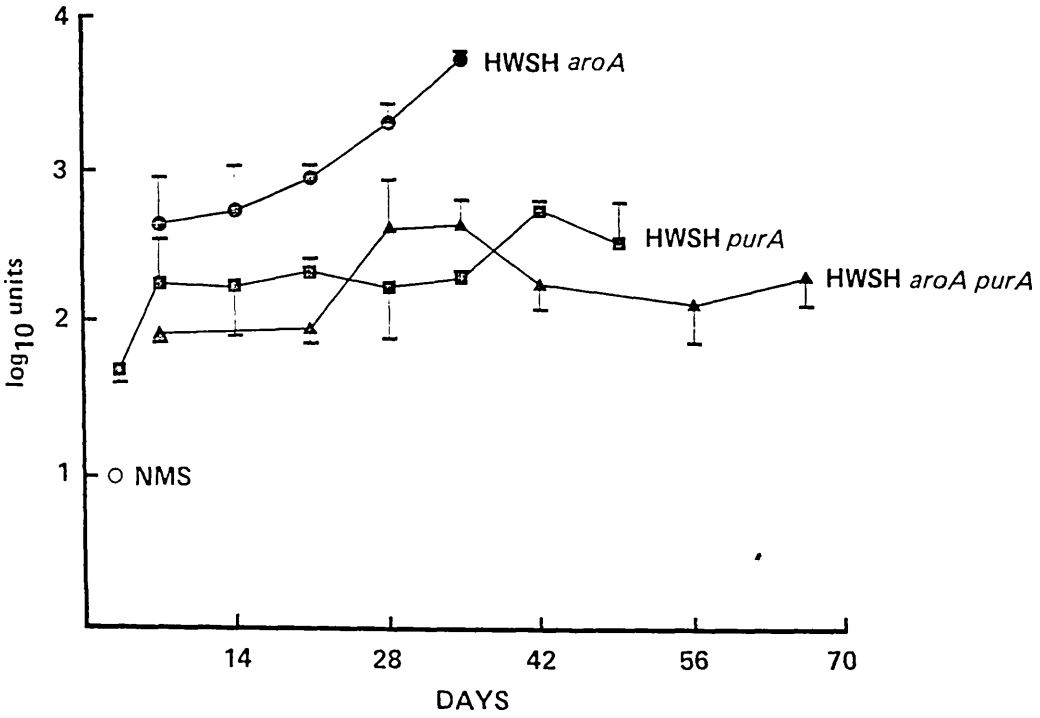


Fig. 5.2 Salmonella specific IgM levels in sera from mice immunised i.v. with HWSH derived mutants. Levels are expressed in arbitrary units with each point representing the geometric mean ± 2se of 4 mice.

5.2 Antibody levels following immunisation with SL1344 derived strains.

Sera were available only from mice immunised with SL3261 and SL1344 purA. IgG levels in the SL3261 immune sera (fig 5.3) were similar to those of the corresponding HWSH aroA sera. Sera were taken not only throughout the SL3261 infection, but for several weeks afterwards. They showed that IgG levels rose rapidly and remained at these high levels over the 12 weeks that sera were taken. The IgM levels (fig 5.4) also rose rapidly but were seen to be declining slowly after the sixth week.

Salmonella specific IgG and IgM in the SL1344 purA sera increased to similar levels to that in SL3261 sera. IgG levels did not maintain the high levels as long as in the SL3261 sera as they were seen to decline rapidly between weeks 5 and 7. IgM levels began to decline after week 4.

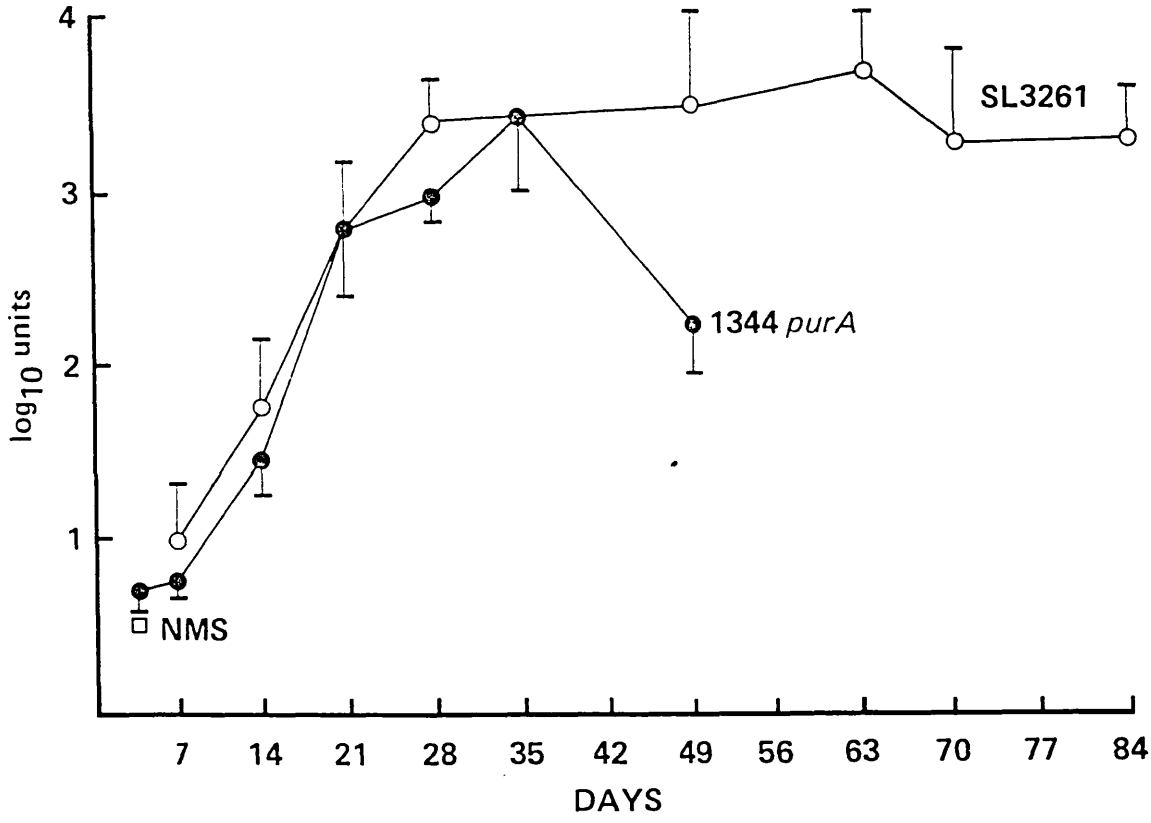


Fig. 5.3 Salmonella specific IgG levels in sera from mice immunised i.v. with SL1344 derived mutants. Levels are expressed in arbitrary units with each point representing the geometric mean ± 2se of 4 mice.

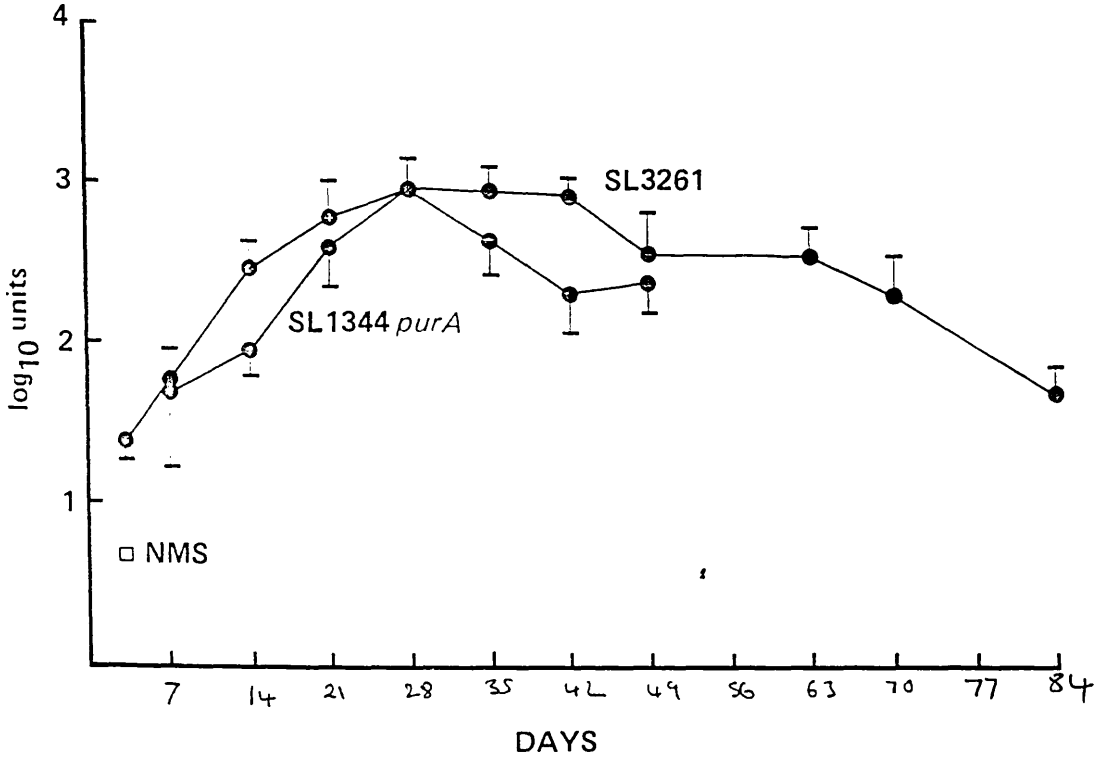


Fig. 5.4 Salmonella specific IgM levels in sera from mice immunised i.v. with SL1344 derived mutants. Levels are expressed in arbitrary units with each point representing the geometric mean ± 2se of 4 mice.

6.0 CELL MEDIATED IMMUNE RESPONSES TO VACCINATION.

6.1 Macrophage activation.

Macrophage activation was assessed *in vivo* by following the ability of the mice immunised i.v. with HWSH *aroA* or HWSH *purA* to control a superinfecting homologous (HWSH), or heterologous (*L. monocytogenes*), challenge in the early stages of the immunising infection. Activation was also assessed *in vitro* by measuring I-A expression.

6.1.1 Resistance to early homologous and heterologous challenge.

6.1.1.1 Characterisation of L. monocytogenes LUGI23.

Groups of five mice were infected i.v. with 10-fold dilutions of *L. monocytogenes* LUGI 23. The LD_{50} was calculated at 28 days to be Log_{10} 3.4. To determine the ideal challenge dose, groups of 15 mice were infected i.v. with approximatly 10^5, 10^4 or 10^3 cfu. The growth of the listeria was followed in livers and spleens over the next 4 days. The 10^5 cfu dose grew rapidly killing all mice by day 4. The 10^4 dose and, even more so, the 10^3 dose produced irregular counts, with some mice controlling the infection and other unable to do so. Therefore, 10^5 cfu dose was taken for all subsequent challenge experiments.

6.1.1.2 Ability to control early salmonella challenge.

Mice immunised with $5.75x10^5$ cfu HWSH aroA and $3.3x10^5$ cfu HWSH purA, were challenged i.v. with approximately $5.5x10^4$ HWSH either 7 or 14 days after immunisation.

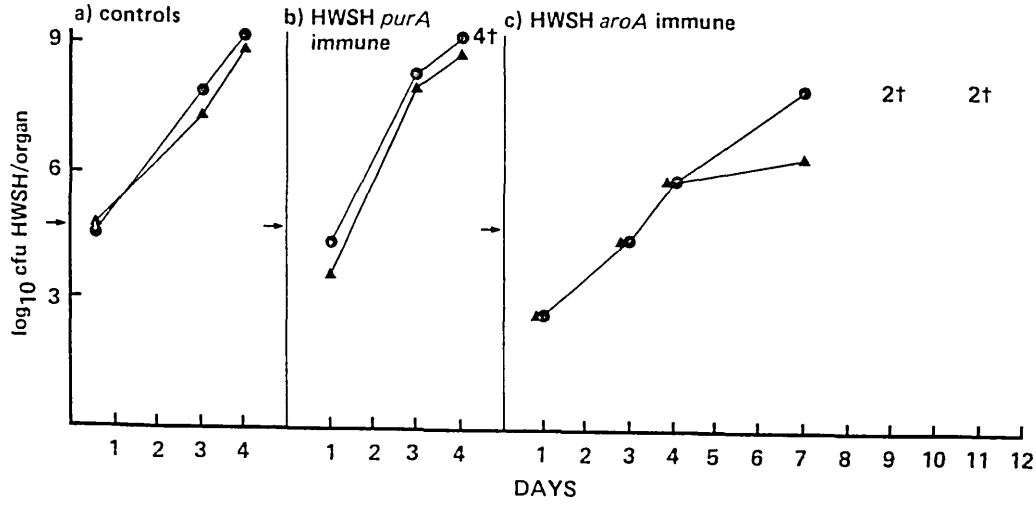
Day 7 challenge. In the control group, the challenge grew rapidly, reaching lethal levels by day 4 (fig 6.1). In the HWSH purA group, the counts on day 1 showed a slightly lower levels of HWSH than in the controls, however, the challenge grew as rapidly as in controls, again reaching lethal levels by day 4. When the aroA immune group were challenged, viable counts on day one showed levels of HWSH 2 logs lower than in controls. The surviving bacteria grew rapidly in the livers and spleens, but at a slightly slower rate than controls. By day 7, there was prounounced splenomegaly, and the mice looked ill. The remaining 4 mice died on days 9 (2 mice) and 11 (2 mice). On autopsy they were found to have gross splenomegaly and often, large hepatic and splenic abscesses. No counts were taken.

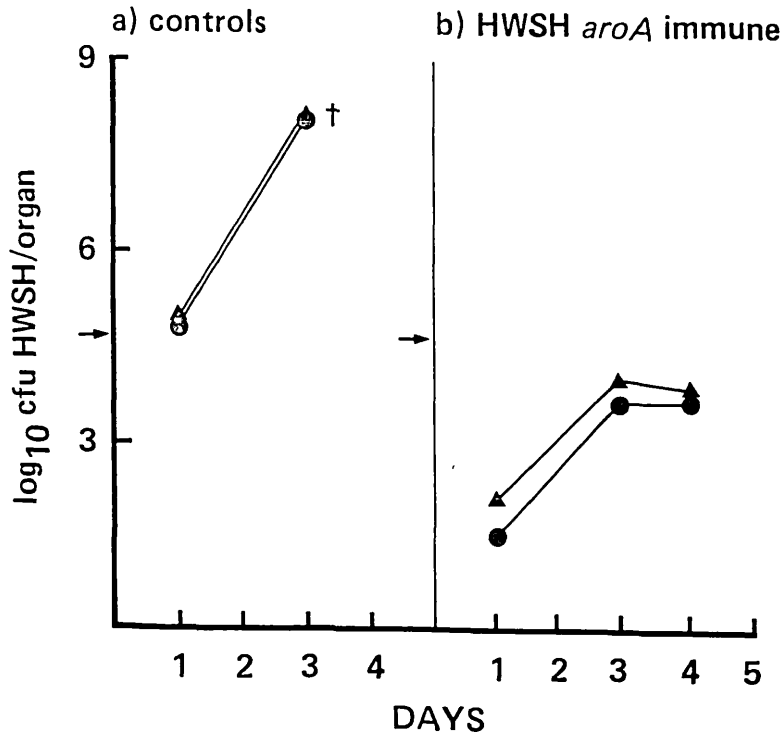
Day 14 challenge. At day 14, only HWSH aroA immunised mice were challenged (fig 6.2). The challenge grew rapidly in controls, killing all mice by day 4. In the aroA group, the challenge was reduced by 3 logs during the first 24 hours. The surviving bacteria grew over the next two days but at about one half of the rate of the growth of the challenge in

Fig. 6.1/ 6.2 Comparison of the ability of HWSH aroA and HWSH purA to induce early immunity to HWSH challenge. Mice were immunised i.v. with 5.75 x 10^5 cfu HWSH aroA or 3.3 x 10^5 HWSH purA and challenged i.v. with HWSH 7 or 14 days later. Points represent geometric mean cfu in livers (●) and spleens (▲). Standard errors were less than 10% of the mean and are not shown.

Fig. 6.1 Day 7 challenge.

Fig. 6.2 Day 14 challenge.


a) controls
b) HWSH purA immune
c) HWSH aroA immune
4†
2†
2†
log10 cfu HWSH/organ
9
6
3
1 2 3 4
1 2 3 4
1 2 3 4 5 6 7 8 9 10 11 12
DAYS


a) controls
b) HWSH aroA immune
†
log10 cfu HWSH/organ
9
6
3
1 2 3 4
1 2 3 4 5
DAYS

unimmunised mice. Between days 3 and 4, growth of HWSH was controlled. No mice died.

6.1.1.3 Ability of mice to control listeria challenge.

Groups of mice immunised with HWSH aroA or purA as described above (6.1.2.2), were challenged i.v. with 10^5 listeria. The growth of the listeria challenge was followed in livers and spleens.

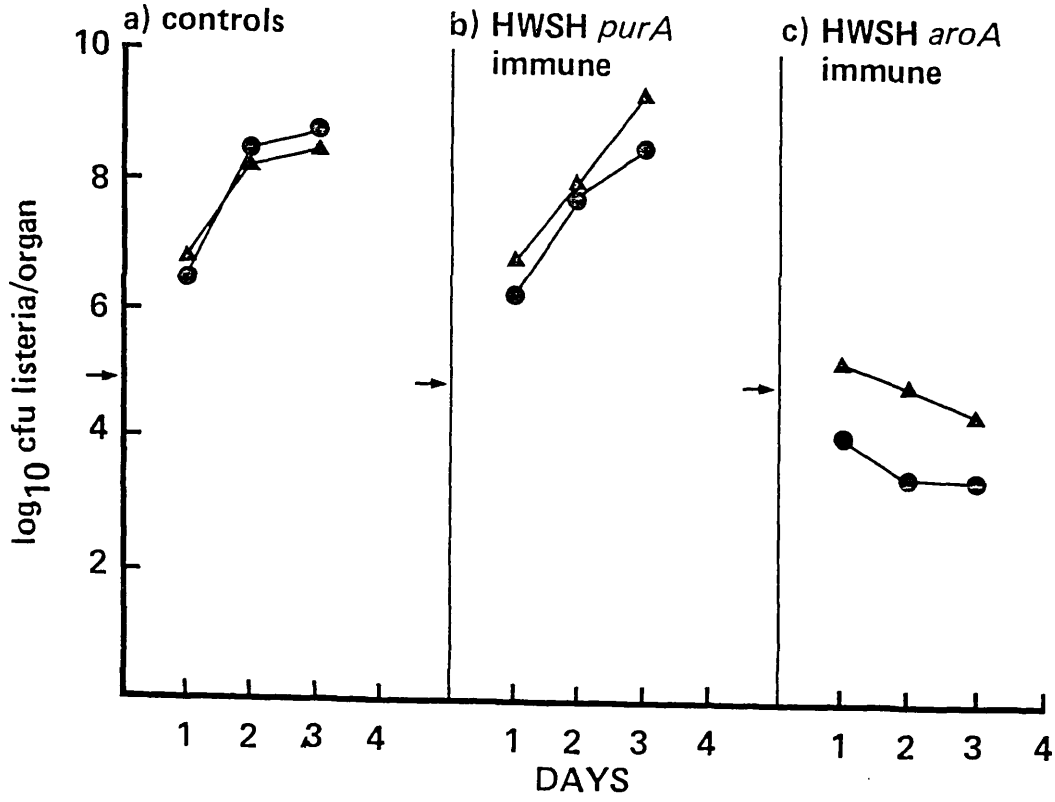
Day 7 challenge. Growth of listeria was similar in both the control and the purA immunised groups (fig 6.3). The challenge grew rapidly to reach levels between $10^{8.5}$ and $10^{9.5}$ in livers and spleens by day 3. The HWSH aroA immune group were capable of controlling the listeria challenge. Viable counts on day 1 showed that, where as in the purA and control groups, the challenge of 10^5 cfu had grown to between 10^6 and 10^7 cfu in livers and spleens, in the aroA group, carriage of listeria was at about 10^5 in spleens and 10^4 in livers. Then, rather than growing, the levels of listeria decreased by 1/2 Log over the next 2 days.

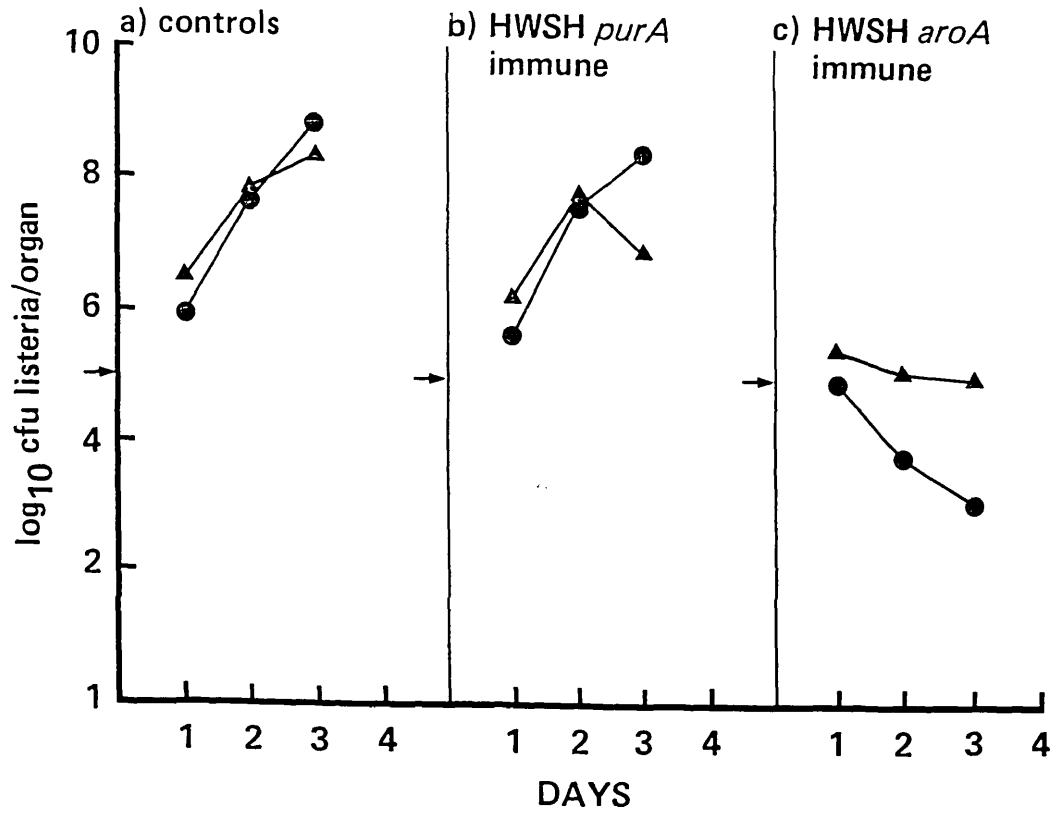
Day 14 challenge. When mice were challenged 14 days after immunisation (fig 6.4), growth of listeria in the HWSH purA group was still similar to that in the controls. The HWSH aroA group were still capable of controlling the challenge. The livers appeared better able to eliminate the challenge between days 1 and 3.

Fig. 6.3/6.4 Comparison of the ability of HWSH aroA and HWSH purA to induce early immunity to listeria challenge. Mice were immunised i.v. with 5.75 x 10^5 HWSH aroA or 3.3 x 10^5 HWSH purA and challenged i.v. with L. monocytogenes 7 or 14 days later. Points represent geometric mean in livers (●) and spleens (▲). Standard errors were less than 105 of the mean and are not shown.

Fig. 6.3 Day 7 challenge.

Fig. 6.4 Day 14 challenge.


a) controls
b) HWSH purA immune
c) HWSH aroA immune
log10 cfu listeria/organ
10
8
6
4
2
1 2 3 4
1 2 3 4
1 2 3 4
DAYS


a) controls
b) HWSH purA immune
c) HWSH aroA immune
log10 cfu listeria/organ
10
8
6
4
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1
1 2 3 4
1 2 3 4
1 2 3 4
DAYS

6.1.1.4 The effect of levels of carriage of the immunising strain on immunity to listeria.

The experiments described above can be criticised on the grounds that, although the same immunising dose of HWSH aroA and purA was used, the resulting levels of carriage in liver and spleens would be different. Attempts were made to immunise mice with doses that would result in similar carriage levels.

Mice were immunised with $7x10^4$ cfu HWSH aroA and $9x10^6$ cfu HWSH purA. Mice were again challenged 7 and 14 days post immunisation. The carriage levels of the immunising salmonella were determined by viable counts on the day of challenge. Unfortunately, the doses chosen resulted in carriage of HWSH purA higher than that of HWSH aroA.

Day 7 challenge. Viable counts on day 7 showed that carriage of HWSH purA was at about 10^5 cfu in livers and spleens, while HWSH aroA was at $10^{3.6}$ in livers and $10^{4.5}$ in spleens. When their ability to control the listeria challenge was compared (fig 6.5), it appeared that the purA immune group were better able to control the challenge. Viable counts on day 1 showed that both groups were better able than control to eliminate the challenge over the first 24 hours. In the aroA group, the challenge then grew between days 1 and 2 at a rate similar to controls, but was controlled between days 2 and 3. In the livers of the purA

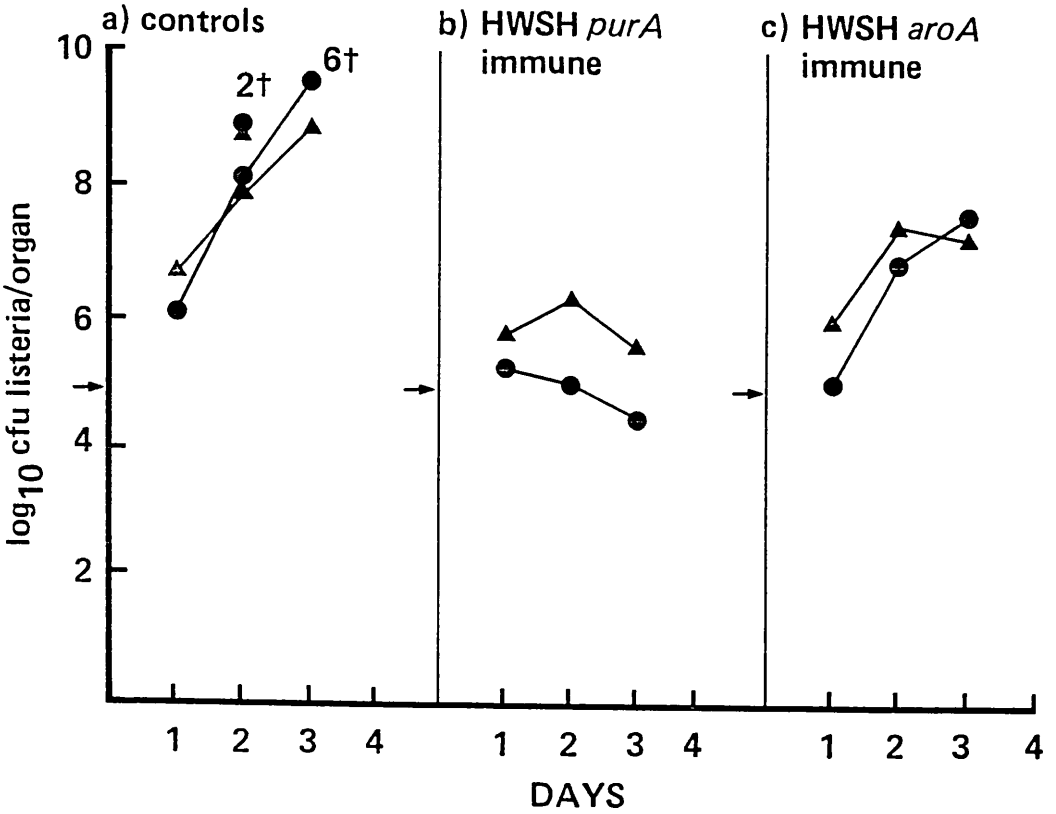
group the challenge was slowly eliminated between days 1 and 3. In the spleens, however, the challenge grew slightly between days 1 and 2 but then was controlled. Elimination had started by day 3.

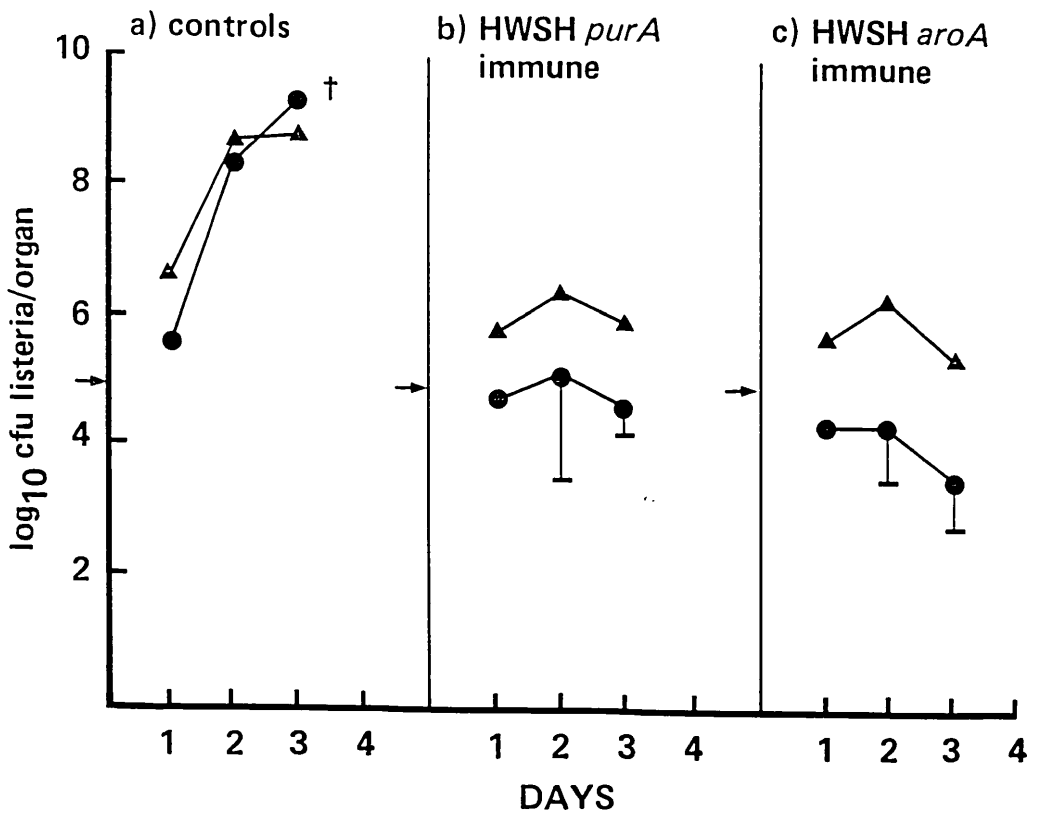
Day 14 challenge. On day 14 the differences in levels of HWSH aroA and purA were much greater than at day 7. HWSH aroA was present at levels lower than HWSH purA by 1.5 logs in livers and 1.25 logs in spleens. Despite these differences, both groups showed similar abilities to control the listeria challenge (Fig 6.6). Both groups eliminated a large proportion of the challenge over the first 24 hours. Livers were better able to do this than spleens. In the spleens of both groups, the challenge grew slightly between days 1 and 2, was controlled, and elimination began between days 2 and 3. In the livers the trend was towards slow elimination of the challenge. The counts were, however, highly variable, with some mice in each group able to control and eliminate the challenge and others unable to do so; the large error bars on some points shown in fig 6.6 reflect this.

Fig. 6.5/6.6 Comparison of the ability of HWSH aroA and HWSH purA to induce early immunity to listeria challenge. Mice were immunised i.v. with 7×10^4 HWSH aroA or 9×10^6 HWSH purA and challenged i.v. with L. monocytogenes 7 or 14 days later. Points represent geometric mean cfu in livers (o) and spleens (▲). Standard errors were less than 10% of the mean unless shown. Error bars represent 2se.

Fig. 6.5 Day 7 challenge.

Fig. 6.6 Day 14 challenge.


a) controls
b) HWSH purA immune
c) HWSH aroA immune
2†
6†
10
8
6
4
2
log10 cfu listeria/organ
1 2 3 4
1 2 3 4
1 2 3 4
DAYS


a) controls
b) HWSH purA immune
c) HWSH aroA immune
†
10
8
6
4
2
log10 cfu listeria/organ
1 2 3 4
1 2 3 4
1 2 3 4
DAYS

6.1.2 In vitro measurement of macrophage activation.

The differences seen in non-specific immunity to listeria suggest that part of the inability of HWSH purA to vaccinate is because they are less able to activate macrophages. To confirm this, in vitro methods were used to measure macrophage activation. To do this macrophages were stained for expression of Ia using biotinated specific monoclonal antibodies and FITC avidin conjugates. First attempts were made by immunising mice i.p. and measuring Ia expression by the peritoneal exudate cells using a fluorescence activated cell sorter. Unfortunately, the results were clouded by the I-A positive sIg positive B-cell population (Data not shown). To overcome this problem, macrophages were enriched from whole spleen cell by adherence to glass or plastic.

Spleen cells were pooled from three mice immunised 7 days earlier with either $1x10^6$ cfu HWSH aroA, $1x10^6$ cfu HWSH purA or three unimmunised controls. The cells were suspended in medium at $2x10^7$/ml and 0.5 ml of this suspension was added to each well of a chamber slide. After incubation for 4 hours at 37°C, non-adherent cells were removed by washing with PBS. The adherent cells were stained as described in Materials and Methods. The numbers of stained cells were counted using a fluorescence microscope and are summarised in Table 6.1.

Two cell morphologies could be seen: smaller rounded cells and, larger cells with an "activated" morphology. I-A antigens were expressed by both cell types. Cells with this "activated" morphology made up 65% of the adherent cells from the aroA mice but were rarely present in control and purA groups. Six percent of the adherent cells from normal mice expressed I-A and, 6.6% possessed surface immunoglobulin. Much greater levels of I-A expression were seen in adherent cells from the aroA immunised mice. Here, 80% of cells expressed I-A and, 21% showed weak fluorescence when stained for surface immunoglobulin.

To check for the specificity of staining, cells from each group were also stained with a monoclonal antibody raised against $I\text{-}A^b$ or with FITC-avidin alone . In each case, ten fields (100-200 cells) were examined, and no fluorescing cells were seen.

The results show that, at the doses given, i.v. immunisation with HWSH aroA activated macrophages, as judged by cell morphology and I-A expression, more effectively than HWSH purA.

Table 6.1

In vitro measurement of macrophage activation.

Expression of Ia and surface immunoglobulin by adherent spleen cells.

Cell type	Antigen	N° cells counted	N° activated	N° +ve morphology
Controls	I-A^d	121	10 (8.25)	7 (5.8)
	sIg	121	8 (6.6)	8 (6.6)
HWSH	I-A^d	140	92 (65.7)	111 (79)
aroA	sIg	100	65 (65)	21 (21)
HWSH	I-A^d	116	2 (1.7)	0 (0)
purA	sIg	164	4 (2.4)	73 (44)

6.2 T-cell mediated immune responses following vaccination.

Groups of three mice were immunised s.c. with ten-fold dilutions of HWSH aroA or HWSH purA. Ten days later the draining lymph nodes were removed and single cell suspensions prepared as described in Material and Methods. Cells were restimulated in vitro with ten-fold dilutions of Heat Killed HWSH (same preparation as used in ELISA) for four days and pulsed overnight with ^{3}H-thymidine. Doses of 10^{6} HWSH aroA and 10^{7} HWSH purA were required to prime T-cells. When the dose response curves of cells from mice immunised with 10^{7} bacteria are expressed graphically (Fig 6.7) it appears that HWSH aroA immunisation primed between ten and thirty times more salmonella specific cells than HWSH purA immunisation.

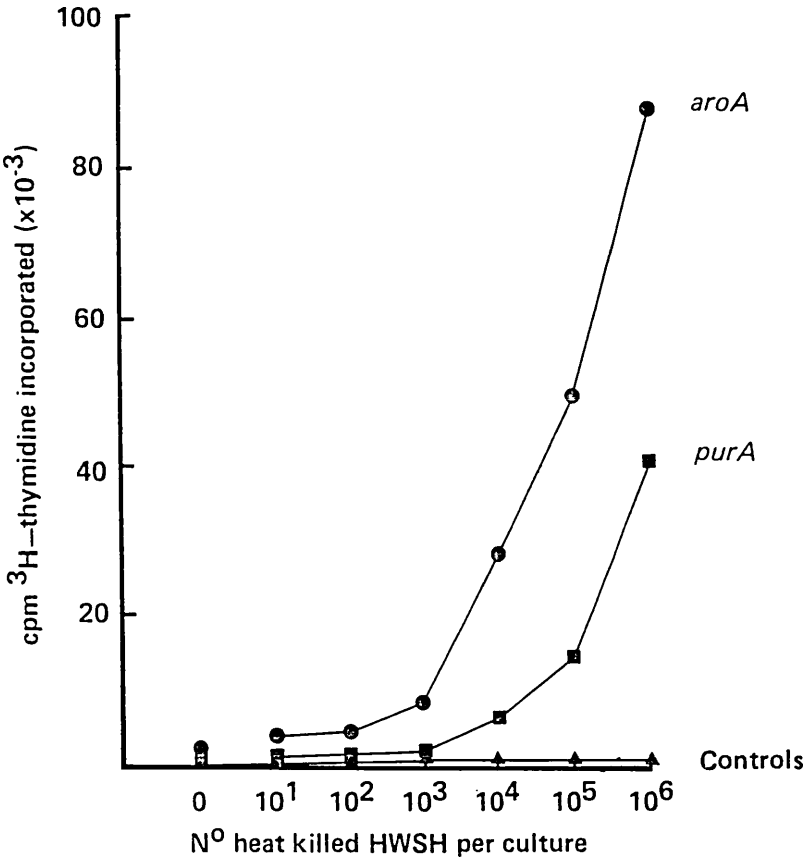


Fig. 6.7 Proliferation of lymph node cells of mice immunised s.c. with 10^7 cfu HWSH aroA or HWSH purA 10 days previously. Cultures were stimulated with varying ammounts of heat killed HWSH and proliferation was measured as ^{3}H-Thymidine incorperation.

6.3 Induction of Natural Killer activity.

Since NK cells have been implicated in macrophage activation in listeria infections (Bancroft *et al*, 1987) and NK activity has been detected in SL3235 immunised mice, it was of interest to see if NK activity was present in HWSH *aroA* or HWSH *purA* immunised mice.

To test this, mice were immunised i.v. with 10^6 cfu of HWSH *aroA* or HWSH *purA* and their spleens removed three days later. Two-fold dilutions of spleen cells were mixed with ^{51}Cr labled YAC-1 cells at effector: target ratios of up to 1: 100. NK activity was expressed as percentage specific lysis after 18 hours incubation. The results, shown in Fig 6.9, show an increase in cell lysis in both the *aroA* and the *purA* immune spleens when compared with the control spleen cells.

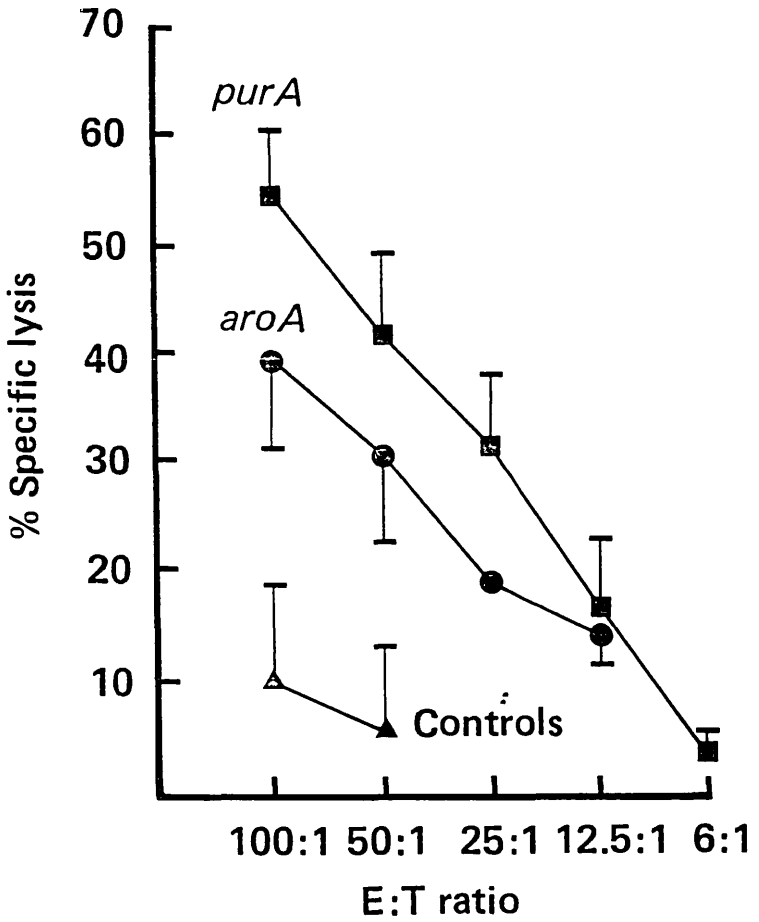


Fig. 6.8 Natural Killer Cell activity in spleen cells from mice immunised i.v. with 10^6 cfu HWSH aroA or HWSH purA three days previously. Each point represents mean specific lysis (± 1SD, n=3) of ^{51}Cr labled YAC-1 cells at varying effector: target ratios.

DISCUSSION

7.1 Attenuation by auxotrophic requirement.

Live vaccines have been found effective for a variety of bacterial pathogens. Most of the vaccine strains, such as those for Brucella melitensis (Eldberg and Faunce, 1957) B. abortus (McEwen and Priestly, 1938; Zdrodowski et al 1957) and tuberculosis (Collins, 1984) are attenuated by unknown mutations. Until recently it has been impossible to create a bacterial strain with a mutation at one genetic locus. Chemical mutagens or irradiation could be used and a bank of mutants screened for the required phenotype. This approach, taken by Bacon et al (1950 ab), is very time consuming (Bacon et al screened 10,000 mutants) and has the disadvantage that a mutant may contain several mutations besides the one selected. This has been found true for S. typhi Ty21a, which contains mutations affecting Vi antigen production, H_2S production, and causing requirements for several amino acids (Hone et al, 1988).

Tn10 imprecise excision can be used easily and reproducibly to inactivate a variety of genes. The main disadvantage of this approach is that the resulting lesion, although often non-reverting, may be a deletion, an inversion, or a more complex rearrangement. Ideally the attenuating lesion in a human vaccine should be genetically defined. If Tn10 imprecise excision is used to construct mutant strains of S. typhi which will ultimately be used in humans, the

lesions created should be characterized in a detailed genetic manner. This can be done by employing Southern Blot analysis using the cloned wild type gene as a probe followed by cloning and sequencing the inactivated gene. An alternative approach is to clone the wild type gene, mutate it *in vitro* in a defined manner, and use the mutated gene to replace the wild type gene on the bacterial chromosome. This approach has been successfully used to create *aroA* mutants of *B. pertussis* (Maskell *et al*, 1988) and *aroC* mutants of *S. typhi* (Dougan *et al*, 1988). When, as in the cases of metabolic pathway genes, an enzyme function has been affected, the activity of the enzyme may be assayed. When specific antisera are available, expression of gene products may also be checked using Western Blotting.

The auxotrophic mutations did not appear to induce any changes in the protein or LPS profiles of the salmonella strains tested. It is important that any candidate vaccine should posess all the antigenic characteristics of the wild-type strain. This is one of the major criticisms voiced against Ty21a which posesses no Vi antigen. One semi-rough HWSH *aroA* *purA* varient was isolated during this study. There is no evidence to suggest that this was in any way due to the auxotrophic lesions. This mutant may have been selected during enrichment on Bochner medium, or during the transduction stage, where too little EGTA in the agar would allow P22 replication, thus giving a P22 resistant isolate a

selectional advantage. The isolation of this mutant shows that it is important to fully characterise any vaccine candidate before vaccination studies are commenced.

The data presented in this thesis support earlier observations that certain auxotrophic mutations attenuate virulent strains of salmonella. Both aroA and pur mutations can attenuate strains but do so to differing extents. purE mutants are attenuated when compared with their parents, however, they are capable of significant growth in vivo, a fact which could exclude the use of such mutations in human vaccine strains. purA mutants are highly attenuated. The different effects of purE compared to purA are due to the respective positions at which the products of each gene act in the purine biosynthetic pathway. The purE gene product acts early in the pathway, before the IMP branch point, and such mutants still have the capacity to scavenge or synthesise purines in vivo. This is supported by the observation that purE, but not purA, mutants can grow slowly in vitro on minimal agar supplemented with liver or spleen homogenates. It is known that IMP is present in mammalian plasma at levels (ca. 1uM) higher than other purines or nucleosides. In man, these levels can increase to as much as 20uM at times of stress such as anoxia (Saugstad and Gluck, 1982) extreme exhaustion (Harkness, Simmonds and Coade, 1983) or severe illness (Grum et al, 1985). All pathogenic protozoan parasites investigated, including the malaria

parasite, Plasmodium falciparum, and Trypanosoma species, are IMP requiring auxotrophs (Gutteridge and Coombs, 1977).

Other lesions in the biosynthetic pathway to IMP can partially reduce the mouse virulence of several strains of salmonella (McFarland and Stocker 1987, Nnalue and Stocker 1987) Y. pestis (Burrows and Bacon, 1954) and Klebsiella pneumoniae (Garber, Hackett and Franklin, 1952), but not Bacillus anthracis (Ivanovics et al, 1968). Mutations in the pathways after IMP are attenuating for many pathogens, including K. pneumoniae, (Garber et al, 1952) and B. anthracis (Ivanovics et al 1968).

The exact reason for the attenuation by aromatic dependence is less clear. It was originally suggested (Hoiseth & Stocker, 1981) that the requirement for DHB, a precursor for the siderophore enterochelin, was the main reason for attenuation. It had been previously reported that enterochelin was necessary for virulence (Yancey, Breeding Lankford, 1979) however, it has since been shown that enterochelin may not be necessary for full virulence (Benjamin et al, 1985). Stocker (personal communication) suggests that the PABA requirement is the key reason for attenuation. He has constructed PABA requiring mutants of S. typhimurium and has found them to be as attenuated as aroA mutants. Stocker suggests that the lack of folate,

leading to a reduction of levels of f-met t-RNA, and so low levels of protein synthesis, is the reason for attenuation.

Although S. typhimurium aroA and S. typhimurium purA derivatives both grow poorly in vivo their growth curves also show some important differences. In the first 24 hours after i.v. infection, the levels of viable purA mutants drop much more than aroA mutants when similar numbers of cfu are administered. After the first 24 hours both purA and aroA mutants establish persistent infections which lasts for several weeks. If aroA and purA mutations are combined in the same bacterium the in vivo behaviour of the resultant the strain differs significantly from strains carrying single mutations. HWSH aroA purA establish a long-lasting, low level persistent infection in the mice quite different from HWSH purA or HWSH aroA. The preliminary characterisation of SL3261 purA suggests that it also establishes a similar long lasting infection after i.v. infection (Fig. 3.5b). Thus it is important to note that data obtained from a strain carrying a single auxotrophic mutation may not be extrapolated directly to a strain carrying two mutations in separate metabolic pathways.

The key role and the ubiquity of the aromatic pathway present the possibility of attenuating pathogens other than salmonella by an aro mutation. Other intracellular pathogens such as Yersinia spp, mycobacteria, brucella and listeria

are obvious candidates. It would be of great interest should an aroA mutation attenuate an extracellular pathogen such as B. pertussis. It is possible that nutrient supplies in extracellular environments may be different to those found in within cells. The aroA gene from B. pertussis has been cloned and sequenced (Maskell et al 1988). Further, this gene has been inactivated by insertion of a kanamycin resistance casette, and re-introduced into wild type B. pertussis, to produce an aroA mutant (Maskell personal communication). Experiments are under way to determine whether this strain is attenuated in mice. Should this strain prove to be attenuated, it may also be possible to attenuate a wide variety of extracellular pathogens, such as Staphylococcus aureus or Neisseria gonorhoea, perhaps leading to a new generation of live vaccines. It is not known whether protozoa, such as malaria or Leishmania possess an aromatic biosynthetic pathway. If they do, aromatic dependent strains may prove to be ideal live vaccines. Work is now under way to determine whether aro genes exist in parasites (Maskell personal communication).

7.2 The efficacy of the mutants as live vaccines.

Different auxotrophic requirements affect not only the degree of attenuation, but also the ability of S. typhimurium mutants to vaccinate. In the oral model,

vaccine efficacy can be ranked: aroA >> purA = aroA purA. In the i.v. system, vaccine efficacy can be ranked: aroA > purA > aroA purA.

One reason for the difference in the oral system is clear: aroA mutants can establish a limited infection in livers, spleens, mesenteric lymph nodes and, peyers patches of orally infected mice (Maskell et al 1987b). When given orally neither purA nor aroA purA strains are capable of doing this (my unpublished observations). The reasons for this inability to colonize the MPS are not known. aroA strains have been shown to be capable of surviving within the gut of orally infected mice (Hormaeche personal communication) and surviving within and adhering to the gut of orally infected, streptomycin treated mice (Nevola, Laux and Cohen, 1987).The survival of purA and aroA purA mutants in the gut has not been studied, but one may speculate that the poor colonisation may be due to an inability to either survive within, adhere to, or invade the gut. Another possible reason is that purA and aroA purA strains , like S. typhi (Collins and Carter, 1978), cannot resist the bactericidal action within the Peyer's patches and mesenteric lymph nodes of orally infected mice. Increased survival may be promoted by pretreating the mice with bicarbonate. This proceedure allows a purA mutant of S. typhimurium C5 to colonise Peyer's patches and mesenteric lymph nodes at levels of up to 10^3 cfu (Attridge personal

communication). However, the poor immune responses in volunteers who ingested aroA purA S. typhi mutants after bicarbonate suggests that in humans this may not be effective. More detailed evaluation of S. typhi auxotrophs in human volunteers is required before a full picture of their behaviour is obtained.

The protection data obtained using oral challenge of mice shows that S. typhimurium strains harbouring purA or aroA purA mutations make poor oral vaccines compared to aroA strains. On no occasion has it been possible to protect mice against virulent challenge using a purA or aroA purA vaccine strain administered orally. These observations may have important consequences for the design of attenuated S. typhi strains for use as live oral typhoid vaccines, which cannot be tested in animal models, especially as some auxotrophic derivatives of S. typhi are now being evaluated in humans. The implications of these results and possible solutions are discussed below.

The reasons for the difference in vaccine efficacies seen in the intravenous model are less evident. It has previously been stated that live vaccines are effective because they persist with in the MPS of immunised animals. Strains which do not persist are poor vaccines (Collins et al, 1966; Collins, 1974; Hormaeche, 1981). The results presented in this thesis show that this is not necessarily so; aroA

mutants establish a persistent infection and are excellent live vaccines, however, purA mutants, given at a similar dose, also establish a persistent infection but are poor vaccines. The levels of persistence for a given dose are lower for purA mutants, however, both purA strains tested persisted for several weeks longer than the isogenic aroA mutants. Further, Killar and Eisenstein (1985) reported that an i.p. dose of 10^4 SL3235 (aroA) would effectively immunised C3H lineage mice, suggesting that levels of carriage are not the prime cause for the poor efficacy of purA mutants.

The magnitude of the immune responses following i.v. vaccination mirrored the vaccine efficacy of the three mutant types, with aroA > purA >. aroA purA. Differences existed in both the humoral and the cell mediated responses to each mutant type. The antibody responses to the protective mutants, such as SL3261, are similar to those reported in CBA mice infected with low dose of a virulent strain (O'Brien et al, 1981). In this study, IgG and IgM responses increased together for six weeks, the IgG response continued to increase while the IgM response plateaued. When vaccine efficacy and antibody responses are compared, it is evident that the effective i.v. vaccines (HWSH aroA, SL3261 and SL1344 purA) gave a far better antibody response than the poor i.v. vaccines (HWSH purA and HWSH aroA purA). It is tempting to attribute this ability or lack of ability to

protect to the antibody response. This hypothesis is further supported by the observation that the protection at day 28 induced by SL1344 purA, corresponds with the peak in the antibody response (Fig. 5.4). Further, both IgG and IgM levels were seen to decline between weeks 5 and 7 (although no later samples were taken). At week 10, when presumably the antibody levels had further declined, only low levels of protection were seen against a C5 challenge (table 4). However, previous studies (Mackaness et al, 1966; Blanden et al, 1966; Collins, 1969a; Saxen, 1984) have shown that antibody plays only a minor role in the i.v. model. Saxen (1984) investigated the effects of passively administered IgG and IgM fractions from a hyperimmune mouse serum on i.v. and i.p. S. typhimurium challenge. IgM was found to be ten times more efficient than IgG at increasing blood clearance of i.v. administered salmonella and their uptake by livers. However, when the subsequent killing of the salmonella in the livers was followed, both IgM and IgG were only capable of slight enhancement. In the i.p. model, both IgG and IgM enhanced clearance and killing by peritoneal macrophages. IgM was, however, 1000 fold more efficient.

Because of the minor role shown for antibody in the i.v. model, it is more likely that the antibody responses, although important, are not the prime cause for the differences in vaccine efficacy. Immunising with the non-protective mutants did induce a good antibody response,

even if only ten-fold of that induced by the protective mutants. This is clear when the ELISA results are examined. Each plate had a reference positive serum. The titre of this serum as assigned an arbitrary value of 10^4 units and the test sera were assigned units in relation to this positive serum. Normal mouse sera gave values of approximately 10 units. Protective mutants gave peak IgG titres of 10^4 units, while the non-protective mutants gave titres of 10^3 units. Unfortunately, as described in materials and methods, IgG and IgM levels were not quantitatively comparable, however, the non-protective mutants did induced high titres of specific IgM, albeit lower than protective mutants.

Indication that protection was due to the combined action of humoral and cellular responses comes from the growth curves of a C5 challenge in HWSH aroA or SL3261 immunised mice. Viable counts one day after i.v. challenge showed considerable inactivation of the challenge. At day 28, a large proportion of the mice sampled appeared capable of inactivating all the challenge of 10^3 cfu within the first 24 hours. At day 70, there was still enhanced inactivation compared to controls but much less than at day 28. This difference suggests that at 28 days the initial clearance is due to a combination of opsonic antibody and activated macrophages, whilst at day 70 the inflammatory macrophage response has died away, leaving only the effects of antibody.

To test cell-mediated responses two isogenic strains, HWSH aroA and HWSH purA were selected. This was because they exhibited such different vaccine efficacies. Differences in the ability to activate macrophages were seen using both in vivo and in vitro assays. In vivo, HWSH aroA was far more effective than HWSH purA at inducing non-specific immunity to listeria. Early resistance to listeria has previously been shown by Eisenstein and co-workers (Eisenstein et al, 1984; Killar and Eisenstein, 1985) although protection was assessed only by mortality following i.p. challenge.

The studies presented in this thesis, show that a single i.v. dose of about $6x10^5$ HWSH aroA induces strong immunity to the listeria challenge, allowing not only control of growth of the challenge, but also its slow elimination from livers and spleens. In contrast, a similar ($3.3x10^5$ cfu) dose of HWSH purA was incapable of inducing any enhanced resistance to the listeria challenge. This inability could be partly overcome by increasing the immunising dose and, therefore, the levels of carriage in livers and spleens. By increasing the immunising dose of HWSH purA to about 10^7 cfu carriage levels could be obtained similar to those seen for aroA mutants when given at doses 10^5-10^6 cfu (see Fig. 3.3). Mice immunised with this dose showed some increased immunity to the listeria challenge (Figs. 6.5/6.6) however, the ability to control the challenge was not as great as in mice which should be carrying similar numbers of an aroA mutant

(Figs 6.3/6.4). This shows that although levels of carrriage in livers and spleen have an effect, other factors exist to make HWSH aroA better than HWSH purA. This is further borne out by the observation that by day 14, mice immunised with $7x10^4$ HWSH aroA had developed similar levels of non-specific immunity to those immunised with 10^7 HWSH purA, even though HWSH aroA carriage levels were 1 log lower.

The increased resistance endowed by a dose of 10^7 HWSH purA may be a result of macrophage activation by the LPS in the bacteria injected. No data is available to compare the effects of a similar dose of killed bacteria. Macrophage activation by low doses of aroA mutants is LPS independent, since it occurs in Lps^d C3H/HeJ mice (Eisenstein et al, 1984; Killar and Eisenstein, 1985; Shafer et al, 1988). The effect of large doses of HWSH purA in C3H/HeJ mice should be studied to further examine the effects of LPS in macrophage activation.

Livers were far more effective than spleens at clearing the challenge. This has been previously reported by Zinkernagel (1976), who found that the livers of S. typhimurium C5 infected mice were better able to eliminate a superinfecting listeria challenge over the first 24 hours after infection than were the spleens. Also, when the growth curves of the aroA and purA strains (Figs. 3.3/3.4) are examined, it can be seen that clearance of the infection always occurs first

in livers. One may speculate that this may be due to different bactericidal capacities of the cells within livers and spleens, or due to differences in the magnitude of the inflamatory response in each organ.

Splenomegaly is a common feature of salmonella infections. Killar and Eisenstein (1985) reported that intraperitoneal infection of C3H lineage mice with an aroA mutant induced splenomegaly which was maximal as the mice began to clear the infection from their spleens. They associated splenomegaly with a period of non-specific macrophage activation, as assessed by immunity to L. monocytogenes. Both HWSH aroA and SL3261 (Table 3.1) induced gross splenomegaly after i.v. infection of BALB/c mice whereas purA and aroA purA mutants induced little splenomegaly. This may indicate that in this system the factors which induce splenomegaly may be related to the mechanisms which induce protective immunity.

As discussed earlier (1.3.3) many workers use DTH as a measure of T-cell mediated immunity in mouse typhoid, although its relevance as a measure of protective immunity is unclear. Since immunisation with aroA mutants is known to induce DTH in some C3H mouse strains, it was of interest to test and compare the aroA purA and double mutants in BALB/c mice. Groups of 8 mice were immunised i.v. with 10^6 cfu and DTH was elicited 21 days later by injecting 10 ug of spent

medium antigen (Plant and Glynn, 1976). The results were not conclusive, however they did suggest that immunisation with both aroA strains induced a DTH response as judged by footpad swelling. Neither histology nor adoptive transfer were performed to confirm that swelling was due to a true DTH response. Mice immunised orally with SL3261 also give a positive DTH response to both salmonella antigens (our unpublished observations; Brown, personal communication) and to cloned hetrologous antigens (Brown et al, 1986). Some footpad swelling was seen in mice immunised with the purA strains, but this was only present at 24 hours having waned by 48 hours. It is known that killed vaccines can induce a 4 hour, Arthus, response (Collins and Mackaness, 1968). No measurements were taken at 4 hours. It is possible that the 24 hour response seen in the purA and aroA purA groups may have been due to an Arthus response which had not yet subsided. The lack of response at 48 hours gives weight to this hypothesis. Unfortunately, time did not permit a repeat experiment with 4 hour measurements.

HWSH aroA and HWSH purA were compared in their ability to prime T-cells. The s.c. route was chosen because it has been shown that the activated macrophages from spleens of mice infected i.v. with salmonella have a supressive effect on T and B-cell proliferation to several mitogens (Lee, Gibson and Eisenstein, 1985; Deschenes et al, 1986). This supression is difficult to overcome since it is not

inhibited by indomethecin, and requires the removal of all macrophages from the cell suspension (Lee *at al*, 1985).

The limited experimental data suggest that HWSH *purA* primes between ten and thirty times fewer salmonella specific T-cells than HWSH *aroA*. Nothing is known about the T-cell subsets involved or their role. Since an antibody response is stimulated, one may assume that some cells are T-helper cells involved in the antibody response to the protein antigens of the bacteria. One can speculate that the differences in the length of persistance of the *aroA* and *purA* strains may be due to a failure of the *purA* strains to induce a full T-cell response. Possibly cytotoxic cells, similar to those seen in listeria and mycobacteria infections (Kaufmann, 1987), are required to lyse chronically infected macrophages. This area requires further study.

When the immune responses to each of the mutants are compared, the reasons for the different vaccine efficacies seen become clear. What is left unanswered, is why one strain induces a protective immune response while another does not. Although *in vivo* persistence at a particular level is essential for stimulating protective immunity, other factors are required. The methods used to construct the mutants should result in strains which are antigenically identical. If one assumes that this is so, one is left with differences in the *in vivo* behaviour of the mutants to

account for the varying vaccine efficacies. When the growth curves of each strain are examined, it appears that both aroA strains and SL1344 purA grow in vivo over the first few days, while HWSH purA and the two aroA purA strains do not. This difference in ability to multiply during the first few days of the infection may be one of the reasons for the different vaccine efficacies. Intracellular growth is essential for induction of T-cell response in listeria (Berche, Gaillard and Sansonetti, 1987). In this study, a non-haemolytic strain of L. monocytogenes, which is unable to survive in vivo, was incapable of inducing a cell mediated immune response, even if levels were kept artificially high by daily i.v. injections. This observation is similar to that of Collins et al (1966) who found that S. pullorum kept at artificially high levels by daily i.v. injections did not induce a cell mediated response. Collins et al proposed that the inabitity of strains such as S.pullorum to induce a cell mediated immune response was because they could not persist in the MPS of infected mice. It is more likely that they do not induce immunity because they do not grow in vivo.

Despite the indications from the growth curves, it is difficult to actually prove that the strains do grow in vivo. Recently, evidence has arisen to suggest that aroA strains do divide in vivo (my unpublished results). These results came from experiments in which aroA S. typhimurium

were being exploited as carriers of foreign antigens (reviewed by Dougan, Hormaeche and Maskell, 1987). A plasmid, pNP2, encoding the nucleoprotein from influenza A virus was introduced into SL3261 (Tite et al, 1988). This plasmid, which allows production of very high levels of nucleoprotein, is very unstable in SL3261. When the growth curve of SL3261 (pNP2) was followed after i.v. infection of BALB/c mice, it was evident that the proportion of plasmid containing (ampicillin resistant) bacteria present in livers and spleens decreased from 100% on day 1 to 0% on day 21. SL3261 which had lost the plasmid (ampicillin sensitive) remained at levels similar to the day 1 levels of SL3261 (pNP2). Such segregation could only occur if cell division had taken place. This observation also suggests that SL3261 divides in vivo for at least 21 days, a period much longer than previously assumed (Hoiseth & Stocker, 1981). Attempts are being made to transfer pNP2 into purA and aroA purA strains to see if such segregation occurs. This may show whether these strains can divide in vivo.

Spleen cells from mice immunised 3 days previously with either HWSH aroA or HWSH purA were tested for NK activity using the NK sensitive cell line YAC-1. Cells from both aroA and purA immune mice showed increased levels of specific lysis. YAC-1 cells are also sensitive to lysis by activated macrophages. This result does not, therefore, formally prove that NK activity was present since lysis may have been by

LPS activated macrophages. This area needs further study, with experiments in C3H/HeJ mice, and experiments including NK resistant targets (such as P815) and known inhibitors of NK activity (such as anti-asialo-GMI: Kasai *et al*, 1980) as controls. NK activity has been reported to be enhanced *in vivo* by infection with the intracellular pathogens *L. monocytogenes* (Holmberg and Ault, 1986) and *Legionella pneumophila* (Blanchard *et al* 1988) and *in vitro* by glutaraldehyde fixed *S. typhimurium* (Tarkkanen *et al* 1986). The *L. pneumophila* induced NK cells (Blanchard *et el*, 1988) both produced and responded to IFN-gamma. An attempt was made to detect IFN-gamma producing cells in the spleen cells tested for NK activity. The attempt was unsuccessful (Data not shown) probably because too few cells (10^6) were tested. Induction of gamma interferon production requires further study.

Intravenous immunisation with HWSH *aroA* induced early immunity to virulent salmonella. The salmonella challenge proved far more difficult to control than the listeria challenge. When challenged 7 days after immunisation, the mice could only slow the growth slightly but by 14 days post-immunisation, the mice could control the growth of the challenge before it reached lethal levels. Immunisation with HWSH *purA* was ineffective. It should be remembered that the HWSH challenge, which represented over $5x10^4$ LD_{50}'s, was a far more substantial challenge than the 80 LD_{50} listeria

challenge, so the protection seen at day 14 is excellent. Zinkernagel (1976) found that CBA mice infected with S. typhimurium C5 developed immunity to listeria before immunity to salmonella challenge. This was interpreted as meaning that control of a salmonella infection requires specific factors other than macrophage activation. The early salmonella challenge experiments confirm this view. When the listeria challenge in aroA immune mice are compared at days 7 and 14 (Figs. 6.3/6.4), it can be seen that the ability to control the listeria challenge is fully developed by day 7, and remains unchanged at day 14. However, the capacity to control the salmonella challenge sucessfully develops between days 7 and 14 (Figs. 6.1/6.2). Much of this increased protection stems from the inactivation of the challenge during the first 24 hours.

The rise in antibody titre seen between days 7 and 14 again suggests that antibody, as proposed by Zinkernagel (1976), is this specific factor. However, antibody is more likely to be one of several factors, rather than the only one. Macrophages activated by infections other than salmonella kill S. typhimurium poorly. Mice infected with listeria show only low levels of non-specific immunity to salmonella (Zinkernagel, 1976). Peritoneal macrophages activated in vivo by BCG or listeria show varying abilities to kill different pathogens (van Dissel et al, 1987). In this study, it was observed that activated macrophages were less

effective at killing salmonella *in vitro* than they were at killing listeria or *Toxoplasma gondii*. Macrophage activation does not necessarily endow bactericidal capacity. It has been shown that the bactericidal and tumoricidal activities of macrophages activated *in vitro* and *in vivo* by either infection or lymphokines could be dissociated (Nacy, Leonard and Meltzer, 1981; Hopper and Cahill, 1983; Campbell, Czuprynski and Cook, 1984; Campbell, Canono and Cook, 1988). This further suggests that specific factors are required to promote efficient killing of salmonella by activated macrophages.

It is possible that macrophages require T-cell derived lymphokines to be stimulated to kill salmonella effectively, rather than just control their growth. The time course of the T-cell responses in salmonella infections has not been studied, however a T-cell response, as judged by DTH, is absent in M525 infected BALB/c mice at day 7, but present at day 14 (Hormaeche *et al*, 1981). Studies with nude mice show that T-cells are essential for the late phase control of *S. typhimurium* infections (O'Brien and Metcalf, 1982). The macrophages activated by T-cell independent mechanisms can control the growth of the salmonella but not clear the infection from livers and spleens. Clearance of *aroA* infections (Fig.3.3; Killar and Eisentein, 1984; O'Callaghan *et al*, 1988) does not commence until late in the second week of the infection, again suggesting that specific factors

become available which enhance the bactericidal activity of the macrophages.

As well as the obvious differences seen between aroA and purA mutants derived from the same parental strain, differences in efficacy were also obvious between the two aroA and the two purA mutants tested. The differences seen between the capacity of SL3261 and HWSH aroA to vaccinate were slight. They may result solely from the different lengths of persistence of the two strains since on day 28, the time of the first C5 challenge, SL3261 was found in livers and spleens at levels at least 1 log higher than HWSH aroA (Confirmed by counts taken at time of C5 challenge - data not shown). This may have led to higher residual levels of activated macrophages in the SL3261 immune mice. This is also suggested by the greater clearance of C5 by SL3261 immune mice seen in the first 24 hours of the infection. It is likely that some activated macrophages were still present, since we have found non-specific resistance to S. dublin challenge in HWSH aroA immune mice at this point which wanes considerably by day 56. The levels, however, cannot be too great since Killar and Eisenstein (1985) found no non-specific resistance to listeria challenge by day 28 of a SL3235 infection in C3H mice and Schafer et al (1988) had similar results when the kinetics of tumor cells killing was followed.

Differences have been reported in the ability of salmonella aroA mutants to immunise cows (Smith et al, 1984a). aroA derivatives were constructed from three mouse virulent S. typhimurium strains of different biotypes : UDC, WRAY and FIRN. The UDC aroA mutant was an effective vaccine whilst the WRAY and FIRN aroA mutants were not. The FIRN biotype is not normally associated with bovine salmonellosis, and this may account for its lack of efficacy but the WRAY parental strain is highly calf-virulent. Both the FIRN aroA and the WRAY aroA (SL3261) mutants are effective as vaccines in mice.

Differences between the protective capacity of the purA mutants were far more pronounced than those between aroA mutants. The growth patterns of the two purA mutants were very similar. However, SL1344 purA and not HWSH purA appeared to grow slightly over the first few days of the infection (Fig. 3.4). SL1344 purA also induced some splenomegaly. The degree of protection induced by SL1344 purA at day 28 was not as great as that induced by SL3261 and varied considerably by day 70, despite the fact that the immunising infection had only recently been cleared (Fig. 3.4). The reasons for this lack of long-term protection are unknown, but may be due to a lack of antibody, or failure to prime a correct memory T-cell response, or may just be because the challenge regime used was too severe to show any protection. Challenge growth curve experiments, similar to

those shown in Figs. 4.3/4.4, may help determine the reasons for the lack of protection.

It has often been assumed that the more virulent the parent strain that a vaccine is derived from, the better the vaccine will be. The data presented above suggest that this is not so. HWSH is more virulent than SL1344 but the auxotrophic strains derived from it are not as effective as live vaccines. The reasons for the differences in the efficacy of vaccines derived from different virulent strains presented here and seen in other studies (Hormaeche, personal communication) are unknown. However, should they also occur in vaccines derrived from S.typhi it could present serious problems. More work should be done to discover the reasons for such differences in the mouse model so that good and bad S,typhi vaccines may be identified at the volunteer study stage. If it is impossible to discover why one strain makes a better vaccine than another, challenge studies in volunteers may be required to test efficacy in order to avoid large field trials with an ineffective preparation. In light of the risks of challenging volunteers, both approaches present ethical problems.

7.3 Experimental models to study vaccine efficacy.

Collins and co-workers (Collins, 1974) have always maintained that the best measure of vaccine efficacy is by following the growth of a sub-lethal challenge. The data presented in this thesis confirm this view. In many challenge experiments, it was possible to compare the LD_{50} of the challenge with its growth in the livers and spleens of mice. It can be seen that, in all cases, in mice which were strongly protected in terms of LD_{50}, the i.v. challenge was almost completely inactivated within the first 24 hours. The surviving bacteria did then grow in livers and spleens for several days before being controlled. Chronic infection always resulted. It therefore seems that, in the i.v. system, much of the protection is due to the ability of the mice to inactivate most of the challenge within the first 24 hours after challenge. A similar view was taken by North (1974). In this study he found that the ability of M. tuberculosis immune mice to control a listeria challenge was dependent on the ability of the activated macrophages within their livers to reduce the challenge to sub-lethal levels within the first 8 hours. This inactivation was dependent on resident non-dividing cells since it was resistant to cortisone and gamma-irradiation. Challenge organisms surviving the initial inactivation grew but were controlled by a de-novo response which was sensitive to cortisone and irradiation.

Whilst it is true that much of the protection seen in the i.v. challenge experiment reported in this thesis is due to this initial inactivation, it must be stressed that this initial inactivation alone would not be sufficient to protect BALB/c mice against virulent salmonella challenge. The challenge dose was reduced to levels of between 10 and 100 cfu at 24 hours. This number of salmonella would be lethal for a naive mouse, as shown by the LD_{50} determinations where doses of <10 cfu consistently killed all mice. The surviving bacteria grew rapidly in livers and spleens of infected mice but, whilst the controls quickly succumbed to the infection, the immunised mice were able to control the infection by about Day 5 suggesting that vaccination had enhanced the capacity to mount a cellular response.

Antibacterial immunity, expressed as constant clearance of the challenge from livers and spleens, as shown by Collins and co-workers, was evident only with listeria challenge. Collins (1974) found that early after vaccination, when the mice were still colonised at high levels, a super-infecting salmonella challenge was rapidly eliminated from the host tissue without growing. This clearance did not affect the behaviour of the immunising infection. As the time between vaccination and challenge lengthened, the ability to immediately control the challenge decreased, and the challenge grew for a period of several days before being

controlled. There are several important differences between this work and the experiments presented above: Collins used out bred CD1 (salmonella resistant) mice, vaccination was with sublethal doses of virulent salmonella which may not have actually cleared from the mice, since the limit of detection was only 200 cfu/organ.

The i.v. challenge system is a very severe test of immunity. When results from i.v. immunisation and challenge are compared with oral/oral experiments, the limitations of the system are evident. Some of the mice immunised with either HWSH aroA or SL3261 died when challenged with doses of C5 which were much lowerthan the calculated LD_{50}. However, when mice immunised orally with SL3261 were challenged orally with SL1344, 57 days later, no deaths occured (Maskell et al, 1987b). Growth of the challenge was quickly controlled by all mice, and levels never went above 10^4 in tested organs. In more recent experiments, (Dougan et al, 1988) it has been shown that BALB/c mice, immunised orally with SL3261 are completely protected against oral challenge, with doses of 10^{10} SL1344, as long as 70 days post-immunisation. Similar high levels of immunity to oral challenge can be seen even 6 months after oral immunisation (Hormaeche, personal communication).

Complete immunity to an i.v. challenge with strains as virulent as those used in this study is too much to expect,

especially in salmonella susceptible mice. A more reasonable test of vaccine efficacy in the i.v. system may be to use a less virulent challenge strain. Large i.v. doses are needed because small doses would be completely eliminated in the first day of the infection, possibly giving a misleading view of the immune responses. The most representative model is the oral model. If the results from the mouse model can be extrapolated to human disease, the immunity seen to an oral challenge with 10^{10} virulent S. typhimurium suggests that aromatic dependent S.typhi will be excellent oral vaccines for human use, especially in light of the normal dose sizes in human typhoid (Blaser and Newman, 1982).

7.4 The future for auxotrophic S. typhi vaccine candidates.

The complete ineffectiveness of both the aroA purA S. typhimurium mutants as oral vaccines puts great doubt on the future use of aroA purA S.typhi as human vaccine. This is further born out by the poor immune responses in human volunteers who took these vaccines orally. While aroA is still a prime candidate attenuating lesion for S.typhi mutants, purA appears unacceptable. It is desirable to have a second attenuating mutation in human vaccine strains. Since the discovery of the ineffectiveness of aroA purA S. typhimurium strains, work has been in progress to find a second attenuating mutation which would be safe and immunogenic both on its own and in combination with aroA.

Since a lesion in another metabolic pathway may present problems similar to those encountered with *purA*, it was decided to introduce a second mutation in the aromatic biosynthetic pathway (Dougan *et al*, 1988). The *aroC* gene, which encodes the enzyme chorismate synthase was chosen. Chorismate synthase catalyses the step in the aromatic biosynthetic pathway after *aroA* (Fig 1.2). *aroC* maps at 47 minutes on the *S. typhimurium* genetic map while aroA maps at 19 minutes (Sanderson and Roth, 1983). An *aroC* mutant of SL1344 has been constructed and an *aroA* deletion was then introduced to give an *aroA aroC* double mutant. Both strains were found to have similar LD_{50}'s and i.v. growth curves to *aroA* derivatives of SL1344. BALB/c mice were immunised orally with SL3261, SL1344 *aroC* or SL1344 *aroA aroC* and challenged orally with SL1344. All three strains gave good levels of immunity to oral challenge both 28 and 70 days post immunisation. Since SL1344 *aroA aroC* proved to be such an excellent vaccine, the *aroC* gene was cloned from *S. typhi* (Charles *et al*, in preparation) and inactivated by insertion of a kanamycin resistance cassette. The inactivated gene was then re-introduced into a *S.typhi aroA* mutant (Dougan *et al*, 1987) to produce a *S.typhi aroA aroC* mutant. The presence of the kanamycin resistance gene in this strain means that it is unsuitable for use as a human vaccine. Work is under way to create an *aroC* deletion with no such antibiotic resistance. Mutants in the *aroD* gene of the aromatic pathway have also found to attenuate *S. typhimurium* (Miller *et al*,

MGG in press). aroD has also been suggested as an attenuating mutation in S.typhi vaccines (Stocker, 1988).

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