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THE B CELL RESPONSE OF THE MOUSE TO ANTIGENS OF A LIVE *SALMONELLA TYPHIMURIUM* VACCINE

BY

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled, "The B Cell Response of the Mouse to Antigens of a Live Salmonella typhimurium Vaccine", submitted by Eleanor Bergen Mackie in a partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

An aromatic-requiring (aro A^-) mutant of a smooth strain of Salmonella typhimurium was used to characterize the B cell response of known genetically susceptible and resistant mice. The use of a viable aromatic auxotroph with smooth lipopolysaccharide (LPS) permitted circumvention of previous problematic areas of immunization of man and animals through the use of killed or rough strains, or toxicity from extracts of Salmonellae. It could be used safely in the living form, permitting limited replication within the host's cells including macrophages, thereby stimulating the entire host's immune repertoire similar to a natural infection.

Through the use of mice chosen for differences at the Ity locus it was possible to compare the B cell response both in whole serum and at the cellular level through lymphocyte hybridoma technology. Test antigens from a two-step sucrose gradient representing principally outer membrane and inner membrane, commercially prepared smooth S. typhimurium LPS, LPS core fractions, and "in house" prepared porin were used in an enzyme-linked immunosorbent assay (ELISA). Through the use of anti-mouse IgG, IgM and IgA, serum titres and the relative distribution of reactive B cells in spleen, mesenteric lymph nodes and Peyer's patches were followed over a forty-five day oral immunization schedule.

The genetically susceptible, homozygous Ity^S/Ity^S, represented by Balb/c, showed no IgA response over the immunization period with a retarded and depressed response to LPS. More than 1000 hybridomas were tested at thirty-two days of the immunization schedule and more than six hundred at forty-five days. The resistant F_1 DBA X Balb/c

(heterozygous Ity^r/Ity^s, Mendelian dominant and same H-2 haplotype as Balb/c) had an intermediate response to LPS. The Ity^r wild-type represented by RBF/Dn had the highest response to LPS although this was still small in proportion to the overall response to antigens of the vaccine bacterium. Of this small fraction of the total, the majority of hybridoma products from RBF/Dn mice were directed toward smooth LPS. There was little response to porin proteins in any of the mice. The greater reaction, both in numbers of reactive hybridoma supernatants and whole serum antibody was to the inner membrane fraction and was especially high for an inner membrane preparation of a rough Rc mutant (uridine diphosphategalactose-4-epimeraseless mutant).

The response was different in time, rate and the production of immunoglobulin class in the three strains of mice when all groups of antigens were considered. The RBF/Dn showed the earliest and most comprehensive response.

The control of B cell responsiveness is considered to be polygenic and unrelated to the Ity locus with the exception of the retarded response of Balb/c mice to LPS and the lack of IgA production by the same mice.

It is proposed that the B cell response is important for a positive outcome of a natural infection and that its effectiveness is exerted later (after two weeks) in the course of the disease. It seems likely that the protective effect of the vaccine bacterium is it's ability to induce antibody to internal antigens.

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ABBREVIATIONS USED IN THIS THESIS

LPS	-	lipopolysaccharide (smooth)
O.M.	-	outer membrane
I.M.	-	inner membrane
I.M.(Rc)	-	inner membrane of the Rc mutant
P.P.	-	Peyer's patches
M.L.N.	-	mesenteric lymph nodes
Ig	-	immunoglobulin
ANOVA	-	analysis of variance
F	-	F-testing
P.W.M.	-	pokeweed mitogen
Spl.	-	spleen
d.	-	day(s)
ELISA	-	enzyme linked immunosorbent assay
I.P.	-	intraperitoneally
ICR mice	-	Institute for Cancer Research Swiss Webster mice

INTRODUCTION

A. Review of the Literature

Murine typhoid, a naturally occurring disease in inbred mice, the causative agent of which is Salmonella typhimurium, provides a natural animal model for typhoid fever in man. Although it has been extensively studied both genetically and antigenically, the immune response of the host to this intracellular pathogen, which is capable of replication within the host's macrophages, has escaped definitive characterization. Some explanations include the fact that pathogenic species for a given host species often produce a disease state which destroys the host before an immune response can be mounted. Carrier strains often lack some of the antigenic structures, preventing definitive proof of beneficial immune stimulation by these structures. Moreover, variability in response to known antigens such as O-antigens, particularly from Salmonella species with tropism for other host species has frustrated attempts to delineate the response with accuracy.

1. Immunologic, genetic and immunogenetic background of mouse resistance to Salmonella typhimurium.

a) History of the concept of genetic control of resistance and susceptibility to Salmonella infections.

In the 1930's, Webster (J. Exp. Med., 57, 793, 1933) used groups of mice rendered inbred through selected brother/sister matings which were either susceptible or resistant to B. enteritidis. Salmonella enteritidis, as it is now known, also causes a disease in mice similar to that caused by S. typhimurium. The selection was based on survival after a

dose of 5 x 10^6 bacteria was administered by gastric intubation in animals which were carefully bred and housed in facilities where any bacterial cross-infection was minimized (i.e. "specific pathogen free" environment). The factor(s) governing resistance were identified as dominant and not sex-linked. Additional experiments demonstrated a dose dependancy.

However, Gowan (Bacteriol. Rev., 24, 192, 1960), and Stadler and Gowan (J. Inf. Dis., 100, 284, 1957), established through more than thirty generations of inbreeding, strains of mice ranging from strongly resistant to highly susceptible with intermediate strains, as well. Gowan concluded that a number of genes influenced resistance and that their combinations contributed to the host range from resistant to susceptible. In the work of Stadler and Gowan, regional body irradiation of five of these inbred strains of mice was used in an attempt to localize the cells and/or organs affected in decreasing the resistance of S. typhimurium for which the mouse strains had been selected. No real conclusions were reached.

Unremarkable progress in the characterization of the genetic control of host responsiveness marked the following decades. Great emphasis was placed on the search for mechanisms of resistance to intracellular pathogens (Blanden, et al, J. Exp. Med., 124, 585, 1966; Rowley, et al, Aust. J. Exp. Biol. Med. Sci., 46, 447, 1968). Interest in attributes of the bacterium required for effectiveness as a vaccine are referred to below, and are reviewed, together with resistance mechanisms, in detail by Collins (Bacteriol. Rev., 38, 271, 1974).

Recently, important extensions of Webster's and Gowan's work have contributed to the study of the genetic characterizations of innate susceptibility and resistance of both inbred and outbred mice to S. typhimurium. They include both non-specific resistance and immune responsiveness.

b) Immunogenetic studies on inbred mouse strains.

In 1972 Robson and Vas (J. Inf. Dis., 126, 378, 1972) reported a systematic study of inbred mice infected intraperitoneally with S. typhimurium showing three patterns of survival. Susceptible mice included C57B1/6j, Balb/c and C3H/HeJ strains. These mice died within a week of infection with doses as low as one to ten bacteria. DBA/2j mice were intermediate and A/J mice were resistant surviving infectious doses of 10^4 or more bacteria beyond twenty-eight days. F_1 crosses in these cases showed survival characteristics similar to that of the resistant parent strains. Phenol-killed bacteria, ribosomal vaccine and live, avirulent S. typhimurium (strain M206 used as test antigen in some of the experiments of this thesis) did not protect the susceptible strains from challenge with virulent S. typhimurium (strain C5, also used as a test antigen in some of the thesis experiments). Plant and Glynn (J. Inf. Dis., 133, 72, 1976) did not observe an intermediate resistance to the same virulent strain of S. typhimurium, but simply susceptible and resistant mouse strains. Examination of the F_1, F_2 and parental backcross generations of one resistant and susceptible combination (CBA and Balb/c matings) showed that resistance behaved as a simple Mendelian dominant. Moreover, it was not linked to H-2 genes. Strains of mice which were

resistant also showed an increased ability to demonstrate delayed-type hypersensitivity suggesting T cell involvement, an observation later corroborated by Killar and Eisenstein (J. Immunol., 133, 1190, 1984) respecting mice in the C3H lineage. However, Hormaeche (Immunology, 37, 311, 1979) had found this variable.

Since the work of Robson and Vas, three loci governing susceptibility to S. typhimurium have been identified. In a series of genetic experiments to characterize gene(s) responsible for susceptibility of mice to S. typhimurium infections, O'Brien and colleagues mapped to chromosome four the gene believed to cause C3H/HeJ mice (one of the susceptible groups of Robson and Vas) to be refractory to the effects of LPS (O'Brien, et al, J. Immunol., 124, 20, 1980). These mice are concomittantly highly susceptible to S. typhimurium infections as also shown by the work of Robson and Vas. The Lps^d allele (for LPS defective) is, however, not linked to the dominant autosomal gene named Ity (for immunity to typhimurium) mapped to chromosome one by Plant and Glynn (Clin. Exp. Immun., 37, 1, 1979). The latter controls susceptibility to S. typhimurium in C57B1 and Balb/c mice. Its exact position on chromosome one was determined by Taylor and O'Brien (Taylor and O'Brien, Infect. Immun., 36, 1257, 1982) and is closely linked to but distinct from the locus controlling natural resistance to Leishmania donovanii (O'Brien, et al, Nature, 287, 440, 1980).

A third locus controlling differential sensitivity of inbred mice to S. typhimurium is the X-linked gene, xid, which renders certain strains of mice carrying this allele (eg. CBA/N) and the F_1 male offspring from

females of the strain B cell defective and Salmonella-susceptible, as well.

Since the nature of immune mechanisms in Salmonella infection is unclear, the xid mice were chosen by O'Brien (O'Brien, A.D., et al, J. Immunol., 126, 1368, 1981) to evaluate the role of antibodies, hence B cells, in resistance to murine typhoid. Any T cell-dependent delayed hypersensitivity involvement would be intact in such mice. The defective male F_1 mice, immunized with killed preparations of a virulent strain of the bacterium did not develop significant amounts of IgG or IgM antibodies but their resistance could be increased to the level of their female counterparts by passive transfer of immune serum from the females and the gamma-globulin fraction extracted from the serum of females. Moreover, the resistance of the F_1 males to subsequent intraperitoneal challenge could be increased by reconstitution with immunologically normal F_1 bone marrow cells. Taken together these experiments were strongly supportive of a significant role of humoral immunity in Salmonella infections.

In separate experiments to test the involvement of the T cell-dependent, cell-mediated immune response in Ity regulated S. typhimurium susceptibility, O'Brien and Metcalf (J. Immunol., 129, 1349, 1982) used athymic nude mice of Ity^r phenotype, infected them intravenously with S. typhimurium and analysed the kinetics of Salmonella replication in their livers and spleens. This was compared with the pattern of bacterial multiplication in euthymic, nu/+, littermate controls. No differences in bacterial growth in spleens and livers were observed between

nu/+ and nu/nu mice in the first thirteen days of infection. This time period covers the early phase of infection during which mice must be able to restrict the growth of S. typhimurium in order not to succumb. Hence, it was concluded that T cell-mediated immunity did not play a role at this early stage of infection thought to be regulated by the Ity gene.

After showing that decreasing the phagocytic capacity of splenic macrophages by administration of silica decreased the LD_{50} to S. typhimurium 100-fold (O'Brien, et al, Infect. Immun., 25, 513, 1979), the level of regulation of the Ity gene was narrowed down to the level of surviving bacteria (i.e. the degree of bacteriostasis) that accumulate within resident macrophages of the liver and spleen (Swanson and O'Brien, J. Immunol., 131, 3014, 1983). In both Ity^s and Ity^r mouse strains, ninety-nine percent of bacteria were cleared from the blood within two hours after intravenous injection. Uptake of S. typhimurium by splenic and hepatic macrophages was similar regardless of Ity genotype. In vivo phagocytosis was followed by a thirty to sixty percent decline in viable bacteria in both instances. However, bacterial numbers in reticuloendothelial organs of Ity^s mice were significantly greater than in Ity^r mice by twenty-four hours after infection. This was interpreted to be a function of the degree of bacteriostatic activity or containment by the macrophages.

The involvement of macrophages in susceptible mice controlled by the Ity gene had also been implicated by others. Hormaeche (Immunology, 41, 973, 1980) demonstrated that S. typhimurium divides faster in the spleens

of naturally susceptible Balb/c than in resistant mice, but could not demonstrate consistently better survival (i.e. from diminished bactericidal activity) in a temperature sensitive, avirulent mutant which could not divide at thirty-seven degrees Celsius (Hormaeche, Immunology, 42, 569, 1981). He had earlier demonstrated differences in net growth rates in livers and spleens of different strains of mice (Immunology, 37, 319, 1979, and Immunology, 37, 329, 1979).

Other factors impinge on resistance and susceptibility and a variety of experiments to identify and characterize them have been performed. Some of those which relate to this thesis work, in particular the polygenic control of antibody responsiveness to Salmonella antigens are described below.

c) Immunogenetic studies on outbred mouse strains selected for specific characters.

In outbred mice, individual genotypes for polygenic characters are normally distributed making the mice an ideal choice for the assessment of natural infections and the immune response to naturally occurring, polydeterminant antigens to which individuals may be "naturally" exposed. In inbred strains, the distribution of individual genotypes becomes compressed with those which occur most frequently in a population becoming preferentially fixed (Georghiu, et al, Clin. exp. Immunol., 59, 177, 1985).

The classic work of Biozzi with respect to one of two major approaches to genes controlling immunoresponsiveness is reviewed in

detail (Biozzi, G. et al, Restricted and general polygenic regulation of antibody responsiveness. Immunogenetics and Immune Regulation, ed. B. Benacerraf. Masson Italia Editori, Milano, 1982). The approach, based on the production, by selective breeding, of high and low antibody responder lines of mice and guinea pigs to natural polydeterminant immunogens such as heterologous erythrocytes, also included Salmonellae. Specifically, flagellar and somatic antigens of Salmonella typhimurium and Salmonella oranienburg were chosen as two chemically different, non-cross reacting antigens on each of two distinct bacterial species of the same genus. The number of generations which were required to reach the selection limit in the breeding program was sixteen and twelve for the secondary response to flagellar and somatic antigens respectively. The selection limit is that generation beyond which further selection does not result in either an increase or a decrease in responsiveness to intraperitoneally administered, killed cells of Salmonellae. The difference in antibody titres between the high and low lines was 90-fold and 85-fold for the two antigens; the estimated number of independent immunogenetic loci by calculating additive variance (heritable portion of cumulative response, both high and low) relative to that of the foundation population, were four to seven for flagellar antigens and two to four for somatic antigens. Various degrees of non-specific effects were evident in responder lines for all selections.

This was carried farther to show that both genetic background and environmental factors modify the phenotypic expression of H-2 associated genes participating in the polygenic control of antibody responsiveness

to Salmonella antigens (Bouthillier, et al Immunology, 46, 67, 1982). The H-2 locus did not intervene in the control of the secondary response to somatic antigens, but an H-2 effect was observed in the primary response in females only. This was measured in interline hybrids: i.e. with a heterogeneous genetic background equivalent to the F_2. In the case of the flagellar antigens, the H-2 locus was responsible, in the secondary response, for about fifty percent of the interline differences in the two sexes. In the primary response it was observed only in females. Moreover, by use of the same variance analysis referred to above, it was shown that the secondary response was subject to simpler control than the primary response. Conditions of immunization and sex affecting the intervention of genes associated with the H-2 locus implied that environmental factors (eg. hormonal) can influence gene interaction in a polygenic system giving rise to preferential expression of some of the relevant genes.

In another system, it was shown that a good anti-Brucella humoral response to intraperitoneal immunization with a low dose (markedly sub-optimal), although inherited as a dominant character, was under polygenic control and independant of H-2 haplotypes or Ig allotypes (Cannat, et al, Ann. Immunol. (Inst. Pasteur), 130C, 675, 1979); that the phenotypic expression of at least one of the genes involved was sex-dependant and influenced by hormonal environmental factors; that one of the sex-limited genes was associated with a "b" coat color gene in C57B1/6 and DBA/2 mice. This is mentioned to emphasize that other intracellular

gram-negative bacterial systems corroborate the findings with Salmonellae.

In the Biozzi high and low antibody responder mouse lines an inverse correlation between macrophage activity (antigen uptake, lysosomal and catabolic activity) and antibody response was demonstrated by Wiener and Bandieri (Eur. J. Immunol., 4, 457, 1974) in a non-bacterial system and by Mouton, et al, (Ann. Immunol. (Inst. Pasteur), 135D, 173, 1984). In the former case, ^{125}I-labeled keyhole limpet hemocyanin, ^{14}C-labeled type three pneumococcal polysaccharide and ^{14}C-labeled levan were used to follow uptake, localization and disappearance in macrophages. Levan, which elicited equivalent IgM synthesis in the two lines had a similar fate within macrophages from the two lines. Keyhole limpet hemocyanin and pneumococcal polysaccharide which evoked maximal and minimal antibody production, respectively, were handled differently: the intracellular digestion of hemocyanin was rapid in macrophages from responder lines and persisted on the membranes of macrophages from high responder lines. More pneumococcal polysaccharide was retained but not on the membranes. Hence, it was postulated that macrophages express genes which control humoral immune responses through the manner of antigen presentation on the membrane. Similarly, Mouton, et al, found that macrophages from low line mice catabolized antigen very rapidly, offering an explanation for the requirement of a higher threshold dose in these lines to invoke antibody production. It also offered an explanation for a greater

improvement of an antibody response when repeated antigen doses were given to low line mice compared with high responder mice.

Moreover, as reviewed by Biozzi (Adv. Immunol. 37, 189, 1984), the high and low antibody responsiveness in the two mouse lines was not, therefore, dependant on the amount of antigen phagocytized in the spleens, but rather on the persistance of the antigen in immunogenic form. Interline differences in antibody production which concerned all classes and subclasses of antibody: IgA, IgM, IgG_1, IgG_2, IgE (reagins), was considered an additional important finding. Macrophage activity confirmed those described by Wiener (above); namely, morphologically greater pseudopod emission in macrophages from high lines and physiologically increased acid hydrolysis in macrophages from low lines. As such, the genetic difference between high and low line macrophages was essentially expressed at the level of control of bacterial growth (bacteriostatic activity) rather than at the level of killing of the ingested bacteria. This corroborated O'Brien's finding in inbred mice of Ity^S genotype, above. Furthermore, in spite of increased antibody producing capacity the high responders were innately much more susceptible to S. typhimurium. Two proposals were put forward: firstly, the genetic defect was phenotypically expressed by a poor innate bacteriostatic activity and/or a lack of activation by T cell-released lymphokines; secondly, when antibodies are produced in large amounts as in the high line, this could reduce the efficiency of the T cell-mediated response.

The point is made in this review by Biozzi, that antibody response to complex immunogens such as microorganisms is similar in various inbred

strains of mice. This is discussed in detail in the DISCUSSION section of this thesis in light of the lack of substantiation of that view with the approach used for this thesis work.

2. Bacterial Strains.

a) Immunization with live strains.

Live vaccines for Salmonella infections have been considered to be the most efficacious. However, great variability in protectiveness has historically frustrated the use of any one strain of a given characteristic for a defined purpose. Some examples of the qualifications thought to be required follow.

Kenny and Herzberg reported, in 1968 (Kenny and Herzberg, J. Bacteriol., 95, 406, 1968) that intraperitoneal, intravenous or subcutaneous immunization with smooth, heat-killed vaccines and a rough live vaccine protected against homologous but not heterologous challenge. In contrast, intraperitoneal immunization with a smooth, live vaccine protected against both. Moreover, the duration of protection conferred by the rough strain vaccines correlated with the endotoxic content of the immunizing strain. Collins (J. Bacteriol., 97, 676, 1969) found that the survival percentage observed in specific pathogen free mice vaccinated by intravenous, intraperitoneal and subcutaneous injection with ethyl alcohol-killed vaccines varied extensively depending on the route of challenge and that the vaccination failed to prevent growth of the challenge organism in vivo. On the other hand, mice which had been vaccinated with living bacteria contained challenge bacteria to the regional lymph node and subsequently eliminated them. Neither route of challenge

which was also by one or the other of the immunizing routes, nor the regimen used in the vaccination had any appreciable influence on the level of antibacterial immunity detected in the organs of the reticuloendothelial system at the time of challenge.

Germanier (Infect. Immun., 2, 309, 1970) investigated the protection induced by different live rough mutants (representing LPS regions Ra to Re) of S. typhimurium on intraperitoneal injection and found that all uridine diphosphate-galactose-4-epimeraseless mutants (Rc) were much more potent immunizing agents than any other mutants. This immunizing capacity was not dependent on the persistence of viable bacteria in the mouse, nor was it correlated with virulence or complexity of cell wall polysaccharide. Similar results were confirmed with subcutaneous, intravenous and oral immunization. It was thought that this outstanding protective capacity was due to the fact that when galactose was supplied exogenously (as occurrs in vivo), smooth cell wall lipopolysaccharides were synthesized, but the mutants were rendered avirulent as a result of galactose-induced bacteriolysis attributed to different activities of galactokinase and galactose-1-phosphate-uridyl transferase. This lysis was considered due to intracellular accumulation of galactose-1-phosphate and UDP-galactose or both in varying degrees depending on the mutant (Germanier and Fürer, Infect. Immun., 4, 663, 1971). Double mutants, in which the phenotypic expression of smooth type was prevented by a secondary mutation were ineffective in protection studies. No crossprotection between S. typhimurium and S. enteritidis which share O antigens one and twelve could be demonstrated (Germanier, Infect. Immun., 5, 792, 1972).

Moreover, comparison with immunization using a killed smooth vaccine strain showed only short term inhibition of multiplication of challenge bacteria. These experiments were of four and eight week's duration and comprised challenge with virulent bacteria two and six weeks after immunization. Classical Widal agglutination tests were done on sera at two weeks after immunization only, with agglutination titres from mice immunized with heat inactivated cells being the highest. Those to viable smooth strains were low to intermediate. The Widal test was considered a measure of antibody production to O antigens.

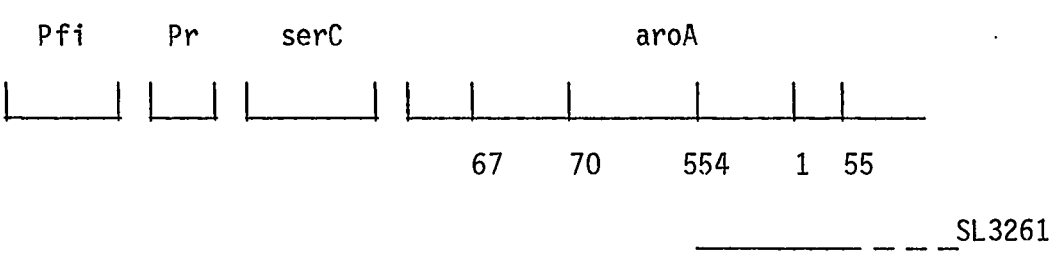
In a different vein, Hormaeche, et al (Immunology, 43, 547, 1981) put forward the argument that lack of immunity when vaccination (intravenous or subcutaneous) was done with living bacteria was due to the use of Salmonella strains which did not establish the carrier state. A strain which did establish the carrier state effectively immunized susceptible strains such as Balb/c against virulent challenge.

b) The vaccine strain of S. typhimurium.

In 1981, Hoiseth and Stocker reported the construction of strains of S. typhimurium with nonleaky, nonreverting blocks in the aromatic biosynthetic pathway (Hoiseth and Stocker, Nature, 291, 238, 1981). These strains were isolated as secondary deletion or deletion-inversion mutations in strains made aroA554::Tn10. That is, the tetracycline resistance transposon, Tn10, was inserted in gene aroA to produce non-reverting, aromatic requiring derivatives of virulent S. typhimurium. Such strains proved to be nonvirulent, it was believed, because of their requirement for p-aminobenzoate and for 2,3-dihydrobenzoate which are not

available in mammalian tissues. Salmonella, unlike vertebrates cannot assimilate exogenous folate and must synthesize it from para-aminobenzoic acid, the absence of which from vertebrate tissues is shown by the efficacy of sulfonamide chemotherapy. The protective capacity of these strains for mice, including the susceptible Balb/c was clearly demonstrated both via oral and parenteral routes. Some of the strains were found effective as live vaccines in mice and calves.

One vaccine strain, SL3261 (a derivative of virulent strain SL1344) was deleted for one segment of gene aroA. This gene, at nineteen minutes on the linkage map (Sanderson and Roth, Microbiol. Rev., 47, 410, 1983) specifies 3-enolpyruvateshikimate-5-phosphate synthetase, which catalyzes a reaction of the aromatic biosynthesis pathway. The diagram which follows gives the inferred deletion and possible extension of the deletion (or an inversion - broken line) relative to a map of the aroA gene. The positions of point mutations in the cluster (indicated by numbers) are shown together with other proximal genes as mapped by Hoiseth and Stocker (J. Bacteriol., 163, 355, 1985). The proximal genes are indicated as follows: Pfi: which determines pyruvate formate lyase, Pr specifies the requirement for pyroxidine and serC which determines the enzyme phosphoserine aminotransferase.



Other nonreverting mutants of aroA554::Tn10 strains have deletions and/or inversions to the right or to the left. Some of these have been used for other experimental purposes (Eisenstein, et al, J. Immunol., 133, 958, 1984) and for evaluation of vaccine efficacy in calves (Smith, et al, Am. J. Vet. Res., 45, 1858, 1984).

B. The Thesis Problem

1. The importance of the humoral B cell response.

The problem of humoral and cellular immunity with their respective contributions in host resistance to facultative intracellular bacterial pathogens such as the Salmonellae (others include Brucellae, Listeriae, and Mycobacteriaceae) has been an enigma for many years. Using the Salmonella system, Mackaness (J. Exp. Med., 120, 105, 1964) addressed three points which affected, and were perceived as peculiar to, the high degree of resistance toward other, unrelated organisms in addition to the pathogen in question. These were: the consistent association with delayed-type hypersensitivity, a form of acquired resistance which could not be passively transferred with serum, and a change in the antibacterial activity of the host's macrophages. At that time (1964) it was suggested that resistance might be due to the interaction of antigen and a specific antibody adsorbed to the surface of host macrophages; and that the antibody involved in the reaction was perhaps identical with the antibody which conferred the state of delayed-type hypersensitivity. On further work (Mackaness, et al, J. Exp. Med., 124, 573, 1966), passive transfer of serum from actively infected or vaccinated animals and immunization with heat-killed organisms, increased the capacity of the host to clear

organisms from the blood, but did not interfere to any significant extent with their subsequent multiplication in the tissues. It was concluded that the resistance of actively infected animals depended on a nonhumoral mechanism capable of destroying organisms from endogenous or exogenous sources. Moreover, it was concluded from further transfer and active immunization experiments, that enhancement of the microbicidal activity of macrophages played a major role (Blanden, Mackaness and Collins, J. Exp. Med., 124, 585, 1966).

In an attempt to characterize the exact involvement of known antigens on Salmonellae, Salmonella enteritidis was used as a challenge strain. It is virulent for mice, causes a disease similar to that produced by S. typhimurium, and shares the same O-antigens as S. gallinarum and S. pullorum which differ greatly in their virulence and immunogenicity for the mouse despite being antigenically alike. In summary, animals vaccinated with live S. gallinarum were capable of eliminating a lethal dose of S. enteritidis, whereas vaccination with living S. pullorum failed to evoke an effective bactericidal response on challenge with S. enteritidis. Killed vaccines of both increased the blood clearance rate but gave only marginal protection against S. enteritidis challenge (Collins, Mackaness and Blanden, J. Exp. med., 124, 601, 1966). Hence, antigenic similarities, at least respecting the O-antigens could not be demonstrated to play a major role in protection against Salmonellosis.

This enigma of humoral and cell-mediated immunity, live vs. killed vaccines, known antigenic similarities, particularly as related to facultative intracellular bacteria is reviewed in detail by Collins (Bacter-

iol. Rev., 38, 1974). It has not been greatly clarified over the years and has remained a great source of concern and investigation in the early 1980's (Hormaeche, C.E., et al, Immunology, 43, 547, 1981, and Killar and Eisenstein, J. Immunol., 133, 1190, 1984). Additionally, Hormaeche demonstrated that the Ity gene was not related to the development of the positive footpad test in mice (Hormaeche, Brock and Pettifor in Genetic Control of Natural Resistance to Infection and Malignancy, E. Skamene, et al, eds., Academic Press, N.Y. 1980). However, as referred to above, O'Brien demonstrated a protective role for antibody and transferred bone marrow cells in xid mice. Hochadel and Keller (J. Inf. Dis., 135, 813, 1977) showed that transferred B cells rather than T cells from mice known to be immune protected unimmunized mice from 50 LD_{50} intraperitoneal challenge. Other experiments have not excluded humoral antibodies even when the objective was to demonstrate the role of cell-mediated immunity as in the experiments of Mackaness and Collins, referred to above.

More recently, Elkins and Metcalf have undertaken a series of experiments to characterize more closely the immune responsiveness to S. typhimurium. The approach was primarily to characterize the antibody response under given conditions.

Elkins and Metcalf used acetone-killed and dried virulent bacteria, which carried O-antigens 1, 4 and 12, to investigate the complexity of antigenic determinants presented on the surface of S. typhimurium. A panel of fifteen monoclonal antibodies which bound LPS were examined for differential binding with the resultant interpretation that epitopes presented by LPS are more complex than indicated by classical serology

(Elkins, K. and Metcalf, E.S., J. Immunol., 133, 2255, 1984). Additionally, it was demonstrated that an anti-lipid A monoclonal antibody did not bind well to purified whole S. typhimurium LPS, indicating the existence of antigenic determinants in purified lipid A (the immunizing antigen) which were not available for binding on the whole LPS molecule or intact bacteria (Elkins and Metcalf, Infect. Immun., 48, 597, 1985).

2. The choice of mice differing at the Ity locus to assess the humoral response.

The approach in this thesis was somewhat different from those described above. Since the genetics of inbred mice, in particular, has been well characterized regarding three genes which control resistance and susceptibility to S. typhimurium, it was thought that the choice of mice endowed with known susceptibility at the Ity locus should localize the infuence of macrophages, the effector cell for this gene, on the intact B cell response. The T cell response should also be intact (Hormaeche, et al, in Genetic Control of Natural Resistance to Infection and Malignancy, 1980). Therefore, T cell aberration would not be a source of distortion of the B cell response. It was not known, whether any altered B cell response might be a consequence of the expression of the Ity gene, its effect on the processing of the whole multideterminant antigen, or its influence on time, rate, and extent of the response. Since O'Brien had shown (O'Brien, et al, J. Immunol., 126, 1368, 1981) in xid mice, above, that humoral immunity played a significant role, it seemed pertinant to characterize this response in greater detail with mice of known Ity genotype.

In order to best represent the B cell response to the natural infection, it was considered essential that immunization be oral to simulate as closely as possible, the route of acquisition of the natural infection. The vaccine strain described above Hoiseth and Stocker, Nature, 291, 238, 1981) was a mutant of a virulent strain and had no change in surface structure and antigenic characteristics. Due to its aromatic auxotrophy, it was unable to multiply within the host sufficiently to overwhelm and cause life-threatening disease, thereby destroying the host before the immune response could be characterized. The bacterium could divide sufficiently to simulate the turnover in a natural infection, exposing internal or external antigens which might be recognized by macrophages or other immunocompetent cells. Any catabolic derivative from the macrophages' bacterial processing would also be available for presentation to T and B cells. If the Ity gene influenced differences in either catabolic derivatives or mode of antigen presentation, a subsequent B cell response should reveal this.

Very little is known about the mechanisms which are operational during macrophage processing. It seems fairly conclusive that at the time of presentation of antigen to the T cell, an antigen fragment or perhaps the native antigen itself is present on the macrophage surface. It is not known whether internalization and proteolytic breakdown are essential or nonessential features of macrophage handling. It is likely that lysosomal proteolysis of complex particulate antigens is mandatory. But it is not conclusive that an intracellular step is required for all antigens. Small peptide antigens may bypass some of the steps involved

in the processing of complex structures like bacteria (Shevach, E.M., in Fundamental Immunology, 1984).

3. The use of monoclonal antibody technology as a tool for cellular dissection of the B cell response.

Polyclonal assessment of the response as the summation of individual cellular responses would be expected to be insensitive. The use of monoclonal antibody technology provides a unique way of assessing individual B cells for their response. With an appropriate choice of a myeloma cell line which does not synthesize immunoglobulin chains as one parent fusion partner, any hybridoma product (antibody) from fusion with an antigen-primed normal B cell must be from that B cell. As such, a population of antibody-producing hybridomas can be assayed for specific reactivity with several antigens. Assessment of immunoglobulin class with enzyme-labeled anti-mouse Ig can be performed simultaneously. Although practical considerations preclude extensive propagation of the entire reactive population, most can be maintained sufficiently for supplementary testing where required, with maintenance of a representative few for ascites production and cryo-preservation. This is unlike plaque techniques for assessment and dissection of the B cell response. These are limited in the number of assays per individual antibody-secreting cell, require many separate assay protocols for inclusion of broadspectrum characterization, hence many more animals as the source of primed B cells. As such, requirements for control of animals and immunization schedules is extended.

Strict control of fusion experiments is, however, a prerequisite. Myelema cells must be carefully cultured so that their established generation time is maintained. They must also be in logarithmic growth phase to ensure optimal fusibility. Reagents which affect these requirements include fetal bovine serum, which must be carefully checked for toxicity and the concentration at which it maintains the generation time of the myeloma cell line of choice. Care must be taken in the use of PEG (polyethylene glycol) as the fusing agent. Certain products can be toxic. Requirements for feeder cells must be strictly adhered to, both to ensure the formation of hybridomas, as well as to maintain subsequent division. Most reagents used in the selection of hybridomas are ultimately toxic. Therefore, strictest attention must be paid in their preparation, as well as their gradual removal as hybrid cells are propagated. The procedures are usually straight forward. A combination of strict attention to detail and the procurement of high quality reagents presently available commercially permits use of this technology to study B cell distribution with reliability.

B cells from any source are adequate in fusion experiments. Hence, it was reasoned, it should be possible to follow the appearance and/or levels of specifically reactive B cells in various lymphoid organs over the period of an immunization schedule which incorporated the time frame in which protective immunity was acquired (determined by Hoiseth and Stocker, Nature, 291, 1981). The Peyer's patches from the first four centimeters of the duodenum were chosen as the most likely source of initial exposure to the antigen on oral immunization. Mesenteric lymph

nodes to which B cells would be expected to migrate via the abdominal lymph duct, and spleen cells were also included. In conjunction with polyclonal antibody assays, these experimental terms of reference should permit one to address the following questions. Does the B cell response occur to a significant extent? If so, at what time and under what conditions? Does it differ in homozygous susceptible strains of mice possessing the Ity^s allele, the effector cell for which is the macrophage, compared with resistant Ity^r strains? Which bacterial antigens are affected, at what time, and which immunoglobulin class is involved? Are there differences in responsiveness and/or numbers of B cells in the lymphoid organs most likely to be involved in antigenic contact?

In the experiments described in this thesis, adult mice at age four to six months were used to ensure complete maturation of immunocompetence. Both oral and parenteral immunization protocols were used with emphasis on the former. Known genetic mouse strains were chosen representing homozygous Ity^s/Ity^s (Balb/c), heterozygous Mendelian dominant Ity^r/Ity^s (F_1 DBA X Balb/c) and wild-type Ity^r/Ity^r (RBF/Dn). These strains also met specific compatibility requirements for hybridoma technology, such as histocompatibility for ascites production (Balb/c and F_1 DBA X Balb/c) and the chromosomal translocation involvement for stability of Ig-producing hybridomas when the FOX-NY myeloma cell line is used (RBF/Dn). Balb/c and F_1 DBA X Balb/c are also identical $H\text{-}2^d$ haplotypes ensuring identical control at the best characterized immune response genes.

The results show that the B cell response exists and is extensive from approximately two and a half weeks onward irrespective of route of immunization. It is not directed toward the O-antigens or the outer membrane as the primary target. Since it was originally anticipated that these antigens should be significantly involved, the B cell response to the outer membrane was analysed in greater detail. It was found that the response, although small in all cases was graded toward LPS. The wild-type RBF/Dn responded earliest and greatest, followed by the resistant F_1 DBA X Balb/c. No IgA was detected from Balb/c mice and F_1 DBA X Balb/c exhibited a depressed IgM response. Very little response was detected to the highly conserved porin proteins of the outer membrane. A large part of the B cell response was directed toward more internally located sites. This was determined by compartmentalizing bacterial test antigens into inner and outer membrane fractions from a two-step sucrose gradient and incorporating commercially available smooth and rough LPS in the test system. Inner membrane preparations reacted with the majority of positive hybridoma supernatants and the most strongly and extensively with whole sera taken from mice at the time of sacrifice for fusions. Differences in time of appearance, numbers and specific Ig-reactive cells in different lymphoid organs were observed between mouse strains.

Contrary to the view put forward by Biozzi (Adv. Immuol. 37, 189, 1984) that inbred mice respond similarly to Salmonella antigens, compartmentalization of bacterial antigens demonstrated differences in the response of the two inbred strains with respect to time, rate, degree and immunoglobulin class as well as the distribution of specifically reactive

cells in lymphoid organs. Differences were even greater when assessment was extended to RBF/Dn mice. Sex-dependent increase in antibody was demonstrated in female F_1 mice, and an increased frequency of greater response in female RBF/Dn. The results parallel the experimental evidence of Biozzi that antibody response to a multideterminant antigen is polygenic as demonstrated by the inclusion of inbred mice of identical H-2 haplotype in the experimental protocol. Additionally, the work delineated some of the bacterial antigen groups which contribute to the differences in response. The results suggest that time and rate of migration of B cells between lymphoid organs are included in the polygenic control of mucosal immunity, although the relationship of this and other differences to the Ity gene was not demonstrated.

The humoral response probably plays a significant protective role in the course of the disease. On initial immunization it is increased after two weeks. This is after the effect of the Ity gene on macrophage activity has taken place. Thus, following the initial requirement for resistance due to macrophage activity, B cell activity must also be efficient in contributing to resistance. T cell immunity, which is also involved in resistance was not studied in this work. The implications of the known effective protection of the vaccine strain is that antibody is preferentially directed to internal antigens under immunizing conditions which simulate the route of a natural infection. Mouse strain variations in responsiveness exist and are discussed in detail.

MATERIALS AND METHODS

A. Mouse Strains

Balb/cbyj and F_1 (DBA/2j) X Balb/cbyj, available from Jackson Laboratories, Bar Harbour, Maine, were initially chosen as the susceptible and resistant strains, respectively (Robson and Vas, J. Inf. Dis., 126, 378, 1972) with respect to the Ity gene on chromosome one of inbred mice. Balb/c are homozygous Ity^S (Ity refers to immunity to typhimurium) and the F_1 cross with DBA/2j results in a heterozygous, Mendelian dominant, Ity^r individual. DBA/2j and Balb/c are of the same $H\text{-}2^d$ haplotype such that immune response genes as defined by response to synthetic polyeptide antigens (Klein, J. Genetic control of immune response. Ch. 17, p. 41, in Biology of the Mouse Histocompatibility Complex, 1975) would not introduce additional variables. Additionally, this genetic combination is sufficiently compatible with most procedures in hybridoma technology, including ascites production to allow a certain amount of strain cross-production without invoking histoincompatibilities.

The RBF/Dn mice originate from a mating of a feral male to a Swiss strain (Hyclone Laboratories THT Taggart Hybridoma Technology Brochure) with subsequent establishment of a breeding colony from the F_1 hybrids. These were also obtained from the Jackson Laboratories. This provided the wild-type gene, Ity^r, for these studies. In addition, it was a decided asset for the use of the FOX-NY myeloma cell line (described below under assessment of the B cell response by the product of lymphocyte hybridomas) in generating stable hybrid cell lines.

Genetically, RBF/Dn mice are homozygous for three Robertsonian chromosome translocations: Rb(8.12)5Bnr, Rb(1.3)1Bnr and Rb(9.14)6Bnr. The first of these genetically links the immunoglobulin heavy chain gene on chromosome twelve with chromosome eight which carries the gene for the enzyme, adenosine phosphoribosyltransferase (APRT) for which the FOX-NY myeloma cell line is deficient (as well as in hypoxanthine phosphoribosyltransferase - see below). These mice were used as young adults at four to six months of age to insure immunologic maturity.

Random bred ICR mice, (Institute of Cancer Research, Swiss Webster strain) available locally from the Faculty of Science Vivarium, were used for the initial evaluation of immunogenicity of different outer membrane preparations.

B. Preparation of Antigens

1. Bacterial strains.

Salmonella typhimurium strain SL3261 (Hoiseth and Stocker, Nature 291, 238, 1981), the aromatic-dependent vaccine strain used for the major portion of this work was kindly provided by Dr. B.A.D. Stocker, Stanford University, Stanford, California, along with the virulent, isogenic parent, SL1344. The latter is referred to as the "grandparent" (Hoiseth and Stocker, 1981) since two steps in producing the vaccine strain were involved. It was made aromatic-dependent by deletion mutation, DEL407 arising from aroA554::Tn10; the transposon having been inserted into gene aroA by transduction with phage P22.

Strain M206, an unrelated avirulent, rough (Rb) carrier strain and strain C5, an unrelated virulent strain were obtained from Dr. Derrick

Rowley, Department of Microbiology, University of Adelaide, Adelaide, South Australia. These strains had been used as vaccine and challenge strains in the determination of resistance of inbred mice to Salmonella typhimurium (Robson and Vas, J. Infect. Dis., 126, 378, 1972).

On receipt of the bacterial strains they were plated out on solid media to determine purity (mainly on blood agar plates and MacConkey medium). Strains SL3261 and SL1344, which both require histidine were also plated on minimal medium with and without histidine. In the case of SL3261, additional supplementation with phenylalanine, tryptophan and tyrosine was made together with p-aminobenzoic acid, 2,3-dihydrobenzoic acid and p-hydrobenzoic acid (B.A.D. Stocker, personal communication, February, 1983). A battery of freeze-dry vials as initial stock was prepared for each strain immediately to serve as a primary stock. From this battery, vials for use in the procedures indicated below were taken for the entire duration of the project. At no time were stock strains used beyond the fifth subculture.

2. Preparation of live vaccine of S. typhimurium for immunization.

S. typhimurium strain SL3261 previously taken from a freeze-dry vial and checked for purity on blood agar, MacConkey and minimal medium with appropriate supplements was grown overnight in nutrient broth. The bacterial cells were spun and washed two times in sterile saline and diluted to concentration: 3×10^7 and 10^8 for oral immunization - see individual experiments below - and 3×10^4 for I.P. injection. A MacFarland nephalometer barium sulfate standard was used as the initial refer-

ence standard. Triplicate plate counts of the final suspension were made as definitive checks on concentration.

3. Preparation of test antigens for ELISA.

a) Evaluation of different methods of outer membrane preparation: exclusion of inner membrane by NADH oxidase assay and SDS PAGE.

Three methods of outer membrane preparation were originally investigated with the objective of determining the simplest method which retained the most physiologic and chemical characteristics of the outer membrane. One method was a simple boiling and differential centrifugation method used for preparation of Neisseria meningitidis outer membranes (Mackie, et al, J. Clin. Microbiol., 13, 449, 1981; Frasch and Chapman, Infect. Immun., 5, 98, 1972) where serotype antigens were used as epidemiological markers. Briefly: exponentially growing cells in nutrient broth were inoculated into two hundred millilitres nutrient broth and grown with constant shaking at thirty seven degrees Celsius for fourteen hours. The optical density at six hundred and seventy five nm. was about 0.7 which corresponded to about 10^7 bacteria per millilitre. These were pelleted at 10,000 X g. for twenty minutes and washed once with saline. Two millilitres sterile distilled water was added per gram wet weight of cells, the suspension boiled twenty minutes and placed on ice immediately. The suspension was centrifuged at 13,000 X g. for twenty minutes to pellet unbroken cells and debris. The supernatant was centrifuged at 100,000 X g. for two hours, washed with d.d. H_2O and recentrifuged at 100,000 X g. for two hours. The pellet was taken up in water. This will be referred to as a water/heat preparation hereafter.

A second method based on differential dissolution of inner and outer membranes of gram-negative bacteria with sarkosyl (Lambert and Booth, FEMS Micro. Lett., 14, 43, 1982) was carried out as follows. Thirty millilitres of an overnight broth growth was reinoculated to two hundred millilitres nutrient broth and grown with constant shaking at thirty seven degrees Celsius for seven hours to an optical density of 0.7 at six hundred and seventy five nm. The cells were pelleted at 10,000 X g. for twenty minutes, washed once in phosphate buffered saline (PBS) and resuspended in ten millilitres PBS. This suspension was sonicated at a setting of 6.5 on a Branson sonifier Model 350 using the microtip for three minutes, centrifuged at 10,000 X g. for twenty minutes and the supernatant recentrifuged for ten minutes. One millilitre of a twenty percent w/v Sarkosyl (N-L sarcosine) was added and incubated thirty minutes at room temperature. This preparation was centrifuged for thirty minutes at 35,000 X g., the pellet washed in PBS and taken up in 0.5 milliliters water.

The third method was a sucrose gradient method which was primarily that of Jones and Osborn (J. Biol. Chem., 252, 7405, 1977) with slight modifications used by Munford (J. Bact., 144, 630, 1980). Exponentially growing bacterial cells were inoculated to nutrient broth, grown overnight with constant shaking to an optical density of 0.7 at six hundred and seventy five nm. and pelleted at 10,000 X g. for twenty minutes. The cells from two hundred millilitres at this density were taken up in three millilitres twenty-five percent sucrose in 0.1M TRIS-HCL buffer, pH 8.0, containing 0.4 ug. DNA'ase and RNA'ase/ml. They were incubated at room

temperature for ten minutes and kept on ice for procedures thereafter. This suspension was sonicated for nine minutes at an amplitude of 6.5 (or put through the French press three times at 15,000 psi), centrifuged at 13,000 X g. for twenty minutes and the supernatant recentrifuged for ten minutes. The resulting supernatant was centrifuged at 65,000 rpm in the Beckman ultracentriguge using the Ti 70.1 rotor. The pellet (total membrane) was taken up in 0.5 to 1.0 milliliter twenty-five percent sucrose containing TRIS-EDTA (0.1 M TRIS-HCl, 0.1 M EDTA, pH 8.0) and layered onto a sucrose gradient. Initially, this consisted of 0.5 milliliters fifty-five percent sucrose, then two milliliters each of fifty percent, forty-five percent, forty percent, thirty-five percent and thirty percent sucrose. After establishing conditions at which the inner membrane was excluded by assaying for NADH oxidase (below) this was modified to a pad of 0.5 milliliter of seventy percent sucrose, two milliliters sixty percent sucrose, three milliliter fifty-five percent sucrose (after experiencing greater recovery in one or the other with different S. typhimurium strains) and three milliliters forty-five percent sucrose. This was spun at 36,000 rpm for eighteen hours in the SW 41 rotor (Beckman ultracentifuge). The forty-five percent layer was removed and the fifty-five and sixty percent layers pooled and recentrifuged in the Ti 70.1 rotor at 65,000 rpm for three hours. The pellet was taken up in TRIS-HCl - usually one - two milliliters depending on the original amount of bacterial cells.

The sucrose gradient preparation only was amenable to assaying for NADH oxidase which was patterned after the method described by Osborn, et

al (J. Biol. Chem., 247, 3962, 1972). 0.2mM dithiothreitol, no more than a week old, outer membrane preparations containing fifty ug. protein and 50mM TRIS-HCl, pH 7.5 were placed in test tubes or cuvettes such that on addition of 0.12 mM DPNH the total volume was one milliliter. The latter was added to initiate the reaction and readings for a decrease in absorbance at 340 nm were done at precise fifteen minute intervals. Diaphorase (from porcine heart) purchased from Sigma Chemical Co., St. Louis, Mo., served as a positive control.

In the initial comparative aspects of outer membrane preparations samples from the above three outer membrane methods were run on two gels. In one case an eleven percent polyacrylamide gel with a six percent stacking gel was loaded and electrophoresed and subsequently stained with Coomassie Blue for proteins (Laemmli Nature, 227, 680, 1970). Secondly, a fifteen percent acrylamide gel with 4M urea as the running gel and six percent acrylamide as the stacking gel was used for LPS separation and stained with silver (Dubray and Bezard, Anal. Biochem., 119, 325, 1982; Tsai and Frasch, Anal. Biochem, 119, 115, 1982) as follows. After electrophoresis the gel was soaked in twenty-five percent isopropyl alcohol and ten percent acetic acid overnight at room temperature. The gel was then soaked in 7.5 percent glacial acetic acid thirty minutes, and placed in 0.2 percent aqueous periodic acid and stored at four degrees Celsius for one hour. It was washed in several changes of d.d. H_2O for three hours, then exposed to freshly prepared ammoniacal silver solution: 1.4 millilitres concentrated NH_4OH (twenty-eight percent), twenty-one millilitres 0.36% NaOH, four millilitres 19.4% $AgNO_3$, 73.6 millilitres

d.d. H_2O. The gel was washed in several changes of d.d. H_2O for two hours and placed in freshly prepared solution of: five milliliters glacial acetic acid, fifty milliliters formaldehyde (thirty-seven percent), one hundred milliliters d.d. H_2O. After development of stained carbohydrates (1,2 - diol groups of glycoproteins and polysaccharides) the gel was fixed in fifty percent methanol.

Subsequent SDS PAGE and silver staining for proteins evolved into the following standard procedure. Outer membrane samples containing thirty ug. protein were placed in 0.1 milliliters sample buffer (Bennett and Rothfield, J. Bact., 127, 498, 1976) made up of 0.05 M TRIS-HC1, one percent (w/v) SDS, 0.002M EDTA, ten percent (w/v) glycerol. Ten microlitres mercaptoethanol was added and the mixture boiled for three minutes. Samples were applied to a gel which contained eleven percent acrylamide as the running gel with a three percent stacking gel (Bennett and Rothfield, above) and set up in an Aquebogue electrophoresis apparatus, model 100 and run at twenty milliamperes in the cold room for approximately three to four hours after which it was fixed overnight in twenty-five percent (w/v) isopropanol and seven percent acetic acid for silver staining of proteins (Hitchcock and Brown, J. Bact. 154, 269, 1983). It was placed in ten percent glutaraldehyde and three percent isopropanol for sixty minutes at room temperature and washed eight times for thirty minutes in d.d. H_2O at room temperature on a rotating shaker followed by a ten minute exposure to silver stain on the rotating shaker. Silver stain was fifty-six milliliters 0.1N NaOH, two hundred and thirty milliliters d.d. H_2O, two milliliters concentrated NH_4OH with about nine

to ten milliliters of twenty percent (w/v) silver nitrate added slowly with shaking. This solution was prepared immediately before use. The gel was washed four times for ten minutes with d.d. H_2O on the shaker and exposed for ten to twenty minutes to the developer made up of fifty milligrams citric acid, 0.5 milliliters formaldehyde (thirty-seven percent) and d.d. H_2O to one litre, also prepared immediately before use. Color development was stopped in a stop bath of four hundred milliliters d.d. H_2O, twenty milliliters of seven percent acetic acid for one hour followed by a wash in d.d. H_2O for a minimum of one hour.

b) Evaluation of immunogenicity of outer membrane preparations and antibody response in random bred mice.

On the premise that any changes in physiologic or immunologic response might be reflected in an undetectable way in test systems, comparative antibody response curves were done on random bred ICR mice with the water/heat preparation and the sucrose gradient outer membrane preparation. Preparations containing fifty micrograms of protein were injected intravenously into twelve mice which were bled, two at a time, at two to four day intervals for twelve days, sacrificed, their spleens weighed and the direct Jerne plaque assay (described below) performed. At nine days a booster of the sucrose gradient preparation was given to three mice (the only extra survivors). Serum from the above bleedings were used for the enzyme-linked immunosorbent assay (ELISA).

The ELISA was performed by applying outer membrane samples containing one ug./ml. protein in carbonate buffer (Voller, et al, Bull. World Health Organ., 53, 55, 1976) to ninety-six well polystyrene plates. The

volume per well was 0.1 millilitre (i.e. 0.1 microgram protein per well). Previous experience (Mackie, et al, J. Immunol., 129, 829, 1982) had shown this to be adequate. After overnight incubation at four degrees Celcius the plates were washed three times with phosphate buffered saline containing 0.05 percent Tween 20. 0.1 millilitre of serum dilutions was added and incubated at room temperature for two hours and washed three times with PBS-Tween. 0.1 millilitre of appropriately diluted (usually 1/1000 to 1/2000) solution of peroxidase-labeled rabbit anti-mouse IgG (purchased from Miles Biochemicals, Elkhart, Indiana) was added and the plates reincubated for two hours at room temperature. After another three PBS-Tween washings, 5-aminosalicylic acid (8 mg./10 ml. in water) was added and a brown color allowed to develop for half an hour.

After the initial titrations of all sera under these conditions, positively reacting sera were used for a re-titration of antigen concentration ranging from one to ten ug./ml. with 0.1 ml./well. This was done to ensure optimal antigen application using the highest reacting sera from the above.

With these positively reacting antisera, the reaction of any given serum was the same for antigens containing protein concentration ranging from one to eight ug./ml. Therefore, the concentration of one ug./ml. was maintained for whole sera. Later, this concentration was increased to 2.5 ug./ml. for the testing of hybridoma supernatants. See below.

These sera served a positive controls until more became available. Likewise, as antibody-producing hybridomas were detected, positively reacting supernatants or ascites fluid were used as positive controls.

The positive reagent controls consisted of the enzyme-labeled anti-mouse immunoglobulin added to the substrate to produce a color. The negative reagent control contained all reagents except the enzyme-labeled anti-mouse immunoglobulin. Polyvinylchlorine microtitre plates gave false positive reactions under these conditions and were not used.

For whole serum titrations, the last tube producing a color intensity of more than 1^+ was considered the end point (gradations being 0 to 4^+ to represent intensity of color; 4^+ being the greatest intensity). In the screening of hybridoma supernatants, a reaction of 1^+ or greater was considered a positive reaction in an all or nothing determination.

c) Toxigenicity of outer membrane (LPS-induced) during immunization.

Since LPS (endotoxin) as a major component of the outer membrane was of interest immunologically, its capability in the preparations to produce illness (fever, malaise, lethargy) or death when injected into the mouse was noted. After intravenous injection (above) of the outer membrane preparations the mice were observed half hourly for two hours and hourly for the remainder of the working day, three times daily up to seventy-two hours, and daily for the duration of the experiment. Mice which became ill over the time of the experiment were observed hourly for more precise determination of time of death.

4. Preparation of flagellin.

The method of Suzuki and Iino (J. Mol. Biol., 81, 57, 1973) was used with minor modifications to prepare flagellin for use in the ELISA as a check against other methods (eg: Jerne plaque assay).

SL3261 was grown in nutrient broth, as above, until the stationary phase was reached (twenty to twenty-four hours). The cells were collected by centrifugation and suspended in water, about ten millilitres for each five hundred millilitres of original medium, and deflagellated in the Sorvall blender at 2000 rpm for five minutes at four degrees Celsius. Cell debris was removed by centrifugation and the detached flagellae in the supernatant were sedimented by centrifugation at 70,000 X g for one hour, washed with water and suspended in water at two to five milligrams protein per millilitre. This was acidified to pH two to three with 1M HCl to dissociate flagella into monomers, allowed to stand at room temperature for twenty minutes and centrifuged at 10,000 X g for ten minutes. The supernatant was neutralized by adding 1/10 of the sample volume of 1M K_2HOP_4, and centrifuged at 70,000 X g for one hour. Flagellin in the resulting supernatent was reconsituted into polymers with 1M $(NH_4)_2SO_4$ at room temperature. The reconstituted polymers were pelleted at 50,000 X g for thirty minutes, resuspended in water and 0.5M $MgCl_2$ added to a final concentration of 5mM and recentrifuged at 70,000 X g for one hour. The pelleted flagellae were washed once with 5mM $MgCl_2$ and resuspended in 5mM $MgCl_2$ at ten to fifteen milligrams protein/ml.

5. Preparation of porin.

The method of Nurminen (FEMS Microbiol., 3, 331, 1978; and J. Bact. 127, 971, 1976) was used for preparation of porins. Large plates of

nutrient agar were inoculated with SL3261 from a recent growth and incubated overnight, about sixteen hours. The bacteria were collected by scraping into 0.01M TRIS-HCl buffer, pH 8.0, spun for twenty minutes at 6000 X g, weighed to get the wet weight of cells and washed twice more with 0.01M TRIS-HCl. Ten millilitres TRIS-HCl with 0.01M EDTA was added per gram of original cells together with 1.3 milligrams lysozyme. The suspension was incubated ten minutes at room temperature. Then 0.4 millilitres of 0.01M $MgCl_2$ and 0.3 milligrams of RNA'ase and of DNA'ase were added with further incubation for five minutes. The suspension was centrifuged twenty minutes at 6000 X g and washed with 0.01M TRIS-EDTA (above), and again with TRIS-HCl containing 5mM $MgCl_2$. Ten millilitres of two percent Triton X-100 in 0.01M $MgCl_2$ was added and the mixture centrifuged at 3000 X g. The supernatant was saved and the pellet re-extracted twice more with Triton X-100. The supernatants were pooled and the pellet saved. The pooled supernatants were centrifuged at 3000 X g for twenty minutes and the resulting pellet, considered to be outer membrane envelopes, was taken up in two percent Triton X-100 in 0.01M EDTA. 0.5 mg. trypsin/35 milligrams protein (as determined by the Lowry method) was added and the samples incubated overnight at thirty seven degrees Celsius. The sample was centrifuged at 20,000 X g for twenty minutes and the pellet resuspended in two percent Triton X-100 and 0.01M EDTA and retrypsinized. The next day samples were respun and pooled as were the supernatants. The supernatants were transferred to an Amicon

apparatus with an XM50 filter, filtered and washed with d.d. H_2O (approximately five times the original sample volume). This precipitated the porin fraction which was suspended in water, spun (20,000 X g for twenty minutes), washed once with water, then twice with 0.05M EDTA which removed some contaminating high molecular weight proteins. The final suspension was made in water. For SDS PAGE, used to check for purity and molecular weight of the resulting protein, an equal volume of ten percent SDS was used to treat an aliquot of the sample since Triton X-100 produced a "smear" on the gel. Samples thus treated had two bands of about 36,000 molecular weight on the electrophoresis gel.

C. Preliminary Delineation of Antibody Response to Outer Membranes in Mice Given Live Vaccine Orally and Parenterally

1. IgG antibody response to outer membrane preparations of vaccine, its isogenic parent and two unrelated virulent and avirulent strains.

Balb/c and F_1 DBA X Balb/c mice were immunized orally and parenterally with live SL3261 S. typhimurium cells at concentrations of 3 X 10^7, orally, and 2-3 X 10^4 intraperitoneally. A male and a female mouse were bled and sacrificed every two to three days over an immunization period of one to forty-one days with a boost at twenty to twenty-four days. Where possible, both male and female control, age-matched, unimmunized mice were sacrificed at the same time. The boost was the same concentration of live bacterial cells as the primary immunization. Spleens were weighed, and the cells from them used for the direct Jerne plaque assay. Visual qualitative estimation of the size of mesenteric

lymph nodes was made on orally immunized of F_1 DBA X Balb/c (graded 1^+ to 4^+ increase in size over the controls). Sera were titrated in serial doubling dilutions against outer membrane preparations of SL3261 (vaccine), SL1344 (isogenic "grandparent"), M206 (unrelated rough carrier) and C5 (unrelated virulent strain). Outer membrane preparations made by the sucrose gradient method, above, shown to be equally reactive with whole serum from concentrations of one to eight ug./ml, were applied to polystyrene plates at one ug/ml. ELISA titrations as described above were carried out.

2. Splenomegaly.

Spleens removed from mice were weighed on a balance permitting estimation of the third decimal place.

3. Differential cell counts determining blast transformation of spleen cells.

Just prior to teasing the spleens apart for the Jerne plaque assay, light imprints were made on glass slides from the cut surface of a small piece of spleen. Alternatively, suspensions of cells were applied to slides and air dried. In either case the slides were fixed for thirty minutes in ninety-five percent methanol and stained by May-Grunwald-Giemsa method or with Wright's stain (Miale, J.B., Laboratory Medicine: Hematology, pp 1208-1209, 1972), and examined for blast cells, mitotic figures, and mature lymphocytes in one hundred cells over single cell areas of the imprints or suspension preparations.

4. Direct Jerne plaque assay.

Based on methods of Schwartz and Braun (Science, 149, 200, 1965; and McAlack, et al, Science, 168, 141, 1970) spleen cell suspensions were

adjusted to contain 1 to 2 X 10^7 nucleated cells per 0.1 ml. and added to two millilitres of 0.7% melted Noble agar maintained at forty-five degrees Celsius. One milligram of DEAE-dextran was added to each tube to inhibit the anticomplementary activity of agar. 0.1 ml. nutrient broth containing 2 X 10^8 S. typhimurium SL3261 cells from an overnight growth was added, mixed and poured on a petri dish containing a base layer of fifteen millilitres Brain Heart Infusion (BHI) agar. When the upper layer had solidified, the plates were incubated for ninety minutes at thirty-seven degrees centigrade. 1.5 ml. of a 1:10 dilution of guinea pig complement (Grand Island Biologicals Company) was spread over each plate. The plates were incubated without inversion at four degrees Celsius for one hour to permit diffusion of complement without bacterial growth. They were then transferred back to thirty-seven degrees Celsius and examined after an additional four and eighteen hours of incubation for zones of growth inhibition or lysis and for concentrated colonies (possible indication of antibodies to flagellae with concentration of bacterial cells by agglutination). Zones of no growth measuring 0.5 to two millimetres were examined under the dissecting microscope for centrally located mononuclear cells.

5. Gross lymph node response.

This was begun with the F_1 DBA X Balb/c and consisted of examining the mesenteric lymph nodes, and in some cases the peripheral lymph nodes,

by inspection. Enlargement relative to control mice was graded qualitatively 1^+ to 4^+, with 4^+ representing maximal enlargement.

6. Male versus female response.

With the exception of the Jerne plaque assay where spleen cells of necessity had to be pooled, all individual measurements for male and female animals were documented. They were averaged for presentation of some data where other experimental terms of reference were measured (eg. antibody titres on given days during the immunization period), but also compared for example, with paired analysis of variance (ANOVA).

7. Shift in antibody reaction to outer membrane test antigens prepared from bacterial cells grown in minimal iron-deficient medium.

The four strains of S. typhimurium described above were grown in minimal (M9, Sokol and Woods, Infect. Immun. 40, 665, 1983) medium deficient in iron. Outer membrane preparations were made as above, compared electrophoretically, and coated on polystyrene plates, as above. Selected antisera from the intraperitoneally immunized F_1 DBA X Balb/c were retitrated against these antigens.

8. Differences in response to heat-killed vaccine.

Ten F_1 DBA X Balb/c mice, five male and five female, were injected with 10^9 heat-killed bacterial cells (sixty degrees Celsius for one hour - Schwartz and Braun, Science, 149, 200, 1965; McAlack, et al, Science, 168, 141, 1970) intraperitoneally. A male and a female were bled by cardiac puncture and sacrificed at two, five, eight and nine days, spleens weighed and Jerne plaque assay performed as above. The

sera were titrated against membrane antigens of the above four bacterial strains.

D. B Cell Response in Selected Peyer's Patches, Mesenteric Lymph Nodes and Spleen Cells as Assessed by the Product of Lymphocyte Hybridomas (Monoclonal antibodies)

1. The Myeloma parent.

The FOX-NY myeloma line available by license from Hyclone Laboratories, Logan, Utah, was used for most of the studies. It is a spontaneous mutant of the NS-1 line (Taggart and Samloff, Science, 219, 1228, 1983), produces no immunoglobulin heavy or light chains and is deficient for adenine phosphoribosyltransferase (APRT) as well as for hypoxanthine phosphoribosyltransferase. It is particularly effective when hybridomas are produced by fusing with mouse cells containing Robertsonian 8.12 translocation chromosomes. With this combination, cell fusion mixtures are exposed to a culture medium that can be utilized only by APRT-positive cells (i.e. normal mouse derived), which results in elimination of both unfused APRT-deficient myeloma cells and nonantibody-producing APRT-deficient hybridomas that arise by segregation of the 8.12 translocation chromosomes containing the APRT genes and the heavy chain immunoglobulin gene. The RBF/Dn mice described above have the 8.12 chromosomal translocation and are also wild type Ity^r. Since the APRT gene is on chromosome eight, AAT selection in addition to HAT selection (i.e. media containing adenine, in addition to aminopterin, hypoxanthine, and thymidine to select against APRT-deficient and HPRT-deficient cells, above) requires that both number eight chromosomes (i.e. from the normal lymph-

ocyte parent) must be lost in order for the hybridoma to become AAT sensitive. Hence, this did not interfere with, but rather enhanced its advantage in use with the Balb/c and F_1 DBA X Balb/c mice as representing susceptible and resistant (Ity^s homozygotes and Ity^r heterozygotes, Mendelian dominant) on co-selection with HAT medium. The myeloma cells were grown at optimal densitites of 10^5 to 6 X 10^5 in standard RPMI-1640 medium (below) and frozen at concentrations between 2 X 10^6 and 10^7 (preferrably 10^7) in freezing medium consisting of: RPMI-1640 plus five percent v/v fetal bovine serum, 10mM HEPES and ten percent DMSO - no antibiotics.

2. Lymphocyte preparation at various times and from different sources.

In all preparations described below, all mice were bled by cardiac puncture and sera stored at minus seventy degrees Celsius for future titrations.

a) Pokeweed mitogen stimulated spleen cells.

Two or three unimmunized mice (preferrably three) were used and the spleens only removed after cervical dislocation for exposure to pokeweed mitogen. Spleen cells obtained by teasing the spleens apart were washed once and adjusted to a concentration of 2 X 10^6 in RPMI-1640 medium containing five percent fetal bovine serum (Myoclone from Grand Island Biologicals Co.) and ten ug./ml. pokeweed mitogen (purchased from Sigma Chemical Co., St. Louis, Mo.). They were incubated (thirty-seven degrees Celsius, 6.5% CO_2, and eighty-five to ninety-five percent relative humidity) in Optilux petri dishes (Falcon plastics available from Fisher

Scientific, Edmonton, Alberta). Exposure to pokeweed mitogen was forty-eight hours at which time the cells were washed twice in RPMI-1640, taken up in ten millilitres RPMI-1640, counted in Turk's solution (five percent acetic acid with methylene blue dye), mixed in appropriate concentrations (see fusion procedure, below) with myeloma cells subsequent to which another washing took place. Sera obtained from these mice served as control, nonimmune sera for future titrations.

b) Lymphocytes from immunized mice.

Mesenteric lymph node cells, spleen cells and Peyer's patch cells from the first four centimeters comprising the duodenum were removed from mice sacrificed by cervical dislocation at twenty-four hours, ten days, twenty-two days, thirty-two days (eight days post boost) and forty-five days (twenty-one days post boost) after primary oral immunization of 10^8 SL3261. They were teased apart in about ten millilitres sterile RPMI-1640. Larger clumps and cell debris were allowed to settle in a twelve millilitre sterile test tube for five minutes. The supernatants containing largely single cell suspensions were transferred to another tube and washed once with RPMI-1640, counted in Turk's solution and mixed in appropriate concentrations with myeloma cells.

3. The fusion procedure.

The procedure was that of de St. Groth and Scheidegger (J. Immunol. Methods, 35, 1, 1980). On the day before fusion the following was made up:

a) Sterile media;

(i) 0.34M (11.6%) sterile sucrose for harvesting macrophages.

(ii) Standard RPMI-1640 medium containing five percent fetal bovine serum, 10mM HEPES, 100 U/ml. penicillin, and 100 ug/ml. streptomycin sulfate.

(iii) Selective medium which was standard plus additives:

4.0×10^{-9}M aminopterin

7.5×10^{-5}M adenine

1.6×10^{-5}M thymidine

10^{-5}M hypoxanthine

(iv) RPMI-1640 containing no fetal bovine serum for the washing of cells immediately prior to and immediately after the PEG treatment.

(v) Fusing solution: 0.5 g. PEG 4000 (Merck, for gas chromatography available through BDH Chemicals Ltd).

0.05 ml. DMSO

0.5 ml. distilled water

Sterilization was by boiling tubes with the above in a water bath for fifteen minutes and reboiling to dissolve any crystals on the day of fusion.

b) One millilitre selective medium was placed in each well of twenty-four-well tissue culture plates which were put in a humidified (eighty-five to ninety-five percent relative humidity) CO_2 (6.5%) incubator at thirty-seven degrees Celsius.

c) Macrophages were prepared from any available strains of mice (strain, genetics were immaterial - de St. Groth and Scheidegger J. of Immunol. Methods, 35, 1, 1980) by flushing their peritoneal cavity with sucrose solution. These were pooled, centrifuged at 800 rpm and resus-

pended in selective medium and the recovered cells counted in Turk's solution under 400 X magnification to differentiate macrophages and lymphocytes in the suspension. The cells were then adjusted to a concentration of 5 X 10^5 or 10^6 (below) per millilitre with respect to macrophages and 0.05 millilitre drops distributed into wells and the culture plates returned to the incubator.

On the day of fusion:

All reagents were warmed to thirty-seven degrees Celsius in a water bath and maintained at this temperature as much as possible.

a) Myeloma cells grown up to confluence were harvested into fifty millilitre conical centrifuge tubes, spun at 200 X g for ten minutes and suspended in ten millilitre RPMI-1640 standard medium. These were counted and added to the lymphocytes prepared from Peyer's patches, mesenteric lymph node cells and spleen cells (above) which was done in parallel with the harvesting of myeloma cells. The proportions of myeloma to mouse lymphocytes was one to five. The mixture of cells was spun again and and washed once in standard RPMI-1640 without fetal bovine serum in a fifty millilitre conical centrifuge tube.

b) For fusion, the pellet was loosened and the entire contents of the tube containing PEG (one millilitre) added over one minute with constant agitation of the tube. The tube was immersed an additional ninety seconds in the water bath keeping the contents swirling all the time making total time of exposure to PEG 2.5 minutes. The fusion was stopped by slowly adding twenty millilitres RPMI-1640 standard medium without fetal bovine serum over a time of three to five minutes, dropwise at

first, and gradually increasing volumes. This was allowed to stand five minutes, then centrifuged at 200 X g, the supernatant discarded, and the cells washed once with standard medium containing fetal bovine serum. A cell suspension in selective medium was made to contain 4 X 10^5 mouse lymphocytes/millilitre and one millilitre volumes were dispensed into the wells of the culture plates making the density of cells per well 2 X 10^5 with respect to mouse lymphocytes and 4 X 10^4 with respect to myeloma cells. Since there are fewer macrophages in mesenteric lymph nodes and Peyer's patches than in spleen these were dispensed into wells containing 10^6 machrophages from the day before. With experience it became apparent from the high number of fusible lymphocytes in spleens on days thirty-two and forty-five that better separation of clones in this culture set-up required fewer cells. Hence, on these days spleen cells were made to 2 X 10^5/ml. with final concentrations per well being 10^5/ml. These were dispensed in wells containing 10^6 macrophages, as well.

4. Maintenance and subsequent propagation of selected hybridomas.

0.25 to 0.3 millilitres fresh selective medium was added to each well on day five and on day ten after fusion. All wells were examined on day fourteen and every two days thereafter up to four weeks. Usually most clones had formed by day fourteen which became the standard time for testing supernatants (below). Additional hybridoma supernatants were tested as new clones appeared and became large enough. The cells from wells with positively reacting supernatants were transferred to other wells if multiple clones had formed in the well of question by taking up

a colony directly with a pasteur pipette and transfering. Further separation as necessary was carried out by limiting dilutions of 0.5 cells per well of microculture plates in forty-eight of the ninety-six wells, three cells per well in thirty-six of the wells, and ten cells per well in twelve wells. Cultures of choice were gradually grown up in three millilitre wells (i.e. wells of twenty-four well culture plates), then thirty-five millimetre petri dishes, followed by one hundred millimetre petri dishes as required with periodic testing for maintenance of antibody production. In the case of Balb/c mice, hybridomas with antibodies of interest were propagated by production of ascites fluid when cells had grown to 2 X 10^6. These cells were injected into two mice given 0.5 millilitres pristane (Hoogenraad, et al, J. Immunol. Methods, 61, 317, 1983) ten to twenty-one days previously. In the case of RBF/Dn mice, cells were cultured to desired quantities (F_1 RBF/Dn X Balb/c for ascites production were unavailable at the time). Cells were cryopreserved as for myeloma cells (above).

5. Testing of Supernatants from Hybridomas as a Measure of Clonal Response.

Ninety-six well polystyrene microtitre plates were coated at a concentration of 2.5 ug/ml. of protein in coating buffer with outer membrane preparations of SL3261 (vaccine), inner membrane preparations of SL3261, inner membrane preparation of an Rc mutant SGSC 227 (obtained from Dr. K.E. Sanderson, Department of Biology, The University of Calgary) and smooth LPS from S. typhimurium purchased from List Biochemicals, Campbell, California. The latter was made up to five ug./ml. in coating

buffer. 0.1 millilitres of all preparations were applied to wells (above). Culture supernatants for antibody testing were removed from their wells and 0.1 millilitres of each placed in three wells of each of the above antigen-coated plates, allowed to react at room temperature for two hours, then reacted with peroxidase-labeled anti-mouse IgG, IgA, and IgM (anti-IgG primarily from Miles Biochemicals and anti-IgA and anti-IgM from Cooper Biomedical, Inc., Malvern, Pa.). The substrate, 5-aminosalicylic acid, was allowed to react for thirty minutes as indicated above for the ELISA. Any supernatants which reacted with the outer membrane, smooth LPS or both were further tested against a panel of LPS region components; smooth LPS from S. typhimurium; Ra, Rb, Rc, Rd, Re and lipid A, all from Salmonella minnesota and obtained from List Biochemicals. E. Coli J5 purchased from List Biochemicals was also included as was the porin preparations described above.

Hybridomas of interest following this initial screening were propagated as indicated and included reactants to each of the antigen groups with each of the three immunoglobulin classes represented where possible.

E. Indirect Fluorescent Antibody to Assess Surface Reactivity of IgA Antibody from RBF/Dn Mice

Bacterial cells (SL3261) grown overnight on a blood agar plate were transferred immediately to a second blood agar plate using a heavy inoculum. After four to six hours incubation they were suspended in water and applied to microscope slides (twenty-five microlitres). Excess liquid was aspirated with a pasteur pipet leaving a thin homogeneous film. The

spots were allowed to dry, heat-fixed as for gram-staining bacterial smears and washed with phosphate buffered saline.

Hybridoma culture supernatants containing antibody were applied (twenty-five ul./spot of bacteria) and incubated thirty minutes in a moist chamber at thirty-seven degrees Celsius. A petri dish with a pair of applicator sticks on moistened gauze to keep the slide off the bottom of the dish served the purpose. The supernatants were removed with a pasteur pipet and slides washed in PBS three times, five minutes apart. The area around the smear was blotted with tissue. Twenty-five microlitres of suitably diluted FITC-labeled anti-mouse IgA (determined by checker board titration) was added to each spot (smear) of bacteria just treated with antibody. The slides were returned to the moist chamber at thirty-seven degrees Celsius for thirty minutes. The excess FITC conjugate was aspirated with a pasteur pipet and the slide dipped in PBS. This was followed by another three washings, five minutes apart with PBS, and a fourth with water. The areas around the smears were blotted as before and a drop of mounting buffer added. A coverslip was placed directly on the smear(s) excluding air bubbles and the slides read for fluorescence. The mounting buffer consisted of ten millilitres PBS containing 100 milligrams para-phenylenediamine (Huff, et al, J. Invest. Derm., 78, 449, 1982) and ninety millilitres glycerol (Baker). The pH was adjusted to 8.0 with carbonate/bicarbonate buffer at pH 9.0. Storage was in a stoppered brown bottle at minus twenty degrees Celsius.

RESULTS

A. Preparation of Test Antigens for ELISA

1. Evaluation of different methods of outer membrane preparation.

Outer membranes from the three methods of preparation described in Materials and Methods were all subjected to SDS PAGE. Initially, although relatively poor gels were obtained, the sucrose gradient and water/heat preparations exhibited the most similar LPS profiles by silver staining. On this basis, since LPS was considered an important immunological component of interest, the sarkosyl preparation was eliminated. With respect to the protein profiles exhibited on SDS PAGE, the sarkosyl and sucrose gradient preparations appeared most similar. Because of this the water/heat extracts were rerun on a gel to compare the protein profiles with the 55%, 45% and 40% fractions of the sucrose gradient. The 55% fraction and the water/heat extract exhibited comparable protein profiles; the 45% and 40% fractions resembling the expected profiles of the inner membrane had initially been positive for NADH-oxidase (Materials and Methods, above). Since an earlier gel had been suggestive of some differences in protein profiles, supporting the notion that heat treatment might alter the preparation, mice (random bred ICR) were immunized with both preparations (Materials and Methods).

2. Evaluation of immunogenicity and survival respecting toxicity of outer membrane preparations.

Figures 1. and 2. show survival and spleen enlargement respectively of mice given outer membrane preparations containing fifty ug. of protein. Figure 1. shows that at this concentration 8% (i.e. one mouse out

of twelve) died early following injection of the sucrose gradient preparation. Presumeably, this was due to the effects of endotoxin since it died at four hours post injection. All mice in the group were ill (malaise, prostration, fever as exhibited by ruffled fur) within one half hour, and extremely so at four hours. With the exception of the one death, all remained ill through forty hours and then recovered. This sequence of events corresponds to the classic description of gross sequellae of endotoxemia (Jawetz, et al, Medical Microbiology, 15th ed., Lange Medical Publications, Los Altos, California, 1982).

The mice given the water/heat extract showed a delayed reaction but with a greater overall death rate. There were no signs of illness until four hours when the mice appeared uncomfortable. They were ill the following day but not as extremely so as those given the sucrose gradient preparation. The first mouse died at forty hours at which time all others in the group were equally as ill as those which had been given the sucrose gradient preparation had been (the latter were recovering at this time). Deaths occurred progressively up to seventy-two hours totalling five mice out of twelve, leaving 60% surviving. The survivors recovered progressively after seventy-two hours.

The spleen weights of the two groups shown in Figure 2 indicated a progressive enlargement over the experimental period in the mice given the water/heat extract, whereas those receiving the sucrose gradient preparation reached a peak at five days with a subsequent gradual decline over the experimental period.

Proliferative response of spleen cells using blast transformation and the enumeration of mitotic figures in spleen imprints, Table 1, appeared to parallel splenomegaly in the general sense that there was a somewhat delayed, but slightly greater proliferative response in mice given the water/heat extract. Note that proliferation is given as the sum of the means of both lymphoblasts and mitotic figures.

The antibody response, as measured by ELISA and shown in Figure 3 with reciprocal assays for the two antigen combinations reflected, in general, the spleen proliferative response. Each point on the figure represents the mean serum titre from two immunized mice sacrificed at two, five, eight and twelve days. Mice given the water/heat preparation showed an antibody response reactive with both antigens to be delayed compared with that of mice receiving the sucrose gradient preparation. Moreover, the reaction of sera from these mice was significantly less to the sucrose gradient preparation as compared with the water/heat preparation which they had received. This suggests an alteration of some sort in the course of preparation and that this alteration is reflected in reactivity with the preparation (sucrose gradient) which produced pathological events of endotoxemia most nearly approximating classical descriptions. Concomitantly, animals which received the sucrose gradient preparations produced antibody earlier (presumably involving an LPS-induced mitogenic response of physiologically primed lymphocytes, as well, at four days). The response was highest at four days, did not vary greatly in reactivity between the two antigen preparations and appeared to be declining by the end of the experiment.

Significant differences existed in the reaction of sera from control, unimmunized mice to the two outer membrane antigen preparations. The higher reaction (two to three doubling dilutions) with the sucrose gradient preparation suggests a reaction with something to which the mouse had been exposed physiologically. From this one might infer that this preparation most nearly simulates a "naturally" encountered antigen. As such the two step sucrose gradient preparation incorporating the 55% and 60% fractions were used as the standard preparation throughout the remainder of the work.

The direct Jerne plaque assay did not show any zones of growth inhibition, lysis of bacterial cells or aggregation of bacterial cells with earlier colony formation over background.

B. Preliminary Delineation of Antibody Response to Orally and Parenterally Administered Live Vaccine

1. IgG antibody response to outer membrane preparations of the vaccine strain, its isogenic parent and two unrelated virulent and avirulent strains.

a) The response of the susceptible Balb/c.

Figures 4 and 5 show the serum antibody response (IgG) of Balb/c mice with time during a thirty-four day immunization when the vaccine strain was given live, parenterally and orally, respectively. Outer membrane extracts used as antigens on the solid phase for ELISA were from SL3261 (vaccine strain), SL1344 (isogenic, virulent parent to the vaccine), C5 (unrelated virulent strain) and M205 (unrelated carrier strain). The parenterally immunized mice were given 2 X 10^4 live bac-

teria rather than the intended 3 X 10^4 (a protective dose established by Hoiseth and Stocker, Nature, 291, 238, 1981) and chosen as a dose with little endotoxic effects. The dosage difference between this and 3 X 10^7 given orally (Figure 5) seemed to be apparent. However, notwithstanding differences in smoothness of curves, an overall similarity was the progressive preferential antibody reactivity with the immunizing vaccine strain in parallel with the unrelated carrier strain. The anticipated equal response to the isogenic parent to which the vaccine strain was similar in every way except for the requirement for aromatic amino acids and vitamins was not realized. Aromatic auxotrophy is unique to the vaccine strain. Hence, the preferential reaction of antibody to outer membranes of the carrier strain (a rough, Rb mutant) was not explained by aromatic auxotrophy. Moreover, a similar pattern of reactivity was also seen with the F_1 DBA X Balb/c.

b) The response of the resistant F_1 DBA X Balb/c.

DBA/2j and Balb/c are of the same H-2 haplotype such that if an immunological outcome of a macrophage defect should be exhibited, it would not be complicated by differences in genetic control over known immune responses (in this case the response to synthetic polypeptides is defined by the immune response genes controlled by the H-2 haplotype). In the cross, DBA/2j (M) X Balb/c (F) the Mendelian dominant Ity^r gene from the DBA/2 would be expected to be the influencing factor in responsiveness if, indeed, macrophages, as the effector cell for this gene, influence some consequential immunologic outcome affecting the B cell response. Figures 6 and 7 show the antibody reactivity of F_1 DBA X

Balb/c sera from parenterally and orally immunized mice. The parenteral dose of 3 X 10^4 bacteria invoked a greater antibody reactivity to the outer membranes of the four strains of S. typhimurium in the latter part of the immunization schedule compared with the oral dose of 6 X 10^7 live SL3261 bacteria. The comparison with the Balb/c in Figures 4 and 5 shows a much greater response of F_1 mice when the vaccine was administered parenterally. The Balb/c received two thirds the intended dose (2 X 10^4 vs. the intended 3 X 10^4 which was received by the F_1's) which may be a partial explanation. Orally, the increase in antibody reactivity from the initial to the final of roughly six dilutions was the same in the two strains although the actual titres were different. However, the pattern of preferential reactivity was the same as for Balb/c. Roughly two dilutions greater reactivity in serial doubling dilutions were exhibited for the carrier and vaccine strains which are avirulent as compared with the virulent strains. In these experiments there was a suggestion of an earlier response, between ten and twenty days in F_1's but a difference relative to the controls was difficult to demonstrate.

Figures 8 and 9 show control reactivity of sera from age-matched unimmunized mice of the two strains. A high background existed, a phenomenon which was also demonstrated in sera collected at specified times during monoclonal antibody assessment where three immunoglobulin classes of antibody were examined. In Figure 8, control Balb/c are age-matched comparable to those in Figure 5. Figure 9 shows the F_1 DBA X Balb/c controls age-matched to the animals in Figure 6. The actual response,

therefore, of antibody production over control, or the natural state of physiologic exposure to Salmonella typhimurium outer membrane antigens was not great. There is no predilection for any of the four different strain antigens in unimmunized control sera.

2. Splenomegaly.

This was not marked in orally immunized mice. Figure 10, a) and c) indicates that spleen enlargement occurred to some extent but not greatly compared to controls. This gross measure of proliferative response appeared to be slightly greater in F_1 DBA X Balb/c test animals compared with Balb/c, relative to comparable control F_1's (Figure 10 c) and d). It must be noted that the initial IP dose given to Balb/c was 2 X 10^4 as opposed to 3 X 10^4. However, the boost at twenty-one days was 3 X 10^4. The booster dose of live vaccine did not enhance spleen enlargement with any of the experimental conditions or mouse strains used.

3. Differential Cell Counts Determining Blast Transformation and Mitotic figures of spleen cells.

Table II shows counts done on imprints made on some of the spleens removed from F_1 DBA X Balb/c mice when the above experiments were performed and before spleen cells were dispersed for the direct Jerne plaque assay. As an index of the proliferative response to supplement data on spleen enlargement, the number of blast cells and mitotic figures were taken as indicators of lymphocyte activation from the quiescent stage through division incorporating mitosis. Table II shows the per cent blast cells, mitotic figures, the combination of the two, and the per

cent mature lymphocytes. $F_{1,17}=15.15$, $p<0.05$ in paired analysis of variance indicated a higher proliferative response in the parenterally injected mice. The per cent of blasts and mitotic figures was as high as forty-five with many between twenty and thirty per cent. The orally immunized mice had a high of thirty-one percent with most less than twenty percent. A relatively high proliferative response at three days may be mitogenic.

4. Gross lymph node response.

On gross examination, there was no appreciable change in the size of the peripheral lymph nodes, specifically the brachial and inguinal nodes. Mesenteric lymph nodes were examined and graded 1^+ to 4^+ in terms of relative increase in size compared to controls when mice were immunized orally. Figure 11 incorporates this relative enlargement with those of RBF/Dn mice examined at the time fusions were done on orally immunized mice (no enlargement of mesenteric lymph nodes occurred in parenterally immunized mice). Under the experimental conditions, a slight delay (four days) in maximal enlargement of Balb/c mesenteric lymph nodes was apparent relative to those of F_1 DBA X Balb/c. Otherwise the pattern of enlargement was comparable to F_1 DBA X Balb/c mice. Both showed an abrupt decline after twenty-one days with a slight lag during the last portion of the immunization schedule. These observations were repeated when the fusion experiments were performed (below). The RBF/Dn (resistant, wild-type Ity^r) exhibited a moderate and sustained enlargement of mesenteric lymph nodes.

5. Direct jerne plaque assay.

No plaques above background were observed at any time. Those few zones of inhibited bacterial growth were examined under a dissecting microscope or placed under low power of a compound microscope to examine for centrally located mononuclear cells. Plaques with mononuclear cells numbered approximately $1/10^8$ spleen cells irrespective of route or time of immunization or strain of mouse.

6. Female versus male antibody response.

Sera from F_1 DBA X Balb/c mice immunized parenterally with live vaccine were compared on the basis of male versus female response when the test antigens were the outer membranes from the immunizing bacterium, its virulent isogenic parent and the unrelated virulent and carrier strains. Figure 12 shows the comparison of all means of male and female test sera as well as control sera (seven female and five males represented in the controls). The test sera show a greater response by females as tested by all four antigens. Reactivity of control sera does not exhibit the same phenomenon.

Later work, Table III, done with the three strains of mice used for assessing the cellular response on oral immunization as well as three additional resistant mouse strains showed that although the females respond more strongly polyclonally in most cases (the notable exception being the two Swiss Webster strains, SWR/j and SW-local) the extent (titre) was significant only in the case of the F_1's (ANOVA of $\log_2$ titres). However, the frequency of greater female response existed for both RBF/Dn mice and F_1 DBA X Balb/c, Table IV. The latter included

the assessment to inner membrane and smooth LPS as well as to outer membrane preparations. It should be noted that Table III gives the overall means to a given antigen group of males and females tested at all times of the immunization schedule. The ANOVA was, nevertheless, done on individual values at individual times; n being reflected in the degrees of freedom indicated for the value of F. It should also be noted that there was no correlation between related strains. For example: DBA/2j do not resemble F_1 DBA X Balb/c; RBF/Dn which have a Swiss Webster derivation do not resemble the two Swiss Webster strains.

7. Shift in antibody rection to outer membrane test antigens prepared from bacterial cells grown in minimal iron-deficient medium.

Since a seeming lack of reaction, or definitely lower than expected reaction to the outer membrane (Figures 4 through 7 and Figure 12) seemed incongruous with the concept that surface exposed antigenic components should meet the host's immune system first, thereby triggering responsiveness, it was considered that the measurement of the response was inadequate. The level of response also seemed incompatible with the level of protection achieved with the immunizing doses (Hoiseth and Stocker, Nature, 291, 238, 1981). Hence, all four strains of S. typhimurium were grown in minimal medium with no iron (Sokol and Woods, Infect. Immun. 40, 665, 1983) in the anticipation that the bacteria would synthesize iron-binding proteins (expected to be of molecular weight of approximately 80K, Bennett and Rothfield, J. Bacteriol., 127, 498, 1976) and perhaps other antigens in larger quantities. As such, an assay with outer membranes containing greater quantities of iron-binding proteins as

antigen on the solid phase of the ELISA might detect antibody previously poorly detected. Figure 13 is a silver-stained polyacrylamide gel of outer membranes of each of the strains grown in nutrient broth and M9 medium containing no iron. It did not show remarkable differences in protein profiles in the two preparations. Minor differences in expression of some of the proteins were discerned on close inspection. Water/heat extracts were also run simultaneously and indicated either lower recovery of, loss of, or diminished expression of some proteins compared with sucrose gradient preparations.

Figure 14 shows that sera taken at ten days and thirteen days post-boost from F_1 DBA X Balb/c immunized parenterally with 3 X 10^4 live SL3261 vaccine and boosted with the same amount, showed no remarkable difference in titre from that shown in Figure 6 at days thirty-eight and forty-one. The difference between tests and controls was non-existent. Note that Figure 14 represents assays on individual male and female mice. In Figure 6, each point represents the mean titre of sera from two mice.

8. Differences in response to heat-killed vaccine.

Insofar as the work to this point indicated a lesser and different mode of immunological response to the outer membrane from that reported with other bacteria, the method of heat killing the vaccine bacteria was altered with subsequent assessment of the response. A single dose of 10^9 heat-killed bacterial cells was given intraperitoneally and the response followed for nine days. Figure 15 a) and b) shows antibody reactivity of mouse sera to outer membranes from bacteria grown in nutrient broth and in minimal, iron-deficient medium respectively. Antibody

reactivity to membranes from bacteria grown in iron-deficient medium was less than to those from bacteria grown in nutrient broth, reactivity with LPS was minimal, no plaques over background in the direct Jerne plaque assay were realized, and splenomegaly was marked (Figure 16). The order of magnitude of splenomegaly was 0.2 to 0.3 gram as early as three days, relative to highs between 0.2 and 0.25 gram (Figure 10) when protective doses of live vaccine were given, usually reached only after ten days. There was no predilection of antibody reactivity to outer membranes from avirulent strains as demonstrated with live bacteria (Figures 4 through 9). Moreover, any differences which might exist paralleled the etiologic origin of the strains - i.e. antibody reacted with the immunizing vaccine and its isogenic parent to a greater extent than to the unrelated strains (Figure 15a). This was not evident when membrane antigens prepared from bacteria grown in minimal iron-deficient medium were used on the solid phase for the ELISA. A possible interpretation of this could be that a killed bacterium is processed differently from a living bacterium by the host's macrophages. This may be especially true since Salmonellae replicate within the host macrophages and a different repertoire of antigens would be turned over.

9. The lack of response to flagellin and to porin.

Those fractions of flagellin prepared as described by Suzuki and Iino (J. Mol. Biol., 81, 1973) which contained a crude preparation of polymer or a partially purified $(NH_4)_2\ SO_4$ treated polymer preparation or monomers were used at five ug/ml. to provide 0.5 ug./well of protein to coat microtitre plates. Selected sera remaining from ICR mice

(orally immunized in supplementary experiments) and Balb/c mice, above, were tested against the preparations. Although not all sera tested were from the same strain of mice, sera representing ten days of immunization through twenty-one days of immunization were tested. One Balb/c serum at thirty-one days (ten days post-boost) was tested. None reacted with any of the flagellar fractions. Antibody to flagellar proteins was, therefore, not detectably produced on oral immunization although strain SL3261 was actively motile. Supernatants from hybridomas generated when spleen cells from unimmunized mice were cultured with LPS as a mitogen also did not react with flagellin. Hence, the high background or "natural" antibody was not to flagellar proteins.

Similar selected sera were tested for reactivity with porin. The highest titre obtained was 1/64. Most were 1/32 or less. Background as represented by sera from unimmunized mice ranged from 1/4 to 1/32. Hence, reaction with porin, a highly conserved outer membrane protein could not be considered great when used as an index of immune response in orally immunized mice.

C. The B Cell Response in Selected Peyer's Patches, Mesenteric Lymph Nodes and Spleen Cells as Assessed by the Product of Lymphocyte Hybridomas after Oral Immunization

1. The S. typhimurium susceptible (Ity^S homozygous) Balb/c.

a) Peyer's patches.

Although there were no hybridomas producing antibodies of interest to the test antigens, Peyer's patches from Balb/c mice gave the most successful fusion rate of any of the three strains tested. Figure 17 a)

shows that the number of fused lymphocytes per 10^6 lymphocytes plated was greater in lymphocytes from Balb/c Peyer's patches than in the other two strains with a significant number at day twenty-two.

b) The mesenteric lymph nodes.

Supernatants from fused cells from mesenteric lymph nodes were tested for reactivity with outer membrane antigens only until day thirty-two at which time the high fusibility of spleen lymphocytes triggered the concept that other antigens were involved. Figure 17 b) shows that fusibility of Balb/c mesenteric lymph node lymphocytes was less than either of the resistant strains of mice with no hybridomas formed at days thirty-two and forty-five (see also Table VI). At days ten and forty-five, fusibility of cells from both Balb/c and F_1 DBA X Balb/c was markedly less than were the mesenteric lymph node cells from RBF/Dn mice per 10^6 cells plated. On day forty-five, this may be related in part to the relative enlargement of mesenteric lymph nodes had gone down dramatically while those of RBF/Dn mice had remained constant.

Table V gives the distribution of reactive hybridomas to different groups of S. typhimurium antigens in per cent of total reactive hybridomas. Of note respecting the mesenteric lymph nodes of Balb/c is that only one at day twenty-two produced antibody reactive with the outer membrane (other antigens were not tested at this stage). Figure 17 a) shows that one out of 2.6 X 10^6 lymphocytes was fused. At days thirty-two and forty-five there were no mesenteric lymph node hybridomas. This is in contrast to the moderate numbers from the resistant F_1 DBA X Balb/c and RBF/Dn. On the same day, with identical experimental reagents and

conditions, hybridoma production from spleens was very efficient, Table VI.

c) The spleen.

The fusibility of spleen cells (Figure 17 c) from Balb/c mice, where done, was a rate per 10^6 comparable to the resistant counterparts. In contrast, however, the total number of hybridomas producing antibody of interest was very high (Tables V and VI), and was in a ratio of 1:1.07 and 1:1.3 reactive hybridomas to total hybridomas on days thirty-two and forty-five respectively. 84% and 93% (Table V) reacted with inner membrane antigens and inner membrane antigens from the rough Rc mutant respectively, on day thirty-two. It did not differ greatly (77.5% and 86%) on day forty-five. There was minimal reaction with the outer membrane at day thirty-two (0.8% of 1456 hybridoma supernatants reacting positively to test antigens) and 13% of the 496 positively reacting supernatants on day forty-five. A total of twenty-nine hybridoma supernatants reacted with LPS on days thirty-two and forty-five (0.2% each day) out of a total of 1952. None of these were IgA secreting hybridomas.

Figure 17 shows that Balb/c unimmunized spleen cells produced the highest number of hydridomas per 10^6 spleen cells after culturing with pokeweed mitogen for forty-eight hours. Only one of these reacted with outer membrane antigens. Other antigens were not included in the screening at this stage. However, the high fusibility suggests a high degree of physiological priming in this strain of mouse, supporting the high background in polyclonal sera (see below).

2. The resistant heterozygous Ity^r, F_1 DBA X Balb/c.

a) The Peyer's patches.

Figure 17 a) shows that no significant number of hybridomas were formed from Peyer's patch lymphocytes of F_1 mice. In fact, only one hybridoma in total was formed. This was at ten days and did not produce antibody reactive with the test antigens. Although the numbers of cells obtained from F_1 Peyer's patches were in an intermediate range, relative to the other two strains, the frequency of hybridomas formed was the lowest over the entire immunization period. Generally, the number of Peyer's patch cells plated was the most variable from any source. These cells were the most difficult to handle and sustained the greatest loss in technical manipulations. The range of plated cells from F_1's was 2.3×10^6 (twenty-two days) to 1.7×10^7 (twenty-four hours). For Balb/c the range was 3×10^6 (forty-five days) to 6.8×10^7 (twenty-two days). For RBF/Dn, the range was 1.25×10^5 (twenty-four hours) to 1.1×10^7 (thirty-two days).

b) The mesenteric lymph nodes.

Figure 18 a), b) and c) gives the clonal B cell response of F_1 DBA X Balb/c and RBF/Dn (both resistant) showing the distribution of Ig-class producing hybridomas. The most noteworthy difference between the two resistant strains was the later acquisition of antibody-producing capacity by F_1's with a gradual increase thereof in cells from the lymphoid organs examined. RBF/Dn showed an earlier antibody-producing capacity with a shift in quality thereof, particularly demonstrated in the mesen-

teric lymph node cells reactive with the test antigens. The details are described below.

Four test antigens, the outer membrane of SL3261, the inner membrane of SL3261 vaccine strain, the inner membrane of an Rc mutant (diphosphate-galactose-4-epimeraseless mutant), and commercially purchased smooth S. typhimurium LPS were used to test IgG-, IgA- and IgM-producing hybridomas. Note that Table V gives the distribution of antibody-producing cells from Balb/c mice with emphasis on thirty-two and forty-five days. Because antigens in addition to the outer membrane were not used until day thirty-two in the Balb/c experiment, the Balb/c experiment is not included in Figure 18.

The numbers of hybridomas formed per 10^6 plated cells from the mesenteric lymph nodes was less in F_1 than in RBF/Dn mice (Figure 17). With the exception of day thirty-two (Figure 18 b) the absolute numbers of hybridomas from mesenteric lymph nodes were also less in F_1 mice (fewer cells were plated, as well). The sequence and rate of generation of antibody-producing cells was from minimal numbers reacting with the Rc inner membrane at day twenty-two (IgG) through a gradual increase in numbers of cells producing antibody to all four groups of antigens reaching a maximum at forty-five days. At thirty-two days a small proportion of IgA producing hybridomas reactive with the Rc inner membrane were detected. At day forty-five IgM-producing hybridomas were also evident. The antibodies from these reacted with the two inner membranes (Figure 18 c). Two hybridoma supernatants with weakly positive IgM reactions in the initial screen to LPS could not be confirmed on retesting with outer mem-

brane components including smooth and rough LPS. Predictably, most of the antibody produced was IgG. It should be noted that Figure 18 gives absolute numbers to total hybridomas formed, as well as absolute numbers of hybridomas producing antibodies of given immunoglobulin classes. In Table V, the data are presented as per cent of total hybridomas producing antibody reactive with all the different groups of antigens. As such there is overlap in the table. For example, antibodies reactive with the inner membrane of SL3261, the immunizing vaccine, were almost always also reactive with the inner membrane of the Rc mutant; antibodies reactive with LPS were almost always also reactive with the outer membrane.

c) The spleen.

Figure 17 c) shows that the spleen of F_1 DBA X Balb/c had a greater ratio of hybridoma formation per 10^6 lymphocytes than did the mesenteric lymph nodes from ten days of the immunization schedule onwards. The increase was progressive, even though it was slight between thirty-two and forty-five days (Figure 18 b) and c) - 310 vs. 369 total hybridomas). This was paralleled by a progressive increase of specifically reactive antibody in hybridoma supernatants to all antigen groups with the exception of LPS which dropped within a very low range, from day thirty-two to day forty-five (by itself this may not be meaningful). More meaningful was the observation that the spleen in F_1 DBA X Balb/c had by far the greater number of activated cells as evidenced by the number of resulting hybridomas producing both specifically reactive antibody and not producing antibody of interest relative to the mesenteric lymph nodes. Note that even though the size of the mesenteric lymph nodes had

decreased by days thirty-two and forty-five (Figure 11), the number of hybridomas from them were moderate as were the numbers of hybridomas producing specifically reactive antibody. Taken together, this suggests that most of the specific antibody-producing cells had migrated to the spleen and that this migration took place without any of the cells being detected in the Peyer's patches and with modest numbers only detected in the mesenteric lymph nodes. Balb/c, above, gave no hybridomas from mesenteric lymph nodes at thirty-two and forty-five days. See Figures 17 and 18 as well as below for comparisons with RBF/Dn mice which represent the wild type of the Ity^r gene. The least number of cells producing antibody was against LPS, followed by the outer membrane, then the inner membrane with the highest reactivity against the inner membrane of the Rc mutant. This was the case for cells from both the mesenteric lymph nodes and from the spleen. This differential reactivity to the different antigen groups was most apparent at forty-five days in both the mesenteric lymph nodes and the spleen.

3. The resistant (Ity^r wild-type) RBF/Dn.

a) The Peyer's patches.

The recovery of cells from Peyer's patches of RBF/Dn mice was the lowest of any of the three strains of mice used (above). Technically they were exceptionally difficult to maintain. Many were lost through both death and excessive self agglutination during manipulations in preparation for fusion. In fact, the entire suspension of cells was lost for the forty-five day fusion in this way. However, at thirty-two days, which also constituted the greatest number of Peyer's patch cells har-

vested (1.1×10^7), five hybridomas were formed. All five reacted with the test antigens: two with the outer membrane, one with the inner membrane and two with the inner membrane of the Rc mutant (Table V). Of the two reacting with the outer membrane one did not react with smooth LPS, any of the core components or with porin, indicating, by elimination, a specificity for other outer membrane components. The other reacted with core components Ra, Rb, Rc, Rd, Re and lipid A.

b) The mesenteric lymph nodes.

Figure 17 b) indicates that the fusibility of lymphocytes from the mesenteric lymph nodes of RBF/Dn was very good in terms of the numbers fused per 10^6 cells plated. By the same comparison, the fusibility of the spleen cells from RBF/Dn mice was lower. See also Table VI. This is the inverse of the situation with F_1 DBA X Balb/c (Figure 17). Figures 18 a), b) and c) show not only many fusible cells throughout the immunization period, but specifically reactive antibody-producing cells including those fused twenty-four hours after the primary immunization in RBF/Dn mice. Antibodies from these fused cells reacted with the outer membrane, inner membrane and the inner membrane of the Rc mutant (the latter were also represented by IgM antibody in additions to IgG). Only antibody to LPS was not represented, a finding which was consistant throughout all these experiments, namely, very little, if any, antibody to LPS from any strain and that only in the latter part of the immunization schedule. By ten days, however, antibody-producing cells from RBF/Dn mesenteric lymph nodes to the inner membrane of SL3261, the immunizing vaccine were very much in evidence (Figure 18 a)). They exceeded

the numbers reacting with the inner membrane of the Rc mutant (the only instance). By day twenty-two this was reversed and more in keeping with the overall findings for the other two strains. At day twenty-two more cells from the mesenteric lymph nodes produced antibody to outer and inner membranes of SL3261 than did cells from the spleen (Figure 18 b)). At days thirty-two and forty-five, cells from mesenteric lymph nodes producing specific antibody were diminished even though fusibility was still high. At day thirty-two most of the fused cells reacted with the inner membrane of the Rc mutant, many of which also reacted with the inner membrane of SL3261. Respecting the latter, IgA-producing hybridomas were in evidence. These were first detected in limited numbers at day twenty-two from both mesenteric lymph node and spleen cells. IgA-producing cells were in slightly greater numbers (the total was small) from mesenteric lymph nodes through day forty-five when the total number of cells producing specific antibody from the lymph nodes was very small compared with the total number fused.

c) The spleen.

RBF/Dn spleens were not fused on day ten so that a comparison with the F_1 DBA X Balb/c cannot be made. Nor can a cellular distribution of hybrid cells producing specific antibody be compared with RBF/Dn mesenteric lymph nodes on this day. However, beginning with day twenty-two a large number of spleen cells were fused. A very high percent of total reactive clones (97.7% - Tables V and VI) reacted with the inner membrane of the Rc mutant. This is also evident from Figure 18 b). By day thirty-two many of the hybrid cells also produced IgM and the number of

IgG antibody-producing cells to the outer and inner membrane also increased markedly. At day forty-five, maximal numbers of splenic antibody-producing cells were realized (MLN antibody-producing cells had gone down markedly). The number producing IgM to the inner membrane of the Rc mutant had gone down, whereas the number producing IgM to SL3261 inner and outer membranes had increased. Hybrid cells producing antibody to LPS were detected in very limited numbers in the spleen at day forty-five only. Note that they were detected from the mesenteric lymph node as early as day twenty-two.

4. The distribution of cells reactive with outer membrane.

Smooth LPS, LPS core components, lipid A and porin were the outer membrane components used to test the supernatants from hybridomas reacting positively with either the outer membrane or smooth S. typhimurium LPS. Table VII, which depicts the number of reactions with each of these components does not distinguish either the original reaction (outer membrane or LPS), the day of immunization, the lymphoid organ source or the Ig class. Some of the supernatants reacted with more than one core component. Sharon, et al (Molecular Immunology, 19, 375, 1982) have shown that monoclonal antibodies, able to bind to linear determinants in the interior of the molecule of dextran, have sites complementary to six, and to seven glucose residues. Hence it is feasible that a single antibody could be involved in reacting with the entire length of the core which spans six linear carbohydrate residues with two additional tertiary carbohydrate residues (Sanderson, et al, Can. J. Microbiol., 1127, 1974). No experimental attempt was made, however, to determine that this was

indeed the case in these instances. Table VII B) merely demonstrates that the reactions took place. Table VII A) illustrates a more dramatic phenomenon, namely, that very few cells from the entire repertoire of the mouse cells at any or all times in the course of an immunization schedule with live vaccine reacted with LPS or a portion thereof. 1.8% of cells in the robust RBF/Dn reacted with LPS and/or porin; the majority being to smooth LPS. This was reduced by one half in the resistant F_1 DBA X Balb/c. Only 0.2% from Balb/c (comprising thirty-two day and forty-five days of the immunization schedule - Tables V and VI) reacted with LPS. Moreover, a miniscule number reacted with porin under these experimental conditions. If one regards the reactions with porin relative to the population of antibody-producing cells compared, then 2/3582 = 0.05% of all antibody generated under conditions simulating natural infections reacted with porin.

5. The polyclonal serum antibody response.

Figure 19 shows the polyclonal serum IgG antibody response of Balb/c,F_1 DBA X Balb/c and RBF/Dn mice in sera taken at the time of fusion. The overall picture was a reflection of the monoclonal but could not, of necessity, distinguish which lymphoid organ contributed the antibody. Refer to Figure 18 for the distribution of antibody-producing cells. Moreover, a high background in the ELISA when using whole serum was evidenced by high controls referred to earlier and partially explainable by previous physiologic priming. For this reason, polyclonally, test animal serum titres appear exaggerated.

The whole serum levels of antibody to LPS in Figure 19 reflected the diminished IgG antibody to LPS after twenty-two days in Balb/c relative to F_1 and RBF/Dn mice. See Figure 18 which indicates that the first appearance of anti-LPS producing hybridomas was on day twenty-two (RBF/Dn). The reaction of whole sera was not higher than background prior to day twenty-two. The reactions with the entire outer membrane did not indicate a lower response in Balb/c suggesting that antibody formed was to non-LPS components of the outer membrane. In fact, IgM antibody (Figure 20) was significantly higher in Balb/c than that of F_1 DBA X Balb/c to outer membranes. Table V does not corroborate this insofar as few hybridoma supernatants tested on days thirty-two and forty-five reacted with the outer membrane. However, spleen cell fusions were not done prior to thirty-two days from Balb/c mice, so it is unknown, in this case, whether anti-outer membrane-producing cells existed in the spleen before this.

In keeping with the fact that Balb/c are not immunologically defective (Kalpaktsoglou, et al, Immunology, 24, 303, 1974; Natsuume-Sakai, et al, Immunology, 32, 861, 1977), the reactions to inner membranes, both from the immunizing vaccine and the Rc mutant were very high. This was confirmed monoclonally for days thirty-two and forty-five. Polyclonally, there was an immediate increase over background at day one, presumeably from mitogenic activation. This was partially supported by the high proportion of hybridomas formed per 10^6 plated pokeweed mitogen stimulated cells (Figure 17). Again, in the latter case, reactions with inner membranes were not assayed for Balb/c. Polyclonally, however, a high titre

of antibody to inner membranes was maintained throughout the immunization schedule, fully equal to and in some instances greater than that of the resistant strains.

Figure 20 shows the IgM response of these mice. The IgM response of Balb/c mice was high, particularly to the inner membrane of the Rc mutant. That of F_1 DBA X Balb/c was relatively low. The difference between them was significant for the outer membrane ($F_{1,11}$ = 13.29, $p<0.05$ in ANOVA) and for the inner membrane of the Rc mutant ($F_{1,11}$ = 7.51, $p<0.025$). The difference between Balb/c and RBF/Dn was also significant for the inner membrane of the Rc mutant ($F_{1,15}$ = 9.72, $p<0.01$) - Balb/c being higher. Kalpaktsoglou, et al, (Immunology, 24, 303, 1974) have shown the hyperglobulinemia respecting IgM in Balb/c mice to be high. This is also shown by the controls in Figure 20 for the two inner membrane preparations. The reason for the immediate increase in titre to the outer membrane and the inner membrane of the Rc mutant is unknown. The possibility of mitogenic stimulation exists (from either LPS or peptidoglycan). Fusible cells from pokeweed mitogen stimulation were high (Figure 17). However, only the outer membrane was used as a test antigen at this stage resulting in the detection of one positively reacting hybridoma. Therefore, confirmation or the lack thereof of mitogenic stimulation of IgM-producing cells was not obtained.

The significant observation in Figure 21 was the absence of IgA over background in Balb/c. It applied to all antigen groups tested. IgA-producing hybridomas were also not found, above.

As substantiated by the occurence of hybridomas producing monoclonal antibodies, Figure 18, differences in rate and time of acquisition of antibody-producing cells to the different antigen groups did exist. Figure 22 shows the polyclonal reflection of this. It shows the acquisition of serum IgG and IgM. For the most part the F_1 DBA X Balb/c were delayed in the generation of antibody, but ultimately did so to equivalent capacity of the RBF/Dn, with the exception of IgM (see also Figure 20 and the reduced number of IgM-producing hybridomas of the F_1 DBA X Balb/c, Figure 18).

Three other resistant strains of mice were titrated for their antibody in the same oral immunization schedule. These were: DBA/2j, the resistant parent of the F_1's, SWR/j (one of the resistant strains used for establishing the genetics of resistance, Lissner, et al, Journal of Immunology, 131, 3006, 1983), and local Swiss Webster (Biology vivarium the University of Calgary). Each bar on the graph in Figure 23 represents the mean serum titre of a male and female mouse. Interestingly, the DBA/2j (which have been classified by some as intermediate in resistance - Robson and Vas, Journal of Infectious Diseases, 126, 378, 1972) did not have increased serum titres until as late as thirty-two days, supporting the monoclonal and polyclonal data for the F_1 DBA X Balb/c, above. This occurred for all antigen groups. The two Swiss Webster varieties (SWR/j and local SW) showed signs of increased titres to the

inner membranes as early as ten days with substantially higher final titres (forty-five days) to LPS and the outer membrane (differences in time and rate indicated for F_1 DBA X Balb/c and RBF/Dn seemed to be confirmed here, as well).

IgM and IgA were inconsistantly detected. In the local Swiss Webster, IgM at 1/64 to inner membrane was detected at forty-five days. SWR/j had a 1/64 titre of IgM to LPS at forty-five days. DBA/2j had weak (1/8) IgA and IgM titres to the inner membrane and the inner membrane of the Rc mutant.

D. Indirect Fluorescent Antibody To Assess Surface Reactivity of IgA Antibody from RBF/Dn Mice

The second antibody which was fluorescein-labeled was polyclonal, prepared in a goat against mouse immunoglobulin and unabsorbed to remove any background reactivity with salmonellae. A high background existed. This was similar to the mouse polyclonal antibody in the ELISA. However, it was possible to distinguish monoclonal antibody reactive with the surface of the bacterium (SL3261, vaccine strain) relative to the background as illustrated in the following schematic using monoclonal IgA antibody from RBF/Dn mice. The intensity of fluorescence is graded 1^+ to 4^+.

Positive Control	Inner Membrane Reactive	No. 1 Smooth LPS Reactive	No. 2 Smooth LPS Reactive	Outer Membrane (non-LPS) Reactive	Negative Control
2^+ to 3^+	1^+	4^+	3^+	2^+	1^+

A degree of variation existed from one monoclonal antibody to the next and from one day's preparation to the next. The relative intensity

was comparable, however. The negative control consisted of simply applying the fluorescein-labeled conjugate to the fixed bacteria on the slide without pretreatment with antibody. This was never definitively negative except at very high dilutions at which the positive controls (known monoclonals which reacted with high intensity in the ELISA to smooth LPS) were also then negative.

Some IgG and IgM monoclonals were also tested in the same way as the IgA indicated in the schematic. For the most part reactions mirrored those indicated. Since IgG monoclonals were more available and greater numbers had been propagated, more could be tested. With these, the greater variability existed in the degree of fluorescence with the outer membrane, non-LPS reactive monoclonal IgG antibody. The range was from no greater than the negative control to the same intensity as the LPS-reactive. Taking into consideration differences in avidity of individual monoclonal antibodies, as well as differences in concentration of non-LPS outer membrane components, this is predictable.

These tests confirmed the anatomic location on the bacteria to which antibody reacted. That is, antibody detected in the ELISA as reacting with the inner membrane from a two-step sucrose gradient neither reacted with nor became adsorbed to the outer surface in such a way as to be detected with fluorescein-labeled second antibody on the intact bacterium. Antibody to outer membrane components were made visible with the second antibody as indicated above.

DISCUSSION

Insofar as this thesis deals exclusively with the assessment of the B cell response to S. typhimurium, reference to cell-mediated, or T cell responses will be made in the discussion below only where clarification of this arm of the immune response impinges on the B cell response. Although the major portion of the B cell response as characterized here appears independent of the Ity gene expression, the effector cell for which is the macrophage, this is discussed in light of the original intention to test the hypothesis of Ity influence on B cell responsiveness.

A. The Test Antigens

1. The membrane preparations.

The two-step sucrose gradient method determined to be the preferred choice as outlined above in RESULTS does have the following weaknesses. Although exclusion of NADH-oxidase activity, or any other inner membrane enzyme excludes contamination by inner membranes from the outer membrane preparation, the reverse is not true under the experimental conditions. That is, the inner membrane fraction can still be contaminated with portions of the outer membrane. In the work of Osborn, et al, (J. Biol. Chem., 247, 3973, 1972), evidence was presented that the synthesis of O-antigen occurs exclusively in the cytoplasmic membrane (i.e. the inner membrane). This places a reservation on some of the thesis observations where reactions were exclusively with the inner membrane as discovered when extensive work showed poor antibody reactivity with the outer membrane. The reservation is that epitopes of the outer membrane may not be

as accessible to antibody in the intact outer membrane as they might be at some stage during assembly on the inner membrane. Hence, what would appear to be a lack of reactivity with LPS, in particular, in the majority of the population of antibodies synthesized, may be an underestimation.

However, if it is an underestimation, the concept that the antibody produced by the host to components of a bacterium which replicates within the host's macrophages, are preferentially directed to more internal components is strengthened. LPS on the inner membrane at various stages of assembly would be expected to expose epitopes subsequently masked in the final product which was used in the whole serum test and hybridoma screening systems. It would also be expected that the quantity of incompletely assembled LPS could vary. It would be more foreign to the host's immune system, thereby invoking a stronger total immune response (both quantitatively and qualitatively). The same argument of greater foreignness or greater recognition of non-self would apply to the total inner membrane including proteins, lipoproteins, enzymes, etc.

In Osborn's work pulse chase experiments showed that O-antigens are rapidly transferred to the outer membrane. Specific activities of enzymes of O-antigen synthesis were fifteen-to thirty-fold higher in the inner membrane fractions which were also enriched for glycosyltransferase activity involved in the biosynthesis of the core region of lipopolysaccharide. In summary, this infers that any cut-off, regardless of precision or number of fractions on a sucrose gradient would still leave an inner membrane fraction (or fractions) which contained outer membrane

components, especially LPS components and their synthetic enzymes. Osborn, et al, (J. Biol. Chem., 247, 3962, 1972), also showed that UDP-sugar hydrolases, ribonuclease I and endonuclease I activities were associated with both.

The main point of the findings in this work is that the bulk of antibody reactivity when immunization is carried out with the live, vaccine bacterium, is with internally located components. That is not to say, that there could not be overlap, or preferential detection with the inner membrane in those cases where there is overlap. The initial conceptual orientation was directed toward the outer membrane. When it became apparent that the response existed but not toward the outer membrane, the remaining fraction, which doubtless also contains some cell wall material was tested. Further purification and fractionation of the inner membrane was not attempted because of the characteristics cited above. Some attempts to distinguish between cell wall and membrane components were made by omitting lysozyme treatment. Lack of change in the pattern of reactivity tended to negate the involvement of the cell wall, although this was not excluded.

2. The reactivity of membrane preparations.

a) Antibody reactivity and survival with immunization.

The initial SDS PAGE with silver staining indicated that the water/heat preparation of outer membranes did, indeed, leave the LPS intact or heat stable as classically accepted (Jawetz, E., et al, Review of Medical Microbiology, 15th ed., 1982; Kenney, et al, Infect. Immun., 1, 41, 1970;

Neter, et al, J. Immunol., 76, 377, 1956). Differences have been observed in the past (Neter, et al, J. Immunol., 76, 377, 1956) in pyrogenicity, ability to adsorb to red blood cells and bacterial strain differences in immunogenicity. SDS PAGE protein profiles of the water/heat treated bacterial membrane preparations were variable. Loss or alteration was not consistantly detectable as indicated in RESULTS. However, Figure 13 does show either reduced quantity, alteration of, or perhaps total loss of some protein components.

One of the objectives in this work was to maintain all extracts, and all immunizing and testing agents in a form most nearly approximating the natural and/or physiologic state in order to assess more closely any possible responses or reactions in natural infections. In the case of the three membrane preparations where the final choice rested between the two preparations made by water/heat extraction and by sucrose gradient both immunogenic and toxicity determinations failed to support the lack of alteration of the outer membrane with heat. Although this is not necessarily identical to the heat treatment of isolated LPS no longer associated with other outer membrane components, the argument of lack of resemblance to the natural state also applied to the purified LPS unless it is subsequently compared by reconstitution in the intact membrane or intact bacterium. Figure 3 shows that the humoral antibody response was not identical when the two preparations were used as immunogens and that "natural" antibody from unimmunized mice did not react as well with the heated preparation as it does with the sucrose gradient preparation. The results in Figure 3 were supportive of the splenomegaly finding of

Figure 2 in that antibody production and reaction were slower with the water/heat preparation and resembled the time course of splenomegaly. Spleen enlargement ultimately exceeds that of the sucrose gradient preparation. The proliferative response of Table 1 was equivocal as far as any distinction between the two preparations was concerned.

Splenomegaly in this case mirrored the survival pattern (Figure 1) insofar as a delayed but greater death rate occurred with the heated preparation. Hence, heat treatment affected both the toxic properties of the outer membrane as well as its immunogenicity. Since toxicity was normally attributed to LPS, it must have been altered either by itself, or, an alteration occurred in its intimate association with outer membrane proteins. Since the heat treatment was to intact bacteria, these concerns apply to the use of heat-killed bacteria as vaccines.

b) Immunologic reactivity with membranes grown in minimal, iron-deficient medium.

Figure 14 indicates that the antisera from mice immunized with intact living bacteria grown in nutrient broth did not react significantly with outer membranes from bacteria grown in minimal, iron-deficient medium. If growth in minimal, iron-deficient medium enhanced the production of iron-binding proteins as well as other antigens (not readily discernable from protein profiles of the outer membrane, Figure 13), then these must be different from, and in addition to, those antigens presented to the host by the living bacterium. Aromatic-requiring mutants exhibit limited division within a given host (Killar and

Eisenstein, Infect. Immun., 47, 605, 1985); approximately four to five divisions in susceptible mice and about two divisions in resistant mice (different bacterial strain from SL3261 as well as different strains of mice). If the acquisition of iron was limited within the mammalian host, these division rates could bring about in vivo iron-binding protein synthesis. If so, however, no real exposure of these new antigens to the host seems to have taken place since a test antigen enriched with certain new antigens prepared to detect such antibody resulted in a negative reaction. Alternatively, these antigens were insufficiently immunogenic to invoke antibody responsiveness under the experimental conditions.

3. The differences in detectable immunological reaction to outer membrane preparations of S. typhimurium strains from different etiological sources.

a) Antisera from mice immunized with the living vaccine.

Because intuitively surface exposed antigens should contact the host's immune system first, and thus be included in the antigen processing system of host's macrophages on a priority basis, the antibody reaction to outer membranes of S. typhimurium was the most extensively characterized. That initial contact with the host, especially with the macrophages, and antigen processing of the contact components are not necessarily sequential events, when the bacterium can replicate within the macrophages, is discussed in detail below. Notwithstanding the presentation of more detailed evidence supporting the latter concept, interesting differences in antibody reactivity to external components, i.e. outer membrane antigens also occurred.

Since the literature describes the use of a variety of different pathogenic and non-pathogenic strains of S. typhimurium in determining the genetics of resistance and susceptibility in mice, two which were frequently used were obtained from Dr. D. R. Rowley, University of Adelaide, Adelaide, Australia. Strain M206 (avirulent) and C5 (virulent) have been used by Robson and Vas (J. Inf. Dis., 126, 378, 1972), Plant and Glynn (J. Inf. Dis., 133, 72, 1976; Genetic Control of Natural Resistance to Infection and Malignancy, p. 133, Academic Press, 1980; Clin. exp. Immunol., 37, 1, 1979), Hormaeche (Immunology, 42, 569, 1981; Genetic Control of Natural Resistance to Infection and Malignancy, p. 121, Academic Press, 1980) and O'Brien, et al, (Cellular Immunology, 67, 325, 1982).

Figures 4 through 9 show IgG antibody response with time in susceptible Balb/c and resistant F_1 DBA X Balb/c with appropriate controls. Both oral and parenteral immunization routes were represented. As indicated in RESULTS the response above background was low when a minimum but totally protective dose was used. In fact the response was out of keeping with total protection as well as with the degree of proliferative response (Table II) and splenomegaly (Figure 10 d)) for parenterally immunized F_1 mice. Some of the early proliferative response in Table II (three days) was doubtless mitogenic, but the sustained increase beyond ten days, supported by splenomegaly, implied antigen activation. Because xid mice are susceptible to Salmonella infections, B cell activation was strongly suggested as was the concept that protection was not exclusively cell mediated. It seemed that some antibody was not being

detected. It should be noted that the proliferative response in the orally immunized animals was markedly less in the spleen compared with parenterally immunized animals.

The low antibody reactivity nevertheless revealed a consistently greater rise in antibody reactivity to the outer membranes of the two avirulent strains than for the virulent strains in the latter portion of the immunization schedule. The time of this phenomenon was not identical in the two strains. It did not occur at all in control mice of either strain and was, thus, due to immunization. The reason for this is unknown. Strain M206 was shown by phage typing to be a rough, Rb mutant (performed through Dr. K. E. Sanderson, Department of Biology, the University of Calgary). Since the isogenic parent to the vaccine, the vaccine strain and the virulent strain C5 are all smooth, rough lipopolysaccharide did not account for the similarities in reactivity with antibody with the two avirulent strains. Moreover, aromatic requirements of the vaccine strain would argue strongly against antigenic similarities with the outer membrane of a rough, unrelated strain.

An interesting aspect from the point of view of host responsiveness is that in F_1 DBA X Balb/c mice the difference was apparent immediately after immunization. It is difficult to compare with accuracy the time of occurrence of increased antibody reactivity to the outer membrane of the avirulent bacterial strains because doses were different for the two mouse strains (Balb/c received 2/3 of the parenteral dose of live SL3261 and one half of the oral dose compared with F_1 DBA X Balb/c). The later response in orally immunized Balb/c mice suggested that doses may

need to be greater in Balb/c to invoke this reaction. That is, the minimal but totally protective dose of 3 X 10^7 bacteria did not bring about this response until later. In the F_1's, a minimally protective parenteral dose and twice the minimally protective oral dose invoked both the mitogenic and antigenic response to this antigen(s). This lends additional support to polygenic control of antibody responsiveness, discussed in greater detail below, in the context of the mouse strains being of the same (H-2^d) haplotype, but with different rates of response. The more insensitive polyclonal reaction, because of a high ELISA background, places a reservation on the significance of the differences in titre in the early stages of the immunization schedule.

The greater reactivity of antibody with the two avirulent strains did not occur when vaccine bacterial cells were killed by heating (below) prior to immunization. Hence, it seems uniquely induced by the use of live immunizing vaccine bacteria. It is doubtless incidental to protection, since Robson and Vas (J. Inf. Dis., 126, 378, 1972) found that live strain M206 did not protect susceptible mouse strains.

b) Antisera from mice immunized with heat-killed vaccine.

The degree of the proliferative response (Table II) and the significant splenomegaly with parenteral immunization were indicators of a B cell response taking place, although a cell-mediated response could still play a major role. Despite the fact that heat-killing seemed to prevent appropriate assessment of a natural response, it was considered important to investigate this response once more in order to compare differences in response for the sake of completeness and for comparison with the work of

others. F_1 DBA X Balb/c, which would be expected to be the most responsive were chosen as the mouse strain.

The direct Jerne plaque assay, did not at any time give a positive assay above controls but had been used by others (Schwartz and Braun, Science, 149, 200, 1965; McAlack, et al, Science, 168, 141, 1970) with Escherichia coli and Vibrio cholerae as a measurement for numbers of spleen cells producing bacteriolytic antibody to outer membranes of these bacteria. Heat-killed, immunizing vaccines of these two bacterial genera, which are extracellular pathogens, had been used to induce antibody production. In the experiments performed here with S. typhimurium, no bacteriolysis or inhibition of growth, nor foci of growth (from antibody to flagellae) were observable in the direct Jerne plaque assay.

A proliferative response to heat-killed bacteria was marked as indicated by splenomegaly (Figure 16) and could not be due to bacterial replication in the spleen. The reactivity of antibody, in this case, was not great and again reflected differences which might be attributed to effects of heat treatment on the immunizing bacterium. The microtitre plates used for the data presented in Figure 15 a) and b) were coated with sucrose gradient-prepared outer membranes of the four different strains of bacteria each grown in nutrient broth and minimal, iron-deficient medium, respectively. Antibody reactivity with homologous strain SL3261 vaccine outer membrane was lower than anticipated. It was highest to the isogenic parent of the vaccine which should be antigenically the same. Reactivity with membranes from nutrient broth grown unrelated strains (C5 and M206) seemed less or equal to the isogenic

strain and homologous strain. Reactivity with LPS was lowest of all. Variation in the pattern of reactivity seemed to exist when tested against membranes from bacteria grown in minimal, iron-deficient medium (Figure 15 b)). However, inspection showed that no reaction occurred at a dilution of greater than 1/32, the level of most control reactions. Hence, heat-killing and a larger immunizing dose (10^9) of the vaccine bacterium did not enhance antibody production, irrespective of test. In the ELISA (Figure 15), the pattern of reactivity simply mimicked more closely the origin of the strains. This may not, by itself be meaningful from the overall point of view of protectiveness.

As alluded to in the introduction, many variations on the use of killed Salmonellae have been used over the years; all with lesser efficacy than the use of living Salmonella (Collins, Bacteriol. Rev., 38, 1974; Eisenstein and Sultzer, in Host Defense to Intracellular Pathogens, Plenum Publishing Co., 1983). Collins presents the argument that killed vaccines are unable to invoke the accelerated recall of earlier cell-mediated immunity when a challenge bacterium enters the tissues. The evidence cited was that, although vaccinated mice, for instance, may survive challenge, viable bacterial counts carried out on the liver and spleen indicate that the animals had, nonetheless, suffered a severe attack of mouse typhoid fever. Genetic control of the mice was not in practice at the time.

When Figure 15 a) is compared with Figure 6 in this work, the antibody titre to the outer membrane did not attain the same level in nine days with an inoculum of 10^9 heat-killed bacteria as it did in thirty-

two days with a primary inoculum and boost each of 3 X 10^4 live bacteria. Strains of mice and routes of immunization (intraperitoneally) were the same. The living bacteria would be expected to die within a few days at most (in both cases the immunogen was the aromatic-requiring vaccine strain). Although one might argue that titres would be expected to rise after the nine days, as well, the lack of involvement of the outer membrane in the humoral response was implicated in this way. Additionally, it suggests that, with time, more or other antigens even from the outer membrane were available for the host's response when the immunizing bacterium is living (the enhanced antibody reactivity with avirulent strains, Figures 4 to 7).

It is generally agreed that nonviable vaccines induce primarily humoral immunity against salmonellosis in the host (Hsu, et al, Can. J. Microbiol., 35, 54, 1985; Eisenstein and Sultzer, in Host Defense to Intracellular Pathogens, Plenum Publishing Co., 1983; Nakoneczna and Hsu, Infect. Immun., 39, 423, 1983). However, the observations here do not indicate that the response is greater than with viable bacteria, the marked splenomegaly notwithstanding. The latter could also have been invoked by endotoxin (LPS) mitogenically.

The low levels of antibody to the outer membrane, as revealed in the present study, do not seem to be bactericidal as assessed by the direct Jerne plaque assay, below. They appear to be incidental to bacteriostatic and bactericidal activity. There was, moreover, a relatively high background (natural) antibody to the outer membrane which was not increased greatly with immunization (reflecting a secondary response),

and it was not naturally protective. Background antibody as assessed on whole serum by ELISA (Figures 8 and 9) was as high in Balb/c (susceptible) as in the resistant F_1 DBA X Balb/c mice. Antibody to the outer membrane may be involved in cell-cell recognition, or opsonization, or it may block uptake by some host cells. Enhanced uptake by peritoneal exudate cells in addition to enhanced blood clearance was reported by Jorbeck, et al, in mice immunized with O-antigen-specific conjugated oligosaccharides (Infect. Immun., 32, 497, 1981). Collins and Eisenstein, above, also review many experiments demonstrating enhanced blood clearance.

4. The lack of responsiveness.

a) The lack of responsiveness in the direct Jerne plaque assay.

This assay, used by others to provide a direct method of enumerating cells producing antibody to E. coli and V. cholerae membranes did not assist in the establishment of any bactericidal or bacteristatic mechanism in the systems used here. Retrospectively, from the monoclonal work discussed below, this could be partially explained by the fact that the majority of antibody-producing cells synthesize antibody to more internally located antigens. Alternatively, if the antigens should , in fact, be components of the outer membrane, they could be masked, as discussed above by subsequent assembly steps in the case of LPS.

In any event, the role of antibody to LPS, or more specifically the O-antigens, has eluded definitive correlation with protection. The work of Collins (Bacteriol. Rev., 38, 371, 1974) in the Salmonella enteritidis system using smooth S. pullorum and S. gallinarum both with the same

O-antigens as S. enteritidis (Topley and Wilson, Principles of Bacteriology, Virology and Immunity, 1983), showed protection by the latter and not the former. Calves, vaccinated with an aromatic- requiring, smooth S. dublin vaccine of common O-antigen 12, when subsequently challenged with S. typhimurium showed some clinical signs of illness: diarrhea, loss of appetite, positive stool cultures, increased rectal temperature. Survival was complete in this instance (Smith, et al, Am. J. Vet. Res., 45, 2231, 1984). Synthetic conjugates of O-antigens with protein exhibited differences in levels of protection depending on the conjugating protein, including porins (Svenson, et al, Infect. Immun., 25, 863, 1979) and the size of the oligosaccharide (Svenson and Lindberg, Infect. Immun. 32, 490, 1981; Jorbeck, et al, Infect. Immun., 32, 497, 1981). Colwell, et al, showed protection by monoclonal antibodies to be greater in C3H/HeN mice than in C3H/HeJ mice (J. Immunol., 133, 950, 1984). Additional variation existed in their reactivities with different S. typhimurium reference strains.

In short, it is possible to force antibody production to LPS and to the outer membrane, but the extent of its protectiveness is uncertain beyond enhanced blood clearance of a subsequent virulent challenge. Organ invasion is not entirely dependent on blood clearance and great variation has been reported depending on mouse strains (Eisenstein, T. K. in Host Defences to Intracellular Pathogens, 1983). On immunization under conditions most closely approximating natural exposure, the thesis work showed that it was not the preferred choice of the host's immune system to produce anti-LPS or anti-outer membrane antibodies.

An alternative explanation for the failure to detect zones of bacteriolysis or growth inhibition was that *in vitro* the rate of accelerated bacterial division (approximately twenty minutes, relative to approximately eight hours *in vivo*) was too rapid for the incorporation of antibody to internally located determinants in such a way as to prevent bacterial growth or cause bacteriolysis. That is, the physical conditions of diffusion of the antibody in soft agar relative to turnover and exposure of internally located sites of the bacteria were not sufficiently synchronized to be detected in this way requiring technical modifications.

b) *The lack of responsiveness to flagellin and flagellae.*

The direct Jerne plaque assay can also be a measure of antibody-producing cells which secrete antibody to flagella (Diener, E., J. Immunol., 100, 1062, 1968; Diener, E. and Armstrong, W. D., Lancet, 2, 1281, 1967). The principle of the test is the adherence of flagellated bacteria to antibody secreting spleen cells in agar, thereby producing foci. The resulting early (less than four hours) colonies or foci of concentrated growth can be observed to a large extent by eye, but must be counted under low power magnification. All direct Jerne plaque assays were observed at four and eighteen hours without the detection of any foci of concentrated growth.

Selected sera as indicated in RESULTS were also tested against semi-purified polymer and monomer fractions of flagellin without observeable reaction in the ELISA. The reason for the lack of response to a protein which is, by itself, highly immunogenic is unknown. Flagellae

are a virulence factor for S. typhimurium (Carsiotis, et al, Infect. Immun., 46, 814, 1984) in C57B1/6j mice (Ity^S/Ity^S homozygous). Further, the flagellae help S. typhimurium survive in the macrophages of these mice (Weinstein, et al, Infect. Immun., 46, 819, 1984). It is unknown, however, whether this holds for all strains of mice, or, if so, how it relates to the failure to invoke a B cell response under natural conditions.

c) The lack of response to porin.

Animals respond to porin proteins when the purified or semi-purified protein is injected as an immunogen (Kuusi, et al, Infect. Immun., 25, 857, 1979). This has been shown to be protective (Kuusi, et al, Infect. Immun., 34, 328, 1981).

Porins are highly conserved proteins among enteric bacteria. It would be surprising if microbes evolved in immunodominant protein readily recognized by host defenses. Antibody could block transmembrane transfer through pores formed of porin protein (Nakae, T., J. Biol. Chem., 251, 2176, 1975; Hancock and Nikaido, J. Bacteriol., 136, 381, 1978; Hancock, et al, Biochemica et Biophysica Acta, 554, 323, 1979) and adversely affect survival of the microbe. With heat-killed outer membrane preparations of Pseudomonas used as an immunogen, sixteen out of three hundred and ninety hybridomas (4.1% - Mackie, et al, J. Immunol., 129, 829, 1982) were panreactive to a panel of twelve Pseudomonas serotypes. Three of these were since identified as recognizing porins (K. Williams and H. R. Rabin, personal communication). Hence, even under these conditions of immunization where porins would be expected to be enriched relative to

the intact bacterium the anti-porin response was limited. In this thesis work, 0.05% (RESULTS, Table VII) of all hybridomas tested after antibody responsiveness began to increase recognized purified porins.

If one accepts the possibility of molecular mimicry and that the host would respond minimally to antigens most similar to self antigens (Mosmann and Longenecker, J. Immunol., 128, 100, 1982; Damian, Am. Nat., 98, 129, 1964) then one would expect the conserved, broadly shared structures on a microbial species which are critical to the viability of the microbe to fall into this category. In the case of Salmonella where a carrier state can be established, and porins are common to all species, a strong immunological reaction from the host to porins is an unlikely phenomenon. It is also logical, therefore, that when antibody is produced experimentally against such a structure, that it would be protective. Mosmann and Longenecker (above) and Cunningham and Pilarski (Eur. J. Immunol., 4, 319, 1974) have shown that immune responsiveness, as represented by antibody responsiveness, of the host is specific for private antigens (i.e. those unique to the multideterminant immunogen) early in the response. It is only later that the response is panreactive, or to those antigens shared by all members of the species from which the multideterminant antigen is derived, usually meaning highly conserved antigens.

The above discussion is directed primarily to the host's response as it affects antigenic properties of outer membrane preparations. It details the impact of different methods of preparation, environmental effects (nutrition) on the preparations, and the effects of different

etiological sources of S. typhimurium. It also suggests a diversity of antigenic determinants on (in) living bacteria not detected with killed bacteria.

When it was discovered that the suspected significant B cell response was, in fact, to more internally located antigens of S. typhimurium, the inner membrane fraction of the vaccine strain SL3261 was added to the assay protocol. Commercially prepared purified smooth LPS was added to distinguish between LPS and other outer membrane components. The inner membrane of an Rc mutant was added through coincidence in the course of investigating the effect of lysozyme treatment on membrane preparations. Initially, it was assumed that the inner membrane from this mutant should be the same except for lysozyme treatment, a concept later dismissed when lysozyme treated and untreated inner membranes of SL3261 reacted identically. Supplementary testing of LPS and porin was with commercially purified, rough LPS and "in house" prepared porin.

The discussion below involves the time, rate and extent of the responsiveness to the antigens in three lymphoid organs on the cellular level, and the total overall response as reflected polyclonally in whole serum. The two strains of mice, Balb/c and F_1 DBA X Balb/c were additionally compared with the wild-type, Ity^r, RBF/Dn, a strain selected primarily for its chromosomal translocation characteristics as opposed to selected inbreeding for immunological characters.

B. The B Cell Response in Selected Peyer's Patches, Mesenteric Lymph Nodes and Spleen

1. The Peyer's patches.

Technical difficulties in handling Peyer's patch cells and bringing them through the entire fusion procedure preclude making definitive conclusions about these cells. No reason is known for greater self-agglutination, for instance, in one strain of mouse compared with another. Yet this was experienced throughout the course of any given experiment. For example, all five fusions involving Peyer's patches from RBF/Dn mice from day one through day forty-five were more difficult to handle than were those from Balb/c mice or F_1 DBA X Balb/c.

Given the differences in difficulties in handling Peyer's patches, Figure 17 a) shows that although a reasonable number of Peyer's patch cells were recovered from the F_1 DBA X Balb/c their fusibility was negligible. This suggests that there was no antigenic or mitogenic (as in twenty-four hours, for example) activation of these cells at any time. In contrast, those from Balb/c mice were reasonably fusible through twenty-two days even though no antibodies of interest were derived from them. The generally responsive RBF/Dn mice, despite small numbers of cells plated, not only produced some hybridomas, but the hybridomas were antibody-producing (day thirty-two).

Due to the problems of assessing Peyer's patch cells, only one of the conclusions derived from the more controllable fusion experiments of the mesenteric lymph nodes and spleen could be supported. That is that the data from this thesis which add to the concept of polygenic control of antibody production also seems to include genetic control of migration of cells from one lymphoid organ to another on oral immunization. The presence of specifically reactive antibody producing cells in Peyer's

patches of RBF/Dn mice at thirty-two days tended to support repopulation after stimulation (Bienenstock and Befus, Immunology, 41, 249, 1980; Bienenstock, et al, Annals of the New York Academy of Sciences, 409, 164, 1983). This would appear to be different from the other two strains.

2. The mesenteric lymph nodes and shifts in cell populations respecting reactions to different antigen groups with time.

Table V, in particular emphasized marked differences in the contribution to total antibody of antibody-producing lymphocytes from the spleen and mesenteric lymph nodes. Although thirty-two days and forty-five days were the only two days in the immunization schedule of Balb/c which allowed comparison with the antigen groups ultimately used, a major point was emphasized. There were no activated lymphocytes of any kind in the mesenteric lymph nodes (Table VI-A). That is, no hybridomas were formed despite the fact that comparable numbers of lymphocytes were plated (Table VI-B) from Balb/c as from the other two strains. It is unlikely that this was a technical artifact since all reagents and materials, including media, PEG, feeder cells in the form of macrophages were identical to those used for the spleen fusions from Balb/c on the same days. In the case of the latter, the fusibility was extraordinarily efficient, with most hybridomas being antibody-producing. This suggested that the total antibody came from spleen cells exclusively at this stage, and none from the mesenteric lymph node cells.

At twenty-two days a total number of sixty-eight fusible mesenteric lymph node cells from three Balb/c mice was realized - a modest amount, when compared with the other two strains where two mice contributed a

good number of lymphocytes (Table VI-B) as well as hybridomas. Even though all antigen groups were not used as test antigens with Balb/c at this stage, the important point is that peak activation had been reached at this stage in Balb/c mesenteric lymph nodes. This peak correlated with the node enlargement (Figure 11) as did the subsequent dropoff in both size of mesenteric lymph nodes (Figure 11 was confirmed in the fusion experiments) and fusibility of cells from them. Days one and ten did have fusible cells but not to the extent of day twenty-two cells. Since the outer membrane was the only test antigen used, with which none of the hybridomas were reactive, the fusibility of activated lymphocytes was the only comparison which could be made for these early fusions.

The most dramatic contrast of the Balb/c mesenteric lymph nodes was with those of the RBF/Dn. The RBF/Dn, chosen as the wild-type resistant had a genetic background of immune responsiveness in addition to the H-2 which would be comparable to the F_2 of Bouthillier, et al, (Immunology, 46, 67, 1982) and Biozzi, et al, (Adv. Immunol., 36, 189, 1984). That is, since the RBF/Dn mice are not necessarily derived from brother/sister matings they maintain a distribution of genes associated with the polygenic control of antibody responsiveness to Salmonella antigens which more closely approximates a normal population distribution. The repertoire of genes would be expected to be more comprehensive compared with totally inbred strains. This seems to be supported by the observation that hybrid cells from RBF/Dn mesenteric lymph nodes showed limited reactivity with the outer membrane as early as day one after immunization (Figure 18a). Presumeably this would be due to mitogenic stiumulation of

previously primed lymphocytes at this stage. Together with reactions to the inner membrane of both the vaccine and the Rc mutant, all fourteen hybridomas (Table VI - A) were reactive after mitogenic stimulation. Moreover, at day ten (Figure 18a) and Table VI-A) the peak fusibility of mesenteric lymph node cells was achieved (again with high specific reactivity) in contrast to the later peak of the Balb/c mice and even later of F_1 DBA X Balb/c mice (thirty two days).

The RBF/Dn mice best illustrated the finer points of distribution of lymphocytes. The specific reactivity of hybridomas could be a marker of their distribution. The RBF/Dn showed the most diverse distribution, as well as the most comprehensive repertoire. Antibody reactivity with all antigen groups except smooth LPS was represented in the limited numbers of hybridomas formed at day one. At day ten the majority of reactive hybridoma supernatants reacted with the inner membrane of the immunizing vaccine strain, SL3261 (Figure 18 a)). This was the only instance that this occurred. It was interpreted as meaning that, at an early stage in the immune response, RBF/Dn mice were endowed with the genetic capacity to recognize specific unique determinants located internally in the vaccine strain. Later as at day twenty-two (also Figure 18 a)), when the ratio shifted in favor of the inner membrane of the Rc mutant, those determinants which were shared were included in the repertoire of antibody-producing cells. If any of these were represented either in greater quantity or were more readily accessible by virtue of the mutant being rough, this would be reflected by greater numbers of cells producing antibody reacting with the Rc mutant inner membrane.

Figure 18 a) also shows that at twenty-two days there was an increased number of cells (approximately thirty out of the eighty-two reactive hybridomas) which reacted with the outer membrane. Of these a small number reacted with LPS. This was in contrast with the single reaction out of sixty-eight fused cells in the case of Balb/c and low fusibility and specific reactivity of the F_1 DBA X Balb/c at this time.

At thirty-two days the B cells reactive with the outer membrane (Figure 18 b)) were no longer readily detectable from the mesenteric lymph nodes in RBF/Dn mice, but they were in the spleen. At forty-five days, although fusibility of mesenteric lymph node lymphocytes was still very efficient (Table VI - A), only 8% of the cells were specifically reactive with any of the antigen groups used here. Of these few, there was a fairly even distribution between outer and inner membrane reactions with fully detectable IgA representation.

In contrast to both the RBF/Dn and the Balb/c, the F_1 DBA X Balb/c showed a prolonged lag time, both in fusibility as well as specific reactivity of lymphocytes from the mesenteric lymph nodes. Table VI-A and Figure 18 a), b), and c) show that there was a progressive increase beginning at twenty-two days of specifically reactive cells representing all antigen groups. Fusibility peaked at thirty-two days and remained at about the same level at forty-five days.

3. <u>The spleen and shifts in cell populations reacting to different antigen groups with time</u>.

The high number of hybridomas on days thirty-two and forty-five derived from Balb/c spleen cells have a proportion of specifically react-

ive supernatants very close to the proportions derived from RBF/Dn spleen cells. However, in Balb/c mice the distribution of these reactive cells is more restricted (Table V) with markedly diminished numbers reacting with the outer membrane, particularly LPS, and proportionately greater numbers reacting with the two inner membranes. Figure 18 a), b) and c) shows the broader repertoire of antibody-producing cells from the spleens of RBF/Dn even compared with the F_1 DBA X Balb/c.

Notwithstanding differences in the magnitude of response between the two strains, spleen cells from RBF/Dn mice at twenty-two days were producing antibody in limited numbers to the outer membrane as well as to the inner membrane preparations. In contrast, although the response of F_1 spleen cells was limited in total at this time, only antibody-producing cells to the inner membranes were detected. On day thirty-two a significant number of IgM -producing cells reactive with the inner membrane of the Rc mutant were detected from the spleens of RBF/Dn mice. At day forty-five, IgM-producing cells were detected as reacting with both inner membrane preparations as well as the outer membrane. By comparison, IgM-producing cells from F_1 DBA X Balb/c were few in number at both thirty-two and forty-five days.

Hence, in a manner similar to the cells from the mesenteric lymph nodes, the spleen cells represent a repertoire which is different from one mouse strain to the next. It is, moreover, different between the

mouse strains of the same H-2^d haplotype. The spleen cell differences between the Balb/c and the F_1 DBA X Balb/c are the peak fusible numbers and peak reactive numbers at day thirty-two for the Balb/c, whereas the peak for the F_1 DBA X Balb/c is at day forty-five or later. Table VI shows the respective percentages of reactive to fusible cells. As well, Table V and Table VII show that the reactions with the outer membrane and LPS of spleen cells from the F_1 were intermediate between the Balb/c and RBF/Dn. With respect to LPS they were roughly four times the number from Balb/c. It was not shown in this experimantal work whether this was a consequence of the Ity gene or not.

However, some of the work of others (Biozzi, O'Brien, above) is supported in that the high B cell response of the Balb/c mice, particularly demonstrated at days thirty-two and forty-five accompanied the susceptibility to S. typhimurium. It is referred to again, below, in the discussion of the polyclonal response. It can be said that this high antibody response, particularly involving IgG, but also IgM (see polyclonal response, Figure 20 showing high IgM titres to all antigens except LPS, and especially to the inner membrane of the Rc mutant) corresponds to the findings of Biozzi, et al, (Adv. Immunol., 36, 189, 1984). The total number of reactive B cells in Tables V and VI came from three mice in the case of Balb/c and from two mice in the case of RBF/Dn and F_1 DBA X Balb/c. On comparing the total number of hybridoma supernatants (representing antibody secreting spleen cells) with the total number of spleen cells plated, the comparisons are as follows. There were five times more Balb/c lymphocytes plated at thirty-two days (peak Balb/c response) than

F_1 DBA X Balb/c spleen cells and 1.8 times more than RBF/Dn spleen cells. The number of reactive hybridomas was seven times more and six times more respectively. Although the F_1 response had not peaked at this point, the RBF/Dn response had. Certainly, those ratios shifted for given days of the immunization schedule, as for instance, day forty-five when the response of the Balb/c was diminishing while that of the F_1 was still increasing. The point to be made is that, even at the cellular level, at the time of peak antibody production, the susceptible mice have a much greater antibody response than do the resistant.

Greater numbers of spleen cells in orally immunized mice were mature (Table II) presumably because maturation took place during migration.

C. The Polyclonal Antibody Response

1. The comparison of IgG, IgA and IgM antibody classes in Balb/c F_1 DBA X Balb/c and RBF/Dn mice.

Figures 19, 20 and 21 give the mean $\log_2$ titre in the ELISA of IgG, IgM and IgA respectively for the three strains of mice. Noteworthy were the diminished IgG response to LPS by Balb/c (Figure 19 a)), the substantial response of Balb/c to all other antigens, both IgG and IgM, and the total lack of an IgA response by Balb/c, Figure 21 (note from RESULTS that no IgA secreting hybridomas were detected in any of the 1952 hybridomas tested from the Balb/c spleens at days thirty-two and forty-five). Balb/c mice have been documented as developing hyperglobulinemia after three months of age (Kalpaktsoglou, et al, Immunology, 24, 303, 1973; Natsuume-Sakai, et al, Immunology, 32, 861, 1977). This is the age at which they were used in these experiments. Indeed, controls were high

from this strain of mouse as well as from the RBF/Dn. However, this was remarkably so for IgM in the Balb/c for the two inner membrane preparations. An increase in Balb/c IgM over the controls was evidenced only for the inner membrane preparation of the Rc mutant. Kalpaktsoglou, et al, (Immunology, 24, 303, 1973) reported hyperglobulinemia in Balb/c attributable to an increase in IgM as well as IgG_1 and partly IgG_{2b}. Natsuume-Sakai, et al, found that IgA was also increased. The latter suggests strongly that the total failure to detect IgA in Balb/c in these experiments, either polyclonally or monoclonally is not due to any genetic inability of the Balb/c to respond by synthesizing IgA. Rather, it would appear to be related to the antigenic system and the consequence of the Ity^s allele and its effect on macrophages. The experimental design did not include confirmation of this. Rightfully, this would include IgA monoclonal antibody and is elaborated in the General Discussion below.

F_1 DBA X Balb/c had diminished IgM response although the response did exist. This confirmed the fewer, although detectable IgM-producing hybridomas (Figure 18).

All immunoglobulin classes from sera of RBF/Dn mice showed increases over controls, once more confirming the monoclonal pattern depicted in Figure 18. Although the titres were not always as high as for the other two strains (particularly the IgM titres to the inner membrane preparations, Figure 20 c) and d), relative to Balb/c) the polyclonal increase in IgG and IgM occurred as early as for the other two strains. Serum IgA

was detectable at day twenty-two before the F_1 DBA X Balb/c where it was detectable at day forty-five. See also Figure 22.

2. Additional comparison of three resistant mouse strains.

To test the hypothesis that individual strains of mice would vary according to the total complement of their genetic background in a polygenic system whole serum antibody was tested from three Ity^r resistant strains of mice which would be expected to be B cell responsive. As such, if the B cell response reflected variations in response, over and above the resistance conferred by the Ity^r allele in any way, it should be most detectable in these mice. One of the strains chosen was the DBA/2j resistant Ity^r parent to the F_1 DBA X Balb/c which Robson and Vas (J. Inf. Dis., 126, 378, 1972) had shown to be intermediate in resistance in their system. In the system of Robson and Vas this strain still survived infectious doses of 10^4 bacteria for more than a week but none survived to day twenty-eight like the extremely resistant A/J mice. Another resistant strain, SWR/j used by Lissner, et al, (J. Immunol., 131, 3006, 1983) should be as resistant as the A/J. Swiss Webster mice from the colony at the Biology Vivarium, the University of Calgary, which would be expected to be random bred but resistant were the third group. The polyclonal IgG antibody response depicted in Figure 23 shows that DBA/2j were the least responsive of these resistant strains and produced antibody above control levels later than did the other two strains. This mirrored the F_1 DBA X Balb/c monoclonal and polyclonal response (Figures 18 through 21). That is, with the exception of a possible mitogenic response at day one (Figure 23 d)) to the inner membrane

of the Rc mutant, detectable B cell response above controls was measured as late as thirty-two days. This applied to all antigen groups.

This may impinge on the findings of Robson and Vas (J. Inf. Dis., 126, 378, 1972) who found DBA/2j intermediate in resistance to incremental infectious doses by measuring death over an experimental period of twenty-eight days, as well as those of Plant and Glynn, who did not find intermediate resistance on the basis of F_1 and F_2 parental backcrosses (J. Inf. Dis., 133, 72, 1976). However, in Plant and Glynn's study the LD_{50} for DBA/2j was the lowest of all resistant strains listed: that is 2×10^5 with other strains having an LD_{50} from 10^6 to 10^7. The possible reconciliation of the two findings may reside in the manner of acquisition of antibody as it affects time to death. Since the Ity gene affects the ability of the macrophages to control the infection early in its course (i.e. the first ten days), the acquisition of antibody early enough, in sufficient quantities may make the ultimate outcome of the disease beyond ten days positive. Examples are an increase in all antibodies by twenty-two days in SWR/j and SW-local and an increase in antibody to inner membrane at ten days. However, if the acquisition of antibody is delayed or reduced, or both, certain numbers of victims could be anticipated later in the course of the disease even though the Ity^r genome rendered the mouse capable of overcoming the disease in its initial stages.

3. The sex-dependent increase in antibody responsiveness in F_1 DBA X Balb/c and RBF/Dn mice.

Bouthillier, et al, (Immunology, 46, 67, 1982) showed that F_1 hybrid females from the Biozzi high and low responder lines, demonstrated a greater phenotypic expression of the dominant responder character of the flagellar and somatic antigens referred to in the introduction. Hybrid lines would be expected to have a more heterogeneous background, more closely approximating random mating populations, than the parental lines. Hence, a variety of gene interactions in a polygenic system, in which gene dosage would quantitatively vary the response, would also be expected. The authors related this to the expression of H-2 linked genes and found it to vary with sex and genetic background.

A criticism has been extended to the use of inbred strains (Biozzi, et al, Adv. Immunol., 36, 189, 1984) on the grounds that genetic homogeneity reduces the extent of interaction between polymorphic alleles, an important source of individual variability. Therefore, results obtained on inbred strains would provide a simplified view of the complex genetic regulation occurring in natural populations of genetically heterogeneous individuals. It is also pointed out by Biozzi that antibody response to complex immunogens such as microorganisms is very similar in various inbred strains because quantitative characters under polygenic regulation present small interstrain variability.

Both results discussed in the preceeding sections of this thesis and those depicted in Tables III and IV do not support this view completely. The serum titres presented according to groups of antigens in Table III,

showing clearly significant differences in male and female response to all groups of antigens in the F_1 DBA X Balb/c, indicate that these differences can be detected with relative ease in inbred mice. Whether or not the combined polymorphic genotypes of DBA/2j and Balb/c interacted in such a way as to attribute this difference to enhancement of H-2 expression, was not determined in these experiments. A possible additional interpretation to the contribution of female hormonal environment in enhanced gene expression, is gene dosage. Both DBA/2j and Balb/c are of H-2^d haplotype, which, combined with a complement of other genes including Ir genes linked to H-2, may provide an additive effect.

In Table III the extent of the increase was shown to be significant for the F_1 DBA X Balb/c mice only. However, when the frequency of females responding more than males was examined, Table IV shows that the frequency in both RBF/Dn and F_1 DBA X Balb/c was fully twice that of all other strains. The degree of response of females over males was not significant for RBF/Dn (Table III) but the number of times of its occurence was. The RBF/Dn would be expected to have a heterogeneous genetic background (see Materials and Methods for description) respecting both immune response genes linked to the MHC locus on chromosome seventeen of the mouse, as well as any other genes permitting interaction. These mice were selected specifically for the chromosomal translocation characteristics described in Material and Methods. The above findings corroborated both those of Bouthillier (above) and those of Cannat (Ann. Immunol. (Inst. Pasteur), 130 C, 675, 1979) in the Brucella system where F_1 and F_2 as well as the backcrosses of the same showed amplified sex-depend-

ent differences both with respect to titre and incidence of positive reactions.

Additionally, all the elegant genetic work of Biozzi was done using agglutination of whole Salmonella cells as a measurement of anti-flagellar antibody, and precipitation of whole cells using the classical Widal reaction for somatic antigens following immunization with killed bacteria. These are insensitive reactions compared with an ELISA and involve two antigen groups for which the host does not readily respond by producing antibody under conditions simulating natural infections. The latter has been discussed above where, in this thesis work, no response at all to flagella was detected and the response to the outer membrane, in particular the LPS component, was markedly diminished. However, by compartmentalizing bacterial antigens into groups according to the anatomic location on or in the bacterium, together with a sensitive test assay, the same differences reported by Biozzi in different mouse strains could be detected readily in inbred mouse strains. It was also possible to show time and rate differentials of response with relative ease.

D. General Discussion

1. Summation of the total B cell response to live Salmonella typhimurium.

When a live vaccine was administered, contrary to expectation, the B cell response of the mouse was not to surface structures of S. typhimurium but rather to internally located sites. The lack of response to outer membrane preparations containing no NADH-oxidase activity, hence uncontaminated with inner membrane, was shown for both orally and parent-

erally immunized mice in whole serum (Figures 4 to 7) at protective doses. The greatest part of the response was to the inner membrane, both polyclonally and monoclonally when a live vaccine was administered orally, the route through which a natural infection would be expected to take place (Figures 18 through 23; Tables III, V, and VII).

In terms of host-parasite relationships with a facultative intracellular bacterium capable of replication within host macrophages, the following may provide an understanding of the lack of response to outermost antigens. The evolution of a wide variety of O-antigens may be a survival mechanism amongst Salmonellae. Instances of molecular mimicry with host tissue would preclude the hosts' mounting an active antibody response against such structures. Also, Salmonella species, causing typhoid fever in different host species may have evolved an O-antigen repertoire which allows specificity of penetration and evasion as opposed to immunogenicity. The greater antibody response to O-antigens would be more likely in those species for which the given Salmonella does not have tropism and in which the disease state is restricted to the gut with no accompanying tissue or organ penetration.

It is possible to generate antibody against these structures as evidenced by the use of O-antigen serotyping procedures for taxonomic classification of Salmonellae. Often typing sera are generated in another species (eg. the rabbit) where fixed, killed cells from a bacterium with tropism for another host species will induce the production of antibody against the O-antigen. Antibody to O-antigens (LPS) has allowed taxonomic schemes such as the Kauffmann-White classification to be

derived (Parker, M.T., chapter 37, in Topley and Wilson's Principles of Bacteriology, Virology and Immunity, p. 332, 1983). These may however, be unrelated to a hosts own response to such an organism; a consideration supported by the persistent unsatisfactory behaviour of most known vaccines and the enormous variability and contradictions in the literature regarding immune response to this ubiqitous, as well as problematic group of bacteria. The O-antigens may, in fact, be involved as recognition sites, either for species tropism or specific organ systems.

If the latter were the case, it is reasonable that, even though the response to LPS is limited (Table VII), it is, nevertheless, greatest in the most resistant strains (the RBF/Dn in this work). Antibody to the O-antigens also occurred earlier in this strain of mouse (twenty-two days). Hence, antibodies produced to the O-antigens may be helpful but insufficient by themselves for complete protection. A genetically resistant strain of mouse might very well overcome an attack of Salmonellosis with antibody to the O-antigens through upgraded phagocytosis, blood clearance and containment of specific antibody-opsonized bacteria within the reticuloendothelial system. It may, nevertheless, pay the price of entry of the Salmonella into the reticuloendothelial system with an accompanying prolonged carrier state (Collins, Bacteriol. Rev., 38, 271, 1974). The genetic gradations of susceptibility may derive little assistance from antibody to O-antigens without the additional antibody to deeper components of the bacterium.

The possibility cannot be excluded that antibody could be made to LPS core components during assembly on the inner membrane, as discussed

previously. Teleologically, this would seem an incomplete protective device for the host since O-antigens are not required for the bacterium to remain viable. They are required to maintain virulence which suggests they are important for either recognition or attachment and penetration; requirements for disease production. Indeed, Colwell, et al, have reported incomplete protection with monoclonal antibodies to different core regions of Salmonella typhimurium (Federation proceedings, 42, 432, abstr. # 822, 1983). Once more, one might reason that this could be helpful but insufficient antibody.

The assay system for identifying positively reacting hybridomas did not, in retrospect, support this possibility. Core components were tested for reactivity against hybridoma supernatants which reacted positively either with the outer membrane as such, or with smooth LPS. No supernatants reacting positively with the inner membrane preparations were tested in this way.

Similar arguments for the evolution of a protective structure for the bacterium can be made regarding flagellar antigens. Notwithstanding the elaborate classifications such as the Kauffmann-White schema, and their usefulness in diagnostic identification, antibody from the vulnerable host is not directed toward these structures under conditions simulating natural infections. As with LPS and porin, it is unlikely that a pathogenic bacterium would evolve a conserved surface structure which would be vulnerable to easy immune attack by a putative host and still maintain its virulent qualities.

The results reported here are not in agreement with those of Metcalf and O'Brien (Infect. Immun., 31, 33, 1981), particularly regarding surface exposed structures. In their experiments mice, also of known genetic resistance and susceptibility, were immunized with living, sublethal doses of a virulent strain of S. typhimurium, intraperitoneally, subcutaneously and intravenously. Antibody production was assayed by whole cell bacterial agglutination, passive hemagglutination, and radiolabeled class-specific anti-mouse immunoglobulin after reacting test mouse sera with whole bacterial cells fixed to microtitre plates (radioimmune assay). That is to say, only bacterial surface antigens were assayed. Immunoglobulin M was detected before immunoglobulin G. IgA was minimally detected. The majority of the antibody detected was directed toward lipopolysaccharide.

The argument is presented that the method of assay in Metcalf's and O'Brien's work provides a system open to bias. Although one would think that the use of living bacteria, regardless of route of immunization, would provide the results obtained in Figures 19 to 23 for LPS and outer membrane, it may be that route of immunization shifted the response to different outer membrane components. ELISA results in this thesis work showed that the total antibody response to the outer membrane after parenteral immunization (Figures 4 and 6) was inordinately low (1/256 to 1/512) compared to titres of greater than 1/5000 in the RIA used by Metcalf and O'Brien with a bacterium which persisted in the mouse. The bacterial persistence may be a partial explanation for the discrepancy in that SL3261 vaccine strain would have a finite lifetime within the host.

Importantly, the high antibody titres in Metcalf's and O'Briens system did not eliminate a sublethal dose, even with time.

2. The relationship of the thesis findings to the protectiveness of the vaccine.

A more appealing argument for the major antibody response of the mouse to deeper inner membrane components is that of greater foreignness of these components to the host. These may or may not be conserved structures. Present knowledge would suggest they are conserved. They would be sequestered structures, ill exposed to any recognition or immune assault by the host in the intact bacterium, but critical for the viability of the bacterium. It is possible that not until the bacterium has gone through attachment and penetration, that the critical consequences of replication within the host macrophages lead to its ultimate vulnerability. Turnover and exposure of internal antigenic determinants on replication allow the host macrophage to recognize previously sequestered structures. Hence, the subsequent rapid mobilization of antibody responsiveness.

If one accepts that at least a partial explanation of the usefulness of a vaccine lies in its ability to prepare the host's immune memory in such a way that rapid recall on subsequent exposure to a virulent pathogen would lead to efficient mobilization of T cell and B cell activity then antibody would certainly play a significant role. If it is against internal bacterial components critical to the bacterium's viability, an immune host could contain an infection within a generation or two of bacterial replication. The above paragraph and the probable sequence of

events in B cell responsiveness as observed in these experiments presents this as a plausible hypothesis.

3. The genetic control of the B cell response as characterized through the use of three mouse strains of known Ity genotype.

The findings in this work suggest that the differences observed in antibody, hence B cell responsiveness between the mouse strains used is the result of polygenic control of immune responsiveness rather than any relationship to the Ity locus. The possible exceptions are the diminished response to LPS and lack of IgA production in Balb/c mice.

The fact that susceptible Balb/c mice can be fully protected (Hoiseth and Stocker, Nature, 291, 238, 1981) without significant response to LPS and without the generation of IgA antibody when a vaccine is administered orally, suggests that the massive response to the inner membrane fractions may be responsible. It is tempting to think that identification of the immunodominant component or components, purification thereof, and use as a vaccine might suffice to protectively immunize all mouse strains regardless of innate susceptibility status. This may be too simplistic.

Since the response even to the inner membrane components is variable as evidenced by the RBF/Dn (Figure 18) in the selectivity of the early response to the inner membrane of the immunizing strain by mesenteric lymph node cells and subsequently including the Rc mutant, this suggests a response to "private" antigen(s) first, followed by a response to "public" antigen(s). Therefore, even though it seems reasonable to think that internal bacterial antigens are primarily conserved, there may be

diversity, as well as differences unique to different strains of Salmonella typhimurium. If so, these have escaped characterization through lack of recognition that the response to an intracellular pathogen is to internally located sites. The one exception to this lack of recognition has been the less than popular acceptance of ribosomal vaccines and "ribosomal protein" vaccines. The work of Bigley (Infect. Immun., 32, 353, 1981; Infect. Immun., 31, 1273, 1981) on the relationship to host resistance of these vaccines, and the effect of antigenic modification of them on rosette-forming cells (a measure of T cell immunity) has not been extended.

An alternative explanation for comprehensive RBF/Dn reaction is that the aromatic-requiring vaccine strain may synthesize quantitatively and qualitatively different proteins compared with the non-aromatic requiring strains. This could affect both cell wall and inner membrane polypeptides. It is, however, unlikely for most proteins.

Nonetheless, the finer resolution of antibody response in the RBF/Dn would not seem to be related to the Ity gene. If it were, one would expect a qualitatively similar pattern in the F_1 DBA X Balb/c even if the response occurred later. Rather, the response seems to be controlled by a greater repertoire of response genes in the RBF/Dn compared with the highly inbred strains. Unfortunately, the initial failure to recognize the antigenic contribution of internal bacterial determinants resulted in failure to characterize the early response in the hyperglobulinemic Balb/c on the cellular level. The observations of these experiments parallel those of Biozzi and colleagues, referred to above, closely.

Precise proof of the exactness of the polygenic control of B cell response would require genetic studies including parental backcross experiments.

Total response of the F_1 DBA X Balb/c seemed to reach that of the RBF/Dn ultimately. The polyclonal response was done twice (Tables III and IV) with similar patterns of response. The time/rate response of IgG and IgM is depicted in Figure 22. The tardiness of the response of the F_1 DBA X Balb/c and that of the DBA/2j cannot be due to the H-2^d haplotype since that was the same as Balb/c which responded well and early to inner membrane antigens as assessed polyclonally (Figures 19 and 20). It must also be independent of the Ity gene since the Ity^r allele would be expected to affect F_1 DBA X Balb/c and RBF/Dn mice similarly if it influenced B cell responsiveness. In short, the F_1 DBA X Balb/c show a B cell response distinct from, and presumably controlled by a repertoire of response genes different from those of either Balb/c or RBF/Dn. These response genes must be in addition to the H-2^d genome and independent of any influence by the Ity gene.

This lends support to the concept put forward by Eisenstein, et al, (J. Inf. Dis., 150, 425, 1984) that mouse strain variability contributes significantly to antibody responsiveness. In Eisenstein's work, mice in the C3H lineage, all histocompatible (H-2^k) but differing in innate susceptibility to S. typhimurium were variably protected by a panel of non-viable vaccines and by passive transfer of hyperimmune serum. The ability to protect was not closely related to the capacity of the mice to make agglutinating or anti-O antibody. Two strains, C3H/HeJ and

C3HeB/FeJ were susceptible because of the LPS^d allele (as opposed to the Ity gene) on chromosome four of the mouse, while the third strain was resistant.

4. The relationship of the thesis findings to the Ity gene.

The only aspect of the B cell response as assessed here which could be considered a consequence of the Ity gene would be the lack of response of Balb/c in forming IgA and the retarded response to LPS. Balb/c are not immunologically defective and Natsuume-Sakai, et al, (Immunology, 32, 861, 1977) have shown them to be hyperglobulinemic respecting IgA as well as IgM and IgG_1. Therefore, the lack of production of IgA under the experimental conditions was not due to an inability to do so. Rather, it seems to be a consequence of the host's interaction with the antigen; in this case determined by the Ity^S allele. An alternative explanation is different patterns of migration of antigen activated lymphocytes. Few were detected in mesenteric lymph nodes of Balb/c relative to the resistant strains (Figure 17 and Table VI). A control mechanism of cell migration may be superimposed over Ity differences.

The experimental work does not show that there is a complete failure to respond by generating IgA. It may be delayed beyond forty-five days. Alternatively, increased and/or more frequent antigen doses might also be required. As noted above, all monoclonal experiments used three times the known protective dose.

In order to address the question of the delayed response to LPS, and/or the lack of response regarding IgA production in this system it would be necessary to use IgA antibody to the LPS in phagocytic studies

from the same mouse strains as well as backcross strains. Some of the monoclonal IgA antibody from the RBF/Dn mice would suffice.

Contradictory reports on uptake and killing of S. typhimurium between Ity^S and Ity^r phenotypes of macrophages have already appeared in the literature. Swanson and O'Brien (J. Immunol., 131, 3014, 1983) reported that the level of Ity regulation controlled the level of bacteriostasis in splenic and hepatic macrophages but that uptake and killing was similar in strains of mice differing at the Ity locus. Later, Lissner, Weinstein and O'Brien (J. Immunol., 135, 544, 1985) reported that the Ity locus regulates microbicidal activity in isolated peritoneal macrophages. This was in keeping with the report of van Dissel, et al, (J. Immunol., 134, 3404, 1985), in which killing by susceptible mouse peritoneal macrophages of Ity^S/Ity^S genotype was much slower compared with Ity^r macrophages under conditions of opsonization, continuous phagocytosis and after in vivo phagocytosis.

This seeming contradiction points to the need to address organ specificity in regard to the phenotypic expression of the Ity^S allele in macrophages. In other systems, heterogeneity has been reported, not only in macrophages from different organ sources (Caignard, et al, Cellular and Molecular Biology, 31, 41, 1985) but in macrophages from the same source (Shellito and Kaltreider, Am. Rev. Respir. Dis., 131, 678, 1985). Moreover, origin and stage of differentiation are doubtless also involved (Jun, De-Ming, Chin. J. Microbiol. Immunol. (Beijing), 4, 305, 1984).

Table I: Proliferative Response of Spleen Cells of Random Bred ICR mice to Sucrose Gradient and Water/Heat Prepared Outer Membranes

	Sucrose Gradient				Water/Heat			
Day/Time	% Lympho-[1] blasts	% Mitotic Figures	Total Pro-[2] liferation	% Mature Lymphocytes	% Lympho- blasts	% Mitotic Figures	Total Pro- liferation	% Mature Lymphocytes
2d. C1[3]	1	1	2	98				
C2	3	1	4	96		See opposite		
T1[3]	18	2	20	80	3	2	5	95
T2	13	2	15	85	12	3	15	85
5d. C1	2	3	5	95				
C2	4	-	4	96		See opposite		
T1	21	2	23	77	24	16	40	60
T2	15	7	22	78	24	14	38	62
8d. C1	3	2	5	95				
C2	9	3	12	88		See opposite		
T1	21	12	33	67	29	7	36	64
T2	37	2	39	61	34	1	35	65
12d. C1	11	1	12	88				
C2	12	-	12	88		See opposite		
T1	23	1	24	76	17	1	18	82
T2	20	-	20	80				
T3	35	-	35	65				

1. The percent is the mean of 2 separate counts of 100 cells per preparation
2. Lymphoblasts plus mitotic figures is considered an index of total proliferative response
3. C = imprints from control mice, T = imprints from test mice.

Table II: Proliferative Response in Spleen of Orally and Parenterally Immunized F_1 DBA X Balb/c Mice with Live SL3261 (vaccine) S. typhimurium, Expressed as Per Cent Lymphoblasts and Mitotic Figures

	Oral Immunization				Parenteral Immunization			
Day/Time	Lympho-blasts	Mitotic Figures	Total Pro-liferation	Mature Lymphocytes	Lympho-blasts	Mitotic Figures	Total Pro-liferation	Mature Lymphocytes
3 d. C1	8	-	8	92	1	2	3	97
C2	3	1	4	96	ND	ND	ND	ND
T1	13	-	13	87	27	-	27	73
T2	30	1	31	69	34	-	34	66
6 d. T1	19	2	21	79	5	-	5	95
T2	10	8	18	82	21	-	21	79
10 d. C1	13	-	13	87	8	-	8	92
C2	14	-	14	86	ND	ND	ND	ND
T1	9	2	11	89	30	2	32	68
T2	15	2	17	83	32	2	34	66
13 d. C1	ND	ND	ND	ND	1	3	4	96
T1	15	5	20	80	38	3	41	59
T2	12	2	14	86	26	6	32	68
17 d. C1	16	1	17	83	4	4	8	92
C2	4	1	5	95	ND	ND	ND	ND
T1	9	1	10	90	30	4	34	66
T2	9	2	11	89	23	2	25	75
20 d. C1	ND	ND	ND	ND	1	-	1	99
T1	8	6	14	86	37	8	45	55
T2	4	3	7	93	31	11	42	58

Legend is the same as for Table I.

ANOVA - Oral immunization vs. parenteral, $F_{1,17} = 15.15$, $p<0.05$

Table III: Mean[1] Antibody Levels of Male and Female Mice of Different Strains to Different Antigen Groups of S. typhimurium

Antigen Group	F_1 DBA X Balb/c[2]				RBF/Dn		Balb/c		DBA/2j		SWR/2j		SW-local	
	F.	M.	F.	M.	F.	M.	F.	M.	F.	M.	F.	M.	F.	M.
O.M.[3]	7	6.5	6	5.1	8.5	7.7	8.4	8.3	4.1	4.2	5.4	6.0	6.1	6.0
I.M.	7.3	7.2	7.3	6.1	9.7	9.3	10.9	10.1	5.7	5.6	6.9	7.3	6.6	6.8
I.M. (Rc)	12.2	10.3	8.6	6.6	12	11.1	13.	12.8	6.4	6.5	6.6	7.5	8.6	8.7
LPS	6.8	4.8	5.4	4.6	5.8	5.7	5.7	5.2	4.8	4.3	5.9	5.9	4.9	5.8
	$F_{1,47} = 16.06$ $p < 0.001$				$F_{1,19}$ = 1.7, N.S.[4]		$F_{1,19}$ = 2.9, N.S.		$F_{1,23}$ = 0.12, N.S.		$F_{1,23}$ = 2.7, N.S.		$F_{1,23} = 0.19$, N.S.	

1. ANOVA was done on individual values at individual times in the immunization schedule. Values given are mean $\log_2$.
2. Two groups of orally immunized F_1 DBA X Balb/c are represented.
3. O.M. = outer membrane of SL3261 (vaccine), I.M. = inner membrane of SL3261, I.M. (Rc) = inner membrane of the Rc mutant, LPS = smooth lipopolysaccharide.
4. N.S. = not significant.

Table IV: Frequency [1] of Female Greater than Male Serum Titres in Different Strains of Mice

	F_1 DBA X Balb/c 1	F_1 DBA X Balb/c 2	RBF/Dn	Balb/c	DBA/2j	SWR/j[2]	SW - local[2]
O.M.	3/6	3/6	3/6	1/6	1/6	0	2/6
I.M.	2/6	3/6	2/6	2/6	1/6	2/6	2/6
I.M. (Rc)	3/6	5/6	4/6	1/6	3/6	1/6	2/6
LPS	2/6	3/6	2/6	1/6	1/6	2/6	0
Total:	10/24	14/24	11/24	5/24	6/24	5/24	6/24

Legend: O.M. = outer membrane of SL3261, the immunizing vaccine, I.M. - inner membrane of SL3261, I.M. (Rc) = inner membrane of the Rc mutant, LPS = smooth lipopolysaccharide.

1. Frequency refers to the number of times for each antigen group tested at 0 d. (control), 1 d., 10 d., 22 d., 32 d., and 45 d. that the female serum reaction was greater than that from the male. In instances where there was no antibody increase over control until day 22, the frequency out of 6 samples for each antigen group cannot exceed 3.

2. The only groups of mice in which the reactions from male sera were occasionally higher than the female.

Table V: Distribution of Reactive Hybridomas to Different Groups of *S. typhimurium* Antigens in Three Strains of Mice

	Total Reactive Hybridomas			% Reacting With O.M.			% Reacting With I.M.			% Reacting with I.M. of Rc mutant			% Reacting with LPS		
	Balb/c	F_1	RBF/Dn	Balb/c	F_1	RBF/Dn	Balb/c	F_1	RBF/Dn	Balb/c	F_1	RBF/Dn	Balb/c	F_1	RBF/Dn
PWM (Spl)	1	40	34	100(1)	10	14.7	ND	45	76.5	ND	90	85.3	**0**	5	0
24 hr. PP:	0	0	0	0	0	**0**	ND	0	0	ND	0	0	**0**	0	0
MLN:	0	1	14	0	0	**2.0**	ND	100(1)	98(13)	ND	0	98(13)	**0**	0	0
10 d. PP:	0	0	0	0	0	**0**	ND	0	0	ND	0	0	**0**	0	0
MLN:	0	0	133	0	0	**3.8**	ND	0	91.7	ND	0	46.2	**0**	0	0
Spl:	ND	0	ND	ND	0	**ND**	ND	0	ND	ND	0	ND	**ND**	0	ND
22 d. PP:	0	0	0	0	0	**0**	ND	0	0	ND	0	0	**0**	0	0
MLN:	1	17	82	100(1)	0	**35.4**	ND	0	8.5	ND	100(17)	64.6	**0**	0	4.9
Spl:	ND	54	217	ND	3.7	**3**	ND	14.8	2.3	ND	81.5	97	**0**	0	0
32 d. PP:	0	0	5	0	0	**40**	0	0	20	0	0	40	**0**	0	0
MLN:	0	77	80	0	10	**2.5**	**0**	**14.3**	**11.3**	0	**90**	**97.5**	0	0	0
Spl:	1456	209	246	0.8	13	**12.6**	**84**	**33.4**	**48**	**93**	**90**	**99.6**	0.2	0.5	0
45 d. PP:	0	0	0	0	0	**0**	0	0	0	0	0	0	**0**	0	0
MLN:	**0**	**66**	**8**	**0**	**22.7**	**50**	**0**	**42.5**	**50**	**0**	**77**	**50**	**0**	0	**12**
Spl:	496	299	335	**13**	**4.3**	**3**	**77.5**	**39.1**	**56.1**	**86**	**96**	**97**	**0.2**	**0.4**	**1.5**

Bold face numerals are used to emphasize comparative differences or similarities.

Table VI: Comparison of Number of Reactive Hybridomas with Total Hybridomas Formed

A. Total Hybridomas and Per Cent Specifically Reactive Hybridomas.[1]

		1 d.	10 d.	22 d.	32 d.	45 d.
Balb/c:	MLN	0/2[2]	0/4[2]	1/68(1.4%)[2]	0[3]	0[3]
	Spleen	N.D.	N.D.	N.D.	1456/1560 (93.5%)	496/640(77%)
F_1DBA X Balb/c:	MLN	1/4	0/1	17/25(68%)	77/88(87%)	66/80(82%)
	Spleen	N.D.	0/14	59/100(59%)	209/310(67%)	299/369(81%)
RBF/Dn:	MLN	14/14(100%)	133/140(95%)	82/100(82%)	80/80(100%)	8/99(8%)
	Spleen	N.D.	N.D.	217/225(96%)	246/251(98%)	335/358(93%)

B. Number of Mesenteric Lymph Node Cells Plated.

	1 d.	10 d.	22 d.	32 d.	45 d.
Balb/c:	3.9×10^7	1.1×10^7	1.8×10^8	8.4×10^7	1.3×10^7
F_1DBA X Balb/c:	7.0×10^7	8.4×10^7	8.0×10^7	8.0×10^7	4.8×10^7
RBF/Dn:	6.0×10^7	1.4×10^8	9.7×10^7	5.2×10^7	1.7×10^7

1. Specifically reactive hybridomas includes reactions with all groups of antigens
2. Only the outer membrane was tested at this stage.
3. No hybridomas were formed - i.e. no detectable activated lymphocytes.

Table VII: Total[1] Reaction with LPS, LPS Core Components and Porin from Hybridoma Supernatants Reacting Positively with Outer Membrane and LPS on Screening

A

	Sm	Ra	Rb	Rc	Rd	Re	A[2]	J5[2]	Porin	Total Clones Involved	% of Total[3] Reactive Clones
Balb/c	1	2	3	2	3	2	2	1	1	4	4/1952=0.2%
F_1DBA X Balb/c	1	2	1	2	1	2	1			6	6/657=0.9%
RBF/Dn	14	1	3	1	3	4	4		1	18	18/973=1.8%

B

	Multiple LPS Reactions[4]:				
	2	3	4	5	6
Balb/c	1	1			1
F_1DBA X Balb/c	3				
RBF/Dn	1	2			1

1. Includes all sources, all time periods of the immunization schedule, and all Ig classes.

2. A = liquid A
   J5 = rough *Escherichia coli* LPS

3. From Table V and includes those times in the immunization schedule from which the first LPS reactions were detected. i.e. 22 d. onwards.

4. This gives the number of hybridoma supernatants which reacted with more than one core component.

Figure 1: Survival of ICR, random bred mice given two outer membrane antigen preparations containing 50 ug. protein:

a water/heat preparation; 

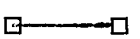
a sucrose gradient preparation; 

Figure 2: Mean spleen weights of mice injected with the antigen preparations of Figure 1. Each point represents the mean of two mice sacrificed on given days. The legend is the same as Figure 1, with controls represented by: 

Figure 1:

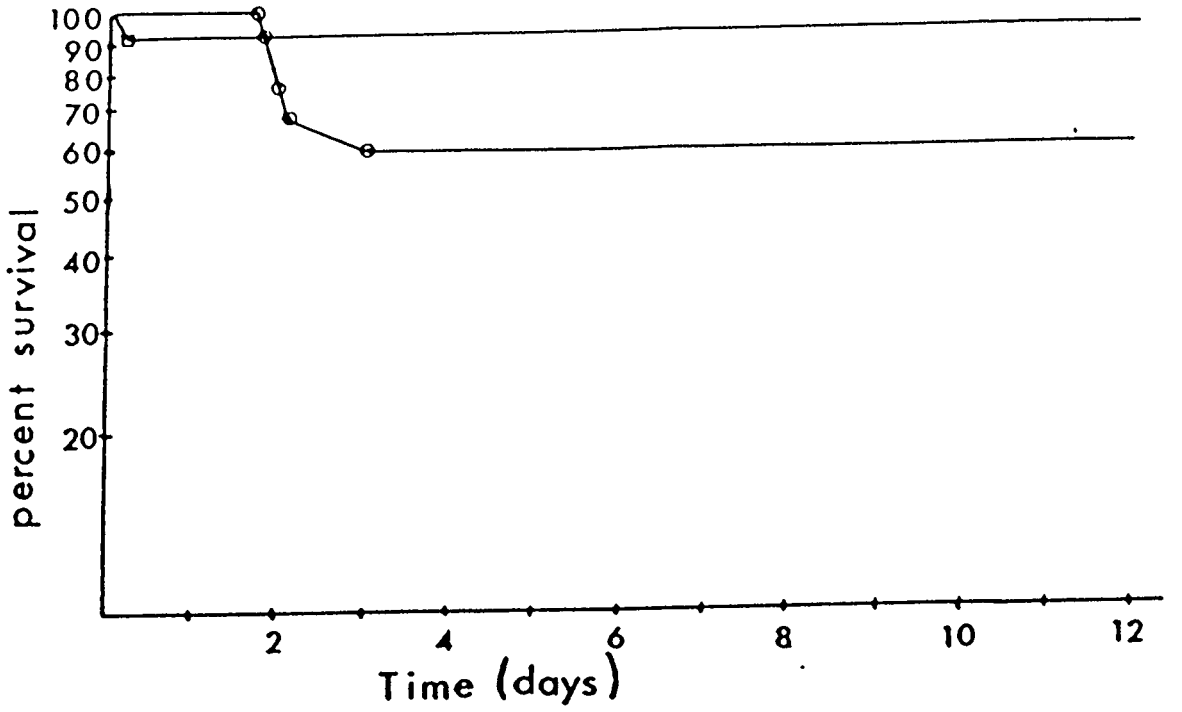


Figure 2:

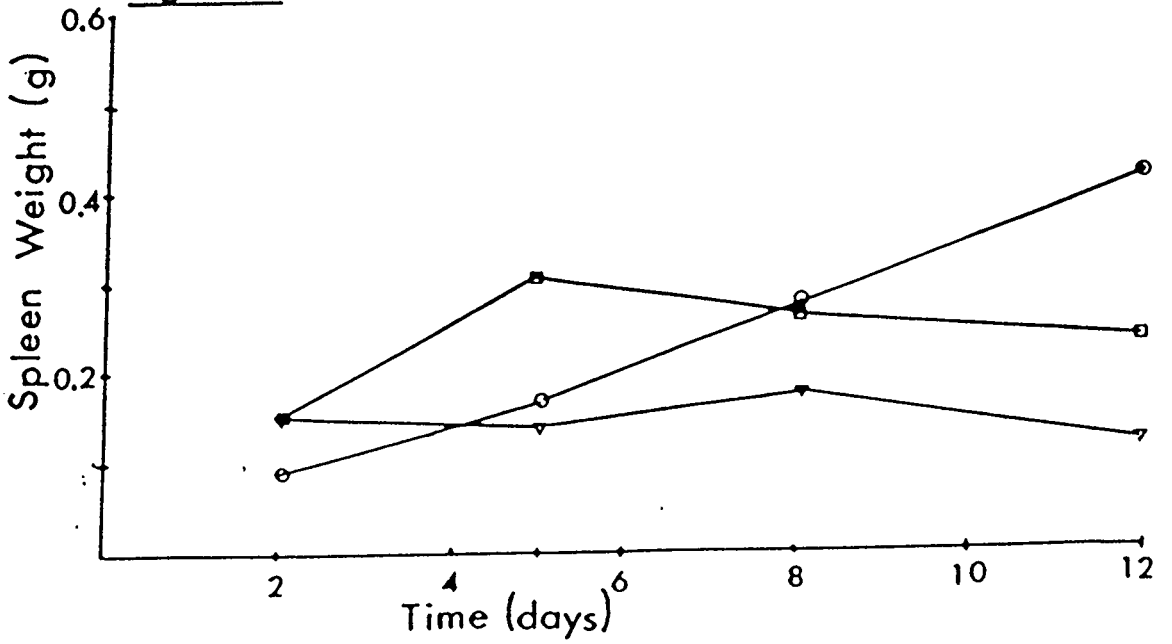


Figure 3: Log_2 of mean ELISA titres of whole mouse sera from the same experiment represented in Figures 1 and 2.

Control sera reacting with the sucrose gradient outer membrane preparation; ▽——▽

Control sera reacting with water/heat outer membrane preparation; ▽— —▽

Sera from mice immunized with the sucrose gradient preparation reacting with:

a) the sucrose gradient preparation □——□

b) the water/heat preparation □—/—□

Sera from mice immunized with the water/heat preparation reacting with:

a) the sucrose gradient preparation ○——○

b) the water/heat preparation ○—•—○

Each point is the mean of the titre of a male and a female mouse.

Figure 3:

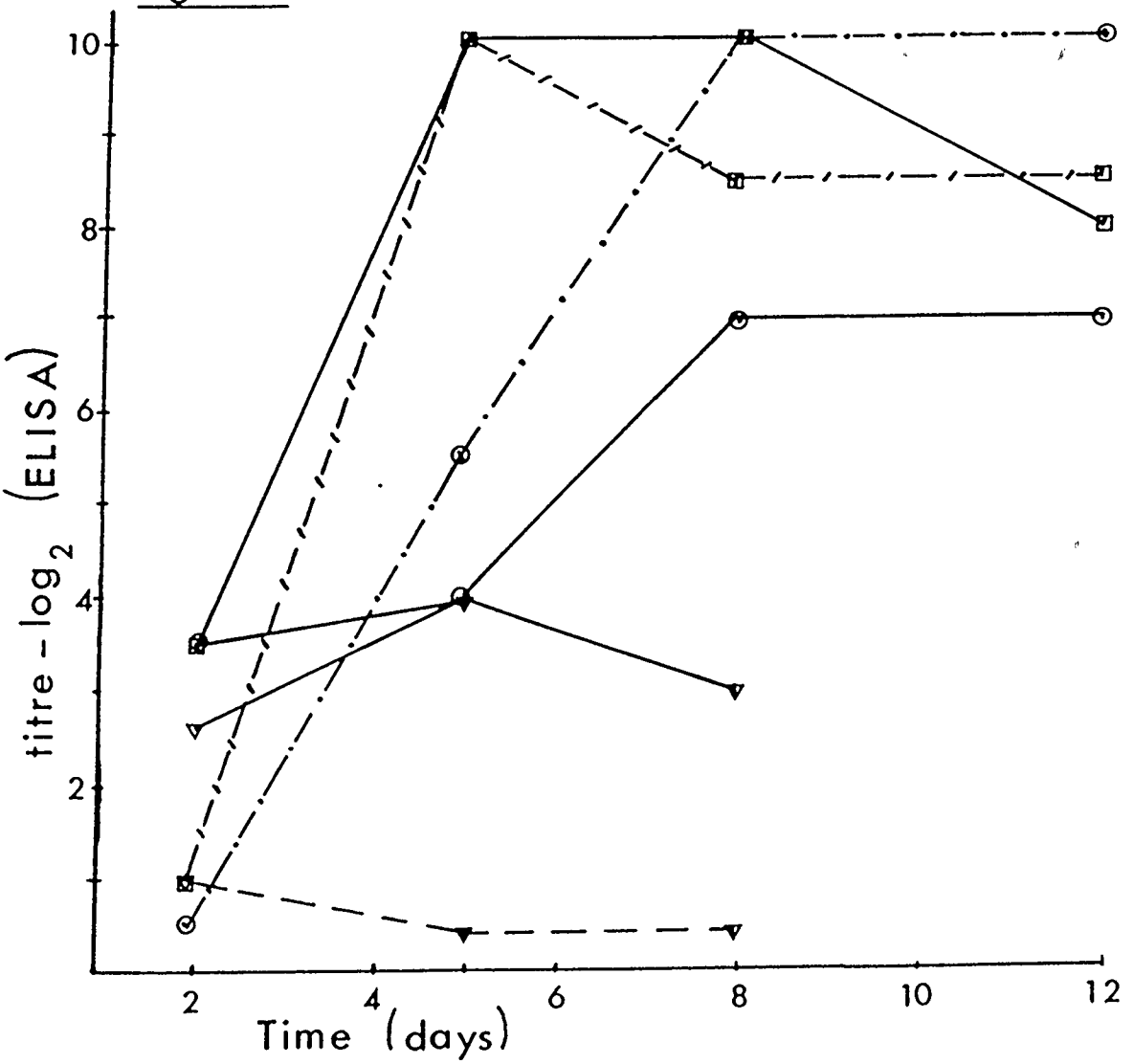


Figure 4: IgG polyclonal response of Balb/c mice given 2 X 10^4 live SL3261, vaccine strain of S. typhimurium parenterally (IP). The reaction with outer membrane antigens from 4 S. typhimurium strains is represented by:

SL3261 (immunizing bacterium) — ○——○

SL1344 (isogenic, virulent parent of SL3261) — ○—·—○

M206 (unrelated carrier strain) — □——□

C5 (unrelated virulent strain) — □— —□

Unimmunized, age-matched controls — ▽——▽

The vertical arrow indicates the time of booster immunization.

Each point represents the mean of two mice; a male and a female.

Figure 5: IgG polyclonal response of Balb/c mice given 3 X 10^7 live SL3261; vaccine strain orally. The legend for the test antigens is the same as in Figure 4.

Figure 4:

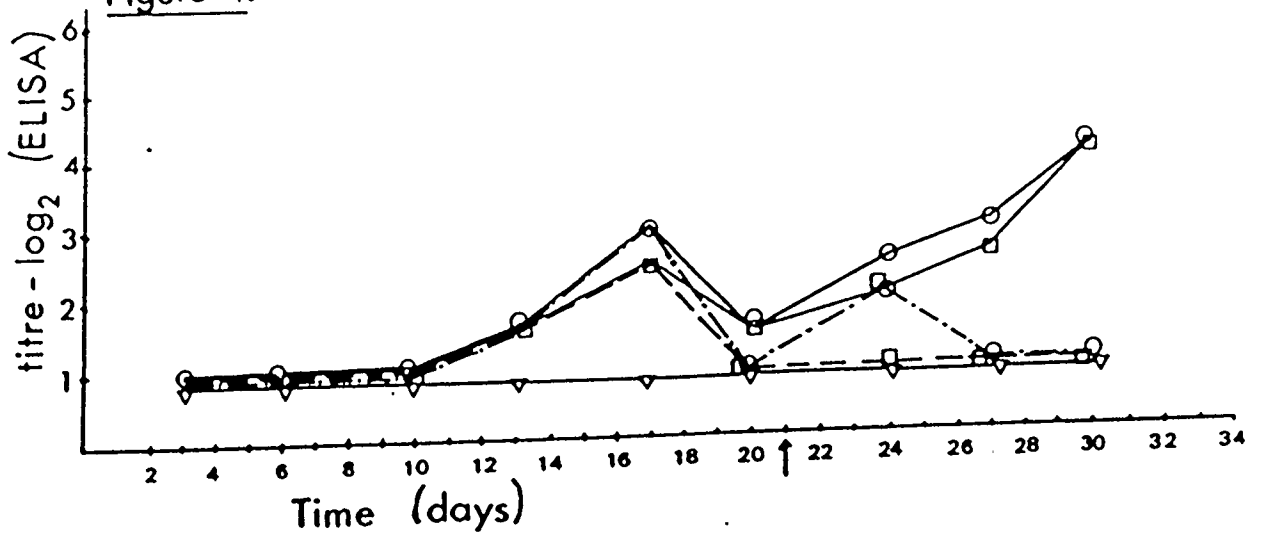


Figure 5:

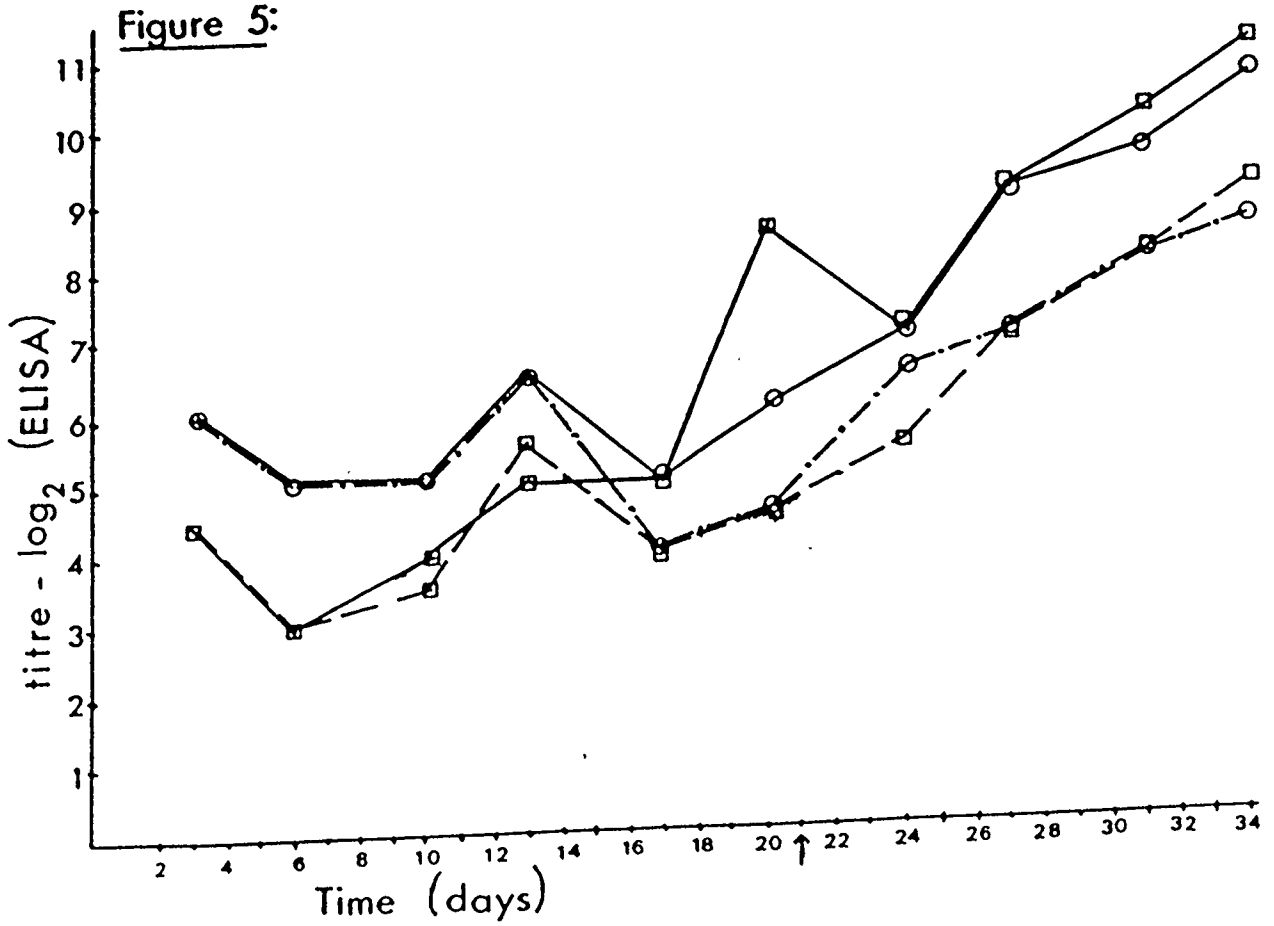


Figure 6: IgG polyclonal response of F_1 DBA X Balb/c mice given 3 X 10^4 live SL3261, vaccine strain, S. typhimurium parenterally (IP). The legend for the reactivity with the four test antigens is the same as for Figures 4 and 5.

Figure 7: IgG polyclonal response of F_1 DBA X Balb/c mice given 6 X 10^7 live SL3261, vaccine strain S. typhimurium orally. The legend for test antigens is the same as for Figures 4, 5 and 6.

Figure 6:

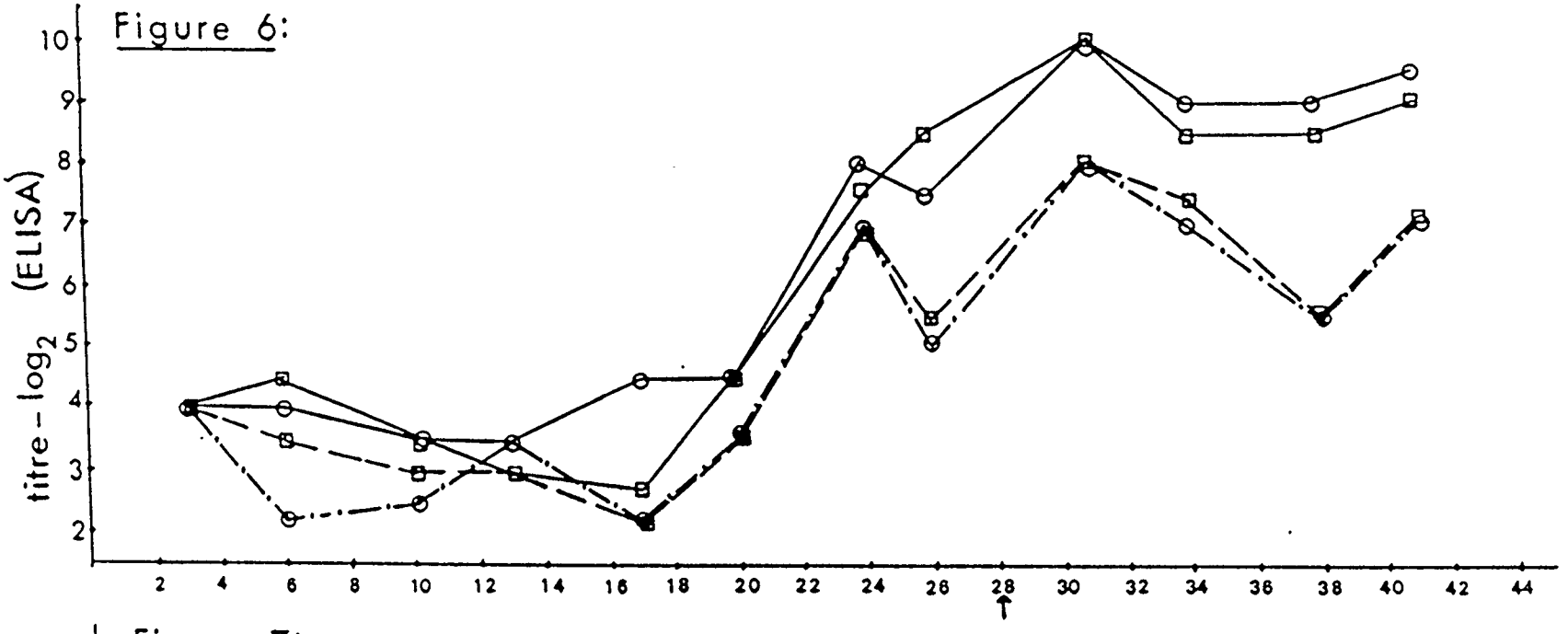


Figure 7:

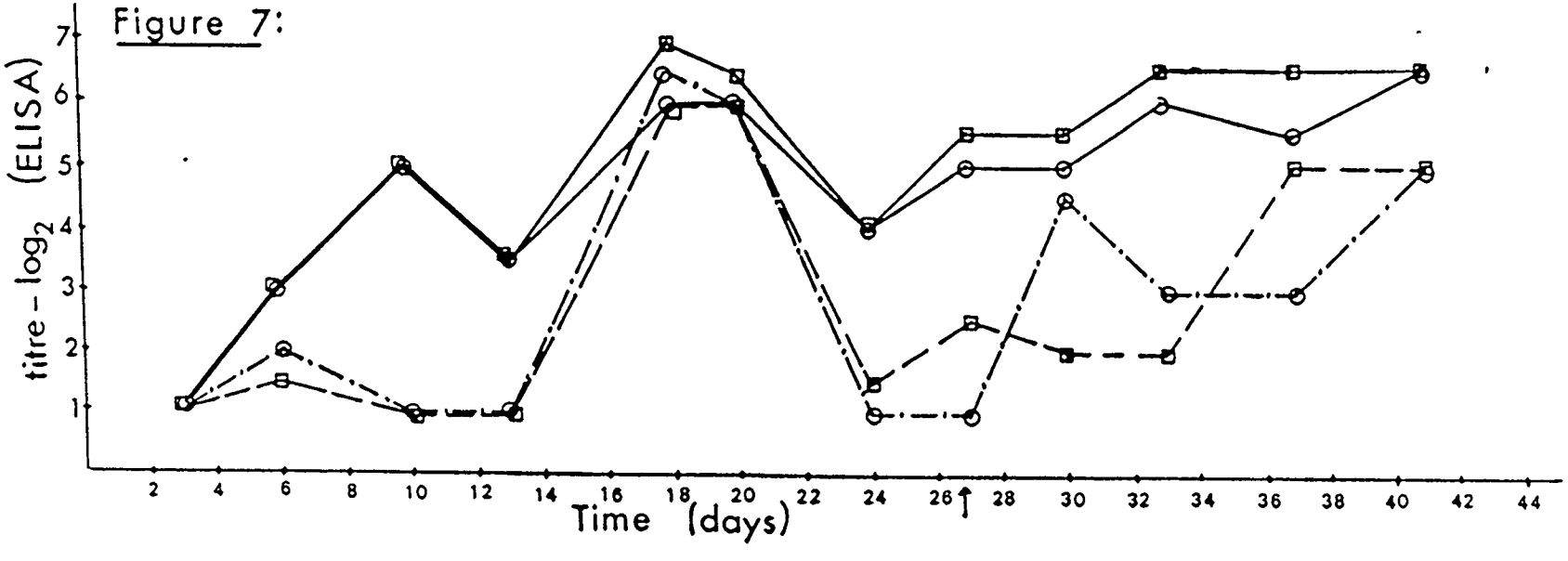


Figure 8: IgG polyclonal reaction of unimmunized Balb/c mice. The legend for the reactivity with the four test antigens is the same as for Figures 4 to 7. The mice are age-matched to those in Figure 5.

Figure 9: IgG polyclonal reaction of unimmunized F_1 DBA X Balb/c mice. The legend for the reactivity with the four test antigens is the same as for Figures 4 to 8. The mice are age-matched to those in Figure 6.

Figure 8:

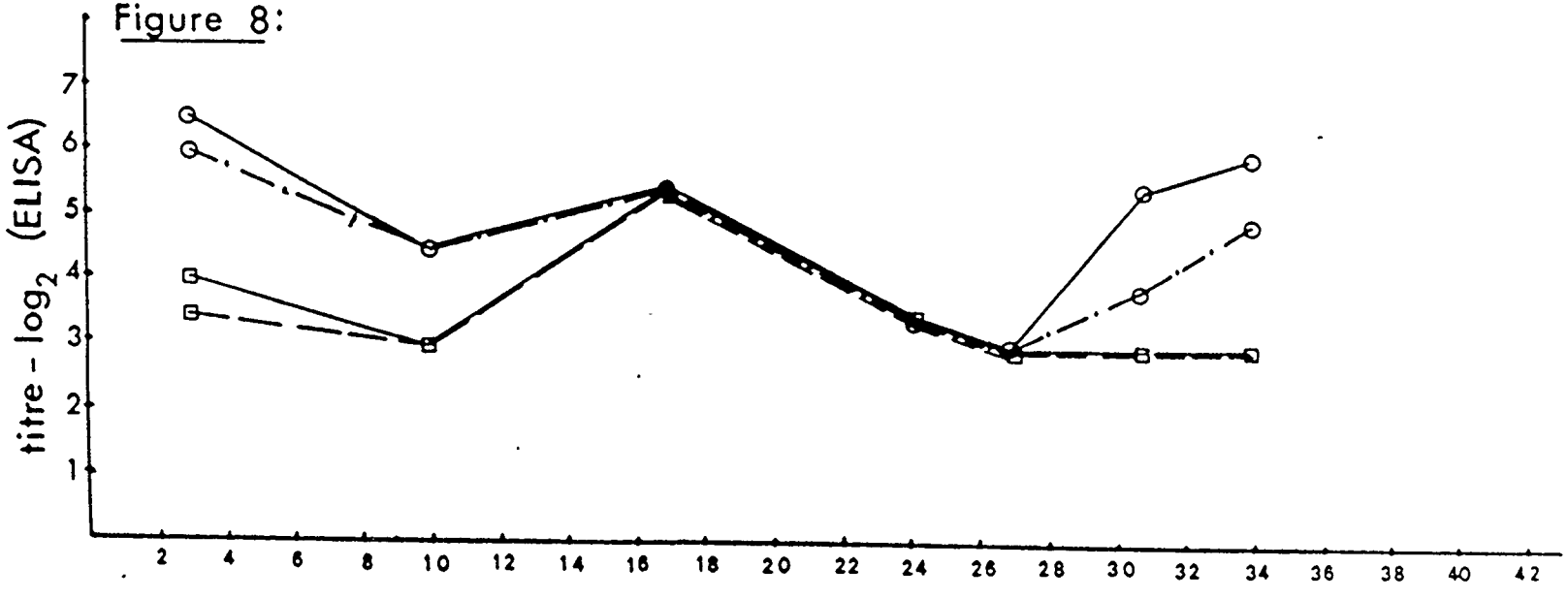


Figure 9:

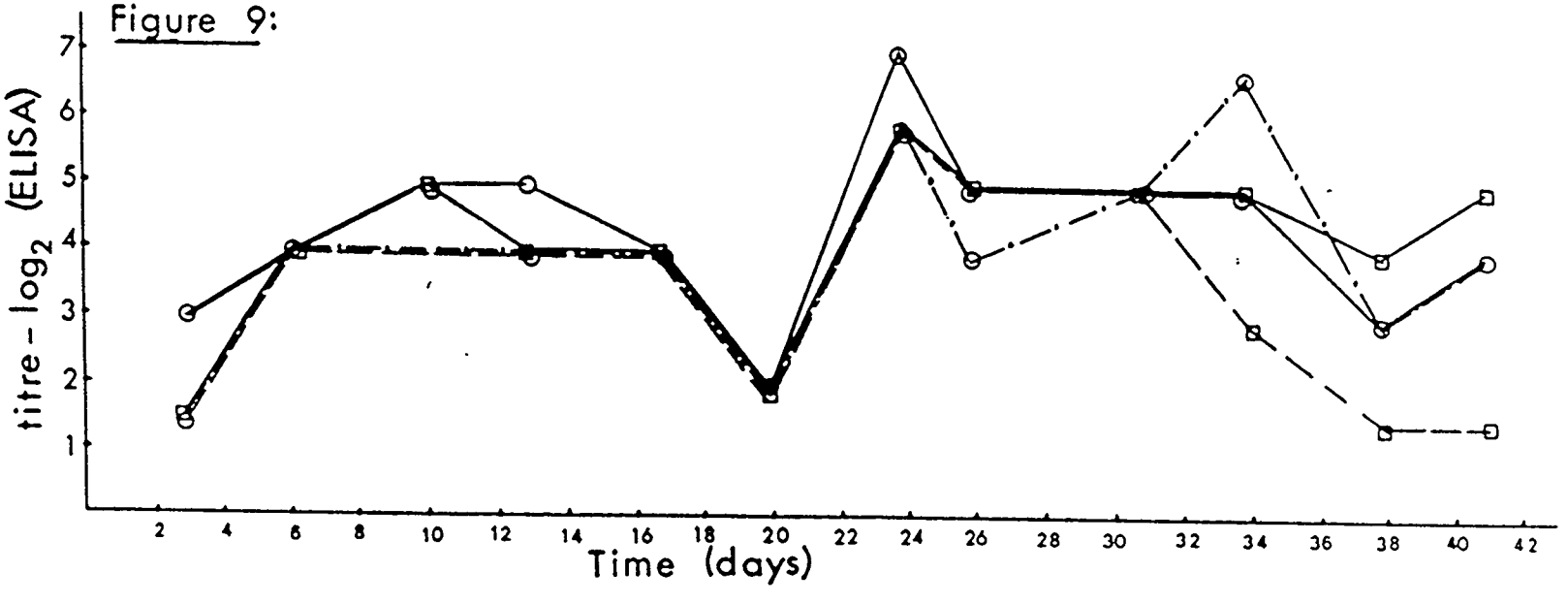


Figure 10: Spleen weights of mice immunized with live SL3261, vaccine strain S. typhimurium.

a) Balb/c - oral immunization

b) Balb/c - parenteral immunization

c) F_1 DBA X Balb/c - oral immunization

d) F_1 DBA X Balb/c - parenteral immunization

In each case test animals are represented by O——O. Control animals are represented by O— —O . Vertical arrows on the X-axis indicate days at which booster doses were given.

Figure 10:

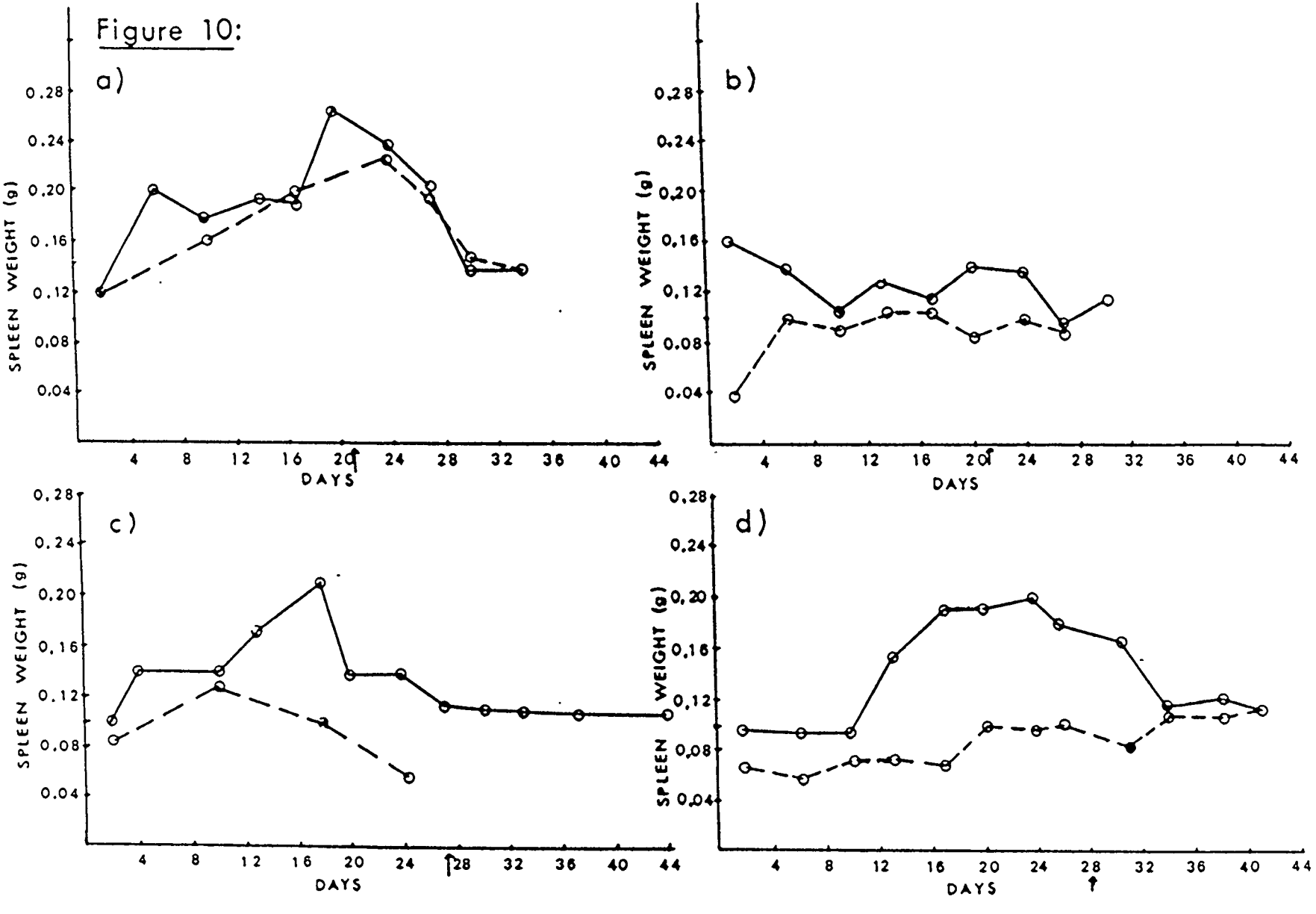

a)
b)
c)
d)
SPLEEN WEIGHT (g)
0.28
0.24
0.20
0.16
0.12
0.08
0.04
4 8 12 16 20 24 28 32 36 40 44
DAYS

Figure 11: Relative enlargement of mesenteric lymph nodes (MLN) of three strains of mice immunized orally with live SL3261, vaccine strain S. typhimurium. 4^+ designates maximal enlargement with gradations from 0 (no enlargement) to 4^+.

Legend: F_1 DBA X Balb/c

Balb/c

RBF/Dn

Figure 12: Comparison of male and female F_1 DBA X Balb/c polyclonal response to SL3261, vaccine strain of S. typhimurium, as tested by ELISA with four outer membrane antigens on the solid phase. M = male, F = female. The left hand panel represents all test sera, the right hand panel represents all control sera. The immunizing dose (same experiment as Figure 6) was 3×10^4, intraperitoneally.

Figure 11:

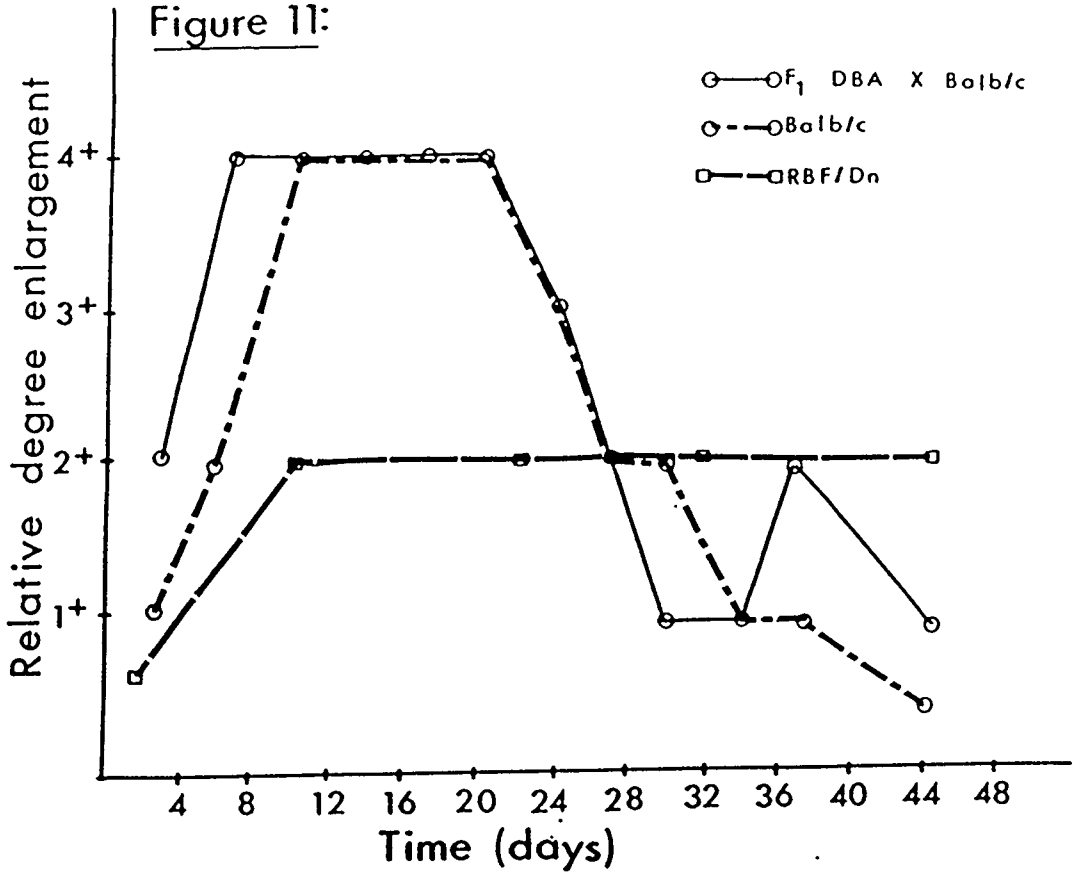
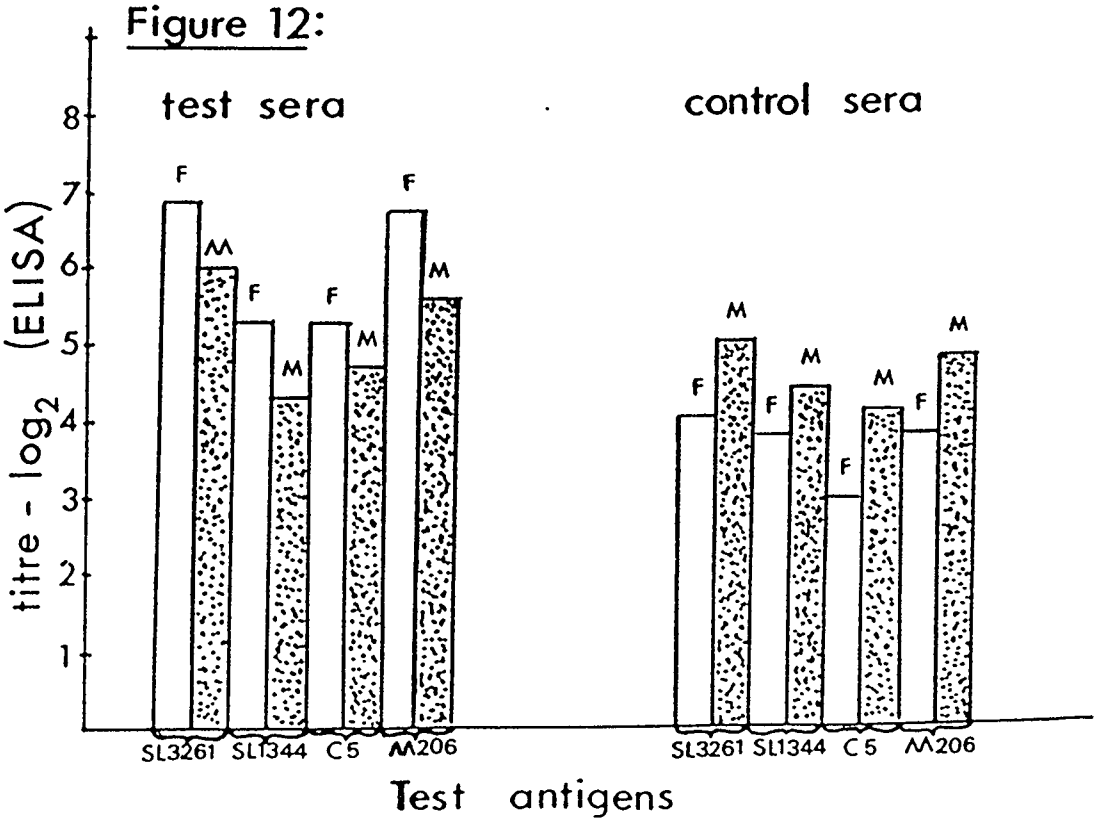


Figure 12:



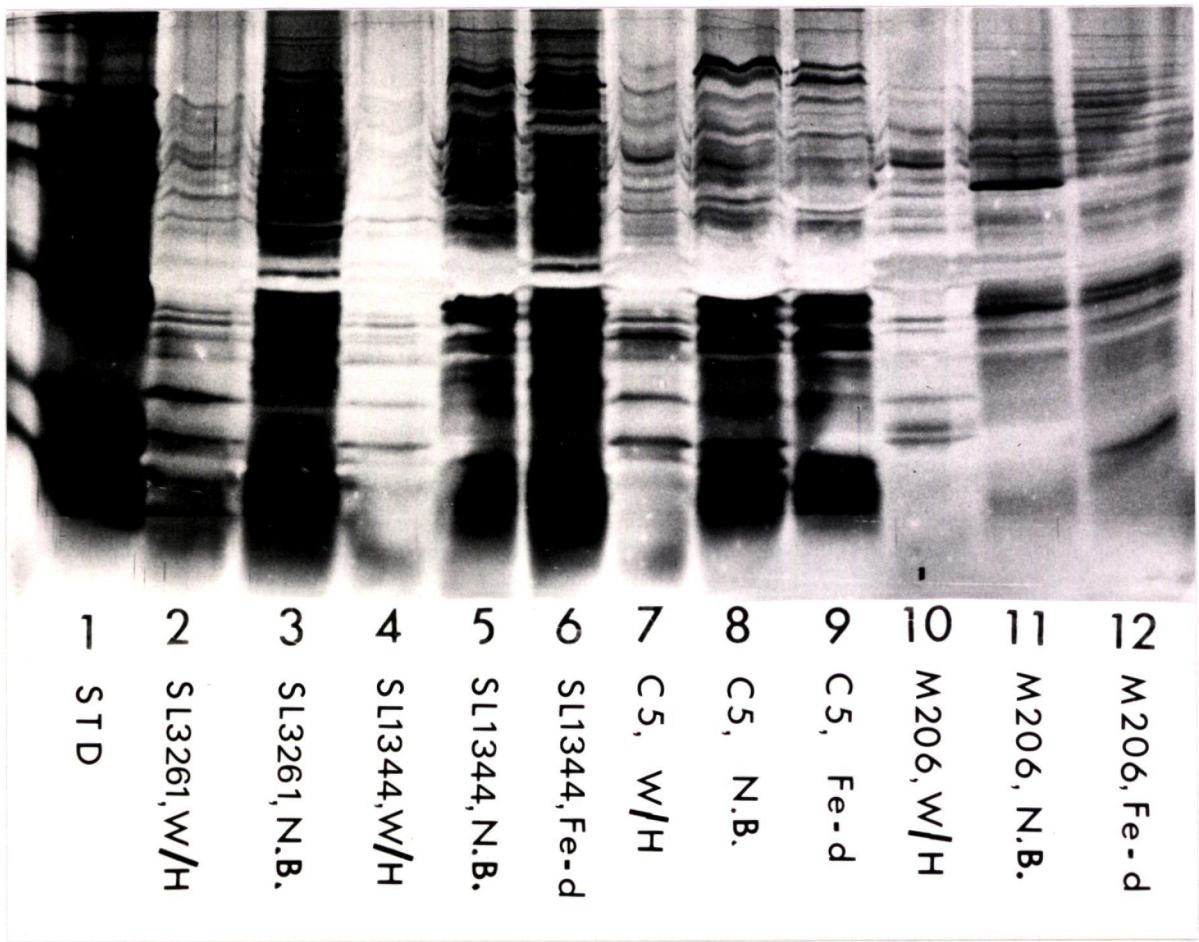


Figure 13: Silver-stained polyacrylamide gel showing protein profiles of outer membranes of four strains of S. typhimurium grown in nutrient broth and minimal, iron-deficient medium. Water/heat extracts of outer membranes are also included. Lanes 1 to 12 are: Standard, SL3261 water/heat extract, SL3261 nutrient broth sucrose gradient extract, SL1344 water/heat extract, nutrient broth and iron-deficient sucrose gradient extracts, C5 in the same order, then M206 in the same order.

Figure 14: Antibody reactivity of sera taken at 10 days and 13 days post-boost (p.b.) from F_1 DBA X Balb/c with outer membranes from four S. typhimurium strains grown in minimal, iron-deficient medium:

F = female, M = male.

One male and one female unimmunized control are represented. Immunization as in Figure 12.

Figure 14:

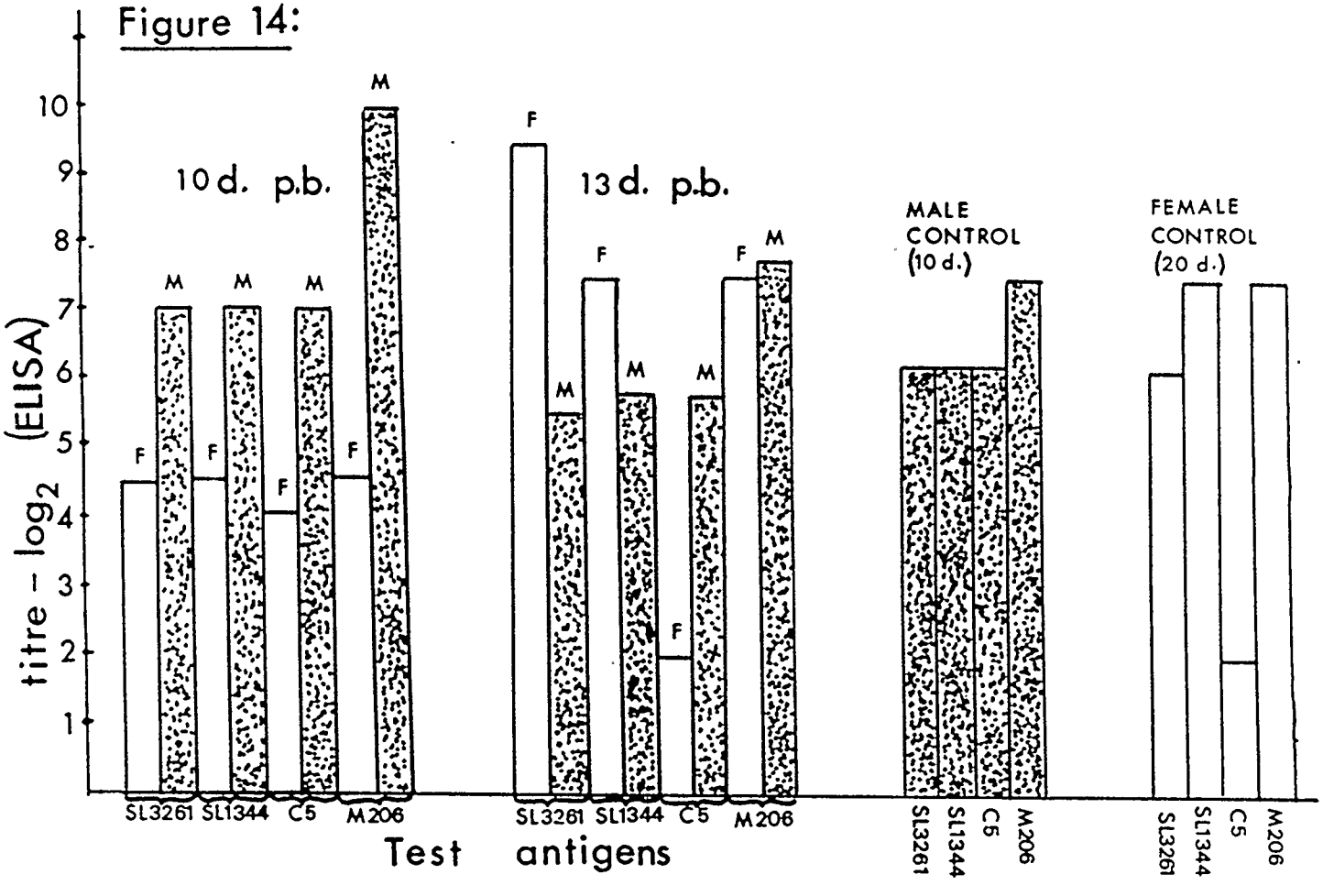

titre - log2 (ELISA)
10 d. p.b.
13 d. p.b.
MALE CONTROL (10 d.)
FEMALE CONTROL (20 d.)
F
M
SL3261
SL1344
C5
M206
Test antigens

Figure 15: IgG polyclonal response of F_1 DBA X Balb/c to 10^9 heat-killed S. typhimurium, SL3261. Figure a) shows the antibody reactivity with outer membranes from four S. typhimurium strains grown in nutrient broth, as well as to smooth LPS. Figure b) shows the reactivity with outer membranes from bacteria grown in minimal, iron-deficient medium.

Legend:

SL3261 (immunizing vaccine strain) – ○———○

SL1344 (isogenic, virulent parent to SL3261) – ○—·—·—○

M206 (unrelated carrier) – □———□

C5 (unrelated virulent strain) – □— — —□

LPS – ▽—/—/—▽

Figure 15:

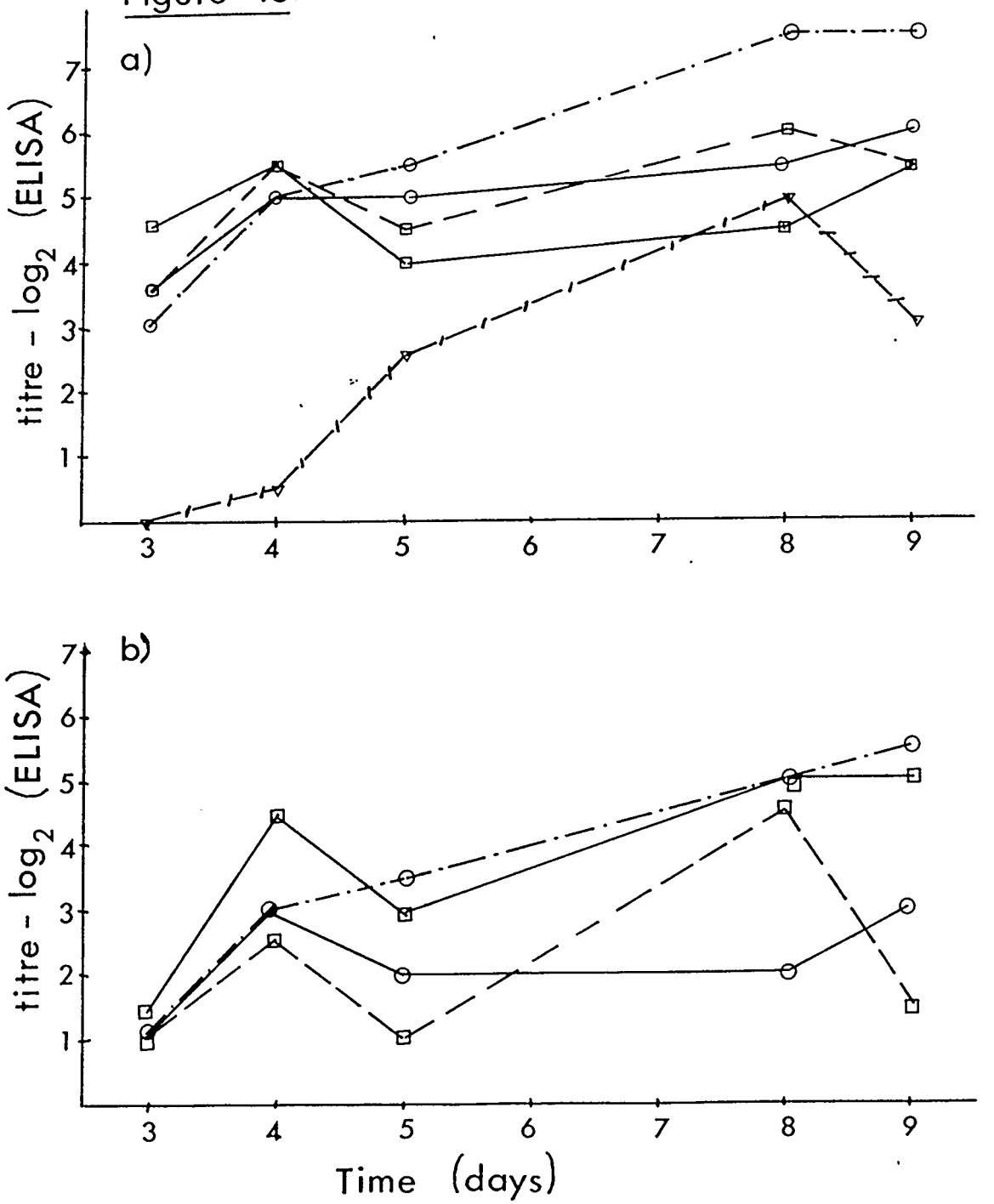


Figure 16: Spleen weights of F_1 DBA X Balb/c mice immunized intraperitoneally with 10^9 heat-killed SL3261.

Legend:

male mice ○——○

female mice □——□

control mice □— —□

Each point represents one mouse.

Figure 16:

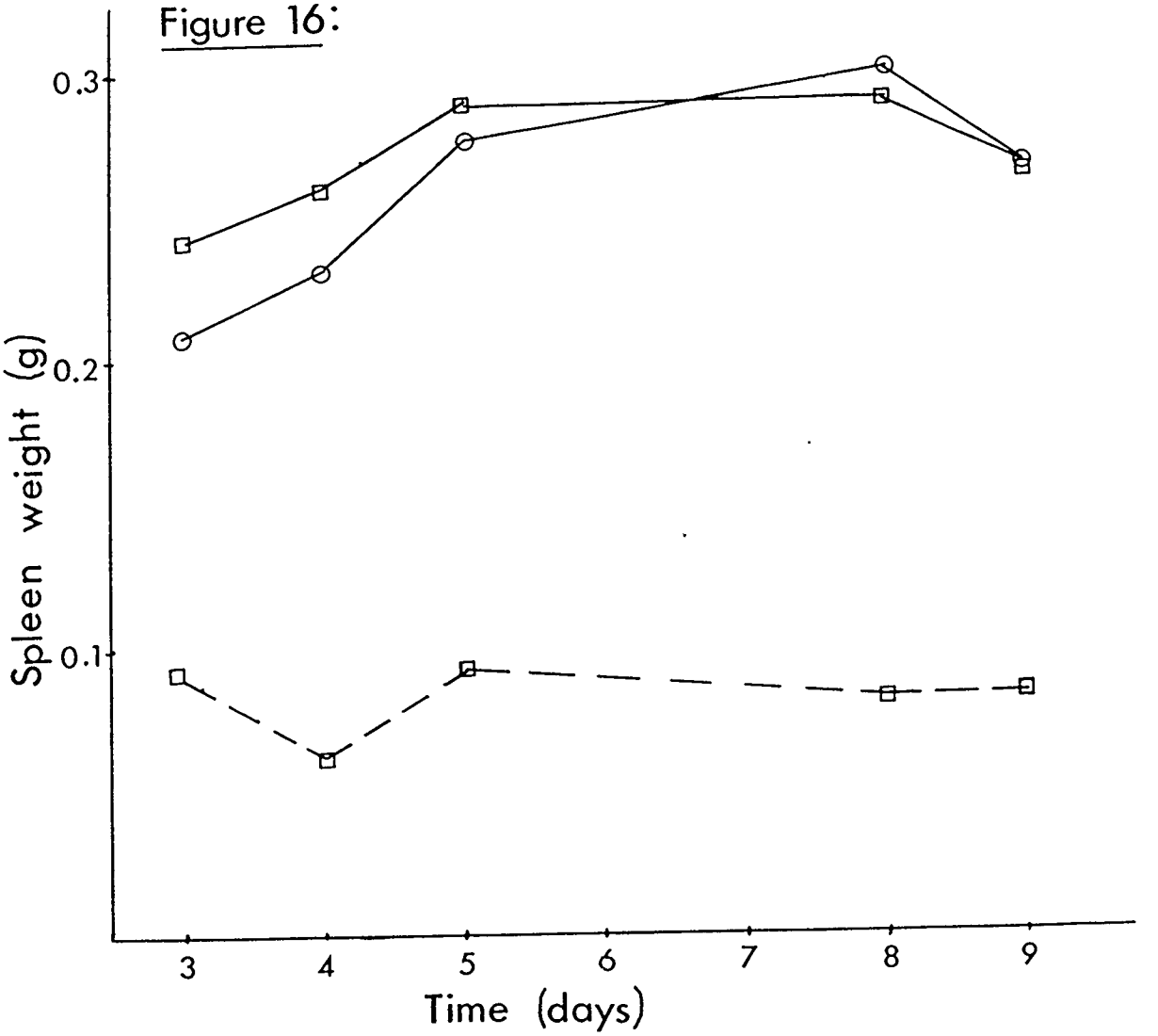


Figure 17: The number of hybridomas formed per 10^6 plated lymphocytes from Peyer's patches (P.P.), mesenteric lymph nodes (M.L.N.) and spleen in Balb/c, F_1 DBA X Balb/c and RBF/Dn mice. PWM refers to spleen cells from unimmunized mice cultured 48 hours with pokeweed mitogen prior to fusion. Spleen cells were not fused (ND = not done) at 24 hours from any mice, or at 10 d. from Balb/c and RBF/Dn mice. The oral immunizing dose was 10^8 SL3261, vaccine strain.

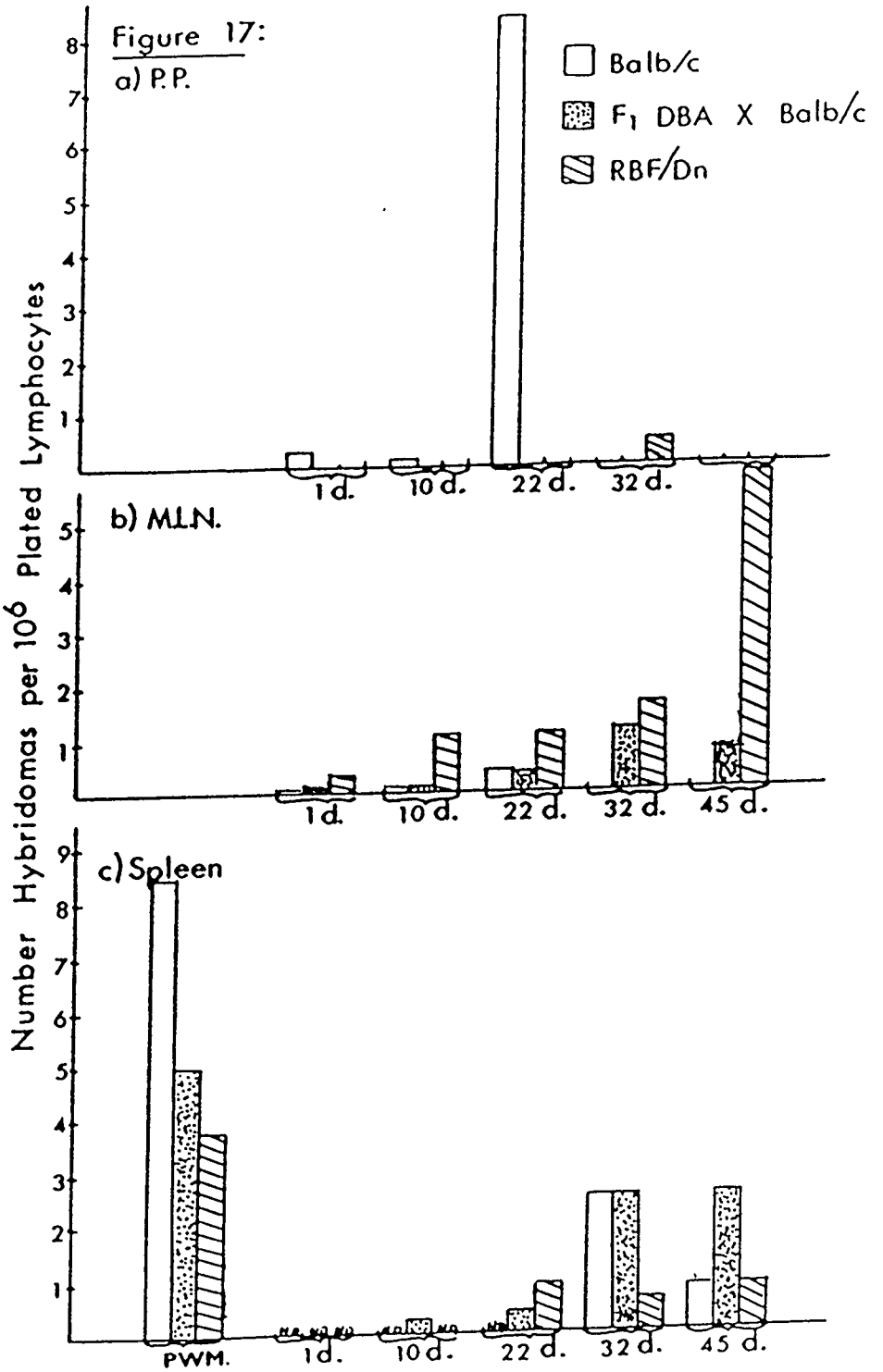


Figure 17:
a) P.P.
Balb/c
F1 DBA X Balb/c
RBF/Dn
Number Hybridomas per 10^6 Plated Lymphocytes
1 d.
10 d.
22 d.
32 d.
b) M.L.N.
45 d.
c) Spleen
PWM.

Figure 18: Clonal B cell response of F_1 DBA X Balb/c and RBF/Dn orally immunized mice (both resistant) showing the distribution of Ig class-producing hybridomas relative to total hybridomas. Hybridomas (hybrids) are expressed as number X 10^2. Figure a) compares the two mouse strains to 22 d. including the mesenteric lymph nodes at day 22. Figure b) shows the comparison through day 32 and Figure c) through day 45, the end of the immunization schedule.

Legend:

PWM = spleen cells from unimmunized mice cultured 48 hours in the presence of pokeweed mitogen prior to fusion.

O.M. = outer membrane of S. typhimurium strain SL3261, the immunizing vaccine.

I.M. = inner membrane fraction from SL3261.

I.M.(Rc) inner membrane of the Rc mutant (diphosphate-galactose-4-epimeraseless mutant)

LPS = smooth lipopolysaccharide from S. typhimurium, commercially purchased and prepared from strain 225.

The last four refer to the solid phase antigens in the ELISA used for screening hybridomas.

The bars representing IgG, IgA and IgM are the same in Figures b) and c) as indicated for Figure a).

The oral immunizing dose was 10^8 SL3261; the mice being identical to those in Figure 17.

Figure 18 a):

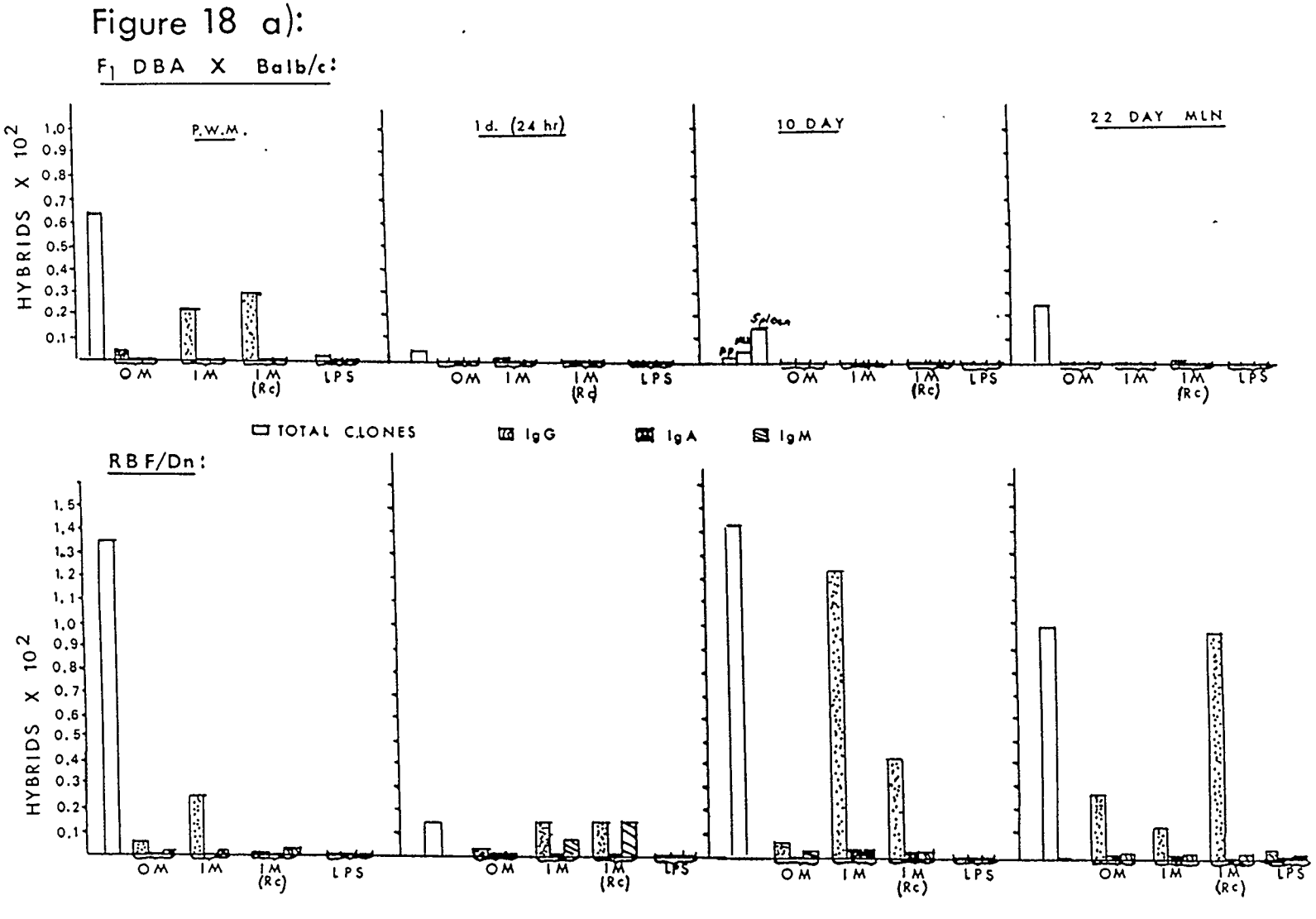

F1 DBA X Balb/c:
P.W.M.
1d. (24 hr)
10 DAY
22 DAY MLN
HYBRIDS X 10^2
OM
IM
IM (Rc)
LPS
Spleen
PP
MLN
TOTAL CLONES
IgG
IgA
IgM
RBF/Dn:

Figure 18 b):

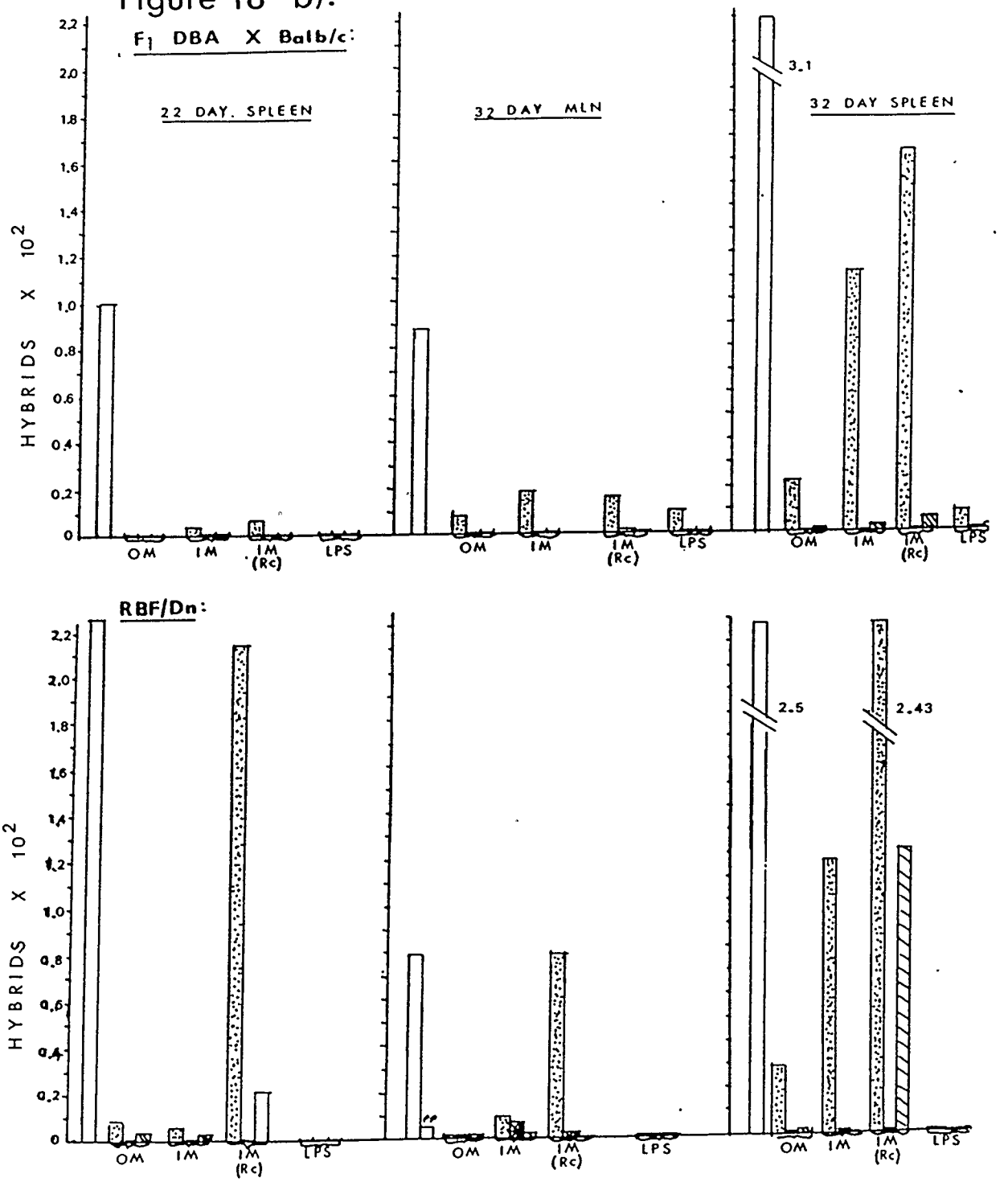

F1 DBA X Balb/c:
22 DAY. SPLEEN
32 DAY MLN
32 DAY SPLEEN
HYBRIDS X 10^2
3.1
OM
IM
IM (Rc)
LPS
RBF/Dn:
2.5
2.43

Figure 18 c):

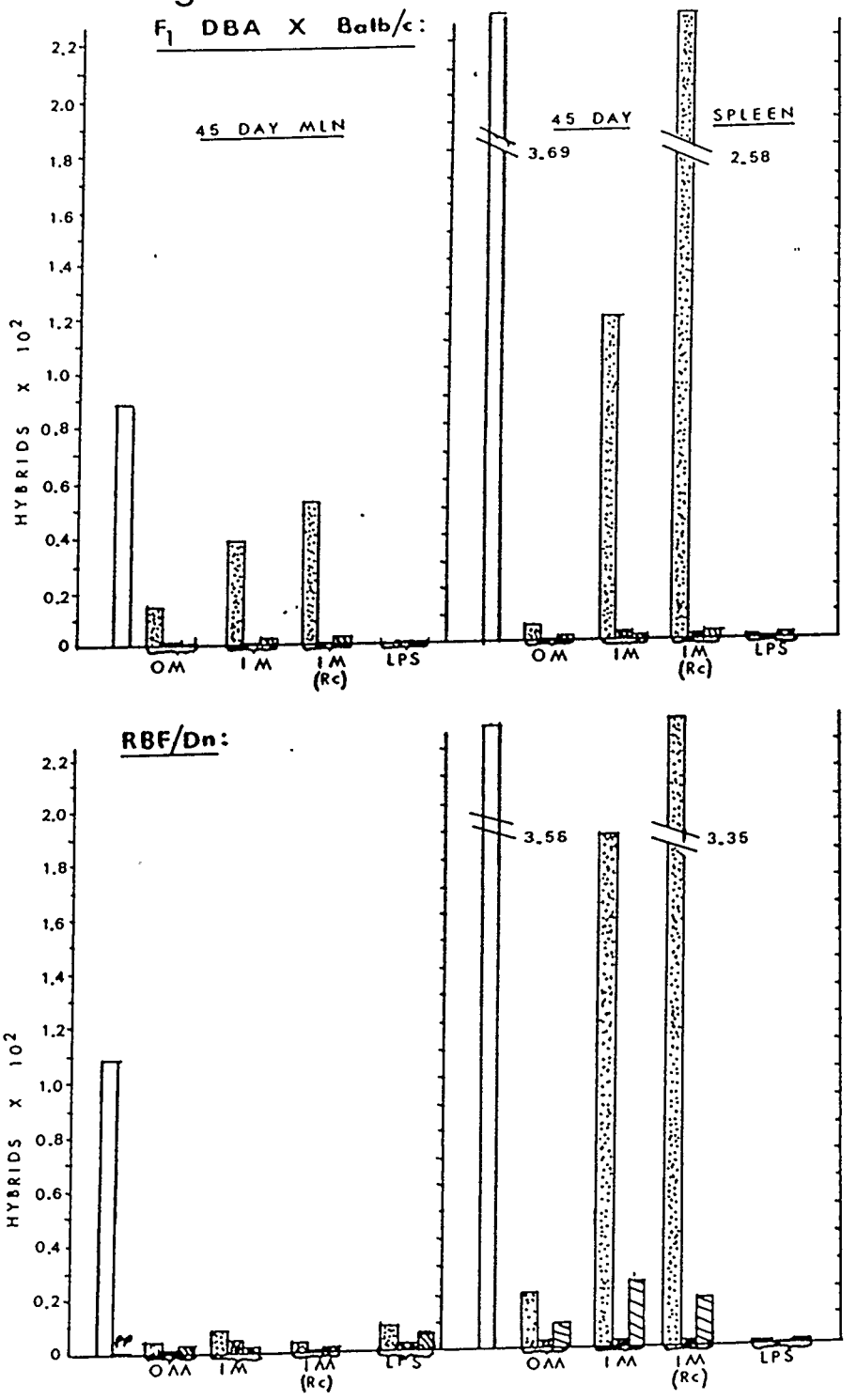

F1 DBA X Balb/c:
45 DAY MLN
45 DAY
SPLEEN
3.69
2.58
HYBRIDS X 10²
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
OM
1M
1M (Rc)
LPS
RBF/Dn:
3.56
3.35
OAA
1A
1A (Rc)

Figure 19: Serum titres of IgG antibody (polyclonal) in the three strains of mice used for the clonal B cell response after oral immunization. These were:

Balb/c - susceptible, homozygous Ity^s/Ity^s

F_1 DBA X Balb/c - resistant, heterozygous Ity^r/Ity^s, same H-2 haplotype as Balb/c

RBF/Dn - resistant, wild-type Ity^r.

The vertical arrows on the X-axis represent the booster dose given at 24 days of the immunization schedule. The abreviations for the solid phase antigens used in the ELISA are the same as those for Figure 18, and the titres to each are represented in Figures a), b), c) and d) of this figure.

Figure 19:

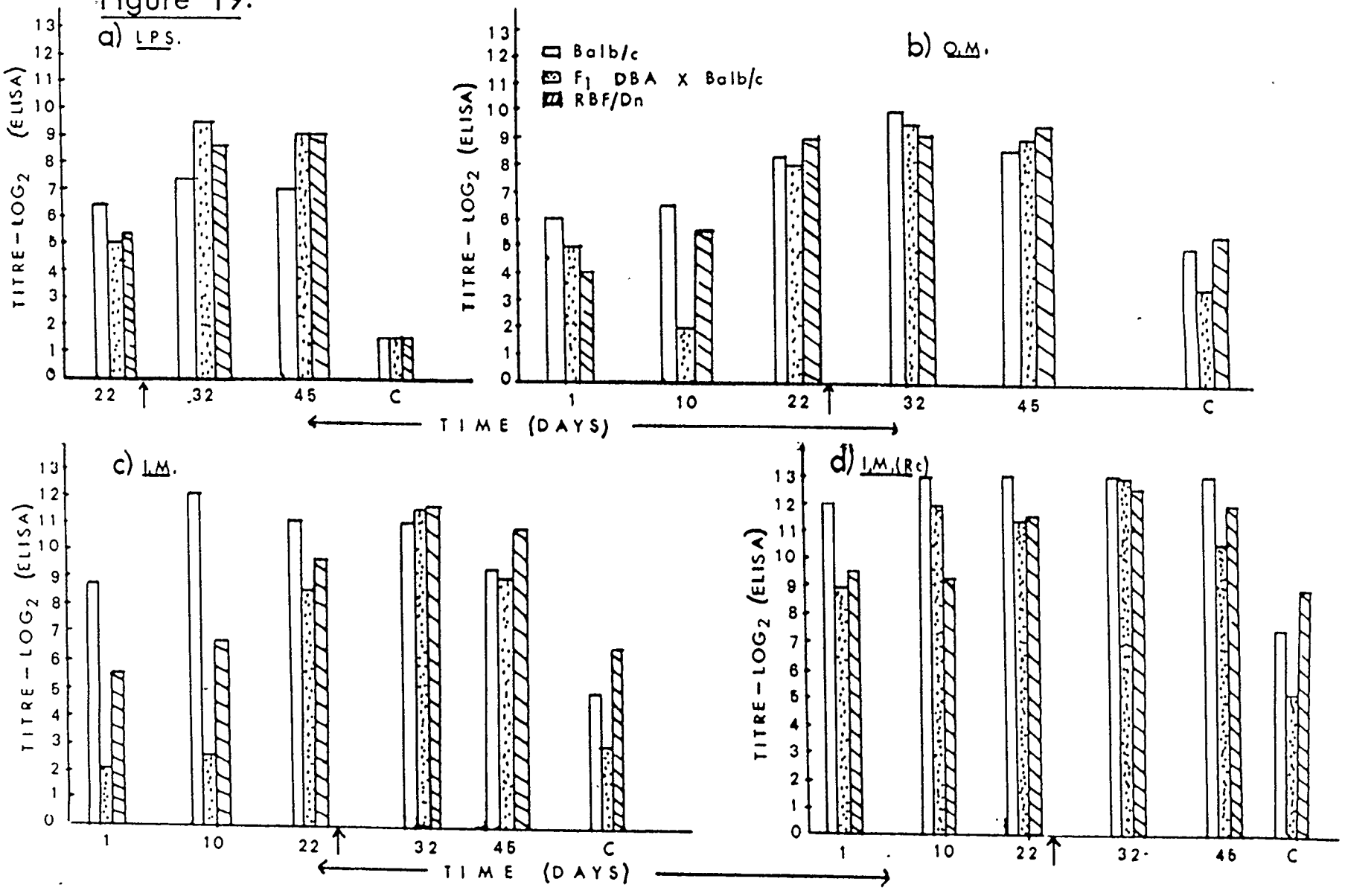

a) LPS.
b) O.M.
c) I.M.
d) I.M.(Rc)
Balb/c
F1 DBA X Balb/c
RBF/Dn
TITRE – LOG2 (ELISA)
TIME (DAYS)
22
32
45
C
1
10

Figure 20: Serum titres of IgM antibody (polyclonal) in the three strains of mice used for the clonal B cell response after oral immunization. They are as indicated in Figure 19. The abbreviations for the solid phase antigens are the same as for Figure 19 with vertical arrows on the X-axis indicating the booster dose at 24 d.

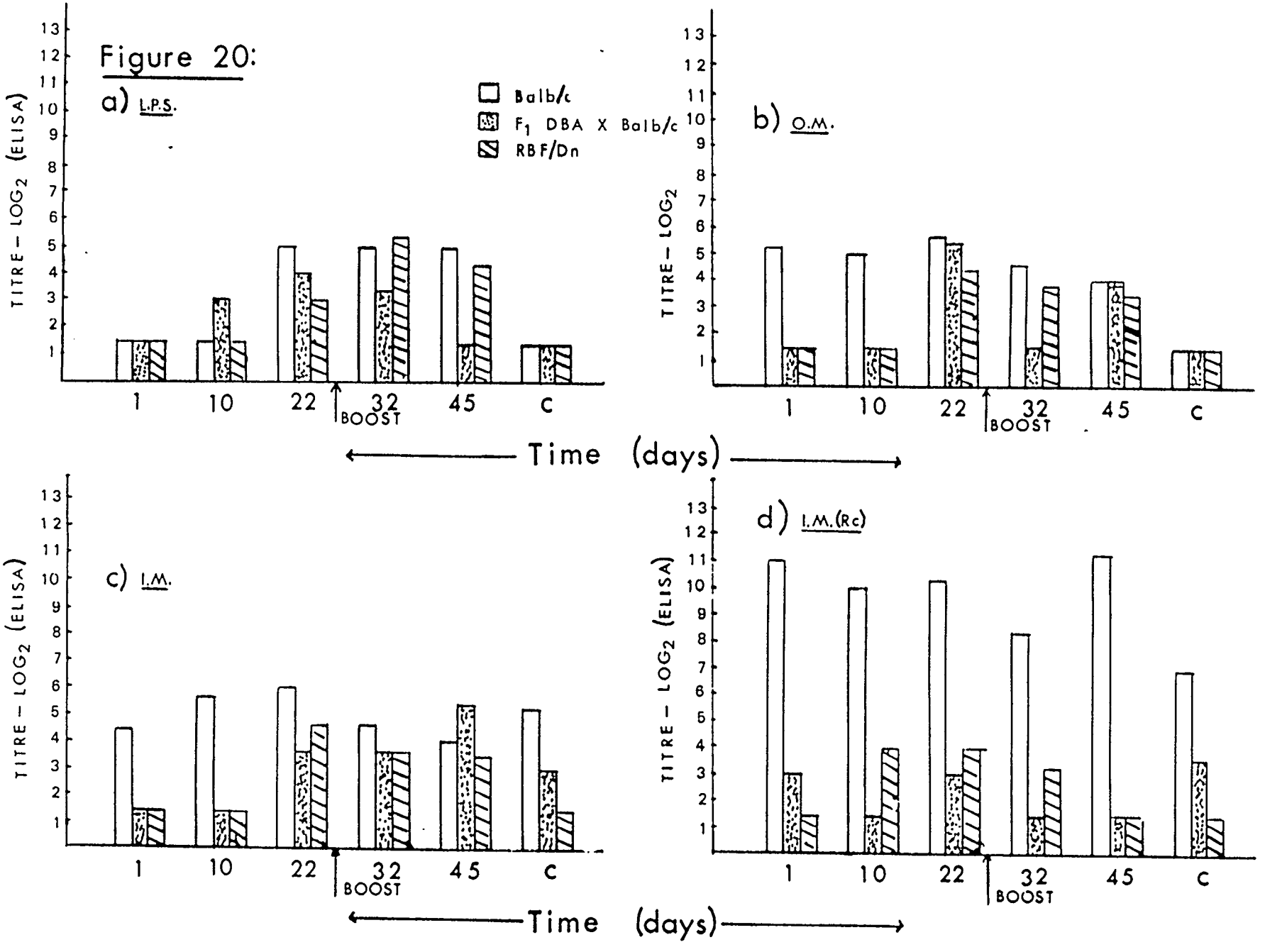


Figure 20:
a) L.P.S.
Balb/c
F1 DBA X Balb/c
RBF/Dn
b) O.M.
c) I.M.
d) I.M.(Rc)
TITRE - LOG2 (ELISA)
1
10
22
32
45
c
BOOST
Time (days)

Figure 21: Serum titres of IgA antibody (polyclonal) in the three strains of mice used for the clonal B cell response after oral immunization. Abbreviations are the same as for Figures 19 and 20.

Figure 21:

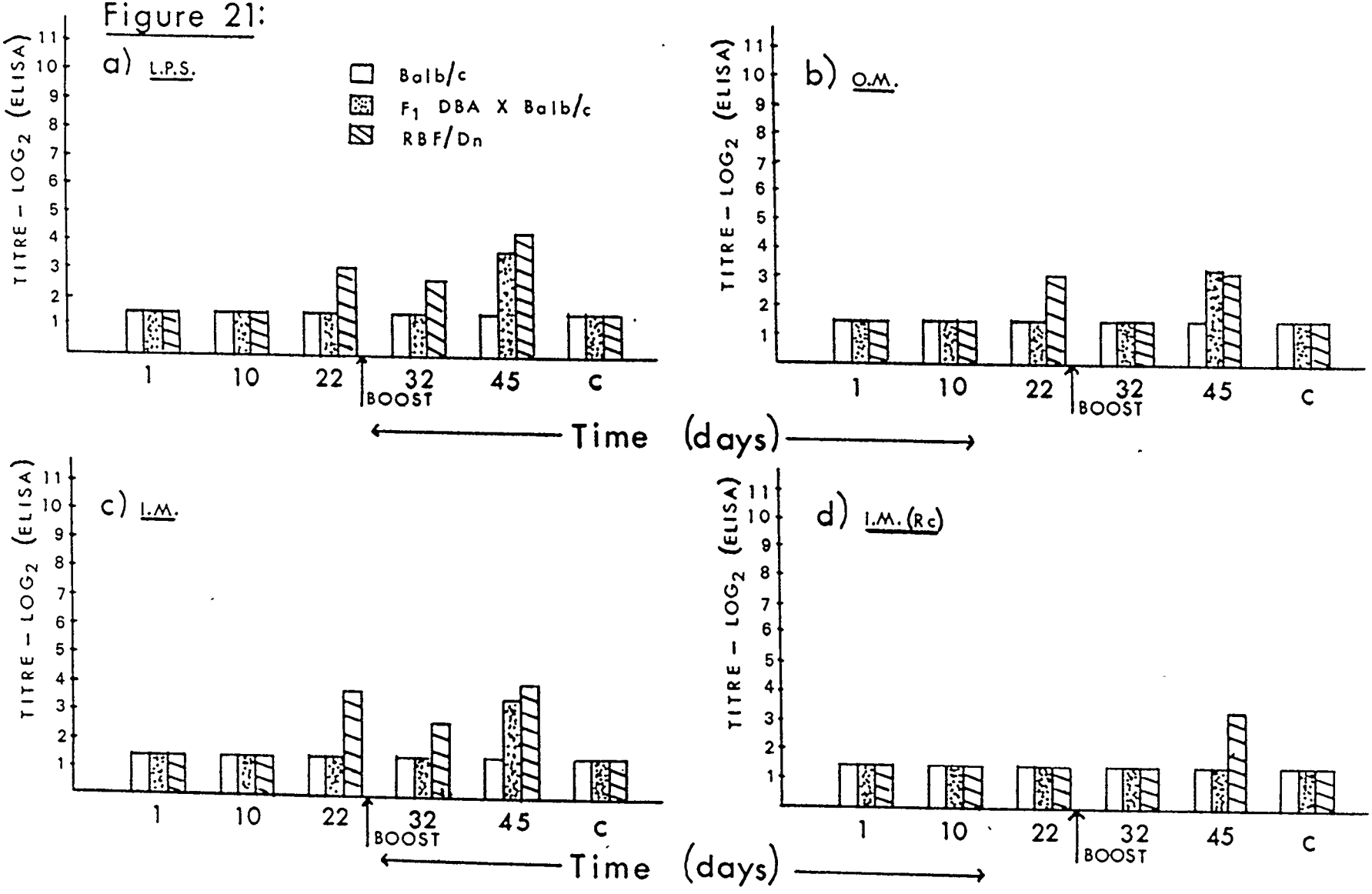

a) L.P.S.
Balb/c
F1 DBA X Balb/c
RBF/Dn
TITRE – LOG2 (ELISA)
1 10 22 32 45 C
BOOST
Time (days)
b) O.M.
c) I.M.
d) I.M. (Rc)

Figure 22: Time sequence of production of IgG and IgM antibody to the four groups of S. typhimurium antigens by the three strains of mice used for assessing the clonal B cell response after oral immunization.

The abbreviations for the antigens are the same as in Figures 19, 20 and 21 for the polyclonal response.

The figure overleaf (Figure 22: continued) shows the time of production of IgM antibody to LPS which was greater, compared to controls, than the acquisition of IgG.

Figure 22:

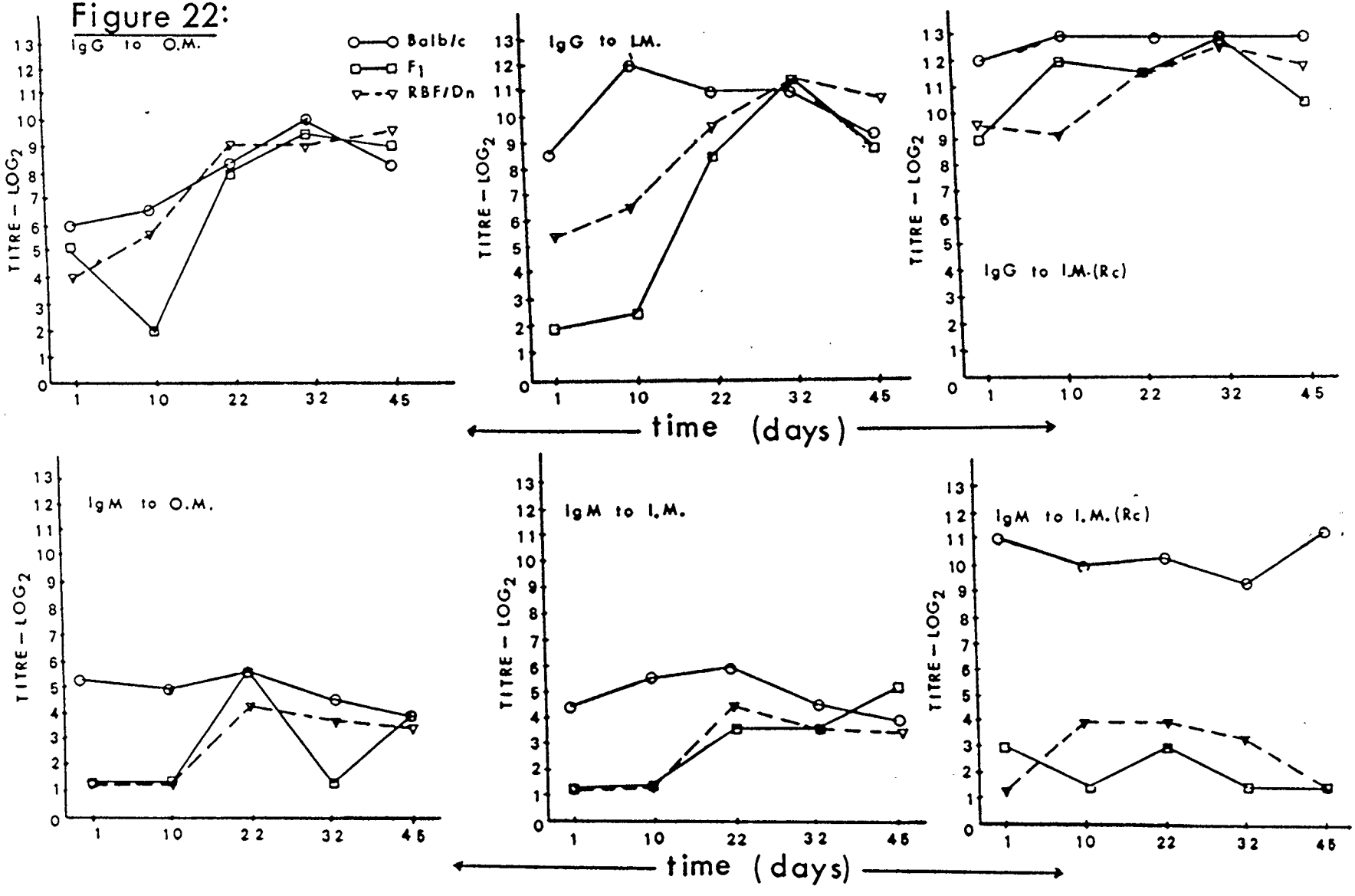

IgG to O.M.
Balb/c
F1
RBF/Dn
IgG to I.M.
IgG to I.M.(Rc)
IgM to O.M.
IgM to I.M.
IgM to I.M.(Rc)
TITRE – LOG2
time (days)

Figure 22 (continued):

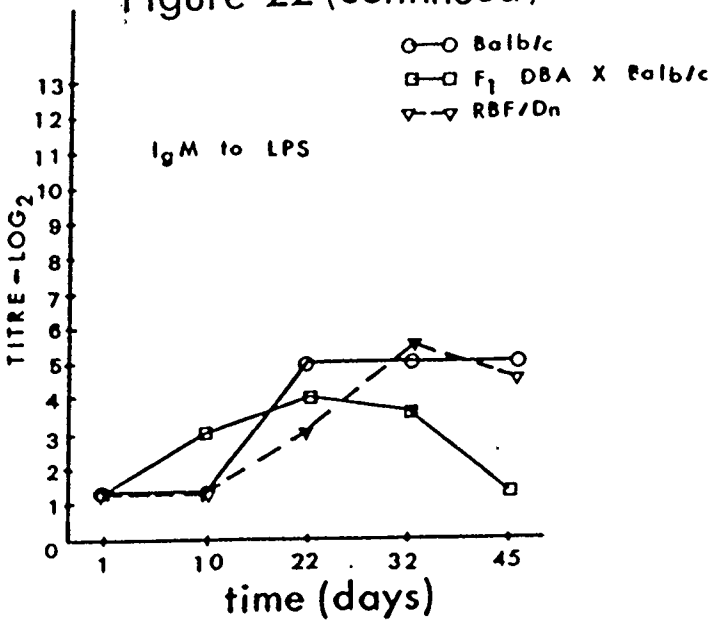


Figure 23: Serum IgG titres (polyclonal) in three additional resistant strains of mice orally immunized and boosted with 10^8 live SL3261.

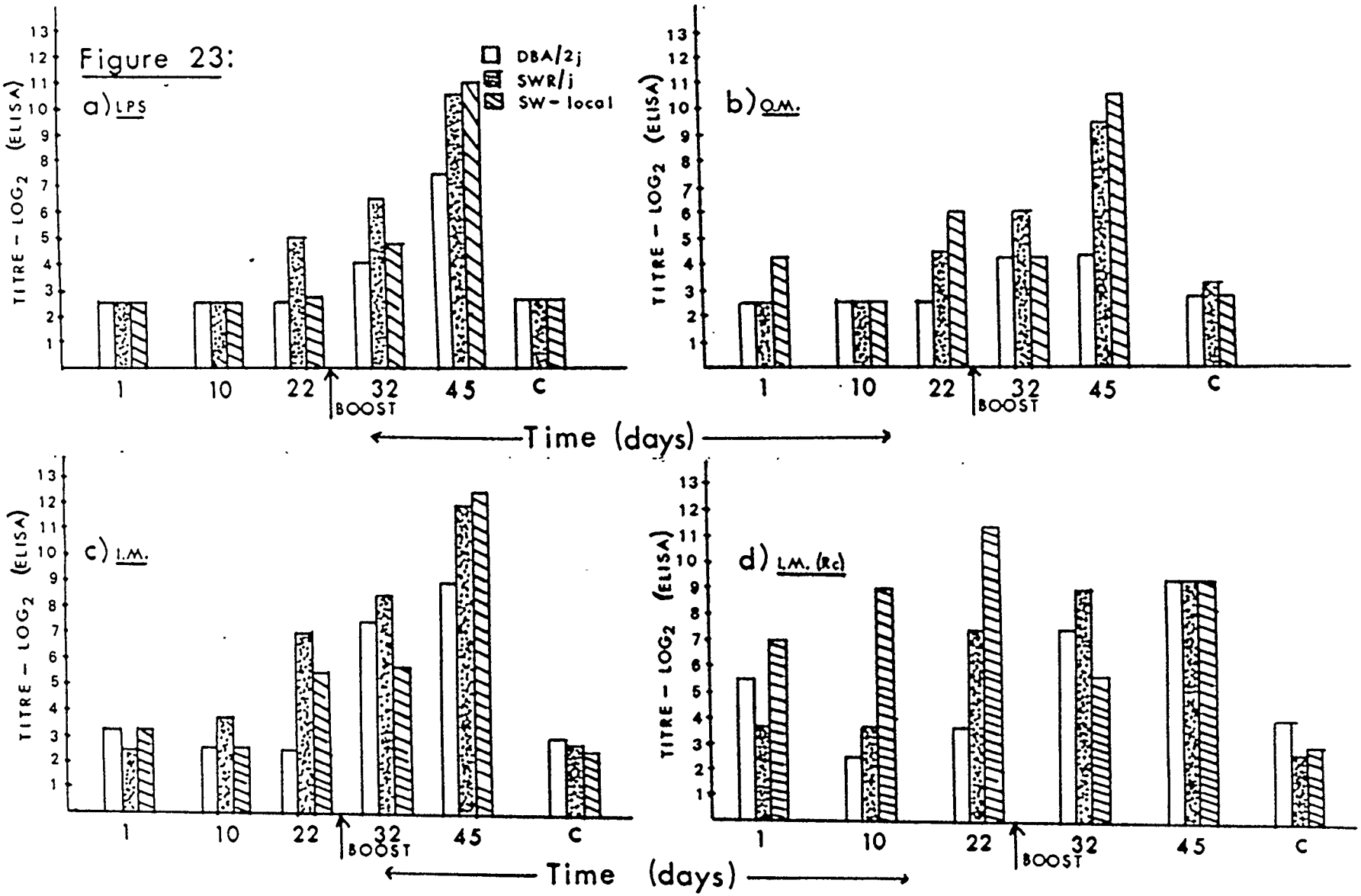
DBA/2j - the paternal parent of the F_1 DBA X Balb/c, same H-2 haplotype as Balb/c, and confers the Ity^r allele in the F_1.

SWR/j - resistant strain on which much of the original Ity genetics was done. See text.

SW-local-random bred Swiss Webster mice from the Biology Vivarium, University of Calgary.

The abbreviations for the solid phase antigens used for the ELISA are the same as for Figures 19 through 22. The vertical arrows on the X-axis also indicate a booster dose given at 24 d.

Figure 23:


a) LPS
b) O.M.
c) I.M.
d) L.M. (Rc)
DBA/2j
SWR/j
SW - local
TITRE - LOG2 (ELISA)
1
10
22
32
45
C
BOOST
Time (days)

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