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Protective Effects of a Supernatant Factor from *Salmonella typhimurium* on *Salmonella typhimurium* Infection of Inbred Mice

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A supernatant factor prepared from 48-h cultures of *Salmonella typhimurium* has been used to immunize mice against subsequent challenge with normally lethal doses of *S. typhimurium*. The mouse strains used, C57BL and BALB/c, were sensitive to *S. typhimurium* with 50% lethal doses of less than 50 organisms. Two doses of supernatant factor, given intraperitoneally 20 days apart, protected mice against a subcutaneous challenge dose 10 days later of 100 50% lethal doses of *S. typhimurium*, resulting in 50 to 80% survival. The viable counts were reduced initially in organs of immunized mice compared with controls, and the multiplication of bacteria was delayed, although the final levels found in the organs would normally have been lethal. Protection obtained was specific for *S. typhimurium* in that no increased survival was shown after *Salmonella enteritidis* challenge of immunized mice. Although lipopolysaccharide was demonstrated in the supernatant factor, lipopolysaccharide alone did not protect challenged mice. Supernatant factor produced delayed-type hypersensitivity reactions in mice sensitized with nonlethal doses of *Salmonella*. The nature of the active factor, found to be partially protein, has yet to be elucidated.

Immunity to systemic salmonella infections was produced early on by means of killed vaccines, but much greater resistance follows recovery from salmonella infection, natural or experimental (28). Postinfection carriers are particularly resistant. They clear organisms rapidly from the blood and have low bacterial counts in the liver and spleen (12). Mice immunized and challenged by genetically distinguishable strains of salmonella eliminate the second infection completely while remaining carriers of the first (19). Previously infected mice successfully resist challenge with 100 or even 1,000 50% lethal doses (LD_{50}) (9, 27).

Because of the obvious advantages, many attempts have been made to develop nonliving vaccines. With such vaccines the mode of challenge is critical. Dead vaccine or bacterial extracts can produce worthwhile resistance (1, 15), but many such experiments have been criticized because the challenge infection was given intraperitoneally (i.p.), that is, in a site where antibodies are particularly effective (19). Phenol-killed vaccines of *Salmonella typhimurium* and *Salmonella dublin* protected mice against i.p. but not oral infection with the homologous strain (20). Topley (26) could not control experimental epidemics of *S. typhimurium* oral infection in mice with dead vaccine. However, dead vaccines can give some protection against subcutaneous (s.c.) challenge (16).

The two types of vaccine differ in their detailed effects. Dead vaccines give good antibody production and hence opsonization but are less effective at controlling bacterial multiplication in the liver and spleen (4, 5, 27), though some control after dead vaccine administration has been claimed (11).

In a systematic comparison, Collins (5) found that live *Salmonella gallinarum* vaccine protected mice against challenge by any route with virulent *Salmonella enteritidis*. Dead *S. enteritidis* vaccine did not protect against intravenous challenge unless vaccination was simultaneously by intravenous, i.p., and s.c. routes. Most protection was achieved against s.c. challenge. Multiplication of the infecting strain in blood and organs was limited after live vaccine but only delayed a day or 2 by dead. Experiments with oral challenge gave similar results (6).

Mackaness and his colleagues (18) hold that effective resistance to salmonella infection in mice is based on cell-mediated immunity induced only by live vaccine. However, there is evidence that antibody, free or cytophilic, may also play a part (24, 25, 29).

The efficacy of live vaccine has been attributed variously to the better production of protective antigen in vivo, its persistence for long periods, and perhaps its particular distribution. None of these suggests that a dead vaccine must inevitably be inefficient, and although cell-mediated immune responses occur more readily after vaccination with live than dead bacteria (7), good delayed hypersensitivity reactions to protein or even simple chemicals are well known.

In view of the importance of cell-mediated immunity in resistance to salmonella infection, it seemed worth investigating the protective effect of a factor known to give delayed hypersensitivity reaction in infected mice. Such a factor was described by Collins and Mackaness (8) in the supernatant of old broth cultures of *S. typhimurium*. Moreover, the degree of delayed hypersensitivity reaction this factor produces in infected mice of different strains is proportional to their resistance (22).

C57BL and BALB/c mice were immunized with the salmonella supernatant factor. They were then tested for delayed hypersensitivity and for their ability to resist s.c. challenge with a virulent strain of *S. typhimurium*. Resistance was assessed both by mortality rates and by ability to control bacterial multiplication in the liver and spleen. Some preliminary data on the nature of the factor concerned are also given.

MATERIALS AND METHODS

Animals. Mice were kept, 10 to 20 in a group, in large cages and allowed unlimited supplies of food and water.

Inbred strains of mice, BALB/c and CBA, were bred in the Animal Department of St. Mary's Hospital Medical School; C57BL/10J mice were obtained from the Laboratory Animal Centre (Carshalton, Surrey).

The outbred strain of albino mice used was that bred at the Wright-Fleming Institute for many years (WFI strain).

Bacterial strains. A virulent strain of *S. typhimurium*, C5, was obtained from D. Rowley (University of Adelaide). *S. enteritidis* var. *danysz* was used in some experiments.

Strains from freeze-dried cultures were maintained on Dorset egg slopes (Southern Group Laboratory, Hither Green Hospital, London) at 4°C and grown overnight in glucose broth (digest broth + 0.1% glucose, obtained from Southern Group Laboratory) before use.

The overnight cultures were diluted, according to their optical density, in 0.9% sodium chloride plus 10% glucose broth (saline/broth) to give the dose required in 0.1 ml. Serial dilutions of this suspension and subsequent plating on MacConkey agar gave an estimate of the viable bacteria injected. Challenge doses were all given s.c.

SF. Supernatant factor (SF) was prepared from *S. typhimurium* or *S. enteritidis* by the adaptation of the method of Collins and Mackaness (8) described in a previous paper (23). The supernatant material from a 48-h culture of the bacteria in Cohn medium (3) was concentrated using a Diaflo ultrafilter of exclusion limit 10,000 molecular weight (Amicon, Lexington, Mass.). Lipopolysaccharide (LPS) was removed by centrifugation at 100,000 × *g* for 2 h.

SF made for experiments with delayed hypersensitivity was standardized to 1 mg of protein per ml by the method of Lowry et al. (17) after partial purification by precipitation with ethanol (23).

SF for use in protection experiments was not treated with alcohol but was concentrated to 1 to 2 mg of protein per ml.

Preparations of supernatant factor were stored at −20°C.

Viable counts in the organs. Viable counts were estimated as described previously (23). The livers and spleens were removed into 10 ml of saline/broth and homogenized in a Colworth Stomacher 80 (A. J. Seward & Co. Ltd., London). Tenfold serial dilutions in saline/broth were plated on MacConkey agar.

Delayed hypersensitivity reaction. The delayed hypersensitivity reaction was tested by footpad injections as described previously (22). Increases in footpad thickness in sensitized mice were recorded at 4, 24, and 48 h after injection of SF and corrected for increases in control footpads receiving saline. The results were also corrected for any increases in unsensitized mice similarly treated.

Experimental procedure. In preliminary experiments, some prolongation of survival was given by one dose of SF given i.p. or s.c. SF given intramuscularly with complete Freund adjuvant was ineffective. The system finally adopted was to give approximately 300 μg of protein per dose, injected i.p. without adjuvant in a volume of 0.2 ml on days 0 and 20. The mice were challenged on day 30 with *S. typhimurium or S. enteritidis* given as a known dose s.c.

C57BL or BALB/c mice approximately 8 weeks old were used for protection experiments and were challenged in groups of 10 or more mice. Deaths were recorded daily for 30 days after challenge.

After challenge, growth of the bacteria in the organs of immunized mice was compared with growth in control mice receiving the same challenge infection. Groups of three to five mice were killed at intervals up to 3 weeks after challenge, and spleen weights and viable counts in the organs were measured.

Delayed hypersensitivity reactions were tested in control and immunized mice on day 30, the usual day of challenge.

LPS content of the SF. Three methods of assay were used. Sheep erythrocytes (SRBC) (Oxoid Ltd., Basingstoke, Hampshire, England) were incubated for 1 h at 37°C with purified *S. typhimurium* SF at a final concentration of 10 μg of protein per μl of packed SRBC. Anti-*S. typhimurium* O serum was then titrated in microtiter trays (Sterilin Ltd., Teddington, Middlesex, England) against 1% suspensions of washed and treated or control SRBC in saline.

Next SF was used to inhibit the hemagglutination of LPS-coated SRBC by salmonella O antiserum.

Glutaraldehyde-treated SRBC were coated with phenol-water type LPS of *S. typhimurium* (Difco Lab-

oratories, Detroit, Mich.). A solution in phosphate-buffered saline of 0.5 ml of LPS (1 mg/ml), 0.2 ml of 1% glutaraldehyde (Kodak Ltd., London), and 0.1 ml of packed SRBC was made up to 2 ml. The cells were incubated with gentle shaking at 37°C for 1 h. The washed, coated SRBC were stored at 4°C as a 4% suspension.

The hemagglutination titer of anti-*S. typhimurium* O serum was tested against a 1% suspension of the SRBC, and the degree of inhibition produced by addition of *S. typhimurium* SF was compared with that produced by LPS solutions of known concentration.

Last, mice were injected with SF by various routes, and serum hemagglutinin titers to LPS-coated SRBC were looked for. C57 mice were injected i.p. with 0.2 ml of purified *S. typhimurium* SF or s.c. with 25 μl into two or four footpads. The antibody titers in the sera were tested against LPS-coated SRBC on days 7 and 14 and repeated on days 21 and 28 after a second injection on day 14.

RNA and protein content of the SF. RNA and protein were removed from the SF by treatment with ribonuclease and either Pronase or trypsin. The enzymes (BDH Chemicals Ltd., Poole, Dorset, England) were added as 50-μl solutions in saline to 450 μl of SF to give a concentration of 1:1 (wt/wt) with the protein in the SF. The solutions were kept at 4°C for 18 h and at 37°C for 1 h before use. Enzyme solutions alone were injected into control footpads, and any swelling produced was used to correct for nonspecific irritation.

The protein concentrations before and after treatment, corrected for the enzyme protein, were determined by Lowry's method (17).

Protein stability was tested by heating the SF for 1 h at 100°C.

RESULTS

Protection of C57BL and BALB/c mice. The protective SF of *S. typhimurium* was given as above, and the mice were challenged with bacteria on day 30 (Table 1). With the challenge doses used (100 or 700 LD_{50}), 50 to 80% of the mice survived at least 30 days. The rest of the mice survived an average of 5 to 10 days longer than control mice, which died between 10 and 14 days after challenge.

A group of protected C57BL mice were tested for reactions to footpad injections of SF on day 30, when the remainder of the mice were challenged with 100 LD_{50} of *S. typhimurium*. The mean corrected increases in footpad size at 24 and 48 h were 0.23 and 0.12 mm, respectively.

Specificity of protection by SF. Replacement of SF by an equivalent (in milligrams of protein per milliliter) amount of bovine serum albumin did not protect C57BL mice or prolong the survival time after a challenge dose of 700 LD_{50}. C57BL or BALB/c mice given two doses of *S. typhimurium* SF and then challenged with 50 or 100 LD_{50} of *S. enteritidis* all died at the same time as the unimmunized controls.

Effect of protective SF on viable counts of challenge organisms in host. Normal and immunized C57BL mice were challenged with 100 or 700 LD_{50} of *S. typhimurium* s.c. The viable counts per liver were followed for 16 days (Fig. 1). Viable counts in the spleens were similar to those in the liver. After both challenge doses the number of bacteria in normal mice rose steadily, reaching 10^3 per liver at day 5 and 10^6 at day 12. Immunized mice given 700 LD_{50} also had 10^3 viable bacteria at day 5 but only a small increase in numbers for the next 4 to 5 days. The counts then rose more sharply, but only reached control level at 16 days. In immunized animals given 100 LD_{50}, no viable bacteria were detect-

TABLE 1. *Survival of mice protected with 2 doses of S. typhimurium SF i.p. on days 0 and 20, and challenged with S. typhimurium s.c. on day 30*

Mouse strain	Challenge dose (LD_{50})	Survival after 30 days: no. of mice/total (%)
BALB/c	100	17/23 (74)
C57BL	100	8/10 (80)
C57BL	700	9/18 (50)

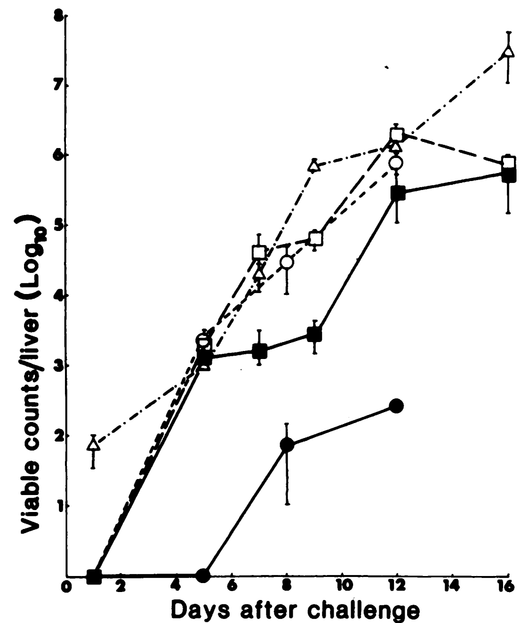

FIG. 1. *Viable counts in the livers of control and immunized C57BL mice challenged s.c. with S. typhimurium. Doses of SF or bovine serum albumin were given i.p. on days 0 and 20, and mice were challenged s.c. on day 30. 100-LD_{50} challenge: (○- - -○) unimmunized controls; (●——●) SF-immunized mice. 700-LD_{50} challenge: (□——□) unimmunized controls; (△-·-△) bovine serum albumin-immunized mice; (■——■) SF-immunized mice. Values are plotted ± standard error of the mean.*

able at 5 days. The numbers rose sharply to 10^2 at day 8 and more slowly thereafter. In mice given bovine serum albumin, the number of bacteria in the organs was similar to that in the controls.

Normal and immunized groups of BALB/c mice were challenged with 100 LD_{50} of *S. typhimurium*. The viable counts per liver are shown in Fig. 2. The bacterial counts in the unprotected mice rose more rapidly than in the C57BL strain and reached 10^6 per liver by day 8. Until day 8 the protected mice showed a 2-log difference in viable bacteria in the organs compared with controls, but the counts continued to increase, reaching levels of 10^8 by day 15. The infection was reduced by day 30, and the mice survived till at least day 50. In contrast, the controls died by day 12 with counts of 10^6.

Response to graded challenge doses. Figure 3 shows the protective effect of two doses of SF given to C57BL mice subsequently challenged with a range of doses of *S. typhimurium*. The SF protected mice against 100 LD_{50} but only led to a longer survival time after greater challenge doses. The degree of prolongation was similar whether 250, 500, or 5,000 LD_{50} were given.

Effect of variation of the protective doses. Groups of 10 BALB/c mice were given either 0.1 or 0.2 ml of SF on day 0, followed by 0.1, 0.2, or 0.3 ml on day 20. Challenge was on day 30 with a dose of 10^3 (100 LD_{50}) *S. typhimurium*. All control mice given only the challenge dose died with a mean survival time of 11 days, compared to 14 to 18 days for the nonsurvivors in the "immunized" groups, of which 10 to 20% survived.

Duration of delayed hypersensitivity reactions. The delayed hypersensitivity reactions in C57BL mice were also tested weekly for 7 weeks after receiving SF on days 0 and 20 (Fig. 4).

Although, as described previously, reactions were never high (22), the 24-h swelling was significant for 6 weeks and then declined. The 48-h swelling was only prominent in weeks 3 and 4.

LPS effects. SRBC coated with SF were not agglutinable with specific anti-*S. typhimurium* O serum.

Injections of SF either into the footpads or i.p. resulted in specific antibody for LPS shown by hemagglutination (Table 2).

SF inhibited slightly the hemagglutination of LPS-coated SRBC by anti-*S. typhimurium* O serum. The titer was reduced from 1,280 to 320 by extract at a concentration of 1 mg of protein per ml, compared with a similar inhibition by a concentration of LPS of 5 μg/ml. The SF therefore contained approximately not more than 5 μg of LPS per ml. SF diluted 1 in 200 was pyrogenic for rabbits, but that diluted 1 in 2,000 was not.

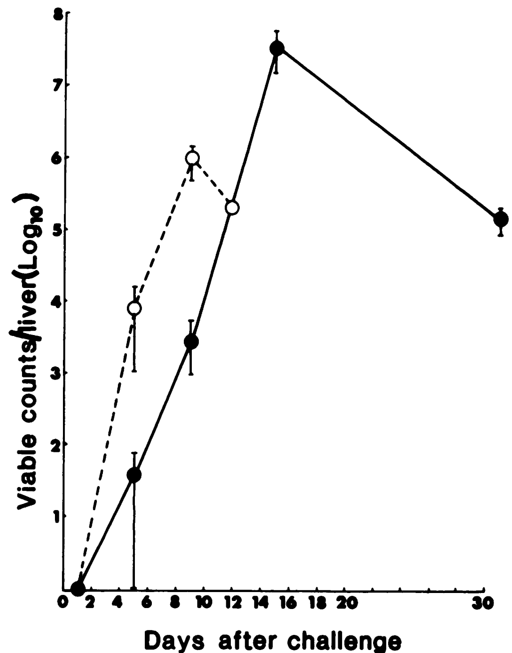

FIG. 2. *Viable counts in the livers of control and immunized BALB/c mice challenged with S. typhimurium s.c. 100-LD_{50} challenge:* (O- - -O) *controls;* (●——●) *SF-immunized mice. Values are plotted ± standard error of the mean.*

Protection by LPS. Two doses of 10 μg of *S. typhimurium*, phenol-water type, LPS given i.p. on days 0 and 20 gave no protection against a subsequent dose of 50 LD_{50} of *S. typhimurium* in C57BL mice, although their serum antibody titers were 640 before challenge.

The level of antibody in individual mice was not correlated with their survival. In similar experiments using Boivin type LPS and a challenge dose of 150 LD_{50}, antibody serum titers were 320 and there was no protection.

Delayed hypersensitivity reactions to LPS. No reactions were obtained after SF injection into the footpads of C57BL mice which had been sensitized with two doses of 10 μg of *S. typhimurium* LPS of either phenol-water or Boivin types.

WFI mice were sensitized with 10^3 *S. typhimurium* on day 0 and challenged in the footpads with LPS (Boivin or phenol-water) on day 8. A 25-μl dose of a solution of LPS in saline (50 μg/ml) was injected. Neither LPS type elicited a delayed hypersensitivity reaction in the sensitized mice.

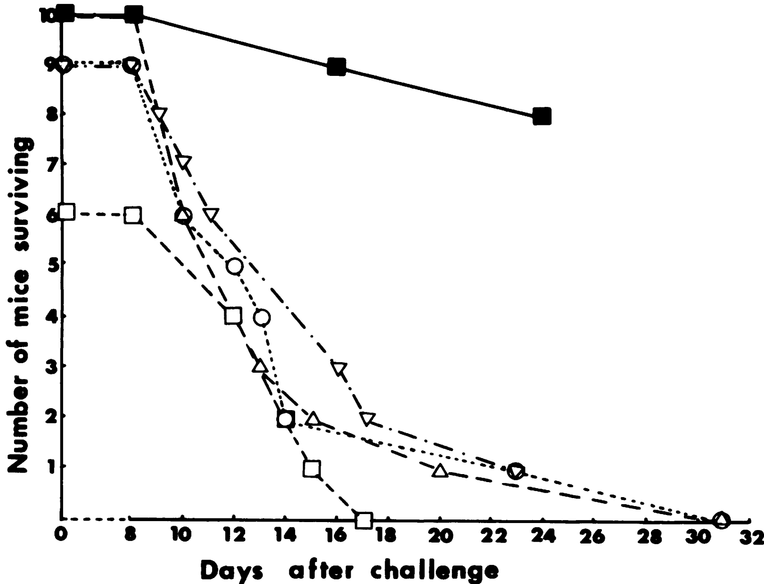

FIG. 3. *Survival of immunized C57BL mice challenged with different doses of S. typhimurium. Challenge doses were given on day 30 after two doses of SF on days 0 and 20. (□- - -□) Controls. Challenge doses: (■——■) 100 LD_{50}; (○· · · ○) 250 LD_{50}; (▽- · -▽) 500 LD_{50}; (△——△) 5,000 LD_{50}.*

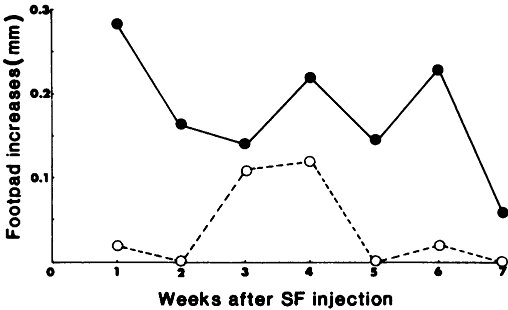

FIG. 4. *Delayed hypersensitivity reactions in C57BL mice after SF sensitization. Mice were sensitized with S. typhimurium SF on days 0 and 20. Values of footpad thickness increases are corrected for control footpads and corresponding increases in unsensitized mice. (●——●) 24 h; (○——○) 48 h.*

Attempted characterization of the immunogen. The SF treated in various ways was tested in infected C57BL and CBA mice for its activity in eliciting a delayed hypersensitivity reaction (Table 3).

C57BL mice normally gave a smaller reaction than CBA mice (22), but in both the extent of the reaction was reduced by Pronase or trypsin treatment of the SF. The activity of the SF was not impaired by ribonuclease treatment, nor by heat, which might have been expected to denature some of the protein content.

Pronase treatment reduced the concentration of protein in the SF by 50%.

TABLE 2. *LPS antibody titers in serum of SF-immunized C57BL mice*[a]

SF injections			Hemagglutinin titer	
Route	Dose (μl)		Day 14	Day 21
	Day 0	Day 14		
Footpad	50	50	20	320
Footpad	100	50	40	640
i.p.	200	200	20	2,560

[a] Day 14 results show the titers before, and day 21 shows those after, the second SF injection.

TABLE 3. *Effect of enzyme treatment of SF on delayed hypersensitivity reactions in CBA mice*

Treatment of SF	Foodpad increase (mm)[a]
None	0.31
Ribonuclease	0.35
Pronase	0.10
Trypsin	0.11
Heat	0.38

[a] Corrected increases in footpad thickness are given as an average of the reactions at 24 and 48 h.

The active fraction passed through an Amicon PM-30 membrane, so that its molecular weight is less than 30,000. The material proved difficult to concentrate or purify since there was a marked tendency for the protein to aggregate and precipitate out of solution.

Attempts to fractionate SF by ammonium sulfate precipitation gave inconsistent results, and column chromatography methods gave low recoveries of protein. Protein determinations by the Lowry method were probably low due to the low content of aromatic amino acids, as observed by absorbance at 280 nm. Table 4 shows a typical analysis of SF.

DISCUSSION

Both BALB/c (LD_{50}, <10 s.c.) and C57BL (LD_{50}, 2×10 s.c.) mice are highly susceptible to challenge with *S. typhimurium* and so perhaps are more difficult to immunize than CBA (LD_{50}, 10^7). It is arguable, however, that there is less room for improvement in the resistance of the latter since a dose of 700 LD_{50} would be entering the purely toxic zone. With two doses of SF, the increased resistance of both BALB/c and C57BL mice enabled 50 to 80% of them to survive a challenge dose of 100 LD_{50} given s.c. Significantly, the protective SF could induce delayed hypersensitivity, which was taken as a convenient indicator of cell-mediated immunity.

After challenge the immunized mice showed a delay in the appearance of salmonellae in the liver and spleen, presumably due to a lower net multiplication rate than in normal mice. Three weeks after challenge, when the control mice had died, the bacterial counts in the organs of immunized mice reached high levels, but the mice could overcome this infection to survive (Fig. 1, 2).

The slowing in the rate of bacterial increase in protected mice around day 6 coincided with the peak development of delayed-type hypersensitivity as shown in the curve for 48-h reactions (Fig. 4). A retardation of growth rate coincidental with the development of cell-mediated immunity was demonstrated by Collins and Mackaness (8). We have previously shown that delayed hypersensitivity to salmonella SF was greater in naturally resistant than in susceptible strains of inbred mice (22). Late deaths in immunized mice occurred at a time when delayed hypersensitivity was waning (Fig. 4).

Collins (7) has argued that antibody may protect mice by enabling them to survive until they can mount a cell-mediated immune response to the challenge infection. Mice which survive an infection are highly resistant to a second challenge. It is difficult therefore to see why infected mice that have survived for 4 weeks or more after challenge should lose their delayed hypersensitivity reactions and die. An intriguing possibility is that, with the immunizing regime used, stimulation of a clone of suppressor cells occurred which overtook and outweighed the immune cell response at around 5 weeks. Such suppressor, or rather regulatory, cells have been demonstrated in s.c. staphylococcal infection of mice. Moreover, in the staphylococcal model the cells suppressed the expression rather than the induction of delayed hypersensitivity. The time scale of course is quite different (10).

The results suggest that the active factor is a relatively heat-stable protein, or protein-hapten complex, with a molecular weight of less than 30,000. Traces of LPS were also present, and in view of the specificity of the protection by the SF a role for LPS cannot be completely eliminated. However, LPS alone was unable to produce either delayed-type hypersensitivity reactions or protection. In ribosomal preparations which give good resistance to systemic salmonellae infections, the precise role of the RNA and protein components is still disputed, but Hoops et al. (13) have shown that, to be effective, the preparation must contain traces of LPS. Johnson (14) further demonstrated the species specificity of protection by ribosomal preparations of *S. typhimurium*. However, Misfeldt and Johnson (21) could only protect the relatively resistant A and C3H strains but not the sensitive C57BL strain of mice. Pure protein fractions of *S. typhimurium* and *Salmonella paratyphi* B or C have been shown to give homologous and some heterologous protection (2).

Experiments are in progress to clarify the role of LPS in the SF. Better protection will depend on the development of more satisfactory separation and characterization of the active factor.

TABLE 4. *Composition of SF*

Component	%
Protein	35.5
Total lipid	0.38
Phosphorus	3.0
RNA	7.5
DNA	0.5
Total carbohydrate	5.8
2-Keto-3-deoxyoctonate	0.24

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