

The immunogenic activity of ribosomal fractions derived from *Brucella abortus*

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SUMMARY

The immunizing activity of ribosome preparations derived from *Brucella abortus* strain 19 cells was examined in guinea-pigs and mice. After subcutaneous injections of *Br. abortus* ribosomes in Freund's incomplete adjuvant, both mice and guinea-pigs developed immunity to challenge by virulent *Br. abortus* 544 organisms which was at least as effective as the protection conferred by live strain 19 vaccine. Both mice and guinea-pigs also developed agglutinating and complement-fixing antibodies and delayed hypersensitivity to *Br. abortus* antigens. Conversely, ribosome preparations elicited delayed hypersensitivity reactions on intracutaneous injection into guinea-pigs chronically infected with *Br. abortus* or *Br. melitensis*.

On injection into rabbits, *Br. abortus* ribosomes incorporated in incomplete adjuvant induced high titres of agglutinins, complement fixing antibodies and precipitins for *Br. abortus* antigens. On immunochemical examination, the ribosome preparations were not grossly contaminated with antigens derived from the cell surface. They were chemically complex, however, and in addition to RNA contained numerous protein components identified by disk electrophoresis. The nature of the components responsible for conferring protection against *Br. abortus* was not determined.

INTRODUCTION

After the demonstration by Youmans & Youmans (1965, 1966*a*, 1966*b*) that immunity to *Mycobacterium tuberculosis* could be stimulated by injection of mycobacterial ribosomes or ribosomal RNA, similar effects were described for ribosomal preparations derived from a variety of bacteria. Thus ribosomal fractions have been shown to stimulate protective immunity to *Neisseria meningitidis* (Thomas & Weiss, 1972), *Salmonella typhimurium* (Venneman & Bigley, 1969; Venneman, Bigley & Berry, 1970; Johnson, 1972, 1973), *Staphylococcus aureus* (Winston & Berry, 1970), *Streptococcus pneumoniae* (Thompson & Snyder, 1971) and *Yersinia pestis* (Johnson, 1972). Furthermore, for those organisms studied, the immunity induced by ribosomal vaccines appeared specific for the species from which the ribosomes were derived (Johnson, 1973).

On the basis of this evidence, it seems probable that protective immunity to a large number of bacterial pathogens could be produced by ribosomal vaccines. In

the present study, the effect of ribosome preparations in stimulating immunity to *Brucella abortus* has been examined.

MATERIALS AND METHODS

Brucella strains

The strains used were derived from the culture collection maintained at this laboratory. They included *Br. abortus* strain 19 (S19), the standard smooth, live vaccine strain; and two virulent strains, *Br. abortus* 544 (544) and *Br. melitensis* H38 (H38).

With the exception of S19, which was grown in continuous culture by the method of Boyce & Edgar (1966), strains for animal inoculation were grown on serum dextrose agar slopes incubated aerobically at 37° C. for 2–3 days. The 544 strain was grown in air enriched with 10% (v/v) CO₂.

Preparation of ribosomes

The method used was a modification of that of Venneman *et al.* (1970). Live *Br. abortus* S19 cells grown in continuous culture were washed by centrifugation in buffered saline (PBS: 0.15 mol./l. NaCl, 0.01 mol./l. phosphate buffer, pH 7.2) at 4° C. and resuspended in a medium containing 0.25% (w/v) sodium dodecyl sulphate (SDS), 0.03 mol./l. MgCl₂, 0.44 mol./l. sucrose, 2 µg./ml. deoxyribonuclease and 0.01 mol./l. phosphate buffer, pH 7.1 (SDS buffer). The suspension was rapidly frozen in an X-press (Biotec, Croydon) pre-cooled to –70° C. and the cells ruptured by 5 cycles of freeze-pressing at 22,000 lb./in.² and –25° C.

The pressed suspension was allowed to thaw at 4° C. and then diluted approximately tenfold in SDS buffer (without deoxyribonuclease but containing 0.004% (w/v) polyvinyl sulphate) and centrifuged at 35,000 g for 30 min. The supernatant was decanted and the pellet discarded. Ribosomes were sedimented from the supernatant by centrifugation at 105,000 g for 3 hr. The pellet was collected, rinsed with SDS buffer and then resuspended in this medium and the suspension clarified by centrifugation at 12,000 g for 30 min. The cycle of differential centrifugation at 105,000 g and 12,000 g in SDS buffer was then repeated. The final suspension of ribosomes in SDS buffer was sterilized by membrane filtration and, after removal of a sample for analysis, emulsified in pre-cooled Freund incomplete adjuvant. This was used for animal inoculation within 30 min. of preparation.

Assessment of protective immunity

Four-week-old albino mice of either sex, weighing *ca.* 25 g. were given two subcutaneous injections of 0.1 ml. of ribosome-adjuvant emulsion. Similar groups of mice received subcutaneous injections of 0.1 ml. vols. of S19 vaccine containing *ca.* 4 × 10⁶ or 4 × 10⁸ viable organisms. Control groups of mice were left uninoculated or else given subcutaneous injections of 0.1 ml. volumes of SDS buffer emulsified in Freund's incomplete adjuvant.

Four weeks after vaccination the mice were challenged by intramuscular injection of *ca.* 1 × 10⁶ viable 544 organisms. Two weeks later the mice were weighed, killed by exsanguination under ether anaesthesia and the spleens excised. Each

spleen was weighed individually and homogenized in 10 ml. vols. of Albimi brucella broth. Replicate volumes of spleen homogenate and serial dilutions of this were plated on serum dextrose agar and incubated for up to 7 days at 37° C. in the presence of 10 % (v/v) CO₂. Representative colonies of brucella morphology were characterized according to recommended procedures (Morgan & Gower, 1966).

A similar experiment was performed in guinea-pigs. Groups of weaned female albino guinea-pigs were given subcutaneous injections of 0.25 ml. volumes of S19 ribosomes in incomplete adjuvant or 0.1 ml. volumes of PBS containing *ca.* 5×10^9 viable S19 organisms. Control groups were left uninoculated or received injections of 0.25 ml. SDS-buffer in adjuvant. Five weeks later the animals were challenged by intramuscular injection of doses of 4.8×10^3 viable 544 organisms. Four weeks after challenge the guinea-pigs were weighed, exsanguinated and their spleens cultured as described for the mice.

Determination of antigenicity

S19 ribosomes emulsified in Freund incomplete adjuvant were injected into mice and guinea-pigs as described above. However, the animals were not challenged but some were exsanguinated 6 weeks after injection and their serum collected and examined for antibodies to *Br. abortus*. The remaining animals were tested for delayed hypersensitivity to brucella antigens using the acid-extracted antigen described by Ottosen & Plum (1949). In the case of the guinea-pigs, 0.1 ml. volumes of antigen were injected intradermally into the depilated left flanks. Control tests were done by injection of 0.15 ml. saline into the depilated right flanks. The skin sites were examined for signs of reaction at 30 min., 2 hr., 7 hr. and 24 hr. after injection. At 48 hr. after injection the animals were killed and the skin test sites excised, fixed in 10 % neutral buffered formalin and paraffin-embedded sections cut and stained with haematoxylin and eosin. The nature of the dermal reaction was then assessed according to the histological appearances.

Intradermal tests in mice were done by injecting 0.025 ml. vols. of antigen into the hind foot pads. The reactions at 24 hr. after injection were assessed histologically as described above for the guinea-pigs.

Intradermal tests with S19 ribosomes were done in guinea-pigs infected 10–12 weeks previously with 544 or H38. The procedures used were identical with those already described.

The antigenicity of S19 ribosomes for rabbits was assessed by injecting two volumes of 0.25 ml. of ribosome-adjuvant mixture by the subcutaneous route. Blood samples were collected before inoculation and at regular intervals after.

Serological tests

The serum agglutination test (SAT), complement fixation test (CFT), 2-mercaptoethanol agglutination test (2MET), Rose Bengal plate test (RBPT) and immunodiffusion test were done according to procedures described or referred to elsewhere (Corbel & Morris, 1974).

Chemical analysis

Concentrations of carbohydrate, protein and DNA were estimated as described by Corbel & Morris (1975). RNA was estimated by the orcinol reaction using yeast RNA as the standard. Lipid was estimated by the hydroxamic acid reaction (Snyder & Stephens, 1959) using glyceryl mono-oleate as the standard. Disk electrophoresis of acid-phenol soluble proteins was done according to Morris (1973). Ribosome preparations for analysis were dialysed against distilled water to remove SDS and sucrose and then dried *in vacuo* over P_2O_5 .

RESULTS

Properties of ribosomal preparations

Although no attempt was made to produce a highly purified ribosomal preparation, the results of chemical analysis showed the S19 ribosomes were not grossly contaminated with lipid, DNA or excessive quantities of carbohydrate. In each case, the concentration of these components did not exceed 5% of the total dry weight. Protein accounted for 26% and RNA for 43% of the ribosomal composition.

On diffusion of ribosomal preparations against antisera to S19 ribosomes or to disrupted *Br. abortus* cells, 1–2 precipitation lines were produced. The characteristic precipitation line formed by the lipopolysaccharide-protein agglutinin of smooth *Brucella* spp. was not produced by ribosome preparations however (Pl. 1, fig. 1).

Disk electrophoresis of acid-phenol extracts of ribosomes showed that they were complex and contained a number of protein components. Some of these were apparently identical with proteins present in extracts of live S19 organisms (Pl. 1, fig. 2).

Assessment of protective immunity

Examination of the numbers of *Br. abortus* organisms present in the spleens of mice 2 weeks after challenge showed considerable differences between the vaccinated animals and the unvaccinated controls (Table 1).

There was little difference between the spleen/body-weight ratios and the mean spleen counts of the mice vaccinated with S19 ribosomes and those vaccinated with doses of 10^6 or 10^8 live organisms. On the other hand the control groups, whether uninoculated or injected with SDS buffer in adjuvant, had consistently higher spleen/body-weight ratios and higher spleen counts than the immunized animals.

Considerable differences were observed in the titres of antibodies to *Br. abortus* produced by the various mouse groups. Thus the mice vaccinated with S19 ribosomes had reciprocal agglutinin titres ranging from 40 to 160. A single animal in this group had no detectable antibodies to *Br. abortus*. Most of these mice also gave low titre reactions in the CFT and the RBPT.

The mice vaccinated with live S19, irrespective of the dose, had reciprocal

Table 1. *The response of vaccinated and unvaccinated mice to challenge with 1×10^6 viable Br. abortus 544 organisms*

Group number	No. mice in group	Vaccination schedule	Mean spleen wt. body wt. \pm S.E.M.	Mean spleen count* and range of counts	Geometric mean titres in serological tests			
					SAT	2MET	RBPT	CFT
1	10	<i>Br. abortus</i> S19 4×10^6 viable cells s.c.	0.0098 \pm 0.0092	5.3×10^3 (130- 5.4×10^5)	1032	806.3	101.6	2155
2	10	<i>Br. abortus</i> S19 4×10^8 viable cells s.c.	0.0127 \pm 0.0089	3×10^3 (0- 2×10^4)	905.1	783.8	80.2	2530
3	14	<i>Br. abortus</i> S19 ribosomes in adjuvant s.c.	0.0071 \pm 0.0064	8×10^2 (0.4 $\times 10^3$)	21.21	20	2.245	4
4	10	Diluent in adjuvant s.c.	0.0111 \pm 0.0017	5.5×10^6 (8×10^5 - 1×10^7)	< 10	< 20	< 2	< 2
5	10	None	0.0164 \pm 0.0055	8.4×10^6 (1.2×10^6 - 2.6×10^7)	< 10	< 20	< 2	< 2

* Geometric mean number of *Br. abortus* organisms per 1 g. spleen tissue.

Table 2. *The response of vaccinated and unvaccinated guinea-pigs to challenge with 4.8×10^3 viable Br. abortus 544 organisms*

Guinea-pig number	Vaccination schedule	Spleen wt. Body wt.	Spleen count*	Reciprocal titres in serological tests				
				SAT	2MET	RBPT	CFT	
1	<i>Br. abortus</i> S19 5×10^9 viable cells s.c.	0.0046	0	40	20	8	400	
2		0.0031	0	40	40	8	200	
3		0.0050	0	80	40	16	400	
4		0.0082	220	40	40	8	400	
5		0.0033	0	20	10	4	200	
6		0.0034	0	20	20	4	200	
7	<i>Br. abortus</i> S19 ribosomes in adjuvant s.c.	0.0041	0	320	160	256	400	
8		0.0035	0	160	80	256	400	
9		0.0063	10	640	640	512	400	
10		0.0047	0	320	320	512	400	
11		0.0039	0	160	80	256	400	
12		0.0068	20	160	80	256	400	
13		Diluent in adjuvant s.c.	0.0064	2.3×10^3	320	320	256	400
14			0.0089	1.6×10^3	640	640	256	400
15			0.0112	580	1280	640	512	800
16			0.0076	3.5×10^3	640	320	256	400
17			0.0125	312	1280	640	512	400
18	0.0087		2.4×10^3	1280	640	512	400	

* Number of *Br. abortus* organisms per 1 g. spleen tissue.

Table 3. *The serological response of rabbits vaccinated with Br. abortus S19 ribosomes*

(Antigen: 500 µg. *Br. abortus* S19 ribosomes in adjuvant s.c.)

Animal number	No. days after vaccination	Reciprocal titres in serological tests			
		SAT	2MET	RBPT	CFT
RR1	0	< 10	< 20	< 2	< 2
RR2	0	< 10	< 20	< 2	< 2
RR1	14	1280	1280	64	800
RR2	14	640	640	64	800
RR1	28	5120	5120	2048	4000
RR2	28	5120	5120	2048	4000
RR1	56	2560	2560	1024	4000
RR2	56	2560	2560	1024	4000
RR1	92	2560	1280	1024	4000
RR2	92	2560	1280	1024	4000
RR1	124	320	320	128	4000
RR2	124	320	320	128	4000
RR1	200	320	320	64	1600
RR2	200	320	320	64	4000

agglutinin titres ranging from 320 to 2560. These sera also gave high titre reactions in the CFT and RBPT.

None of the challenged control animals which had remained unvaccinated or been injected with SDS-buffer in adjuvant had significant antibody titres at the time of death, although they had large numbers of virulent *Br. abortus* organisms in their spleens. It is probable that the number of organisms inoculated was insufficient to provoke an appreciable antibody response within the period of observation.

The results of the vaccination and challenge experiments in guinea-pigs (Table 2) were qualitatively similar to those in mice. Thus those guinea-pigs vaccinated with S19 ribosomes in adjuvant had spleen/body-weight ratios and spleen counts equal to or lower than those of the animals vaccinated with live organisms. In contrast, the control animals had high spleen counts of virulent *Br. abortus* organisms and their spleen/body-weight ratios were also consistent with infection.

The results of the serological tests on the guinea-pig sera were less clear-cut than those on the mice, probably because of the longer interval between challenge and bleeding of the guinea-pigs. Nevertheless, the agglutinin titres observed in the S19-vaccinated animals were all lower than those observed in the challenged, unvaccinated controls. There were, however, no appreciable differences between the agglutinin titres of animals vaccinated with S19 ribosomes and those vaccinated with the diluent-in-adjuvant control preparation.

Assessment of antigenicity

The antibody titres to *Br. abortus* observed in rabbits at various times after injection of S19 ribosomes in adjuvant are summarized in Table 3. They show that the ribosomes were highly antigenic in this species and induced significant titres of agglutinins and complement fixing antibodies to *Br. abortus*.

In the rabbit, guinea-pig and the mouse, predominantly reduction-stable agglutinins were produced indicating that ribosomes evoked mainly IgG antibodies. The rabbit antisera also produced several precipitation lines on diffusion against ultra-sonicates of *Br. abortus* cells. These included an intense line immediately adjacent to the antigen well, corresponding to the lipopolysaccharide-protein agglutinin of *Br. abortus*. With extracts of S19 ribosomes 1-2 precipitation lines distinct from the agglutinin line were produced.

In the rabbit, antibodies to *Br. abortus* persisted at appreciable levels for at least 6 months after injection of S19 ribosomes. The duration of the serological response in the other species was not determined.

Tests for delayed hypersensitivity

S19 ribosomes evoked typical delayed hypersensitivity reactions on intracutaneous injection into mice infected with *Br. abortus* or guinea-pigs infected with either *Br. abortus* or *Br. melitensis*. In mice the reactions were characterized histologically by extensive infiltration of the dermal and subcutaneous tissues by mononuclear cells of predominantly histiocytic type. Many lymphocytes were also present (Pl. 2, figs. 1 and 2). In the guinea-pig the response consisted of erythema and thickening at the test site 24 hr. after injection. The histological appearances were similar to those seen in the mouse foot-pad reactions and consisted of extensive infiltration of the dermal and subcutaneous tissues by mononuclear cells (Pl. 3, figs. 1 and 2). Injection of S19 ribosomes into uninfected guinea-pigs or mice provoked little visible reaction at 24 hr. Histologically, slight infiltration by polymorphonuclear leucocytes was visible.

Mice or guinea-pigs injected with S19 ribosomes in adjuvant developed typical delayed hypersensitivity reactions to *Br. abortus* antigen prepared according to Ottosen & Plum (1949). Histologically, the appearances were identical with those seen in brucella-infected animals (Pl. 4, figs. 1 and 2).

DISCUSSION

Numerous attempts have been made to protect animals against brucellosis by immunization with cell-free extracts of *Brucella* organisms (Paterson, Pirie & Stableforth, 1947; Markenson, Sulitzeanu & Olitzki, 1959; Smith, Keppie, Pearce & Witt, 1962; Rasooly, Olitzki & Sulitzeanu, 1966; Ellwood, Keppie & Smith, 1967). In these cases immunity was successfully induced in guinea-pigs and mice by antigens derived from the cell surface of the organism. All of these immunizing preparations also induced antibodies to *Brucella* spp. and some degree of delayed hypersensitivity to brucella antigens.

In the present study, immunity to challenge with virulent *Br. abortus* was induced by vaccination with internal components of the cells of an attenuated *Brucella* strain. Nevertheless it cannot be concluded from this that the protective antigen was necessarily a structural component of the ribosomes which formed the bulk of the material used for immunization.

Although chemical analysis failed to reveal gross contamination with the

lipopolysaccharide-protein O agglutigen of *Br. abortus*, both the method of preparation of the ribosomal fraction and the agglutinin response evoked by it were consistent with the presence of this antigenic component in the vaccine. However, the failure to detect agglutigen in immunodiffusion tests performed on the ribosomal fraction indicated that it could only have been present in low concentration. The concentrations which could have been present in the ribosomal vaccine were certainly lower than those needed to produce effective immunity in guinea-pigs and mice in other studies (Paterson *et al.* 1947; Ellwood *et al.* 1967). In view of this, the nature of the protective component in the ribosomal vaccine must remain speculative.

Similarly, the mechanisms responsible for the immunity elicited by the ribosomal preparation are equally obscure. Although evidence has been presented which suggests that immunity to brucellosis is largely a cell-mediated process (Mackaness, 1964) humoral factors may also play a role (Ralston & Elberg, 1971). As the ribosomal preparation induced formation of a variety of antibodies as well as delayed hypersensitivity to *Br. abortus*, its immunizing activity could have been attributable to either humoral or cell-mediated processes, or possibly a combination of both. The observation that protection was unrelated to antibody titre in the mice at least, suggested that cell-mediated processes may have been more significant. Cell-transfer studies on ribosome-stimulated immunity in other infections have shown that the process is cell-dependent (Venneman & Berry, 1971).

The fact that immunity to a wide variety of unrelated bacteria can be specifically produced by injection of their ribosomes suggests that the immunogenic activity is related to some general property of these structures. It is possible that the ribosomes might exert adjuvant activity by binding and stabilizing protective antigens released from other parts of the cell during the disruption process. It is also possible that protective antigenic components may be synthesized on the ribosomes before incorporation in other structures such as the cell wall. The concentration of such components on the ribosome might present them in a highly antigenic form.

Although the ribosomal preparations used in the present study were highly effective in immunizing guinea-pigs and mice against *Br. abortus*, no conclusions can be drawn about their immunogenic potential for other species. Experience with other cell-free *Brucella* antigen preparations has shown that the results obtained with these in cattle may not be consistent with their activity in guinea-pigs (Paterson *et al.* 1947; Sterne, Trim & Broughton, 1971). However, it would seem that the immunogenic activity of *Brucella* ribosomes in large animals would be worthy of examination.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Immunodiffusion of S19 ribosomes (S19R) and ultrasonically disrupted *Br. abortus* S19 (BA) against rabbit antisera to S19 ribosomes (aRR) and *Br. abortus* 544 live cells (a544). A number of precipitation lines are visible in the reaction between BA and aRR and a544. The dense precipitation arc (lps) immediately adjacent to the antigen well (BA) corresponds to the lipopolysaccharide-protein agglutinin of *Br. abortus*. In the reaction between S19R and the antisera, 1-2 precipitation lines are visible but none corresponds to the agglutinin.

Fig. 2. Disk electrophoresis of acetic acid-phenol extracts of (A) *Br. abortus* S19 whole cells and (B) ribosomes.

PLATE 2

Fig. 1. Section through left hind foot-pad of a mouse previously infected with *Br. abortus* 544. The sample was taken 24 hr. after local injection of 0.15 mol./l. NaCl. There is no evidence of a cellular reaction. H and E, $\times 250$.

Fig. 2. Section through the opposite hind foot-pad of the mouse examined in Fig. 1 (above). The sample was taken 24 hr. after local injection of S19 ribosomes. A typical delayed hypersensitivity reaction involving infiltrations of predominantly mononuclear cells is evident. H and E, $\times 250$.

PLATE 3

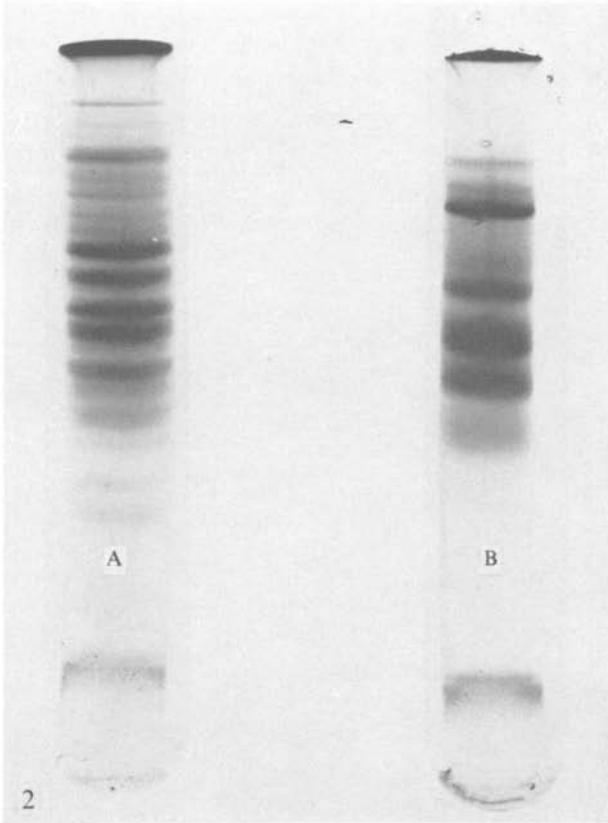
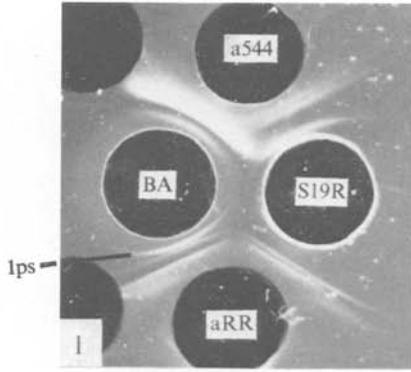
Fig. 1. Section through skin of a guinea-pig injected with *Br. abortus* 544. The sample was taken from the left flank 48 hr. after intradermal injection of 0.15 mol./l. saline. There is no evidence of cellular infiltration of the dermal or subcutaneous tissues. H and E, $\times 100$.

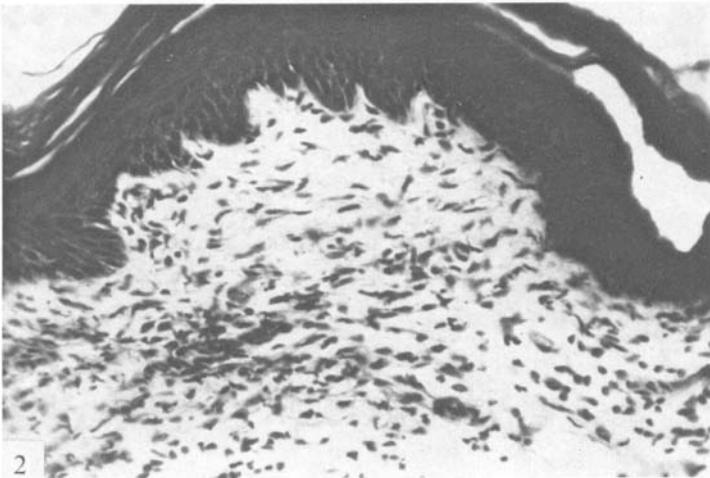
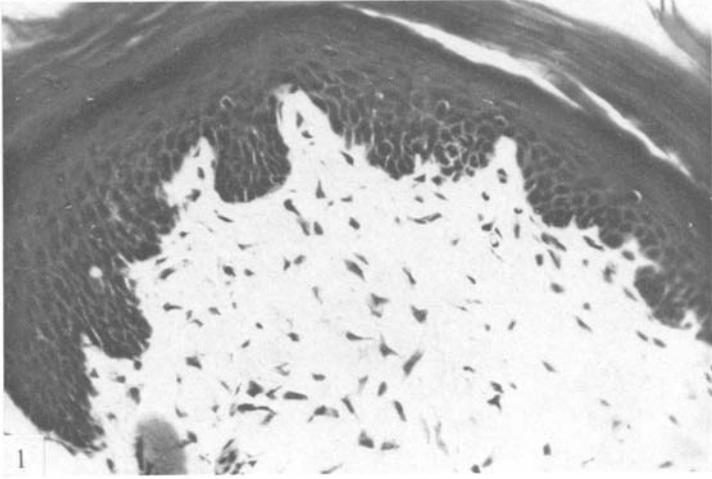
Fig. 2. Section through skin of the guinea-pig examined in Fig. 1 (above). This sample was taken from the right flank 48 hr. after intradermal injection of S19 ribosomes. The dermal and subcutaneous tissues are extensively infiltrated by mononuclear cells. H and E, $\times 100$.

PLATE 4

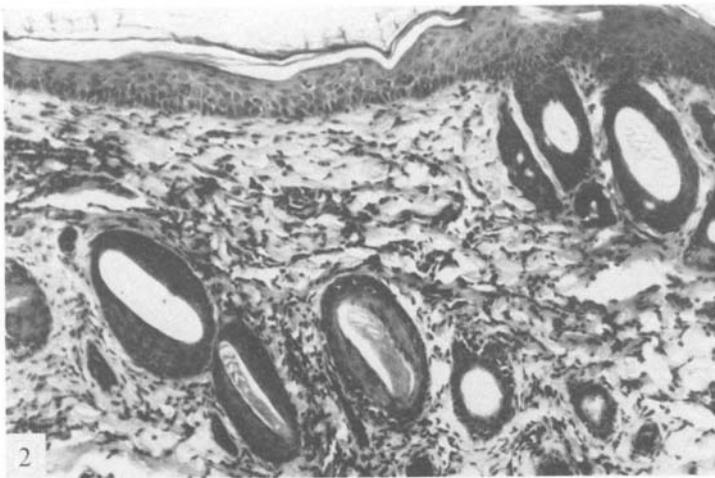
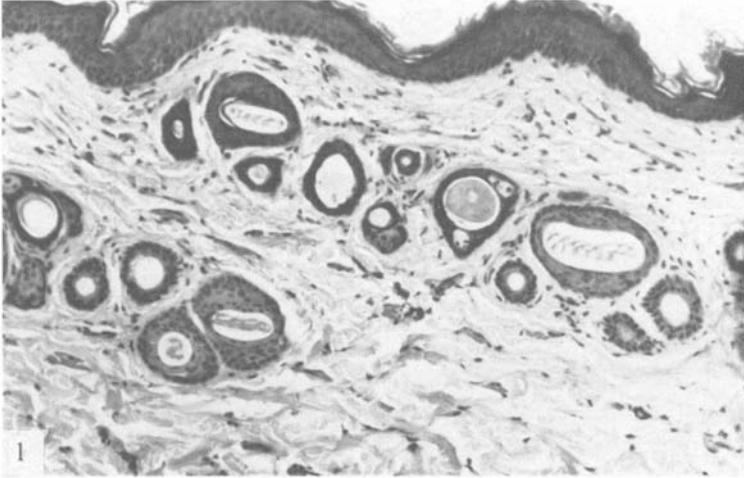
Fig. 1. Section through skin of a guinea-pig vaccinated with S19 ribosomes. The sample was taken from the left flank 48 hr. after intradermal injection of 0.15 mol./l. NaCl. There is no evidence of a cellular reaction. H and E, $\times 100$.

Fig. 2. Section through the skin of the opposite flank of the guinea-pig shown in Fig. 1 (above). The sample was taken 48 hr. after intradermal injection of *Br. abortus* antigen. An intense cellular reaction involving predominantly mononuclear cells is evident. H & E, $\times 100$.

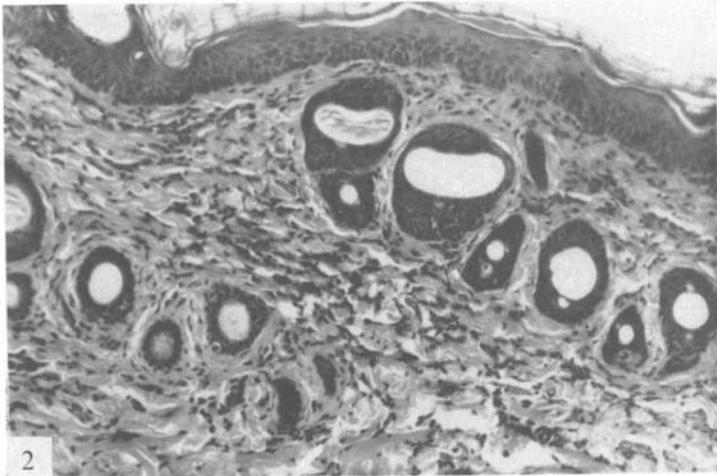




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