TISSUE CULTURE STUDIES ON BACTERIAL ALLERGY IN EXPERIMENTAL BRUCELLOSIS

I. THE EFFECT OF *BRUCELLA SUI*S WHOLE ANTIGEN ON CULTURES OF SPLEEN FROM NORMAL AND *BRUCELLA*-INFECTED GUINEA PIGS

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Tissue culture methods have proved valuable in studying the delayed type of allergic response to infectious disease.

After the appearance of the classic work of Rich and Lewis (1, 2) on the specific cytotoxic action of tuberculin, most reports have dealt with experimental and clinical tuberculosis. An exception is the work of Moen (3, 4) who reported that a specific cytotoxic effect could also be demonstrated in guinea pigs experimentally infected with group C hemolytic streptococci. Cells from infected animals showed an increased sensitivity to antigens prepared from different strains of such hemolytic streptococci, but not to antigens from group B strains or to extracts of alpha-type hemolytic streptococci. The toxic action of the antigen on sensitive cells was completely neutralized by an homologous antiserum in two experiments, and partially neutralized in four others.

Under proper experimental conditions, a cytotoxic response has been demonstrated to be more reliable than the intradermal test to indicate an infectious allergy of the tuberculin type. Cellular sensitivity has been demonstrated in the absence of a positive intradermal reaction in very young animals by Aronson (5) and in other anergic states by Heilman and Feldman (6). Furthermore, Aronson (7) and others have shown that tissue culture methods may be used to distinguish infectious allergy of the tuberculin type from anaphylactic states and the Arthus phenomenon.

Previous experience by one of us in studies on experimental tuberculosis had shown that cellular sensitivity to tuberculin could be demonstrated with regularity (8). The present report is part of a study on bacterial allergy in experimental brucellosis, a disease which is accompanied by a well marked and persistent allergic response of the delayed type. This study is concerned with the reaction in tissue culture of spleen from normal and *Brucella*-infected guinea pigs to different concentrations of a heat-killed suspension of *Brucella suis*.

Materials and Methods

Bacterial Culture.—*Br. suis* strain 364¹ was used to produce brucellosis in guinea pigs. The growth from a 72 hour culture on Albimi brucella agar was suspended in Albimi brucella broth. The density was adjusted to approximately 3×10^8 organisms per ml. using a MacFarland nephelometer.

Animals.—Adult male and female guinea pigs weighing from 300 to 400 gm. were inoculated subcutaneously in the right femoral region with 0.1 ml. of a broth suspension of *Br. suis* containing approximately 30 million cells. Previous studies indicated that *Br. suis* strain 364 usually produced observable infections in guinea pigs in the dose employed. Uninfected adult guinea pigs of both sexes provided a source of normal splenic tissue as well as blood for use in the preparation of tissue culture media. Blood from *Brucella*-infected guinea pigs was also used in the preparation of media for experiments on the effect of antibody on the cytotoxic reaction. Animals were fasted from 24 to 36 hours prior to obtaining blood. Cardiac puncture was carried out under ether anesthesia. At the time tissue was removed from an infected animal for *in vitro* studies, fragments of the spleen were cultured in a Castaneda flask (9) containing Albimi brucella agar and broth. Bacteriologic cultures were incubated at 36.5°C. for 21 days and examined for the growth of *Br. suis*. Agglutination tests were carried out on blood serum from each animal.

Antigen.—A smooth culture of *Br. suis*, strain 364, was used in the preparation of whole cell antigen employed in tissue culture studies. Growth from a 72 hour culture on Albimi brucella agar was suspended in sterile Tyrode's solution. The density was adjusted to approximately 1.2×10^9 bacteria per ml. by nephelometric methods. The unwashed suspension was heated at 70°C. for 45 minutes. Sterility tests were carried out and portions were sealed in glass ampoules and stored at -40°C. Intradermal tests were performed on normal and brucella-infected guinea pigs with different concentrations of the heat-killed antigen. Guinea pigs used for intradermal testing were not employed in cytotoxic studies.

Tissue Culture Methods.—With minor variations the tissue culture techniques and quantitative growth determinations were similar to those previously described (8). The quantitative tissue culture methods used in this study are adaptations of those described by Moen and Swift (10).

Media.—Fresh tissue culture medium was prepared for each experiment. Plasma was obtained by the addition of 0.015 ml. of heparin in 0.85 per cent NaCl solution (200 units per ml.) for each milliliter of blood. Serum-chick embryo extract was made by extracting 8 day chick embryos with guinea pig serum in the proportion of one embryo to 3 ml. of serum. Dihydrostreptomycin was added to culture media to yield a concentration of 100 μ g. per ml. to prevent the extracellular growth of *Br. suis* in tissue cultures of infected spleens. In order to reduce the amount of saline in the medium to a minimum, a 2 per cent solution of dihydrostreptomycin in Tyrode's solution was added to culture medium in a proportion of 1 to 200. At the time cultures were made, appropriate concentrations of antigen in Tyrode's solution were added to test cultures to comprise one-twentieth of the volume of the medium. A similar amount of Tyrode's solution was added to control cultures.

Explants.—The spleen of a normal or an infected guinea pig was used in comparative tests. Fragments measuring approximately 1.5 to 2 mm. in diameter were placed in two to four matched groups of 12 fragments each. One group of 12 fragments was used in control cultures and the remaining groups comprised the test series to which different concentrations of antigen were added.

¹ Obtained through the courtesy of Dr. W. W. Spink, Department of Medicine, University of Minnesota Medical School.

Tissue Cultures.—Cultures were placed in D5 Carrel flasks and consisted of 0.5 ml. of plasma, 1.0 ml. of tissue extract, and four explants of spleen placed in the medium before clotting occurred.

Quantitative Studies.—The extent of migration of small wandering cells was determined at 24 hours of incubation. The average radius of the migration zone was determined for each explant with an ocular micrometer at a magnification of 60 (62.5 ocular micrometer units equal 1 mm.). The average value was determined for each series of 12 explants and the variation within the series was expressed as the standard error of the mean. The migration of large wandering cells or macrophages was determined in a similar manner at 96 hours of incubation and was expressed in ocular micrometer units. Statistical analysis of the data and probability values were determined by the methods described by Treloar (11).

The toxicity of the antigen was determined by comparing the extent of cellular migration in test cultures with that observed in control cultures. The results were expressed in terms of a cytotoxic index in which a value less than 0.90 usually indicated toxicity. The relative effect of the antigen on normal tissue and on the tissue of infected animals was expressed in terms of a comparative cytotoxic index. A value less than 0.90 usually indicated specific toxicity of the antigen for the infected animal's tissue. The formulas used in these calculations are presented in the tables.

Histologic Methods.—Cultures of spleen for histologic examination were made on 22 mm. round coverslips by a method previously described (8). At different intervals during incubation representative cultures from each experimental series were fixed in Zenker-formol solution and sections were prepared by the method described by King (12). Sections were stained by modifications of the eosin-azure and Dominici techniques.

Agglutination Tests.—Serial twofold dilutions of guinea pig serum or tissue culture medium were made in 0.85 per cent NaCl solution in amounts of 0.5 ml. To each was added 0.5 ml of a suspension of *Br. suis* strain 364 in a solution of 0.85 per cent NaCl and 0.5 per cent phenol. The density of this suspension was adjusted to approximately 2.2×10^8 bacteria per ml. by nephelometric methods. Tests were also carried out with known positive and negative sera. Tubes were shaken, incubated in a water bath at 37°C. for 48 hours and observed for agglutination.

EXPERIMENTAL

Guinea Pigs Infected with *Br. suis*.—The experimental brucellosis produced with *Br. suis* strain 364 assumed different degrees of severity. Cachectic animals or those suffering from terminal stages of the disease were not used. The bacteriologic and serologic data on guinea pigs used as a source of tissue for *in vitro* studies are presented in Table I. The spleens of *Brucella*-infected animals were soft in consistency and were from two to several times larger than normal. Enlarged lymph nodes were observed in most of the animals examined during the first few weeks after inoculation. A swelling of one or more joints occurred in over half of the infected guinea pigs. A unilateral epididymitis was present in one instance. Detailed microscopic examination for evidence of brucellosis was not carried out. The existence of infection was proved in each instance by a positive agglutination test and/or recovery of *Br. suis* from the spleen.

Dermal Sensitivity to the Heat-Killed Antigen of *Br. suis*.—The intradermal inoculation of 0.1 ml. of a suspension containing 1.2×10^6 cells of *Br. suis*

caused reactions visible 48 hours later in *Brucella*-infected guinea pigs but not in normal guinea pigs. The areas of induration and erythema caused by this amount of antigen in sensitive guinea pigs had an average diameter of 1 cm.

Tissue Culture Studies.—1. Toxicity of the antigen for cultures of normal spleen:

Normal splenic tissue was grown in a medium prepared with the blood of normal guinea pigs. Each experiment consisted of a series of control cultures and one to three series containing different concentrations of antigen.

The results of individual experiments are presented in Table II. As was expected, a wide variation in the extent of cellular migration was observed in experiments carried out on different days. The variation within each experi-

TABLE I
Brucella-Infected Guinea Pigs Used in Tissue Culture Studies

Guinea pig No.	Experiment No.	Duration of infection	Culture of spleen for <i>Br. suis</i>	Reciprocal of agglutinin titer for <i>Br. suis</i>
		<i>days</i>		
1	7	32	Positive	Not done
2	8	46	Positive	10,240
3	9	62	Positive	1,280
4	10	116	Negative	10,240
5	11	86	Positive	10,240
6	12	125	Negative	10,240
7	13	76	Contaminated	1,280
8	14	122	Positive	2,560
9	15	84	Positive	1,280

mental group, however, was not great. Large wandering cells were more sensitive than leucocytes to the toxic effect of the antigen. A concentration of 2.4×10^6 bacteria per ml. resulted in a decrease of about 30 per cent in the migration of normal macrophages, with well marked degenerative changes observable in unstained cultures. A concentration of 1.2×10^6 bacteria per ml. caused a significant but moderate degree of inhibition. Half the amount of antigen (6×10^6 bacteria per ml.) caused a slight degree of inhibition in one of two experiments.

2. Toxicity of the antigen for cultures of spleen from Brucella-infected guinea pigs:

Explants of spleen from infected animals were grown in normal medium containing different concentrations of antigen, as well as in normal medium without the addition of antigen.

The results of growth measurements, presented in Table III, indicate that macrophages of *Brucella*-infected guinea pigs were also more sensitive than

leucocytes to the toxic effect of the antigen. In addition, the migration of macrophages from infected animals was inhibited to a greater degree than that of macrophages from normal animals by similar amounts of antigen. For example, a concentration of 2.4×10^6 cells of *Br. suis* per ml. did not restrict the migration of normal cells but decreased the migration of macrophages from infected guinea pigs by approximately 30 per cent. The results of the first two groups of experiments are summarized in Table IV in which the cytotoxic index

TABLE II

Effect of Different Concentrations of Heat-Killed Br. suis on Migration of Cells from Explants of Normal and Infected Guinea Pig Spleen

Values are average radius of the migration zone in ocular micrometer units.

Experiment	Antigen: bacteria per ml.	Leucocytes		Cytotoxic index	Macrophages		Cytotoxic index
		Control cultures	Test cultures		Control cultures	Test cultures	
1	1.2×10^7	$164 \pm 4^*$	$102 \pm 10^\dagger$	0.62§	175 ± 4	41 ± 3	0.23
	2.4×10^6		126 ± 5	0.77		118 ± 5	0.67
	1.2×10^6		145 ± 5	0.88		148 ± 4	0.85
2		165 ± 5			231 ± 5		
	4.8×10^5		162 ± 3	0.98		222 ± 5	0.96
	2.4×10^5		165 ± 6	1.00		216 ± 9	0.94
	1.2×10^5		163 ± 4	0.99		232 ± 5	1.00
3	4.8×10^5	194 ± 6	174 ± 5	0.90	247 ± 6	222 ± 7	0.90
4	6×10^5	223 ± 5	223 ± 8	1.00	332 ± 4	334 ± 6	1.01
5	1.2×10^6	86 ± 2	82 ± 1	0.95	180 ± 8	154 ± 6	0.86
6	6×10^5	125 ± 5	121 ± 3	0.97	167 ± 4	150 ± 3	0.90

* The value following the $\pm$ is the standard error of the mean.

† Values in italics show significant variation from controls.

§ Cytotoxic index = $\frac{\text{Average migration cultures} + \text{antigen}}{\text{Average migration controls}}$.

obtained in each group of experiments is arranged in order of decreasing concentrations of antigen. Although the effect of the same amount of antigen varied somewhat in different experiments, it was possible to observe concentrations that caused a minimal, moderate, and severe toxic effect on cells from normal or infected animals. Thus, under these experimental conditions, the toxicity of the whole antigen was roughly ten times as great for the tissue of infected animals as for tissue from normal animals. For convenience the term "*Brucella*-sensitive cells" will hereafter designate cells from guinea pigs with experimental brucellosis.

3. *Histologic studies:* A representative experiment in which stained sections were prepared from tissue cultures after different periods of incubation, is reported below.

TABLE III
Effect of Different Concentrations of Heat-Killed *Br. suis* on Migration of Cells
from Explants of Spleen of Guinea Pigs Infected with *Br. suis*
Values are average radius of the migration zone in ocular micrometer units.

Experiment	Antigen: bacteria per ml.	Leucocytes		Cytotoxic index	Macrophages		Cytotoxic index
		Control cultures	Test cultures		Control cultures	Test cultures	
7	6×10^4	$179 \pm 7^*$	$151 \pm 5^\dagger$	0.84§	193 ± 6	157 ± 5	0.81
	6×10^5		137 ± 5	0.77		136 ± 5	0.70
	6×10^6		79 ± 3	0.44		67 ± 5	0.35
8	1.2×10^5	155 ± 5	152 ± 5	0.98	259 ± 9	225 ± 8	0.87
	2.4×10^5		126 ± 5	0.81		174 ± 7	0.67
	4.8×10^5		116 ± 6	0.75		166 ± 8	0.64
9	4.8×10^5	165 ± 5	111 ± 6	0.67	260 ± 5	159 ± 5	0.61
10	1.2×10^6	133 ± 5	76 ± 5	0.57	167 ± 5	70 ± 4	0.42
11	1.2×10^6	152 ± 3	104 ± 3	0.68	320 ± 7	122 ± 4	0.38
	2.4×10^6		93 ± 3	0.61		95 ± 4	0.30
12	2.4×10^4	229 ± 6	233 ± 5	1.02	291 ± 5	295 ± 6	1.01
	6×10^4		228 ± 6	1.00		293 ± 6	1.01

* The value following the $\pm$ is the standard error of the mean.

† Values in italics show significant variation from controls.

§ Cytotoxic index = $\frac{\text{Average migration cultures} + \text{antigen}}{\text{Average migration controls}}$.

Fragments of spleen from a normal guinea pig and a guinea pig infected 3 months previously with *Br. suis* (guinea pig 6) were grown in a normal medium and in medium containing 1.2×10^6 heat-killed cells of *Br. suis* per ml. Cultures from each experimental group were fixed in Zenker-formol solution at 4, 8, 12, 24, and 48 hours of incubation. Sections stained with eosin-azure were used to observe cells in the explant whereas those stained by the Dominici technique were used for studying the morphology of cells in the migration zone.

The spleen of the infected guinea pig was soft and enlarged $2\frac{1}{2} \times$. Microscopic examination showed changes characteristic of brucellosis, namely,

hypertrophy of the white pulp, and a uniform increase in the number of reticulum cells in the pulp spaces. Granulomas were not seen, and areas of necrosis were not observed.

Histologic examination of tissue cultures of normal spleen in a medium free from *Brucella* antigen revealed the following sequence of events. At 4 hours of incubation polymorphonuclear leucocytes were present in the migration zone, and at 8 hours lymphocytes also appeared, comprising about 15 per cent of migrating cells. The proportion of lymphocytes to other migrating cells increased to 40 per cent at 12 hours at which time a few of the granulocytes had degener-

TABLE IV
Effects of Br. suis Antigen on Migration of Cells from Cultures of Spleen of Normal and Brucella-Infected Guinea Pigs

Concentration of antigen	Spleen of normal guinea pigs cytotoxic index		Spleen by <i>Brucella</i> -infected guinea pigs cytotoxic index	
	Leucocytes	Macrophages	Leucocytes	Macrophages
Bacteria/ml.				
1.2×10^7	<i>0.62*</i>	<i>0.23</i>		
6.0×10^6			<i>0.44</i>	<i>0.35</i>
2.4×10^6	<i>0.77</i>	<i>0.67</i>	<i>0.61</i>	<i>0.30</i>
1.2×10^6	<i>0.88</i>	<i>0.85</i>	<i>0.57</i>	<i>0.42</i>
1.2×10^6	<i>0.95</i>	<i>0.86</i>	<i>0.68</i>	<i>0.38</i>
6.0×10^5	<i>0.97</i>	<i>0.90</i>		
6.0×10^5	<i>1.00</i>	<i>1.01</i>	<i>0.77</i>	<i>0.70</i>
4.8×10^5	<i>0.98</i>	<i>0.96</i>	<i>0.75</i>	<i>0.64</i>
4.8×10^5	<i>0.90</i>	<i>0.90</i>	<i>0.67</i>	<i>0.61</i>
2.4×10^5	<i>1.00</i>	<i>0.94</i>	<i>0.81</i>	<i>0.67</i>
1.2×10^5	<i>0.99</i>	<i>1.00</i>	<i>0.98</i>	<i>0.87</i>
6.0×10^4			<i>0.84</i>	<i>0.81</i>
6.0×10^4			<i>1.00</i>	<i>1.01</i>
2.4×10^4			<i>1.02</i>	<i>1.01</i>

* Values in italics indicate a significant degree of inhibition of migration.

ated. Eosinophiles were occasionally seen in cultures of normal tissue but represented less than 1 per cent of cells in the migration zone. At 12 hours a definite thinning-out of cells in the explant had occurred. Most of the remaining leucocytes had degenerated, but the connective tissue elements in the explant were intact. At 24 hours a few macrophages were seen in the migration zone, and there was a slight increase in the number of degenerated granulocytes, although most of the leucocytes appeared normal. Within the explant there was an increase in connective tissue cells and in the number and size of large phagocytic cells, especially at the periphery. At 48 hours many macrophages appeared in the migration zone and dead cells and cellular debris had largely been removed from the area by the large phagocytic cells. A few degenerated leucocytes re-

mained in the outer migration zone. Fibroblasts were seen at the edge of the explant, but fibroblastic extension into the plasma clot was not observed (Fig. 1).

The appearance of cultures of normal tissue to which antigen was added was similar to that of control cultures. The granulocytes were somewhat more ameboid than in control cultures and macrophages appeared earlier and were somewhat more numerous. A few macrophages were present at 12 hours, and at 24 hours comprised 4 per cent of migrating cells. About the same number of degenerated leucocytes was observed in cultures containing antigen as in control cultures. At 48 hours a few of the macrophages in cultures containing antigen had degenerated but most of them appeared normal. Further proof that these cells were viable came from the observation that much of the cellular debris in the inner migration zone had been removed (Fig. 2).

During the first 12 hours of incubation cultures of *Brucella*-sensitive spleen without added antigen were similar in appearance to cultures of normal spleen. The only differences noted were a moderate increase in the number and size of migrating lymphocytes early in the incubation period, and a consistent increase in the number of eosinophiles (2 to 3 per cent). At 24 hours the proportion of dead leucocytes was greater than in cultures of normal tissue, and macrophages were much more numerous, comprising 12 per cent of migrating cells. At 48 hours many fibroblasts were growing into the plasma clot and intact macrophages were observed throughout the migration zone. Cellular debris was almost completely absent except for a few degenerated leucocytes at the periphery of the migration zone (Fig. 3).

The addition of antigen to tissue cultures of spleen from the infected animal caused profound cytologic changes. At 4 hours the migrating granulocytes were more ameboid than in control cultures and at 8 hours many had degenerated. At this time a few macrophages were seen near the explant. At 24 hours the degeneration of migrating leucocytes was almost complete, and there was an accumulation of cellular debris and erythrocytes throughout the migration zone. A few macrophages were present but most of them showed toxic changes such as loss of normal nuclear structure, an eccentric placement of the nucleus, and vacuolization of the cytoplasm. Connective tissue cells in the explant also showed abnormal nuclear structure and evidence of cytoplasmic degeneration. At 48 hours an almost complete degeneration of all cells had taken place, both in the explant and in the migration zone. Growth of fibroblasts into the medium was not observed. The presence of the heat-killed cells of *Br. suis* could not be detected in the sectioned material, either in the phagocytic cells or in the fibrin network. It was apparent, however, that the presence of antigen in the concentration employed had a profound toxic effect on cells of the sensitized animal. Similar experiments with splenic tissue from other *Brucella*-infected guinea pigs gave comparable results (Fig. 4).

4. The Effect of Antibodies on the Specific Cytotoxic Reaction:

It was difficult to prepare satisfactory tissue culture media from the blood of infected animals because of the increased concentration of lipides in many of the specimens. Even though cloudy sera were discarded, accumulations of lipoid material often developed on the surface of cultures causing degeneration of the cells. Because cultures were coded before measurements were made, experiments were accepted or rejected on the basis of the general

TABLE V
Effect of Antibodies on Specific Cytotoxic Activity of Heat-Killed Br. suis

Experiment	Antigen: bacteria per ml.	Normal medium		Medium containing antibodies	
		Normal spleen	Infected spleen	Normal spleen	Infected spleen
1. Migration of leukocytes. Values given are average radius of the migration zone at 24 hours in ocular micrometer units					
13	None	125 $\pm$ 5*	170 $\pm$ 3	112 $\pm$ 5	146 $\pm$ 3
	6 $\times$ 10 ⁵	121 $\pm$ 3	157 $\pm$ 5	107 $\pm$ 3	147 $\pm$ 5
14	None	86 $\pm$ 2	135 $\pm$ 3	94 $\pm$ 3	137 $\pm$ 4
	1.2 $\times$ 10 ⁶	82 $\pm$ 1	105 $\pm$ 3	88 $\pm$ 2	121 $\pm$ 3
15	None	109 $\pm$ 4	175 $\pm$ 5	127 $\pm$ 3	160 $\pm$ 2
	1.2 $\times$ 10 ⁶	91 $\pm$ 4	113 $\pm$ 4	117 $\pm$ 3	130 $\pm$ 4
2. Migration of macrophages. Values given are average radius of the migration zone at 96 hours in ocular micrometer units					
13	None	167 $\pm$ 4	247 $\pm$ 6	139 $\pm$ 5	217 $\pm$ 3
	6 $\times$ 10 ⁵	150 $\pm$ 3	164 $\pm$ 7	141 $\pm$ 5	164 $\pm$ 7
14	None	180 $\pm$ 8	234 $\pm$ 6	170 $\pm$ 4	220 $\pm$ 5
	1.2 $\times$ 10 ⁶	154 $\pm$ 6	120 $\pm$ 4	176 $\pm$ 5	156 $\pm$ 4
15	None	161 $\pm$ 6	325 $\pm$ 6	162 $\pm$ 3	271 $\pm$ 9
	1.2 $\times$ 10 ⁶	136 $\pm$ 4	153 $\pm$ 5	152 $\pm$ 4	197 $\pm$ 6

* The value following the $\pm$ is the standard error of the mean.

appearance of all the cultures. Three experiments were carried out in which normal spleen and that from an infected animal were grown in a normal medium and a medium prepared with blood from infected animals. Similar amounts of antigen were added to half of the normal medium and half of the medium containing antibodies. Two different concentrations of antigen were employed in separate experiments, one that had a toxic effect on cells of *Brucella*-infected guinea pigs, but not on normal cells (6×10^5 bacteria per ml.), and a second concentration that was definitely toxic for normal cells and markedly toxic for sensitive cells (1.2×10^6 bacteria per ml.). The first concentration was used to detect any specific toxic substance that might be present in the blood of infected animals. The second concentration was used to demonstrate antitoxic activity.

The results of measurements of cellular migration at 24 and 96 hours are presented in Table V. The relative toxicity of the antigen for normal and sensitive tissue in normal medium and medium containing antibodies is presented in Table VI. The agglutinin titers of tissue culture media containing *Brucella* antibodies were as follows: Experiment 13, 1-1280; Experiment 14, 1-5120; Experiment 15, 1-1280. In Experiment 13, agglutination tests were made on the supernatant fluid from cultures after 4 days of incubation. The presence of added *Brucella* antigen did not decrease the titer of the medium containing

TABLE VI
Effect of Antibodies on Specific Cytotoxic Action of Heat-Killed Br. suis

Experiment	Antigen: bacteria per ml.	Normal medium			Medium containing antibodies		
		Cytotoxic index		Relative cytotoxic index	Cytotoxic index		Relative cytotoxic index
		Normal spleen	Infected spleen		Normal spleen	Infected spleen	
<i>1. Toxicity of antigen for leucocytes of normal and Brucella-infected guinea pigs</i>							
13	6×10^5	0.97*	0.92	0.95‡	0.96	1.01	1.05
14	1.2×10^6	0.95	0.78§	0.82	0.94	0.88	0.94
15	1.2×10^6	0.84	0.64	0.76	0.92	0.81	0.88
<i>2. Toxicity of antigen for macrophages of normal and Brucella-infected guinea pigs</i>							
13	6×10^5	0.90	0.66	0.73	1.01	0.76	0.75
14	1.2×10^6	0.86	0.51	0.59	1.04	0.71	0.68
15	1.2×10^6	0.84	0.47	0.56	0.94	0.73	0.78

$$* \text{Cytotoxic index} = \frac{\text{Average migration cultures} + \text{antigen}}{\text{Average migration controls}}$$

§ Values in italics show significant variation from controls.

$$\dagger \text{Relative cytotoxic index} = \frac{\text{Cytotoxic index infected tissue}}{\text{Cytotoxic index normal tissue}}$$

antibodies. Similar tests were not carried out on incubated cultures in Experiments 14 and 15.

The variation in migration of normal and *Brucella*-sensitive cells in the two media was about the same as that observed in previous experiments with normal medium alone. Antigen in a concentration of 6×10^5 bacteria per ml. did not decrease the migration of normal or *Brucella*-sensitive leucocytes, but showed a definite toxic effect on macrophages which was slight for normal cells and marked for sensitive cells. The presence of antibodies in the medium decreased the toxicity of the antigen for both normal and *Brucella*-sensitive cells. When antigen in a concentration of 1.2×10^6 bacteria per ml. was employed the presence of antibodies gave partial protection to both types of cells but this appeared to be greater for cells from infected animals than for normal cells.

This was particularly evident in Experiment 15. The possibility existed that the apparent difference in antitoxic activity was influenced by the fact that the initial toxicity of the antigen was greater for *Brucella*-sensitive cells than for normal cells allowing a greater opportunity for protection. As a result, two experiments were carried out employing normal guinea pig spleen and high concentrations of *Brucella* antigen (4.8×10^6 -bacteria per ml.). Duplicate tests were performed in normal medium and medium containing antibodies. In both experiments the toxicity of the antigen was slightly less in the presence of antibodies than in normal medium, but the differences were not great enough to be significant, indicating that the protective action of immune serum for normal cells was inadequate in the presence of large amounts of antigen. Agglutination tests on the supernatant fluid from cultures after 4 days of incubation showed that the amount of antigen used in the last two experiments did not decrease the titer of agglutinating antibodies for *Brucella*.

DISCUSSION

It is of some interest to compare the relative cytotoxic effect of *Br. suis* with that of other bacterial products used in tissue culture studies.

A valid comparison of results obtained with different culture methods is difficult; however, general experience has shown that large amounts of old tuberculin (O.T.) are necessary to demonstrate a specific cytotoxic effect, and that the toxicity of O.T. for normal tissue is relatively great due to the presence of impurities. Tissue culture methods similar to those used in the present studies were employed by Heilman and Seibert (13) to observe the effect of purified fractions of tuberculin on tissue cultures of spleen from normal and tuberculous rabbits. The concentrations of purified protein derivative of tuberculin (P.P.D.) that gave a specific cytotoxic effect *in vitro* were several times greater than those used for intradermal testing. A concentration of P.P.D. that was toxic for normal cells, however, was more than six times as great as that required to elicit a similar degree of toxicity for sensitive cells. In experiments with extracts of group C hemolytic streptococci Moen found that a concentration of 1-6000 of the extract was toxic for sensitive cells, but only slightly toxic for normal cells (3). The amount of streptococcal antigen suitable for demonstrating dermal sensitivity in infected animals was 0.1 ml. of a 1-200 solution (3).

The tenfold difference in toxicity of *Br. suis* for normal and sensitive cells compares favorably with the results previously reported with P.P.D. (13). The selective toxicity of the whole *Brucella* antigen was also evident from the microscopic examination of stained cultures. The toxic changes observed were qualitatively similar to changes previously described as resulting from the action of tuberculin on cultures of tuberculin-sensitive tissue (8). The specific toxic action of *Br. suis*, however, was more striking than that of O.T. Concentrations of *Brucella* that caused early and extensive degeneration in sensitive cultures had little or no effect on normal cells. As in Moen's study with strepto-

coccal extracts, the toxicity of the whole *Brucella* antigen in tissue culture compared favorably with its activity in the intradermal test.

The specific cytotoxic action of tuberculin has not been shown to be decreased by the presence of serum and plasma from tuberculous animals (1) or by serum from rabbits inoculated with heat-killed tubercle bacilli in petrolatum in which precipitins for O.T. are present in high titer (14). In this connection the recent reports of Fong, Schneider, and Elberg are of particular interest (15, 16). They reported that serum from rabbits immunized with BCG protected immune monocytes (derived from rabbits immunized with BCG) but not normal monocytes from the degenerative effects of virulent tubercle bacilli. They also observed that immune monocytes were more susceptible to the effect of O.T. and P.P.D. than were normal cells, but immune serum had no effect on the cytotoxic action of these substances. The same investigators also observed a protective effect against the degenerative action of virulent tubercle bacilli on immune cells by heterologous serums (anti-*Salmonella* and anti-ovalbumin) as well as with homologous serums. This was observed only during the first 24 hours of the incubation period. On further incubation only the homologous antiserum protected immune monocytes against the destructive action of virulent tubercle bacilli.

In the present study homologous antiserum decreased the toxicity of *Br. suis* for normal cells as well as for *Brucella*-sensitive cells, although the protective effect was greater for *Brucella*-sensitive cells than for normal cells. The results of studies presented by Moen do not indicate whether immune serum had an effect on the toxicity of the streptococcal antigen for normal tissue (4). The protection of sensitive cells by immune serum in Moen's study appears to be similar in degree to that observed in the present study.

The experiments on the effect of antibody presented here are of a preliminary nature only and do not provide information on the specificity of the reaction. The observations on the toxicity of the whole bacterial cell will serve as a basis of comparison for similar studies on the effect of brucellergen and other fractions derived from *Brucella*.

SUMMARY

Tissue culture methods have been used to investigate infectious allergy in experimental brucellosis. A study was made of the effect of whole cell *Brucella* antigen on cultures of spleen from normal and *Brucella*-infected guinea pigs. The degree of toxicity was based upon the inhibition of migration of wandering cells and upon the morphologic appearance of stained sections of tissue cultures at different periods of incubation. A suspension of heat-killed *Br. suis* was more toxic for splenic cells from guinea pigs infected with *Br. suis* than for normal splenic cells. Macrophages were more sensitive than leucocytes to the toxic action of the antigen. The degenerative changes observed in *Brucella*-sensitive cells exposed to the antigen were similar to the degeneration previously observed in cultures of tuberculin-sensitive cells in the presence of tuberculin. The specific toxicity of the whole *Brucella* antigen, however, was more marked than

that of tuberculin. Preliminary experiments indicate that serum and plasma containing specific antibodies obtained from *Brucella*-infected guinea pigs reduce the toxic effect of the antigen in cultures of both normal and *Brucella*-sensitive cells. The protective action of the homologous antiserum was greater for *Brucella*-sensitive cells than for normal cells.

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EXPLANATION OF PLATE 32

Sections of tissue cultures of guinea pig spleen fixed in Zenker-formol solution after incubation 48 hours. Modification of the eosin-azure stain.

FIG. 1. Tissue culture of normal spleen showing an area of the explant. Small and large wandering cells appear in the migration zone. $\times 320$.

FIG. 2. Tissue culture of normal spleen in medium containing 1.2×10^6 heat-killed *Br. suis* per ml. The appearance is similar to that of control tissue cultures except that the macrophages appear more ameboid in cultures with *Brucella* antigen than in control cultures. $\times 320$.

FIG. 3. Tissue culture of *Brucella*-infected guinea pig spleen showing fibroblasts and wandering cells in the migration zone. $\times 320$.

FIG. 4. Tissue culture of *Brucella*-infected guinea pig spleen in medium containing 1.2×10^6 heat-killed *Br. suis* per ml. Destruction of cells in the explant and in the migration zone. $\times 320$.

(Heilman *et al.*: Bacterial allergy in brucellosis. I)