CYTOMETRIC ASSAY OF TOXICITY OF BRUCELLA ANTIGENS FOR SENSITIZED AND NON-SENSITIZED CELLS FROM THE GUINEA PIG*

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The specific cytotoxic effect of heat-killed *Brucella suis* and brucellergen on spleen fragment cultures from *Brucella*-infected guinea pigs has been described by Heilman, Howard, and Carpenter (1, 2). It was observed that macrophage migration from spleen explants obtained from sensitized hosts was significantly inhibited by exposure to the appropriate antigens. In addition, morphologic evidence of cell destruction was demonstrated. Brucellergen, prepared from all three species, was less toxic than the whole cell antigen, and no demonstrable differential effect of the individual antigens from the three species was noted.

Study of delayed type reactions using the plasma clot method is associated with significant limitations, such as lack of a continuous quantitative measurement of cellular death and the relatively small number of explants available from the individual animal. An alternative experimental system, using trypsinized spleen cells, makes available relatively large numbers of isolated macrophages for culture in tubes, thereby increasing the number of assays possible with cells from the individual test animal. It also provides a method of quantitation with less inherent variance and more direct correlation with cell survival.

This report is concerned with the application of this method to the assay of the cytotoxic effects of *Brucella* antigens on spleen cells of the sensitized and non-sensitized guinea pig.

Materials and Methods

Methods for the assay of cytotoxicity of *Brucella* antigens have been previously reported (1), and will be described only in instances of significant departures from the technique used in the previous study.

Bacteria.—Brucella suis (strain 364), *Brucella abortus* (strain 191, National Institutes of

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Health), and *Brucella melitensis* (strain 181) were used to produce experimental brucellosis in guinea pigs. Aliquots of the inoculum were prepared from a 72 hour growth of each species and subsequently suspended in 0.85 per cent sodium chloride solution. The concentration was then adjusted to approximately 2.4×10^9 bacteria per ml, using a McFarland nephelometer.

Guinea Pigs.—Twenty male guinea pigs weighing from 300 to 400 gm were used. Seven were used as uninoculated controls, and the remaining thirteen were divided into three groups. Six were injected with *Br. abortus*, four with *Br. suis*, and three with *Br. melitensis*. Each animal was inoculated intraperitoneally with approximately 1×10^6 *Brucella* cells of a single species (Table I). 6 weeks after inoculation tests for dermal sensitivity with 0.1 ml brucellergen (Merck, Sharpe, and Dohme) were performed. At autopsy, blood was obtained by aseptic

TABLE I

Immunologic Response in Brucella-Infected Guinea Pigs Used in Tissue Culture Studies

Guinea pig No.	Inoculated strain	Duration of infection	Dermal sensitivity (area erythema)	Culture of spleen for *Brucella*	Agglutinin titer
		days	*mm*		
597	*Br. abortus* (NIH 191)	67	15 × 20	positive	—
593	" "	82	20 × 16	positive	1:640
594	" "	125	5 × 6	positive	1:2560
598	" "	137	18 × 17	negative	negative
596	" "	147	16 × 16	positive	1:1280
653	" "	57	13 × 12	positive	1:10240
479	*Br. melitensis* (NIH 181)	360	positive	positive	1:2560
589	" "	216	14 × 16	positive	1:1280
648	" "	68	13 × 15	negative	1:2560
644	" "	115	28 × 28	positive	1:10240
602	*Br. suis* (NIH 364)	112	13 × 16	positive	1:2560
601	" "	119	13 × 15	positive	1:640
599	" "	188	15 × 15	positive	1:5120

cardiac puncture and cultured by Castaneda's method. Standard agglutination tests for *Brucella* infection were performed on the serum from each animal.

Antigens.—Three antigens were employed as follows: (*a*) a whole cell antigen from *Brucella suis*, (*b*) brucellergen (Merck, Sharpe, and Dohme), and (*c*) a lipopolysaccharide (Difco Laboratories) derived from *Br. abortus*. The whole cell antigen was prepared from cultures of *Br. suis* and killed by suspension in 0.67 per cent phenol for 1 month at 4°C. The cells were thoroughly washed in 0.85 per cent physiologic salt solution, lyophilized, and stored at 4°C. Stock antigen solutions were prepared in concentrations of 2.4×10^9 cells per ml.

Tissue Culture Methods.—The spleen was removed aseptically from the ether-anesthetized guinea pig. Using aseptic technique, the spleen was minced with scissors, washed 3 times in Hank's balanced salt solution (BSS), and transferred to a trypsinizing flask containing 250 ml of 0.2 per cent trypsin solution in phosphate-buffered saline (3). After trypsinization for 10 minutes by gentle rotation with a magnetic stirrer, the resultant cell suspension was centrifuged at 700 RPM for 30 minutes at 4°C. The supernatant solution was removed with a pipette and the packed cells resuspended and washed twice with warm BSS. The washed cells were then suspended in 3 ml BSS, and the concentration determined by counting in a hemocytometer (4).

The final tissue culture medium consisted of 30 per cent pooled homologous fresh serum from healthy guinea pigs, 59 per cent Hank's BSS, and 1 per cent antibiotic solution containing penicillin, dihydrostreptomycin, and neomycin, each in concentrations of 125 μg per ml, and bacitracin in a concentration of 1.25 units per ml.

The suspensions were then diluted to 5×10^6 cells per ml of tissue culture medium. The cell survival index (CSI) was computed by dividing the mean cell count of the culture tubes of the test set by the mean cell count of the control culture tubes.

Histologic Studies.—Coverslips in Leighton tubes were fixed in methanol and stained by Giemsa's and by hematoxylin and eosin methods.

TABLE II

Effect of Phenol-Killed Br. suis on Cultured Trypsinized Cells from Normal Guinea Pigs

Guinea pig No.	Cell count of control aliquot of spleen cells	Antigen:bacteria per ml.	Cell count of test aliquot of spleen cells	Cell survival index*
576	152 ± 43	1.2×10^5	183 ± 57	1.19
547	86 ± 9	1.2×10^6	63 ± 6	0.73
613	263 ± 34	1.2×10^6	220 ± 27	0.84
		1.2×10^7	206 ± 35	0.78
525	204 ± 43	1.2×10^7	149 ± 38	0.73
609	168 ± 21	1.2×10^6	174 ± 38	1.03
616	272 ± 41	1.2×10^7	215 ± 21	0.79
617	207 ± 14	1.2×10^5	201 ± 13	0.97
		1.2×10^6	194 ± 27	0.94
		1.2×10^7	173 ± 9	0.84

* Cell survival index = $\frac{\text{Average cell count of test culture}}{\text{Average cell count of control culture}}$.

RESULTS

Normal spleen yielded from 1 to 1.5×10^8 cells, and the infected spleens from 2 to 3×10^8 cells, the cell yield showing a correlation with spleen size. The usual distribution of cell types was 70–80 per cent small lymphoid cells, 15–25 per cent large mononuclear cells, and 5 per cent segmented leucocytes.

Macroscopic evidence was observed on the wall of the culture tube of a marked increase in cell numbers within 48 hours. Over 90 per cent of these cells were macrophages which rapidly phagocytized the disintegrating small lymphocytes, polymorphonuclear leucocytes, and eosinophiles. Fibroblasts constituted less than 5 per cent of the 48 hour cell population. When the tissue culture medium was regularly changed at 48 hour intervals the relative distribution of cell types showed no significant change until the 5th to 7th day, when overgrowth by fibroblasts became evident. A similar evolution of cell populations was observed in cultures of cells from both control and infected animals.

Cytologic Changes. In the presence of whole *Brucella* cells, lipopolysaccharide,

TABLE III

Effect of Phenol-Killed Br. suis on Trypsinized Spleen Cells from a Normal Guinea Pig (No. 613)

Phenol-killed *Br. suis* cells	Tube No.	Cell counts					Mean ± SD
0, (control)	16–1	292	292	270	302	318	263 ± 39
	16–2	250	168	280	266	364	
	16–3	260	290	262	260	180	
	16–4	218	252	242	258	250	
	16–5	250	266	250	246	290	
1.2×10^6	18–1	250	180	196	202	182	220 ± 26
	18–2	174	218	198	242	220	
	18–3	274	236	216	242	220	
	18–4	232	242	244	198	240	
	18–5	200	248	218	180	232	
1.2×10^7	17–1	170	216	170	220	218	206 ± 34
	17–2	196	214	164	216	228	
	17–3	236	216	234	238	256	
	17–4	150	180	238	210	188	
	17–5	142	160	218	194	290	

TABLE IV

Effect of Phenol-Killed Br. suis on Trypsinized Spleen Cells from an Infected Guinea Pig (No. 599)

Phenol-killed *Br. suis* cells	Tube No.	Cell counts					Mean ± SD
0, (control)	55–1	214	268	224	192	320	257 ± 40
	55–2	280	216	248	254	256	
	55–3	310	316	250	240	226	
	55–4	182	280	312	280	272	
	55–5	272	278	266	296	182	
1.2×10^6	57–1	58	53	51	28	8	32 ± 15
	57–2	32	21	12	21	21	
	57–3	26	71	48	32	31	
	57–4	17	25	31	16	25	
	57–5	36	45	35	28	40	
1.2×10^7	56–1	8	13	7	6	0	10 ± 9
	56–2	20	28	44	5	4	
	56–3	8	11	14	5	0	
	56–4	12	5	10	8	3	
	56–5	3	0	14	0	0	

TABLE V

Effect of Phenol-Killed Br. suis on Cultured Trypsinized Cells from Brucella-Infected Guinea Pigs

Guinea pig No.	Cell count of control aliquot of spleen cells	Antigen:bacteria per ml	Cell count of test aliquot of spleen cells	Cell survival index
597	139 ± 33	1.2×10^5	23 ± 2	0.17
		1.2×10^6	17 ± 7	0.12
593	165 ± 38	1.2×10^4	115 ± 26	0.70
		1.2×10^5	68 ± 17	0.41
		1.2×10^6	81 ± 4	0.49
594	363 ± 64	1.2×10^6	132 ± 8	0.36
598	202 ± 38	1.2×10^7	174 ± 36	0.85
596	417 ± 83	1.2×10^6	273 ± 25	0.66
		1.2×10^7	211 ± 21	0.51
653	273 ± 22	1.2×10^5	128 ± 23	0.47
		1.2×10^6	77 ± 25	0.28
		1.2×10^7	16 ± 7	0.06
479	139 ± 54	1.2×10^4	116 ± 37	0.85
		1.2×10^5	18 ± 8	0.13
589	249 ± 20	1.2×10^6	69 ± 12	0.28
		1.2×10^7	72 ± 4	0.29
648	128 ± 18	1.2×10^7	82 ± 24	0.64
644	155 ± 15	1.2×10^6	170 ± 30	1.10
		1.2×10^7	173 ± 7	1.12
602	100 ± 19	1.2×10^4	55 ± 7	0.51
		1.2×10^5	38 ± 9	0.35
		1.2×10^6	23 ± 4	0.22
		1.2×10^7	9 ± 4	0.09
599	257 ± 40	1.2×10^6	32 ± 15	0.12
		1.2×10^7	10 ± 9	0.04
601	84 ± 16	1.2×10^6	26 ± 7	0.31
		1.2×10^7	7 ± 3	0.08

and brucellergen, normal spleen cells from healthy animals showed no significant morphologic changes and appeared similar to those in the control tubes without antigen (Fig. 1) (Table II). When antigen was added to cells from infected animals, no change was evident for 6 hours. After 24 to 48 hours, however, over 80 per cent of the macrophages became detached from the cell wall and showed evidence of rapid cytolysis (Fig. 2). Intermediate reactions of the

TABLE VI

Interrelationship between Cell Survival Index and Brucella Strains, Agglutinin Titers, Duration of Infection, and Spleen Size of Infected Guinea Pigs

	Cell survival index (Antigen: phenol-killed *Br. suis* 1.2 × 10^6)			
	0 to 0.25	0.26 to 0.5	0.51 to 0.75	0.76 to 1.0
Inoculated Strain				
Br. abortus	1	3	1	1
Br. melitensis	1	1	1	1
Br. suis	2	1	0	0
Agglutinin titer				
0	0	0	0	1
1:640	0	2	0	0
1:2560	2	2	2	0
1:5120	1	1	0	1
Duration of infection				
Less than 90 days	1	2	1	0
91 to 150 days	1	2	1	2
Over 151 days	2	1	0	0
Size of spleen				
Normal size	0	0	2	2
Enlarged	0	4	0	0
Marked enlargement with perisplenitis	4	1	0	0

macrophages were characterized by swelling with loss of normal contour and the formation of large aggregates of cells (Fig. 3).

Cell cultures from three of thirteen infected guinea pigs showed only mild or moderate changes following exposure to whole cell *Br. suis* antigen (Table I). The animals from which these cell cultures were obtained had little or no clinical evidence of infection. No weight loss occurred, the agglutinin titers were low, and the spleens appeared normal.

Quantitative Studies. When manipulation of trypsinized cells was held to a minimum, the evolution of the cell populations showed relatively little variance within the same group of culture tubes.

The addition of $\frac{1}{10}$ volume 1.2 × 10^6 phenol-killed *Brucella* cells per ml to the culture tube containing trypsinized splenic cells from normal guinea pigs

was associated with a slight, but significant decrease in the number of viable cells (Table III). A 10-fold increase in antigen concentration, however, gave no evidence of significant additional cytotoxicity. The cell survival index (CSI) remained close to "1" in such assays.

Similar assays in cultures from infected animals were associated with a 10- to 20-fold increase in cytotoxicity and a very small CSI. In addition, a comparable 10-fold increase of antigen resulted in evidence of significant additional cytotoxicity (Table IV).

Considerable variation was observed in the sensitivity of tissue cell cultures from the individual infected animals to the cytotoxic effects of the same amount of antigen (Table V). Using an antigen concentration of 10^6 bacterial cells per ml, three animals had a CSI less than 0.25, five between 0.26 and 0.50, and two greater than 0.50.

No significant relationship among the three species of *Brucella* antigen fractions, duration of infection, agglutinin titer, and sensitivity of the culture to cytotoxic effects of the antigen was apparent. Although the number of animals observed was small, tissue cell cultures from guinea pigs with marked splenic enlargement exhibited the greatest degree of sensitivity to the cytotoxic effects of the antigens (Table VI).

DISCUSSION

The use of trypsinized cells as an alternative quantitative tissue culture technique to measure the cytotoxic effects of *Brucella* antigens for cells grown from hosts previously sensitized to *Brucella* infection has been demonstrated to be a satisfactory procedure. It provides data equivalent to the previously described use of splenic explants (1, 2). The relative simplicity and sensitivity of the trypsinized cell culture technique suggest its potential value as an experimental tool in the assay of antigens as well as the study of tissue immunity.

Similar techniques have been used for the study of the host-parasite relationship in infectious diseases (5, 6). One method used cells from peritoneal exudates from animals previously sensitized with *M. tuberculosis* or with the BCG strain of the organism. Exclusive of an initial decrease in cell number following addition of antigen in the above study, no cytotoxic effects on either sensitized or normal macrophages could be demonstrated after 72 to 96 hours of incubation. It has also been shown that immune monocytes have the ability to suppress the intracellular growth of *Brucella in vitro*, whereas the organism proliferates in cultures of normal monocytes under similar conditions, (6–10).

The merits of an *in vitro* experimental system for quantitative assay of the allergenicity of various antigens and their subfractions are evident. The continuous elaboration of antigen from microorganisms multiplying *in vivo* provides a more "physiologic" stimulus for the immunization or sensitization of the host cells than does the injection of non-viable and possibly modified material

grown outside the host. Possible differences in manifestations of cellular sensitivity induced by infection with live bacteria and immunization by killed cultures and their biochemical fractions are currently under study.

Aside from the practical value of this technique in the assay of the potency of antigens and their fractions, this experimental system may also be of use in study of cytopathic changes due to the cytotoxic effects of an antigen on the sensitized cell. Preliminary studies with the electron microscope have shown reproducible morphologic alterations of subcellular structures which are related to the amount of antigen required for production of cytotoxic effects and which differ from the cytotoxic effects produced by larger doses of antigen on the non-sensitized cell.

SUMMARY

1. A significant cytotoxic effect of trypsinized, sensitized cell cultures was observed when re-exposed to *Brucella* antigens *in vitro*.

2. The cytometric assay of the toxic effects of *Brucella* antigens using trypsinized cells from sensitized animals provides an objective measure of induced cellular hypersensitivity.

We are grateful to Difco Laboratories for supplying the *Brucella* lipopolysaccharide, to Mr. Timothy Dodge, Medical Illustrations Department, Veterans Administration Hospital, Long Beach, California, who provided the illustrations, and to Dr. David Imagawa, Departments of Pediatrics and Infectious Diseases, School of Medicine, University of California, Los Angeles, for technical advice on tissue culture methods.

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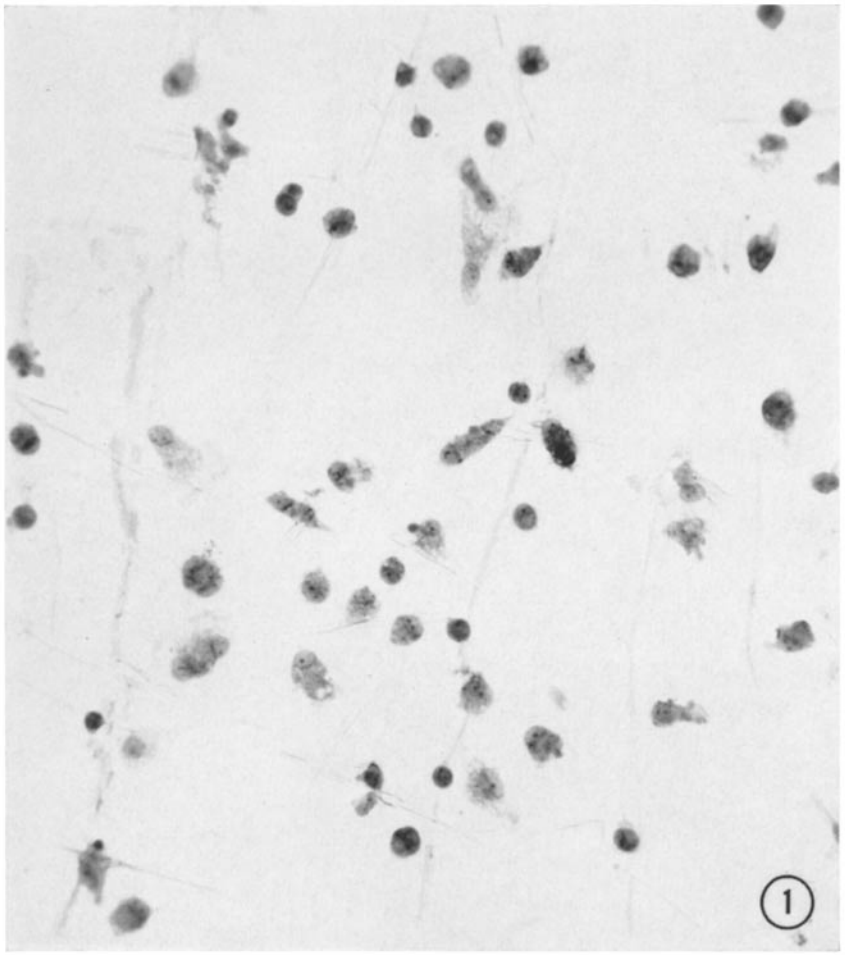
EXPLANATION OF PLATES

PLATE 57

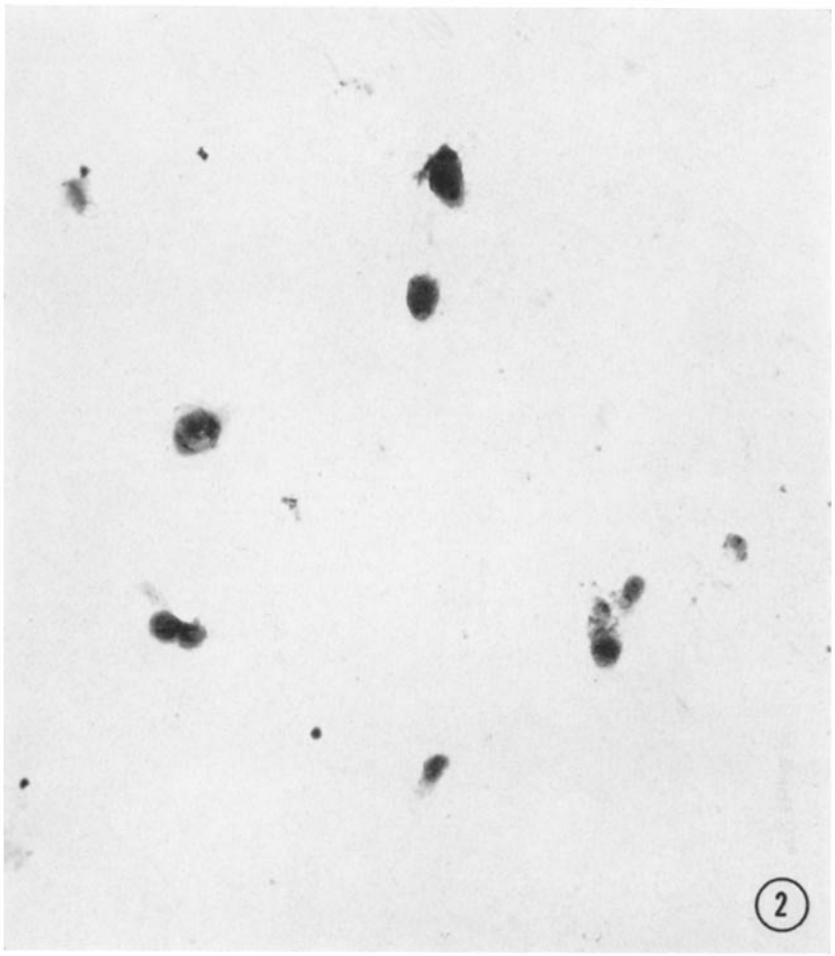
FIG. 1. Normal guinea pig spleen cells in tissue culture. × 350.



(Carpenter *et al.*: *Brucella* antigens)

PLATE 58

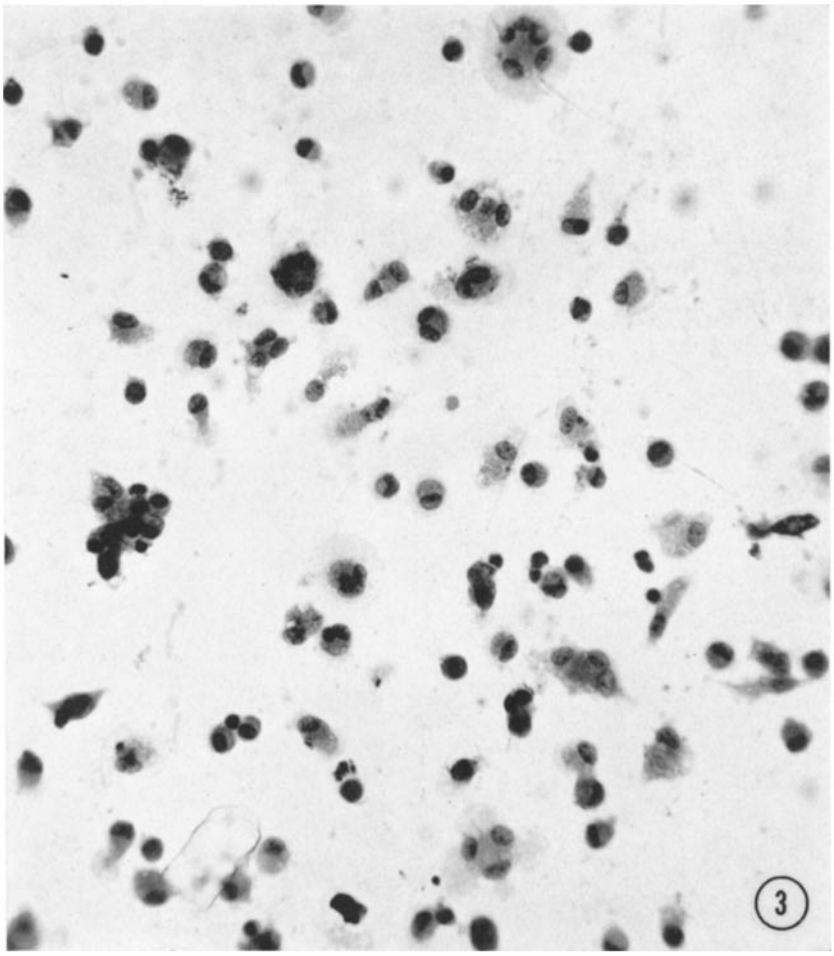
FIG. 2. Effect of brucellergen on sensitized guinea pig spleen cells, showing cytolysis and decrease in cell count. × 350.



(Carpenter *et al.*: *Brucella* antigens)

PLATE 59

FIG. 3. Intermediate reaction of sensitized spleen cell culture to *Brucella* antigens, showing loss of cell contour and agglutination. × 350.



(Carpenter *et al.*: *Brucella* antigens)