PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE, 146 635-642 (1974)

In Vitro Phagocytosis of Transitional Phase Bacterial Variants Utilizing Autoradiography[1] (38162)

GERALD DOMINGUE, KEITH LLOYD,[2] AND JORGEN U. SCHLEGEL

Department of Surgery, Section of Urology and Department of Microbiology and Immunology, Tulane University School of Medicine, 1430 Tulane Avenue, New Orleans, Louisiana 70112

There is a need for experimental studies to determine the exact role of cell wall deficient variants in the pathogenesis of various diseases. The recovery of variants from clinical specimens is not totally sufficient evidence to implicate them in infectious processes, particularly when isolation is coupled with the concomitant presence of antimicrobials which bring about inhibition of cell wall synthesis and their inducement into aberrant forms. Whether organisms persist as cell wall deficient variants *per se* and account for persistent, progressive infection either through reversion to classical parent organisms or through persistence in the form of stable nonreverting variants are fundamental and unsolved issues. Since phagocytosis is a basic host defense mechanism, whether variants (stable and unstable) are phagocytized and possibly survive engulfment warrants extensive exploration. Although phagocytosis has been well investigated with ordinary bacteria, there have been few studies with bacterial variants (1, 2).

Developing a technique of surface phagocytosis, by employing autoradiographic localization of tritiated thymidine (H^3) labeled transitional phase variants (3), we were able to compare the *in vitro* phagocytosis of these organisms with the classical bacteria from which they were derived in the presence and absence of specific opsonins.

Materials and Methods. Media and reagents. L-form broth (LFB) was prepared according to the method of Gutman *et al.* (4) with 10% horse serum (Grand Island Biological Co., Grand Island, New York), heat inactivated at 56° for 30 min. Hanks' Balanced Salt Solution (HBSS) was obtained in standard strength from Grand Island Biological Co., Grand Island, New York. HBSS with 0.1 *M* sucrose was prepared by adding 34.2 g sucrose per liter of HBSS and sterilized by passage through a 0.22 Millipore filter. Caseinate was prepared by dissolving 12 g casein (Nutritional Biochemical Co., Cleveland, Ohio) per 100 ml distilled water at 100° and adjusting the pH to 7.4 with NaOH. This solution was sterilized by autoclaving for 30 min at 120° and stored at 4° in sterile test tubes. Thymidine (methyl-H^3) was supplied by Amersham-Searle of Arlington Heights, Illinois. Specific activity was 114 mCi/mg of thymidine. Serum was obtained by cardiac puncture of anesthetized rats. The blood was allowed to clot at room temperature for 1 hr and refrigerated 4–5 hr for clot retraction. Serum was separated, pooled, and stored at −20°. Specific antisera for opsonization were prepared by injecting classical heat-killed *E. coli* and whole and Waring Blendor disrupted variants into New Zealand white rabbits as follows: 0.2 ml on alternate days iv for three injections followed by 1 ml on day 8. Three days after the last injection maximum agglutination titers of 1:256 were achieved. These antisera were used for the opsonic tests.

Phagocytic slide chambers. A slide chamber was devised for use in phagocytic experiments by affixing plastic cylinders, measuring 14 mm in i. d. and 13–15 mm in height, to glass slides with Permount adhesive (Scientific

[1] This investigation was supported by a Grant from the Veterans Administration Hospital, New Orleans, Louisiana.

[2] Research trainee in Dr. Domingue's laboratory USPHS Training Grant TO 1 AMO 5607.

Copyright © 1974 by the Society for Experimental Biology and Medicine Printed in U.S.A.
All rights reserved.

Products) and allowed to dry 24 hr prior to use in phagocytic experiments. The slides were stored in large plastic Petri dishes and gas sterilized.

Following the phagocytic experiment, the adhesive is easily removed from the plastic cylinders with toluene. The glass slides were cleaned by removing excess dried adhesive with a no. 11 scalpel blade and rinsed in methanol.

Preparation of labeled variants and bacteria. Variants. 24 hour cell wall deficient variants were used in all experiments. Four to five colonies of a stock culture of *E. coli* 04 growing on blood agar were transferred to 10 ml of LFB containing tritiated thymidine, 10 μCi/ml, and incubated at 37° for 24 hr. The resultant 24-hr variants were centrifuged at 77*g* for 20 min, separated, and resuspended in HBSS with 0.1 *M* sucrose × 2. The labeled variants were counted in a hemacytometer chamber under the phase microscope and diluted to 2.5×10^7 or 5×10^7/ml for phagocytic experiments. At the time of final dilution, the appropriate serum was added to a 10% concentration and 100 units/ml of penicillin for staiblization.

Escherichia coli 04. Four to five colonies of the stock culture of *E. coli* 04 were transferred to 10 ml of LFB and incubated 18–20 hr at 37°. 0.2 ml of this broth culture was transferred to 10 ml of fresh LFB with thymidine (methyl-H^3), 10 μCi/ml. This culture was incubated at 37° for 2.5–3 hr, centrifuged at 2820*g* for 15 min at 0°, and resuspended in HBSS with 0.1 *M* sucrose × 2. An aliquot was diluted, stained in solution with methylene blue, and counted in a hemacytometer counting chamber. The bacteria were diluted to a final concentration of 2.5×10^7 bacteria/ml with 10% horse serum added for the phagocytic experiments.

Polymorphonuclear leukocytes (PMNs). Rat peritoneal pmns used in all experiments, were obtained as follows: 15 ml of 12% caseinate solution were injected ip into 250 g Sprague–Dawley rats. 16–20 hr later, the rats were anesthetized and 20 ml of HBSS containing 10 units/ml heparin was injected ip. The abdomen was massaged gently, opened aseptically, and the resultant peritoneal exudate pipetted into sterile tubes. The cells were centrifuged at 200*g* for 5 min and resuspended in HBSS × 2. An aliquot of cells was diluted with 0.1 *N* HCl for counting in a standard hemacytometer chamber. After counting, the cells were adjusted to 5×10^6 cells/ml for the phagocytic experiments. Viability was assessed by the trypan blue dye exclusion test and was always found to be in excess of 95%.

Cellular monolayers were prepared by adding 1.0 ml of leukocytes (5×10^6 cells/ml) to the slide chambers and incubated for 1 hr at 37°. After incubation, the supernate was removed and the resultant monolayers washed gently × 3 with chilled HBSS. Control slides were stained with Giemsa and counts consistently showed 85–90 polymorphonuclear leukocytes with the remainder of cells being either large monocytes or lymphocytes.

Phagocytosis. After washing the cellular monolayers in the slide chambers, a 1.0 ml aliquot of the variant or classical bacterial suspension was added to the monolayers and incubated at 37°. Phagocytosis was measured in the presence of 10% pooled rat serum or specific antisera to *E. coli* 04 variants or clasical organisms. Penicillin (100 units/ml) was added to prevent reversion to parent bacteria during the course of the experiment.

Aliquots were also removed at each time interval from all surfaces and examined under the phase microscope for the presence of variants. Cultures on biphasic media were also performed. Slides were removed at 0, 30, 60, and 180 min, washed gently with chilled HBSS, air dried, and fixed in absolute methanol for 5 min. Control slides with variant suspensions were allowed to dry after removing the supernate without washing to ascertain that labeled variants were indeed present in the test suspensions.

After autoradiographic coating, developing, and staining with Giemsa, the slides were examined for phagocytosis. Three hundred leukocytes were counted in each slide and the number with ingested organisms noted. After observing background counts and control slides, it was decided that an accumulation of four or more silver granules overlaying a polymorphonuclear leukocyte was indicative of phagocytosis. Applying this criterion to multiple control slides, false positives of only 0–1 cell per 300 polymorphonuclear leukocytes counted were seen. The phagocytic index is defined as the percentage of polymorphonu-

clear leukocytes containing ingested organisms.

Autoradiography. After careful cleaning of slides with absolute methanol, an autoradiographic emulsion was applied. Slides were coated with Kodak NTB-2 nuclear emulsion by a hand-dipping technique and allowed to dry for 45 min vertically in an atmosphere of 70–75° and 50–60% relative humidity.

After drying, the slides were placed in black plastic slide boxes with 10 g of Drierite (W. A.

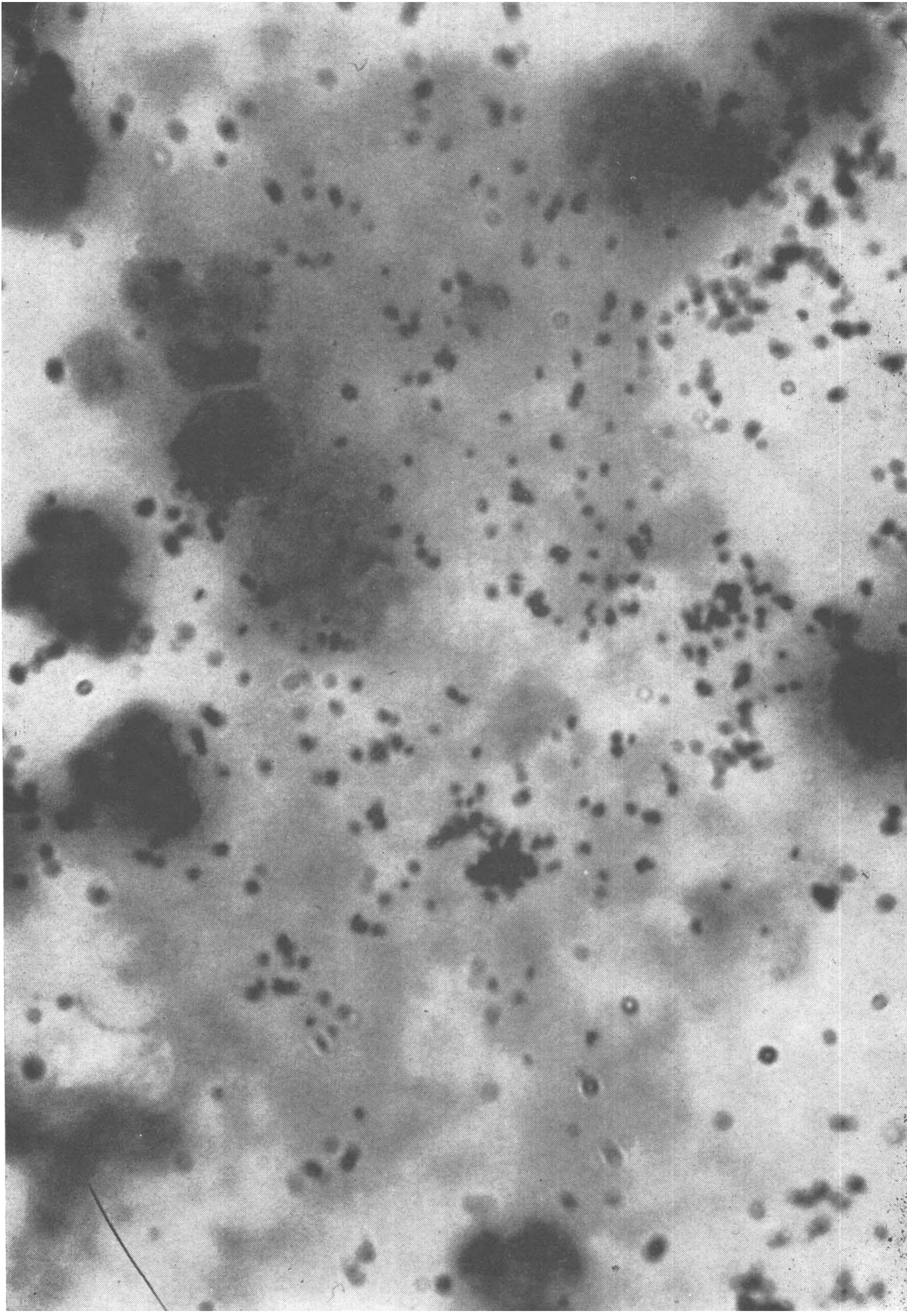

FIG. 1. Autoradiographic phagocytic mixture of variants and leukocytes prior to washing. (Note presence of extracellular stained organisms.) × 1000.

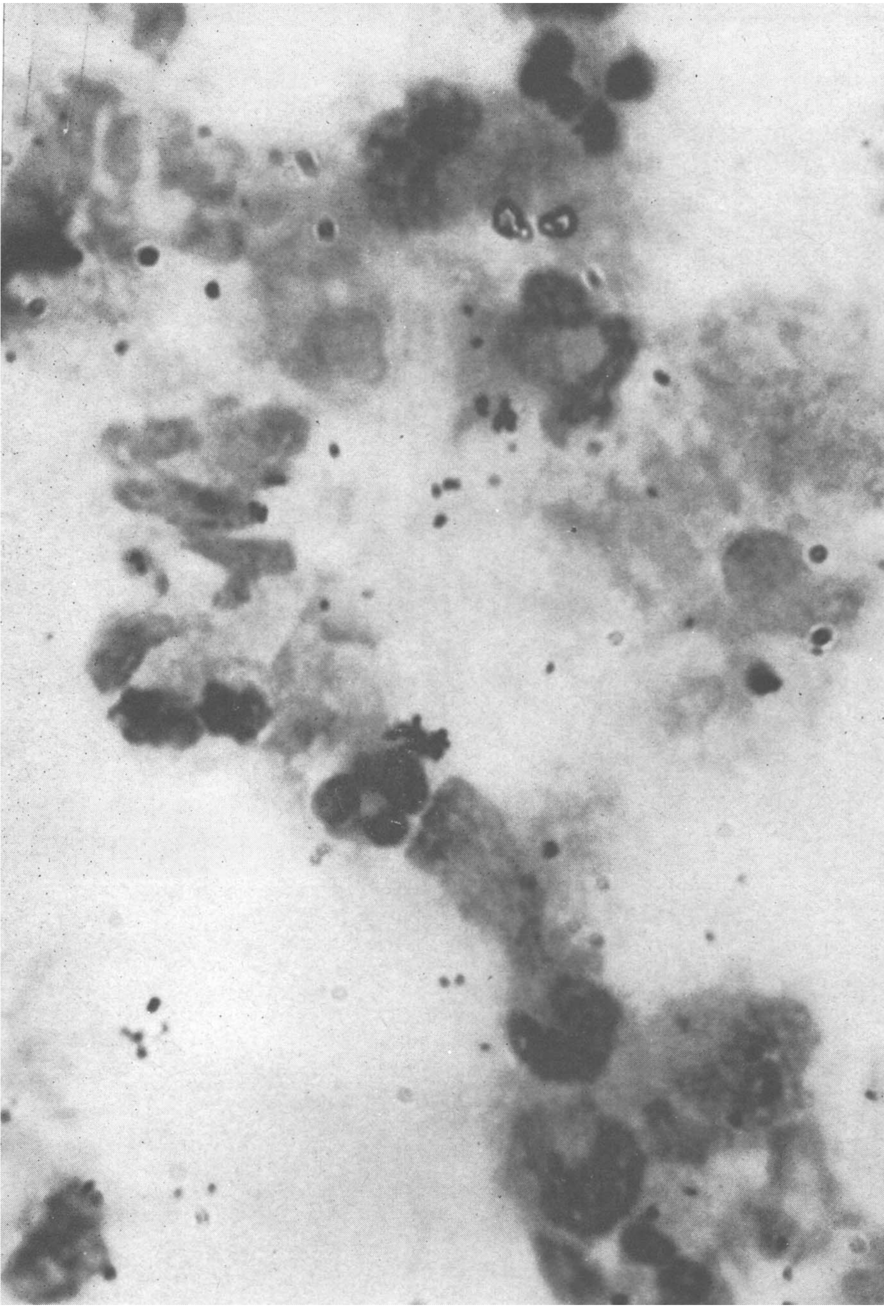

FIG. 2. Autoradiographic phagocytic mixture of variants and leukocytes after washing. (Note absence of stained organisms.) × 1000.

Hamand Drierite Co., Xenia, Ohio) and allowed to expose for a period of 30 days at 4°.

Slides were developed in Kodak Dektol developer for 2 min at 17–18°, rinsed in distilled water 15–30 sec, and fixed in Kodak acid fixer for 5 min. After fixation, the slides were rinsed in slowly running distilled water for 15 min, dehydrated in 95% ethanol for 2 min, and absolute ethanol for another 2 min. Slides were allowed to dry before Giemsa staining.

Results. Employing an autoradiographic method for localization of intracellular microorganisms, a marked difference in phagocytic rates for *E. coli* 04 and its wall defective variant was noted (Figs. 1–3). The results of multiple experiments summarized in Table I indicate that the phagocytic indices for variants were depressed at all time periods, and the addition of specific antisera did not significantly alter these rates. In contrast, type

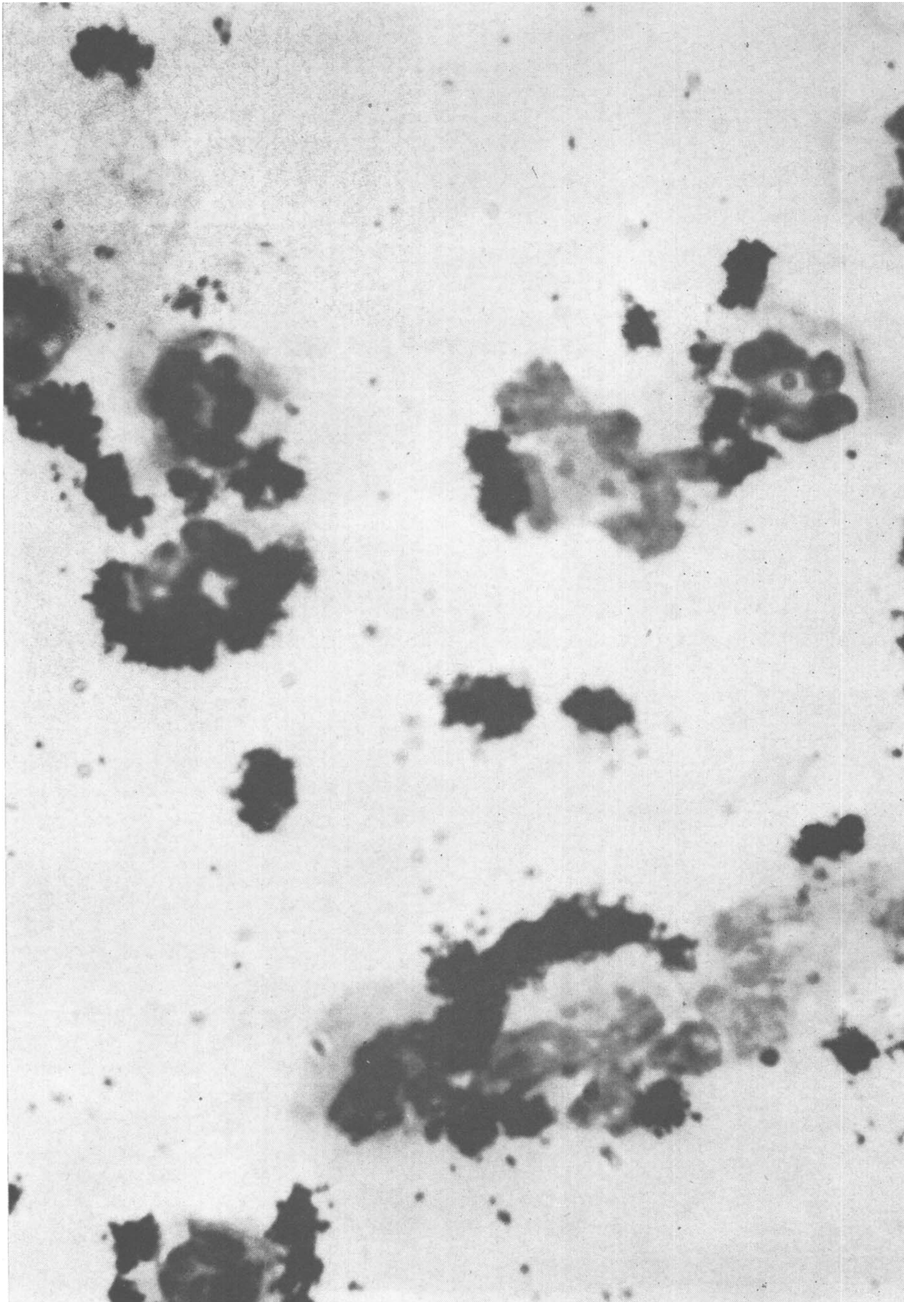

FIG. 3. Autoradiographic phagocytic mixture of classical *E. coli* 04 and leukocytes after washing. (Note heavy stained intracellular organisms.) × 1000.

TABLE I. Phagocytosis of Classical and Variant Forms of *Escherichia Coli* 04

Test serum	Test organism		Phagocytic indices[a] 300 PMNs counted			
		Time	0	30	60	180
Pooled rat serum	*E. coli* variants		1.7	5.0	5.7	11.7
	E. coli (classical)		1.3	15	42	NC[b]
Antiserum to variants	*E. coli* variants		2.0	5.3	6.7	9.0
	E. coli (classical)		3.0	44	60	NC
Antiserum to *E. coli*	*E. coli* variants		2.6	10.6	8.0	10.7
	E. coli (classical)		4.6	45	55.5	NC

[a] Phagocytic Index: Percentage of polymorphonuclear leukocytes containing ingested organisms.
[b] NC—Not countable due to destruction of leukocytes.

specific antiserum to the classical organism caused the expected opsonization of *E. coli* 04 for phagocytosis.

In the initial experiments, a ratio of five microorganisms per phagocytic cell was used. This number was doubled to see if phagocytic rates could be improved, but only slight increases were achieved as is shown by the data in Table II.

In control slides of polymorphonuclear leukocytes without organisms and in slides with variants present, the morphological integrity of the leukocytes was maintained throughout the periods of study. However, in the slides with classical *E. coli*, the leukocytes were losing morphologic detail and decreasing in numbers by as early as 30 min. In many of these experiments, the slides were uncountable by 1 hr and at 3 hr, virtually all cells had been lost from the monolayer.

At each time interval, aliquots of variants were removed from the monolayer and examined by phase-contrast microscopy as were aliquots from the control chambers without leukocytes, and no observable difference in variant morphology could be determined. Aliquots were also cultured on biphasic media without penicillin yielding growth of *E. coli* 04 colonies which were comparable in numbers to control tubes containing variants but devoid of leukocytes. This provided indirect evidence of the viability of L-forms throughout the period of study since the unstable 24 hr variants readily revert to the parent organism in biphasic media after removal from the penicillin containing environment. These transitional variants which were developed in broth cultures did not have the ability to grow on solid media as typical "fried egg" colonies.

Discussion. It was elected to perform these experiments with transitional phase variants

TABLE II. Comparison of Phagocytic Rates with Varying Concentrations of Variants

Test serum	Ratio of variants to phagocytes		Phagocytic indices[a] 300 PMNs counted			
		Time	0	30	60	180
Pooled rat serum	5 to 1		1.6	4.5	5.0	11.6
	10 to 1		0.3	5.0	9.0	6.3
Antiserum to disrupted variants	5 to 1		2.6	5.0	5.6	5.6
	10 to 1		0.3	7.0	9.0	8.3
Antiserum to whole variants	5 to 1		1.3	6.0	7.0	1.3
	10 to 1		—	5.0	9.0	6.7

[a] Phagocytic Index: Percentage of polymorphonuclear leukocytes containing ingested organisms.

rather than stable L-forms since these organisms would probably be the first subjected to host defense mechanisms under conditions allowing for formation of variants *in vivo.* They most likely represent the earliest types of variant forms which are found in clinical specimens. Hence, the ability of these organisms to resist or survive phagocytosis might determine in part their ability to survive in host tissues. These studies have shown a marked difference in phagocytic rates for *E. coli* 04 and its wall-defective variant. Furthermore, the variants did not seem to exert any demonstrable cytopathogenic effect on the cells. Additionally, the presence of specific opsonins did not significantly alter the rate of phagocytosis of the variants. This is especially convincing evidence that these transitional phase variants were less susceptible to phagocytosis since it has long been known that the presence of specific opsonins enhances phagocytic rates with classical bacteria. It is possible that specific receptor sites on the surfaces of transitional phase variants which may interact with certain molecules on the surfaces of polymorphonuclear leukocytes are lacking and may result in the variants not adhering to the leukocytes. This lack of adherence could influence the extent to which phagocytosis occurs. Since variants undergo various morphologic changes in their life cycles (Green, Heidger, and Domingue, manuscript in preparation), they may well vary widely in their ability to attach to different surfaces. This obviously could influence the extent to which organisms might colonize particular sites and persists. In studies of interactions of mycoplasma with mammalian cells, Zucker-Franklin, Davidson, and Thomas (5) have observed at the electron microscopic level an intimate relationship which mycoplasmas have to the plasma membrane of HeLa cells. They suggested that cytopathogenic effects exerted by mycoplasma on some tissue cultures was attributable to membrane damage. They were able to show that the mycoplasma studied were avidly phagocytosed by neutrophils and eosinophils with accompanying degranulation of white cells. Interaction of mycoplasma with monocytes and lymphocytes (6) indicated that on morphological grounds, the cells which were able to take up mycoplasma could not be distinguished from cells which were incapable of this function and following phagocytosis neither the cell nor the mycoplasma showed any morphologic alterations over the 3-hr observation period. Harwick *et al.* (2) in their *in vitro* system have offered lower chemotatic activity as a possible explanation for lesser phagocytosis of bacterial variants.

It would be of interest to determine whether extracts of PMN leukocytes have specific bactericiidal activity for transitional phase bacterial variants. Dajani and Ayoub (7) have reported that extracts of human PMN leukocytes showed a marked mycoplasmacidal effect on three mycoplasma species.

Techniques of surface phagocytosis have been known for sometime (8, 9) and the present one was developed principally as a way of easily separating nonphagocytized variants from polymorphonuclear leukocytes in monolayer. This allowed for less artifact of superimposition for the autoradiographic technique. Autoradiographic localization of variants was chosen because of prelminary studies which showed an inconsistent staining pattern of variants. Nuclear labeling with tritiated thymidine proved to be a useful method for localizing these wall-deficient organisms.

The pathogenicity of bacterial variants has not yet been established. Freeman and Rumack (10) showed that in the case of *Brucella,* the L-form was phagocytized at a greater rate and is apparently more cytopathogenic for monocytes *in vitro* than is the parent *Brucella.* Braude (11) has shown that L-forms of *Proteus* with their retained urease activity are capable of survival and of producing bladder calculi in rats. Although several investigators have indicated an association between L-forms and human disease (4, 12, 13), their role *per se* as disease producing agents has not been determined. Recent data in our laboratory indicate that cell wall deficient organisms can survive in tissue for prolonged periods without causing detectable histopathology (14). The present observations showing a decreased rate of phagocytosis for *E. coli* 04 variants may aid in explaining the ability of an organism to survive in the host at a time when it may be growing slowly and present only in relatively small numbers.

Further changes in the life cycle of transi-

tional phase variants may well give rise to forms which can be phagocytized and persist intracellularly. This is presently being investigated in our laboratory utilizing electron microscopic techniques.

Summary. Autoradiographic localization of tritiated thymidine labeled *E. coli* 04 transitional phase variants in an *in vitro* phagocytic mixture containing polymorphonuclear leukocytes has revealed a significantly lower phagocytic index for these cell wall deficient organisms when compared to the parent bacteria from which they were derived. Although type specific antisera caused expected opsonization of the classical bacteria, there was no marked effect with the variants. The variants did not produce any detectable cytopathogenic effect on the leukocytes whereas the parent organisms were clearly toxic. The ability of such variants to resist phagocytosis may thereby provide an advantage for survival of an organism in the host and possibly lead to microbial persistence.

1. Lisowski, J., Wieczorek, Z., and Kantoch, M., Nature (London) **183**, 1828 (1959).

2. Harwick, H.J., Barajas, L., Montgomerie, J.Z., Kalmanson, G.M., and Guze, L.B., Infect. Immun. **5**, 976 (1972).

3. McGee, Z.A., Wittler, R.G., Gooder, H., and Charache, P., J. Infect. Dis. **123**, 433 (1971).

4. Gutman, L.T., Turck, M., Petersdorf, R.G., and Wedgewood, R.J., J. Clin. Invest. **44**, 1945 (1965).

5. Zucker-Franklin, D., Davidson, M., and Thomas, L., J. Exp. Med. **124**, 521 (1966).

6. Zucker-Franklin, D., Davidson, M., and Thomas, L., J. Exp. Med. **124**, 533 (1966).

7. Djani, A.S., and Ayoub, E.M., J. Immunol. **102**, 698 (1969).

8. Wood, W.B., Smith, M.R., and Watson, B., J. Exp. Med. **84**, 387 (1946).

9. Foley, M.J., Smith, M.R., and Wood, W.B., J. Exp. Med. **110**, 603 (1959)).

10. Freeman, B.A., and Rumack, B.H., J. Bacteriol. **88**, 1310 (1964).

11. Braude, A.I., Ann. N.Y. Acad. Sci. **174**, 896 (1970).

12. Domingue, G.J., and Schlegel, J.U., J. Urol. **104**, 790 (1970).

13. Chattman, M.S., Mattman, L.H., and Mattman, P.E., Amer. J. Clin. Pathol. **51**, 41 (1969).

14. Domingue, G., Turner, B., and Schlegel, J. U., Urology **3**, 288 (1974).

Received Dec. 3, 1973. P.S.E.B.M. 1974, Vol. 146.