THE FATE OF BACTERIA WITHIN PHAGOCYTIC CELLS

II. The Modification of Intracellular Degradation*

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The preceding paper outlined the over-all fate of various classes of bacterial macromolecules following intraleucocytic residence (1). Under the *in vitro* conditions employed, up to 70 per cent of the total isotope of uniformly labeled organisms was degraded into acid-soluble fragments within 180 minutes of incubation. This report represents an extension of these studies in which the influences of both serum and cellular factors on the breakdown of phagocyted bacteria was evaluated. It deals specifically with the effects of immune serum, "immune cells" and selected metabolic inhibitors which block glycolysis and respiration.

Materials and Methods

Procedures for obtaining PMN leucocytes and macrophages, determining phagocytosis, labeling organisms, fractionation and assay of leucocyte-bacteria suspensions were identical with those described in the previous publication (1). The concentrations of phagocytes, fresh serum, and bacteria in the reaction mixtures were the same as in the previous article (1).

Preparation of Antigens and Immune Serum.—Strains of various bacteria were grown for 16 hours with constant shaking in complete medium (1). The cells were harvested by centrifugation, washed twice with pyrogen-free saline and inactivated at 85°C for 40 minutes in a water bath. After another saline wash they were resuspended to an optical density of 0.3 at 450 mμ with a Junior Coleman spectrophotometer.

Rabbits weighing 3 to 4 kg were injected intravenously (0.5 ml antigen) every 3rd day for a total of seven injections and bled by cardiac puncture on the 7th day after the last injection. Serum was obtained and immediately stored at −20°C in small aliquots.

Other variables in experimental methods will be described in Results.

RESULTS

A. The Influence of Immune Serum on the Degradation of Bacteria within PMN Leucocytes and Macrophages.—Previous studies have suggested that immune serum in the presence of bacteria-leucocyte mixtures enhances the killing of microorganisms within phagocytic cells and in some cases increases the rate of degradation of ingested particles (2, 3). In view of these studies it was of interest

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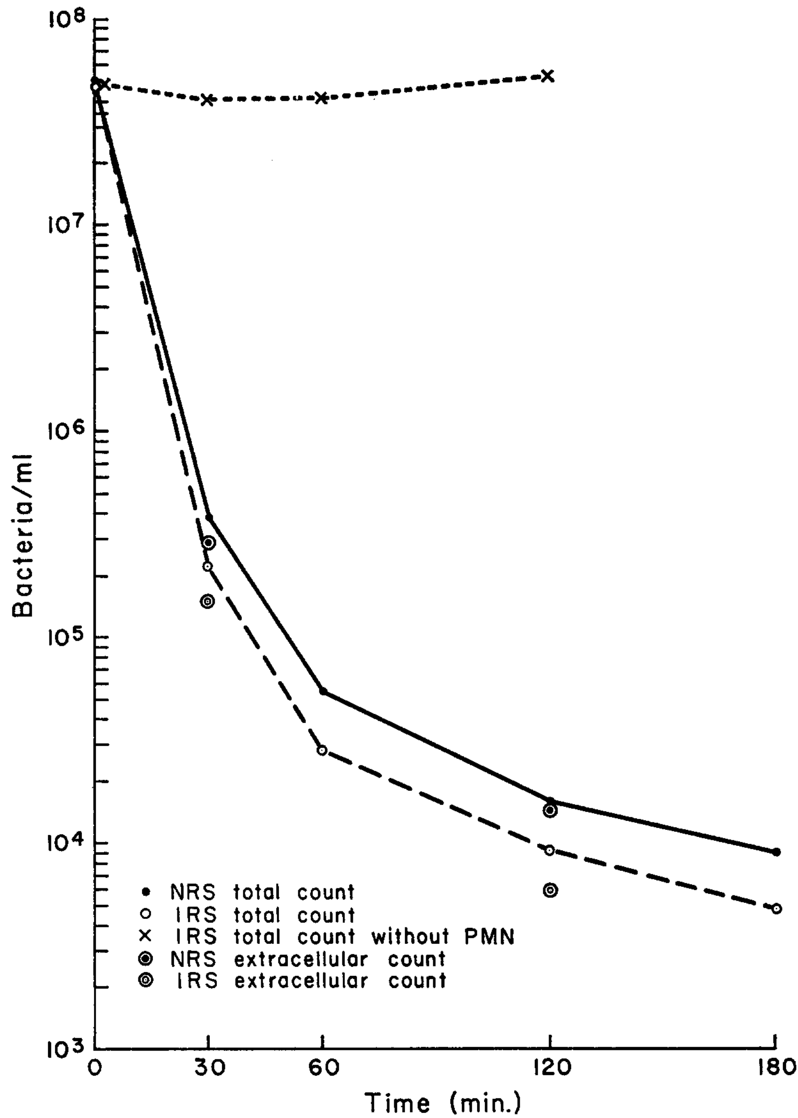

FIG. 1. The phagocytosis and killing of *E. coli* by polymorphonuclear leucocytes in the presence of normal and immune rabbit serum.

to evaluate the degradation of labeled bacteria in the presence of immune serum once ingested by PMN leucocytes and macrophages, and compare their destruction to that occurring with normal serum. In order to carry out these experiments it was necessary to employ bacteria which would be ingested and killed by leucocytes at the same rate in the presence of either normal or immune serum.

The prototype organism which met these criteria was a strain of *Escherichia coli* K-12 although other organisms will be described in succeeding sections.

Fig. 1 presents the interaction of *E. coli* with rabbit PMN leucocytes in the presence of normal rabbit serum and immune serum. The phagocytosis and killing of *E. coli* in both instances was rapid and within 30 minutes less than 1 per cent of the bacteria remained extracellularly. In the presence of 10 per cent fresh immune serum but in the absence of leucocytes the population of viable organisms remained stable. The same results were obtained when rabbit peritoneal exudate macrophages were employed. It was concluded, therefore, that any differences in the degradation of *E. coli* would be the result of intracellular processes rather than on the differential uptake and inactivation of the organism.

Preliminary experiments on P^{32}-labeled *E. coli*-PMN suspensions revealed that immune serum inhibited the formation of acid-soluble degradation products. More complete studies in which all four fractions were compared in simultaneously performed experiments are presented in Fig. 2. The lower portion of the figure presents the over-all conversion of bacterial P^{32} into an acid-soluble form within the PMN. In the presence of normal serum a typical result was obtained with *E. coli* and after 180 minutes of incubation more than 50 per cent of the total P^{32} of the organism had been degraded to an acid-soluble form. With immune serum there was a marked decrease in the formation of acid-soluble products during the first 60 minutes. Thereafter, the rate of formation was similar to the suspensions containing normal serum. An examination of the acid-insoluble fractions revealed certain interesting effects. The hot TCA fraction, which contained primarily nucleic acids and was the chief contributor to the acid-soluble breakdown pool, remained unaffected for the first 30 minutes and then gradually lost isotope. Of perhaps more interest were the interactions between the alcohol-ether and residue fractions. In the presence of immune serum there was a more rapid decrease in alcohol-ether P^{32} during the first 30 minutes with a concomitant increase in the P^{32} content of the residue fraction. This shift occurred during a period in which there was little or no change in the over-all content or distribution of P^{32} between the acid-soluble and -insoluble compartments. Within the next 30 minutes *residue* radioactivity was abruptly lost and this process continued during the remainder of the experiment. With immune serum and labeled *E. coli*, but in the absence of leucocytes, a similar shift of radioactivity to the residue fraction was noted which remained elevated during the next 1.5 hours. No similar change was observed when normal rabbit serum was employed. Identical results were obtained with non-viable organisms, indicating that the shift in radioactivity was not related to metabolic events.

A similar inhibition of intracellular degradation was noted with other organisms and with C^{14}-labeled bacteria as well. These results are presented in Table I in terms of the rate of formation of acid-soluble products in the presence of

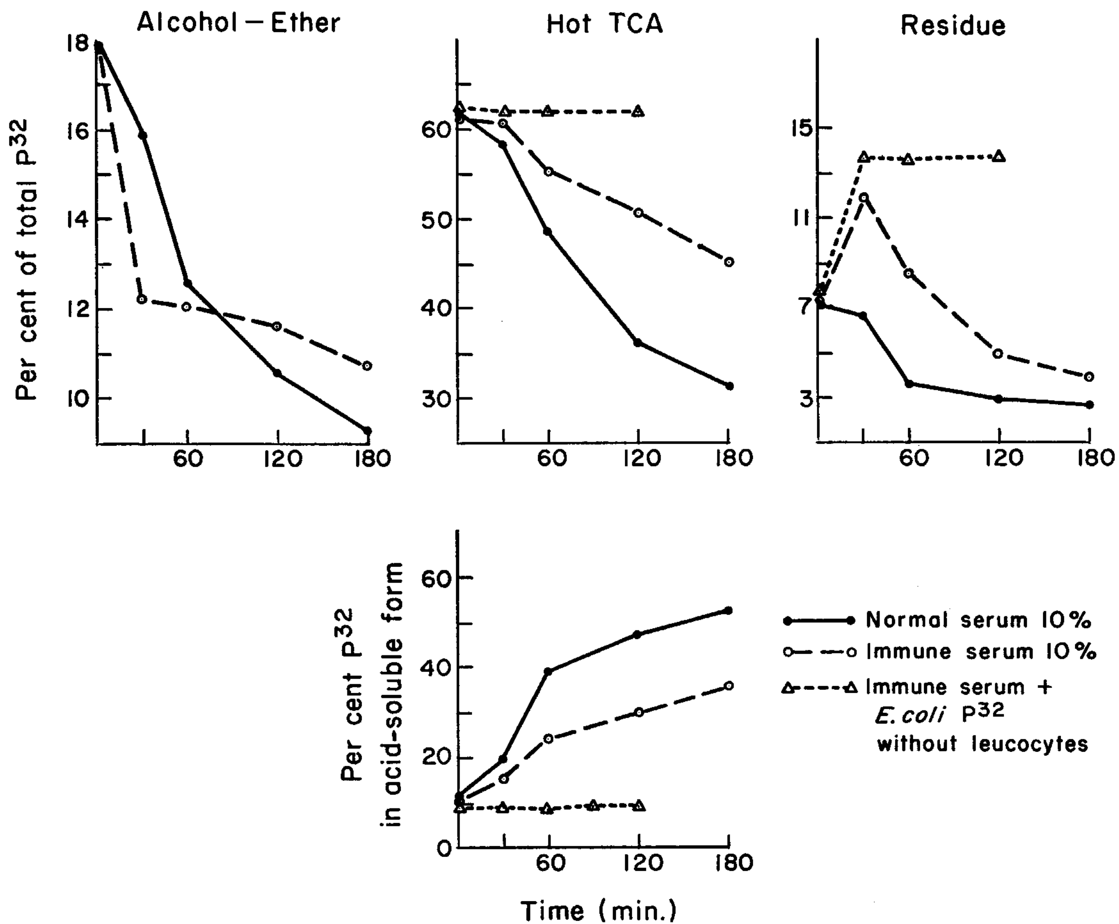

FIG. 2. The influence of immune serum on the degradation of P^{32}-labeled *E. coli* by polymorphonuclear leucocytes.

normal serum. In each case inhibition was noted, ranging from 30 to 50 per cent of the control values. The influence of immune serum on macrophage-bacteria interactions was essentially the same.

Additional experiments showed that immune serum could be considerably diluted and still inhibit the production of acid-soluble products. Fig. 3 represents one such experiment in which definite inhibition occurred out to a 1/500 dilution. The absorption of immune serum with homologous heat-killed bacteria removed the inhibiting properties (compare *C* and *G*). The limiting dilution of this particular lot of immune serum represented 2 to 4 agglutinating units. The fractionation of immune serum with $(NH_4)_2SO_4$ (4) revealed that the inhibiting component resided in the gamma globulin–rich fraction. The pretreatment of

TABLE I

The Influence of Immune Serum on the Formation of Acid-Soluble Compounds

Bacteria	Label	Leucocyte‡	Per cent reduction in presence of immune serum*			
			30 min.	60 min.	120 min.	180 min.
E. coli	P^{32}	PMN	42	56	71	65
E. coli	P^{32}	MN	67	58	51	45
E. coli	C^{14}	PMN	42	51	48	41
S. albus	P^{32}	PMN	57	60	66	72
S. albus	P^{32}	MN	65	61	54	49
S. albus	C^{14}	PMN	48	53	56	44
S. aureus	P^{32}	PMN	50	51	44	39

* Degradation to acid-soluble compounds in presence of normal rabbit serum = 100 per cent.

‡ PMN, rabbit polymorphonuclear leucocytes; MN, rabbit macrophages.

bacteria with either immune serum or the globulin fraction, followed by their addition to leucocytes in the presence of fresh normal serum resulted in the same type of inhibition.

The specificity of the inhibiting factor was examined in experiments in which various combinations of labeled bacteria, immune serum, and PMN leucocytes were employed. One such study is presented in Table II. Immune serum prepared against *E. coli* inhibited the P^{32} *coli*-PMN interaction but did not materially influence the fate of *Staphylococcus albus*. A similar specificity was noted with *S. albus* immune serum. This experiment in addition to illustrating the specificity of the reaction also indicated that immune serum did not contain any non-specific cytotoxic material which influenced the function of the cells.

Microscopic observations on bacteria-PMN suspensions in the presence of either normal or immune serum showed that the distribution of bacteria within leucocytes was identical in both cases. In addition, an evaluation of the de-

granulation phenomenon revealed no significant difference in either the rapidity or extent of degranulation following the ingestion of bacteria in the presence of normal or immune serum.

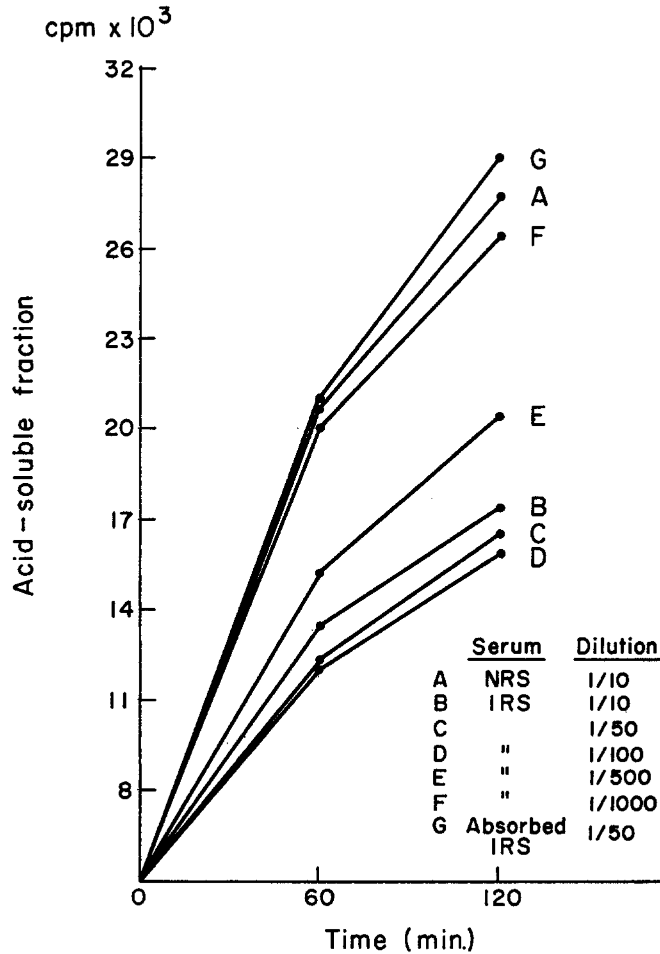

FIG. 3. The effect of dilution on the inhibition of intraleucocytic degradation by immune serum.

B. The Influence of PMN Leucocytes and Macrophages from Immune Animals on the Intracellular Degradation of Bacteria.—A number of authors have appeared to show that phagocytic cells obtained from immunized animals were altered in that they limited the intracellular multiplication of bacteria or in certain cases contained larger quantities of hydrolytic enzymes (5, 6). It was of interest, therefore, to evaluate the ability of both types of phagocytes from

immunized rabbits to degrade the homologous organism in the presence and absence of immune serum.

Rabbits were immunized intravenously with heat-killed *E. coli* or *S. albus* according to the schedule outlined in Materials and Methods. Ten days after the seventh injection either PMN or macrophages were obtained from the peritoneal cavity. The cells were carefully washed and resuspended in the routine medium containing either normal or immune fresh rabbit serum. P^{32}- or C^{14}-labeled bacteria were then added and the tubes rotated in the usual manner (1). Aliquots were removed at intervals up to 180 minutes and fractionated. The rate of phagocytosis and intracellular killing was evaluated under each set of experimental conditions by described procedures (1). The rate of formation of acid-soluble degradation products served as the index of intracellular degradation. Cells from non-immunized animals were obtained each day and compared simultaneously with those from immunized rabbits.

In a series of three separate tests with both PMN and macrophage derived from immunized animals there was no significant difference in the rate of formation of acid-soluble degradation products in the presence of normal rabbit serum. When immune serum was present in the incubation mixture, specific inhibition occurred, and was of the same magnitude in the presence of cells from normal and immunized animals. Under these conditions, therefore, cells from immune animals did not display any unusual capacity to degrade phagocyted bacteria.

TABLE II

The Specificity of the Inhibiting Influence of Immune Serum on Intraleucocytic Degradation

Serum 10 per cent final concentration	*S. albus* P^{32} + rabbit PMN acid-soluble fraction		*E. coli* P^{32} + rabbit PMN acid-soluble fraction	
	60′	120′	60′	120′
	CPM	CPM	CPM	CPM
Normal	10,300	12,230	4,130	6,270
S. albus immune	7,200	8,900	4,080	6,120
E. coli immune	10,180	11,980	3,020	3,920

C. The Influence of Metabolic Inhibitors on the Intracellular Degradation of Bacteria.—Studies on the metabolic requirements of phagocytosis in both PMN leucocytes and macrophages have indicated that glycolysis serves as the main source of energy for this process (7–9). It has not, however, been possible as yet to evaluate the role of energy metabolism, if any, in the subsequent destruction of the ingested particle. This point is of some interest in that a number of laboratories have, in part, equated the metabolic state of the host cell with the resistance of experimental animals to infection (10, 11).

Iodoacetate and arsenite were employed as inhibitors of glycolysis at final concentrations of 2×10^{-4} and 2×10^{-3} M. Cyanide was used under the conditions described previously (7). P^{32}-labeled *E. coli*, *B. subtilis*, or *S. albus* were added to suspensions of either PMN leuco-

cytes or macrophages and incubated for 30 minutes to allow phagocytosis to occur. The cells which contained ingested, labeled bacteria were then sedimented at 500 RPM and resuspended in medium containing the inhibitors. Incubation was then continued and at intervals up to 3 hours, aliquots were removed and fractionated. Inhibitor-free controls were always included in each test. In certain instances the inhibitors were present throughout the entire incubation period and during the initial phagocytic interval.

The results of such experiments clearly indicated that neither iodoacetate, arsenite, or cyanide had any influence on the rate of formation of acid-soluble fragments from the labeled bacteria, once these organisms were within the phagocytes. When iodoacetate and arsenite were present during the entire incubation period, phagocytosis was blocked as evidenced by the stability of the extracellular bacterial counts. Under these conditions no significant change occurred in the distribution of bacterial isotope in the extracellular organisms. These findings applied to both PMN leucocytes and macrophages. It should be emphasized that iodoacetate and arsenite were present at concentrations which reduced glycolysis more than 95 per cent, whereas cyanide inhibited leucocyte respiration at least 70 per cent.

DISCUSSION

The material in immune serum which delayed intraleucocytic breakdown of bacteria was presumably a globulin directed against a superficially located antigen of the bacteria, combining with it during the opsonization process. This material, which was presumably antibody, was present in high titer in immune serum and was specific to the extent that it had inhibitory action only with the homologous, labeled organism.

The mechanism by which specific antibody inhibited the degradation of intracytoplasmic bacteria is not completely clear. From the data presented on *E. coli*-phagocyte interactions it appeared that globulin could combine with a surface component, either when added to the complete system or when the organism was pre-opsonized and then added to leucocyte suspensions. Current hypotheses concerning the chemical composition of the surface of Gram-negative bacilli suggest that a lipoprotein layer overlies the more deeply situated cell wall lipopolysaccharide (12). Assuming that globulin would combine with the most accessible portion of the bacterial surface it is reasonable that a lipoprotein-globulin complex was formed. Certain evidence obtained from the isotope experiments suggests that this may be the case. The composition of the alcohol-ether–extractable fraction of *E. coli* is composed of lipid as well as a protein moiety (not labeled with P^{32}) which behaves differently from the majority of protein in the residue fraction. This is true not only in terms of its solubility in ethanol but in its metabolic properties as well (13). A portion of this material may represent the external lipoprotein layer of the bacteria. The combination of globulin with the surface component might therefore change the solubility

properties of the lipoprotein, rendering it non-extractable with lipid solvents so that the complex would then be found in the final residue fraction. The shift of P^{32} from the alcohol-ether to residue fractions is consistent with this hypothesis. The interaction of immune serum with bacteria in the absence of leucocytes resulted in a similar redistribution of isotope which, however, remained in the residue fraction throughout the period of incubation. In contrast, the isotope in the residue fraction was rapidly lost when the organism resided within the cytoplasm of both the macrophage and PMN leucocyte. This suggested that the globulin complex was degraded by phagocyte proteases with subsequent release of acid-soluble degradation products. In this regard, unpublished experiments had demonstrated that rabbit PMN leucocyte cathepsin is capable of degrading isolated rabbit gamma globulin *in vitro*. As soon as the complex is degraded within the leucocyte, bacterial nucleic acids were then hydrolyzed, the over-all rate of formation of acid-soluble fragments approaching that seen with normal serum. These data suggest that globulin coats the surface of the bacterium, thereby protecting it for a short period from leucocyte enzymes. Of some interest, is the fact that immune globulin does not protect the microorganism from the bactericidal properties of leucocyte cytoplasm during the same period. This difference is as yet unexplained.

The lack of any demonstrable effect of immunization on the degradative properties of peritoneal phagocytes is of some interest in view of the findings that macrophages obtained from stimulated animals contain larger amounts of certain hydrolytic enzymes (14, 15). These differences at best represent a two-fold increase in activity and would not in themselves be apt to alter intracellular degradation. The unstimulated cell apparently has sufficient enzyme to cope with the digestion of foreign particles. These experiments would also preclude the production of "adaptive" systems in phagocyte populations in response to prior experience with the specific agent. The ability of "immune" macrophages to limit the growth of certain intracellular agents may therefore be unrelated to their content of hydrolyses and depend on other more specific bactericidal mechanisms.

Previous studies from this laboratory demonstrated that certain agents which stimulated leucocyte glycolysis and/or respiration were associated with an increased capacity of these cells to ingest particles (16, 17). However, no data could be obtained on the ability of such cells to kill phagocyted bacteria, since the normal cell was highly efficient in this regard. The present technique offered certain advantages in evaluating the relationship between energy metabolism and bacterial destruction. The evidence obtained in this study indicated that potent inhibitors of both glycolysis and respiration were without effect on degradative processes once the bacteria had been within the cell for a 30 minute period. In view of the properties of leucocyte granules this is not surprising since none of the hydrolases are dependant upon energy-yielding mechanisms

for their function. Glycolysis could conceivably have been a source of lactic acid and therefore of a higher hydrogen ion concentration within the cell, thus favoring the activity of many of these enzymes which have acid pH optima. Nonetheless, degradation proceeded at the usual rate in the absence of glycolytic activity. It is of course possible that enzymatic processes other than those related to lysosomes may also influence the destruction of particulate material within the cytoplasm of phagocytic cells.

SUMMARY

The influence of immune serum, PMN leucocytes, and macrophages from immunized animals and metabolic inhibitors on the intraphagocytic degradation of isotopically labeled bacteria has been evaluated.

Immune serum specifically delayed the degradation of a variety of P^{32}- and C^{14}-labeled organisms within both types of phagocytic cells. The active principle in immune serum was found to be a globulin which could be removed by adsorption with the homologous organism. The inhibiting action of immune serum was thought to be related to its combination with the bacterial surface and the subsequent temporary protection of the bacteria from leucocyte enzymes.

PMN leucocytes and macrophages obtained from immune hosts did not differ from normal cells in their ability to degrade homologous, labeled bacteria. Immune serum had the same inhibiting influence in the presence of "immune" cells as with cells from non-immunized hosts.

Iodoacetate, arsenite, and cyanide at concentrations which inhibited the glycolysis and respiration of both PMN leucocytes and macrophages had no influence on the rate of degradation of isotopically labeled bacteria engulfed by these cells. This implied that following the initial phagocytic events, the degradation of bacteria within leucocytes is not dependent upon the major pathways of energy metabolism.

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