Mechanisms of Immunity in Typhus Infections

IV. Failure of Chicken Embryo Cells in Culture to Restrict Growth of Antibody-Sensitized *Rickettsia prowazeki*

C. L. WISSEMAN, JR., A. D. WADDELL, AND W. T. WALSH

Department of Microbiology, School of Medicine, University of Maryland, Baltimore, Maryland 21201

Rickettsia prowazeki, pretreated with typhus immune human serum, readily infects, and grows in, chicken embryo cells in culture. This finding is similar to those of previous studies which showed that typhus rickettsiae, pretreated with immune serum, grow in cells of the yolk sac of embryonated hen eggs and in the cells of the midgut of the human body louse. In contrast, identically treated typhus rickettsiae were destroyed by human macrophages in culture. Collectively, these observations seem to support an emerging concept that the fate of antibody-sensitized typhus rickettsiae depends upon the presence or absence of certain specialized properties of the host cell into which they gain entrance—nonphagocytic cells or "nonprofessional" phagocytic cells versus certain kinds of "professional" phagocytes. The phenomena involved probably have an important bearing on the mechanisms of the persisting infection and the nonsterile immunity which characterizes convalescence from typhus fever in man. They also form the basis for certain practical technical innovations in the laboratory.

Studies on humoral immune mechanisms following typhus infections (1, 5, 6; C. L. Wisseman, Jr., D. W. Krause, I. B. Fabrikant, and P. A. Machowiak, J. Infect. Dis., submitted for publication) have revealed that human typhus immune serum (i) confers strong passive protection on animals against virulent challenge, (ii) exerts no direct rickettsiacidal action but strongly opsonizes *Rickettsia prowazeki* and *R. mooseri* (*R. typhi*) for phagocytosis by both polymorphonuclear leukocytes and the monocyte-macrophage series, and (iii) prepares typhus rickettsiae for retention and destruction within vacuoles of human macrophages, which otherwise are unable to restrict rickettsial growth. Thus, immune serum and macrophages, neither of which is sufficient alone to account for the remarkable protective action of immune serum passively transferred to the intact animal, appear to work in concert to destroy the typhus rickettsiae.

On the other hand, unrestricted growth of antibody-sensitized typhus rickettsiae occurs in the yolk sac cells of the embryonated hen egg, in the midgut cells of the human body louse, *Pediculus humanus humanus L.* (2), and in the rare cell in cultures of macrophages derived from human peripheral monocytes (6). This suggests that the intracellular fate of an antibody-sensitized rickettsia may depend upon the specific properties of the kind of cell into which

it gains entrance, i.e., it is a host cell-determined phenomenon. However, the possibility exists that the yolk sac and louse gut cells represent very special cases in which extracellular enzymes might digest away or alter the immunoglobulins on the rickettsial surface, thus freeing it to penetrate the cells unimpeded. Therefore, to test the evolving hypothesis regarding the determining role of the host cell on the fate of antibody-sensitized rickettsiae, experiments were performed with cultures of cells not uniquely specialized for either phagocytic or digestive roles, i.e., chicken embryo fibroblasts (8). This report is restricted to observations on the intracellular fate, within chicken embryo (CE) cells in cell culture, of *R. prowazeki* which had previously been exposed to normal (NHS) or immune (IHS) human serum or to methylated bovine serum albumin (MBSA). The kinetics of rickettsial uptake by CE cells in stationary cultures and the influence of immune serum and other substances upon uptake are the subjects of a separate report (C. L. Wisseman, Jr., A. D. Waddell, and W. T. Walsh, manuscript in preparation).

MATERIALS AND METHODS

Rickettsiae. Three seed lots of yolk sac (ys)-passage virulent *R. prowazeki*, Breinl strain, were used in these studies. (i) Seed PPP 11/70 (163rd ys passage), used in some slide chamber experiments, was par-

tially purified by the batch sucrose procedure (Wisseman et al., unpublished method), resuspended in sucrose PG solution (3), quick-frozen with a dry ice-alcohol mixture in flame-sealed glass ampoules, and stored at -70 C prior to use. (ii) Seed McD-6/29/71, a partially purified preparation of the 164th ys passage of *R. prowazeki*, Breinl strain, prepared essentially as in (i) above, was used in other slide chamber experiments. This seed, through extensive characterization in other studies (2), contained $10^{10.37}$ rickettsial bodies, $10^{10.49}$ mean egg infective doses (EID_{50}), and $10^{7.92}$ plaque-forming units per ml. (iii) Seed 2F, used in the plaque technique, was a 20% suspension of infected yolk sacs in sucrose PG solution which was ampouled, frozen, and stored as described above.

Miscellaneous reagents. NHS, lot AF-30, was a pool of sera from healthy adult male medical students with no history of exposure to typhus or of immunization against typhus and was devoid of typhus group complement-fixing (CF) antibodies. IHS consisted of various lots (V-19443, V-19504) of "late" serum drawn from a single solidly immune person whose sera were used in previous studies in this series (1, 2, 5, 6; Wisseman, Krause, Fabrikant, and Machowiak, J. Infect. Dis., submitted for publication). This serum has been shown to prepare both *R. mooseri* and *R. prowazeki* for intracellular destruction by human macrophages in vitro (6). MBSA (Calbiochem, grade B) was dissolved in tissue culture medium (0.2 mg/ml).

Tissue culture techniques. All tissue culture procedures were carried out without antibiotics. The basic slide chamber cultures with irradiated secondary CE cells as well as the plaque technique, which was adapted from Wike et al. (10), are described in detail in another publication (C. L. Wisseman, Jr., A. D. Waddell, and W. T. Walsh, J. Infect. Dis., submitted for publication).

(i) Medium and serum. For the slide chamber studies, the medium consisted of N-2-hydroxyethylpiperazine-N'-2'-ethanesulfonic acid (HEPES)-buffered M-199 medium with Hanks salts (Gibco) containing 10% fetal calf serum (FCS) (Gibco). For studies involving the plaque technique, the medium used was that recommended by Wike et al. (10), i.e., M-199 containing 5% FCS and buffered to pH 7.5 with $NaHCO_3$.

(ii) Cells. Cells from 9- to 11-day-old chicken embryos, used throughout these studies, were derived either from embryonated eggs from conventional flocks fed on antibiotic-free feed (Truslow Farms) or from eggs from leukosis-free flocks (SPAFAS).

Primary cells were obtained using conventional trypsinization methods (7). Secondary CE cells were obtained as previously described (Wisseman, Waddell, and Walsh, J. Infect. Dis., submitted for publication) except that HEPES-buffered M-199 medium with 10% FCS was employed. X-irradiated, secondary CE cells were used exclusively in the slide chamber studies in order to obtain nonmultiplying, well-separated cells with a minimum of debris to facilitate microscope examination (Wisseman, Waddell, and Walsh, J. Infect. Dis., submitted for publication).

(iii) Slide chamber technique. Lab-Tek tissue culture slides with eight chambers (Lab-Tek Products, no. 4808), containing X-irradiated secondary CE cells as described above, were employed for studies involving microscope examination and counting of rickettsiae in the following manner.

NHS or IHS in a dilution of 1:2.5 or MBSA (0.2 mg/ml) was mixed with an equal volume of the desired dilutions of rickettsial suspension (PPP-11/70) which had been determined by preliminary tests. The mixtures were incubated in tubes at ambient temperature (about 24 C), with occasional gentle agitation, for 60 min. The medium was removed from the chambers, and 0.3 ml of the appropriate mixture was added to each chamber. After 120 min of incubation at 32 to 34 C, the inoculum was removed, the cells were washed twice with growth medium, and 0.3 ml of fresh medium was added to each chamber. The slide chambers were then incubated at 32 C. At 2, 24, 48, 72, and 96 h, three slides representing three chambers for each condition were removed and processed for examination. The slide chamber cultures were fixed, stained, and counted as previously described (12). (It is impossible to ascertain by light microscope examination of these preparations if a rickettsia falling within the borders of a cell after the 2-h incubation is within the cytoplasm or is merely attached to the surface. Subsequent evidence for proliferation of the cell-associated rickettsiae suggests, however, that either they had already gained entrance into the cells or were irreversibly committed to entry into the cell. Only fibroblast-like cells were counted. The subpopulation of large round cells with obvious phagocytic activity, possibly macrophages, was excluded.) A total of 300 cells was counted for each time period and condition and (i) the percentage of cells infected, (ii) the average number of rickettsiae per cell, and (iii) the average number of rickettsiae per infected cell were recorded.

(iv) Plaque technique. The plaque technique for quantitating rickettsiae was adapted from Wike et al. (10) and Wisseman et al. (Wisseman, Waddell, and Walsh, J. Infect. Dis., submitted for publication) to examine possible opsonization or neutralization of *R. prowazeki* by plaque enhancement or plaque reduction. *R. prowazeki*, Breinl strain (seed 2F), was mixed with 3.7% brain heart infusion broth (BHI) (BBL) to yield 10^{-5}, 10^{-6}, and 10^{-7} dilutions. Equal volumes of the desired rickettsial dilution in BHI and IHS (1:2.5), MBSA (0.2 mg/ml), NHS, or growth medium were mixed and incubated at ambient temperature for 30 min with frequent gentle mixing. CE cell monolayers in the T-30 flasks (Falcon) were inoculated, overlaid with agarose and incubated for plaque formation as previously described (10; Wisseman, Waddell, and Walsh, J. Infect. Dis., submitted for publication).

RESULTS

Effect of pretreatment of *R. prowazeki* with IHS and MBSA on infection of, and growth in, irradiated CE cells in slide chamber cultures. The intracellular fate of *R.*

prowazeki pretreated with NHS, IHS, or MBSA was determined by counts of rickettsiae in the nondividing irradiated CE cells in samples taken at 24-h intervals for 96 h (Fig. 1). On the basis of the detailed study of the growth cycle of *R. prowazeki* in irradiated CE cells (Wisseman, Waddell, and Walsh, manuscript in preparation), it has been established that the observations of the first 36 to 48 h were confined largely to a single infection cycle, which might be expected to reasonably reflect the influence of pretreatment on the interaction between the CE cells and the rickettsiae.

The rickettsiae appeared to behave within the CE cells in qualitatively the same manner regardless of the nature of the pretreatment of the rickettsiae, i.e., NHS, IHS, or MBSA. The differences which do appear are quantitative and are related to the enhanced entry into cells by pretreatment with IHS or MBSA, as reflected by the initial percentage of cells infected. Once within CE cells, however, the rickettsiae appeared to multiply at similar rates regardless of the kind of pretreatment (see average number of rickettsiae per infected cell). Thus, it is clear that irradiated CE cells do not have the capacity to destroy or restrict the intracellular growth of *R. prowazeki* which have been pretreated with typhus immune human serum or with MBSA; instead, entry of the rickettsiae into these cells and their arrival at an intracellular site conducive to multiplication are actually enhanced.

Effect of pretreatment of R. prowazeki with IHS or MBSA on the capacity of R. prowazeki to form plaques in CE cell monolayers. Preincubation of the rickettsiae with serum from a subject solidly immune to epidemic typhus did not interfere greatly with the capacity of the organisms to produce plaques on the CE cell monolayers; i.e., it exerted no dramatic "neutralizing" action (Table 1). The mean plaque count obtained when the results of all three replicates were combined was somewhat lower than that of the control, with a difference significant at the 5% level in the *t* test. However, the possibility that the immune serum caused some clumping of organisms cannot be excluded. In any case, plaque formation by antibody-sensitized rickettsiae in CE cell cultures was in fact remarkably efficient, results which lend support to the conclusions derived from the slide chamber experiments described above that these CE cells do not have the capacity to destroy or seriously limit multiplication of antibody-sensitized rickettsiae or, conversely, antibody did not interfere appreciably with the capacity of the rickettsiae to establish infection and produce plaques in CE cell cultures.

In contrast, pretreatment of the rickettsiae with MBSA increased the number of plaques (significant at the 0.1% level); however, the resulting enhancement of plaquing efficiency was not sufficient to reduce appreciably the discrepancy of about two orders of magnitude between the number of rickettsial bodies in the suspension and the plaque-forming units (2).

DISCUSSION

This study has shown that *R. prowazeki*, previously incubated with IHS, infects CE cells in culture with an efficiency which even exceeds, under certain conditions, that of rickett-
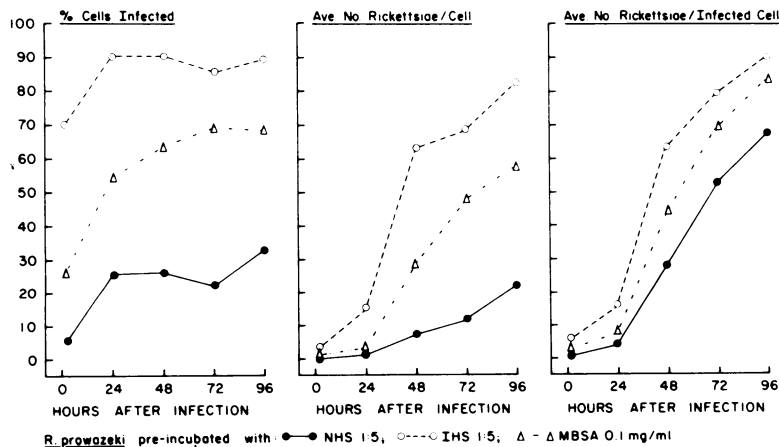

FIG. 1. *Growth of R. prowazeki pretreated with NHS, IHS, or MBSA in irradiated CE cells in slide chamber cultures.*

TABLE 1. *Effect of preincubation of R. prowazeki, Breinl strain, with typhus immune human serum or methylated bovine serum albumin on capacity to form plaques on chicken embryo cell monolayer cultures*

Treatment[a]	Inoculum[b]	Mean PFU			
		Expt 1	Expt 2	Expt 3	Total[c]
Control (TC medium)	10^{-5}	174.6	201	180.3	**184.4**
	10^{-6}	20.6	15	19.5	**18.7**
Typhus immune human serum	10^{-5}	133	167.5	187.3	**161.6[d]**
(V-19444) (1:5 dilution)	10^{-6}	15.3	13.7	18.5	**15.4**
Methylated bovine serum albumin	10^{-5}	266.6	242	211	**243.5[e]**
(0.1 mg/ml)	10^{-6}	32.6	25.3	20.7	**25.9**

[a] *R. prowazeki* incubated with medium, immune serum, or MBSA (all given in final concentration) for 30 min at room temperature prior to inoculation onto chicken embryo cell monolayers.

[b] Final dilution of seed no. 2F, *R. prowazeki*, Breinl strain.

[c] Mean plaque-forming units (PFU) from individual plaque counts in all three replicates.

[d] The difference between this value and that of the control is significant at the 5% level by the *t* test.

[e] The difference between this value and that of the control is significant at the 0.1% level by the *t* test.

siae not sensitized with IHS and is capable of subsequent growth within these cells to an extent comparable to that of nonsensitized organisms. These findings with CE cells are consistent with previous observations that antibody-sensitized *R. prowazeki* can infect, and grow in, cells of CE yolk sac and of the midgut of the human body louse (2; Wisseman, Krause, Fabrikant, and Machowiak, J. Infect. Dis., submitted for publication). The present findings are particularly significant because, in possible contrast to the louse midgut cells and possibly the yolk sac cells as well, the CE cell system is not likely to exhibit strong extracellular proteolysis which might have digested away the antibody coating prior to entry into the cell. Previous studies (Wisseman, Krause, Fabrikant, and Machowiak, J. Infect. Dis., submitted for publication) have shown that IHS serum does not have any detectable rickettsiacidal action.

The findings with the CE cells in cell culture, yolk sac cells of the embryonated egg, and midgut cells of the louse stand in marked contrast to those with human macrophages in cell culture in which antibody-sensitized *R. mooseri* (6) and *R. prowazeki* (L. Beaman and C. L. Wisseman, Jr., submitted for publication) are destroyed after ingestion. These observations suggest that the fate of antibody-sensitized typhus rickettsiae depends upon the kind of cell into which they gain entrance. It would appear that only certain kinds of cells, specialized as "professional phagocytes" (9), may have the capacity to destroy antibody-sensitized rickettsiae. In the case of human macrophages in cell culture, it has been shown that antibody-sensitized *R. mooseri* are retained in phagocytic vacuoles where they are destroyed, whereas in the absence of antibody, the organisms rapidly escape from the vacuoles into the cytoplasm where they grow (1).

One explanation for the difference in fate of antibody-sensitized rickettsiae in CE cells as compared with human macrophages might lie in differences in the mode of entry into the cell. We have growing evidence that typhus rickettsiae can enter the cytoplasm of cells by two different mechanisms: (i) direct penetration through the cell membrane into the cytoplasm, in a manner similar to that described by Cohn et al. (4) for *R. tsutsugamushi* (C. L. Wisseman, Jr., A. D. Waddell, and W. T. Walsh, manuscript in preparation) and (ii) phagocytosis followed by rapid escape from the phagocytic vacuole, as demonstrated with *R. mooseri* in human macrophages (1). Antibody might be expected to interfere both with direct penetration in which uptake would be inhibited, or escape from phagocytic vacuoles, which might lead to intravacuolar destruction, as in the case of human macrophages, if the cell possessed appropriate lysosomal, enzymatic, or other antimicrobial apparatus (professional phagocytes).

Phagocytic activity by CE cells in culture has been reported to be very low (8). In another study (C. L. Wisseman, Jr., A. D. Waddell, and W. T. Walsh, manuscript in preparation) on the kinetics of rickettsial uptake by CE cells and mechanisms of entry, evidence is presented that the usual mode of entry of non-antibody-sensitized *R. prowazeki* into these cells is most likely by direct penetration through the cell membrane into the cytoplasm. Therefore, the observed enhanced uptake of antibody-sensitized rickettsiae by CE cells in slide chamber cultures, but not in the plaque system, was unan-

ticipated and is not yet fully explained.

Regardless of the mechanisms involved, which also are considered in the separate study on uptake, the end result is that typhus immune serum does not interfere with the capacity of *R. prowazeki* to infect, grow in, and destroy CE cells in culture. Ongoing studies with mammalian cells of different origins suggest that this phenomenon is not unique to CE, yolk sac, or louse gut cells. Indeed, the capacity to destroy antibody-sensitized typhus rickettsiae may be limited to certain specialized cells, some professional phagocytes.

These findings, together with those previously reported for macrophages (5, 6), have both theoretical and practical implications. On the one hand, they are consistent with our developing concepts about the mechanisms of nonsterile immunity and persisting infections in man and animals. On the other hand, they provide the basis for technical improvements in methods for detecting, isolating, and quantitating *R. prowazeki* from antibody-containing in vitro and in vivo systems as well as for detecting certain kinds of immune bodies simply by selecting a host cell with the appropriate characteristics.

ACKNOWLEDGMENTS

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