THE CYTOKINETICS OF MONOCYTOSIS IN ACUTE SALMONELLA INFECTION IN THE RAT*

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Acquired resistance in tuberculosis (1), brucellosis (2), listeriosis (3), and salmonellosis (4) is primarily cell mediated. The macrophages, which act as effectors of this type of antimicrobial immunity are derived mainly from circulating monocytes (5). The abundance of macrophages in tissue lesions may be due in part to the state of delayed-type hypersensitivity (DTH)[1] which develops during these infections (6). In this connection, there is evidence that a high proportion of the macrophages which accumulate within a DTH reaction site arise from the further differentiation or maturation of monocytes that emigrate from the blood (7). The expression of peripheral delayed hypersensitivity is, in fact, dependent upon the presence of such macrophages in the test sites (8, 9). Monocytes in most mammalian species constitute only a small proportion of the circulating white blood cells. It follows that in systemic infections of this type the demand for macrophages within infective foci could deplete the blood of monocytes unless compensatory mechanisms were operative. Still, there is little information concerning the activity of such mechanisms within the monocyte system. The strongest evidence for an operative homeostasis is in fact the constancy of the blood monocyte count. Most evidence on this point is inferential and based on data derived from studies of nonspecific inflammation. Thus, virtually nothing is known concerning the capacity of the monocyte system to meet the demands imposed by infection or the means by which the delivery of monocytes can be increased in such circumstances.

The present studies were therefore addressed to the problem of monocyte mobilization in response to Salmonella infection in the rat. This model was chosen on the basis of unpublished observations that the rapid development of immunity in rats infected with *Salmonella enteritidis* was associated with abundant granulomatous accumulations of macrophages in the liver and spleen, an absolute monocytosis in the blood, and the development of high levels of cutaneous DTH. The cytokinetic changes underlying the obvious and rapid mobilization of monocytes in these animals were

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[1] *Abbreviations used in this paper:* DTH, delayed-type hypersensitivity; [^{3}H]TdR, tritiated thymidine; LBN rats, Lewis and Brown Norwegian F_1 hybrid rats; T, half time; T_G, generation time.

investigated by means of pulse labeling with tritiated thymidine ([^{3}H]TdR) combined with autoradiography. At least three different means of increasing the output of monocytes into the blood were detected.

Materials and Methods

Rats.—Male (Lewis × BN)F_1 hybrid (LBN) rats (180–200 g) derived from highly inbred Lewis and Brown Norwegian parents in closed colonies maintained at the Trudeau Institute were used in all experiments. They were fed a conventional commercial diet and permitted water ad lib.

Infectious Agents.—Salmonella enteritidis NCTC 5694 was obtained from the National Type Culture Collection, Collindale, England. A streptomycin-resistant variant, able to grow in the presence of 20 μg of drug (10), was used for reinfection. Conditions of maintenance and subculture of these organisms have previously been described in detail (11). The number of viable bacteria in the challenge inocula was checked by plating suitable dilutions of the bacterial suspensions on tryptose-soy agar and counting the number of colonies after overnight incubation at 37°C.

Infection and Isotopic Labeling.—Three panels of 18 rats were infected intravenously with 0.2 ml saline suspensions containing about 10^6 live *S. enteritidis* (0.2–0.5 LD_{50}) either 2 days before, 2 days after, or at the same time as they received a single intravenous (i.v.) injection of [^{3}H]TdR in a dose of 1 μCi/g body weight (specific activity 3 Ci/mM; New England Nuclear Corp., Boston, Mass.). Tail vein blood for white blood cell (WBC) counts and smears was taken daily for 6 days immediately before the sacrifice of three rats from each panel. A noninfected control group of 18 rats was sampled in the same way. 10 days after initiating the primary infection, an additional panel of 18 rats was reinfected, this time with an i.v. dose of approximately 10^6 streptomycin-resistant *S. enteritidis* (12). This panel was not isotopically labeled, but specimens for hemacytometry and bacteriology were taken as before.

Host Response.—The immune response to Salmonella infection was evaluated on the basis of the number of viable *S. enteritidis* in the liver and spleen and the development of DTH. Bacterial enumeration was carried out by colony counts on tissue homogenates as previously described (11). DTH was evaluated by measuring the increase in thickness of flank skin 24 h after intradermal injection of partially purified *S. enteritidis* antigen (12).

White Blood Cell Counting.—Nucleated cells were counted in tail vein blood using a Coulter Counter model B (Coulter Electronics Inc., Hialeah, Fla.). Differential counts were carried out on blood smears from the same source after staining with May-Gruenwald-Giemsa. Counts corresponding to the time of infection (day 0) were performed just prior to inoculation. The experimental design resulted in overlapping data from panels of infected rats at many of the intervals. Because of close agreement between these data, the medians of the pooled values at each point were used to determine the trend in WBC counts.

Autoradiography.—Smears of tail vein blood were air dried, fixed in absolute methanol, and accumulated until the end of the experiment. They were dipped in the same batch of Kodak NTB2 liquid emulsion, dried in a humidified chamber, and exposed in light-tight boxes at 4°C for 14 days. The slides were then developed in Kodak D19 at 17°C and stained with May-Gruenwald-Giemsa at pH 5.8. Labeling indices (percent of monocytes labeled) and grain counts were determined from these specimens after correction for background. At least 200 monocytes were counted per sample. Background was evaluated by counting the grains in ten fields, each 100 square microns in area, and taking the average. This measurement was facilitated by the use of an eyepiece and stage micrometers. In most instances the background was < two grains. The definitive grain count for determining the labeling index was set at two grains above the average background.

Cytokinetic Determinations.—The application of [^{3}H]TdR to the study of kinetics of hemopoiesis and other types of cellular proliferation is well established and discussions of the theory

and practice of this technique can be found in a number of sources (13–15). Calculations in the present experiments were made with the knowledge that monocytes rarely divide in the blood (7). The half time of monocytes in the blood was estimated from the daily fall in the percentage of labeled monocytes starting from the point of their unequivocal decline. The population with > six grains after initial correction for background seemed to be most suited to these autoradiographs, thus reducing the weighting effect imposed by the accumulation of cells with low grain counts. When plotted on semilogarithmic coordinates, labeled monocytes disappeared from the blood in an exponential manner. Regression lines were fitted to these data by the method of least squares using a Compucorp 445 statistical calculator (Computer Design Corp., Los Angeles, Calif.). The logarithmic equations derived from the regression data were converted to the exponential form $N_t = N_0e - \lambda^t$ (λ = fractional loss constant) from which the equations for the half time, $T = \ln 2/\lambda$, and the average lifespan, $\tau = T/\ln 2$ can be derived. The generation time, T_G, for dividing monocyte precursors was estimated according to the method of Clarkson et al. (16) by determining the halving time of the regression line fitted to a semilogarithmic plot of the median grain counts of the blood monocytes. The mathematics are thus identical with those above and $T_G = T$, the halving time. As a general assessment, the determinations of T and T_G by these methods probably result in overestimation (15). The significance of the regression data was tested using appropriate standard methods including Student's t test, and analyses of variance and covariance (17).

RESULTS

Following the intravenous injection of about 10^6 viable *S. enteritidis* into normal LBN rats, there was a steady decline in the number of organisms recoverable from the liver and spleen (Fig. 1). By day 3, less than 10% of the challenge inoculum could still be detected in vivo. Viable Salmonellae were distributed in the liver and spleen in approximately equal numbers at this time, and both populations declined at comparable rates to reach levels of 100 bacilli or less per organ by day 14. Although the infecting organisms were rapidly inactivated in the liver and spleen, bacterial persistence was demonstrated in the lymph nodes and kidneys for periods exceeding 6 mo (11). *S. enteritidis* infection in the rat thus runs a nonprogressive but protracted course leading to an apparently stable carrier state. The accelerated elimination of the reinfecting drug-resistant *S. enteritidis* (Fig. 1) indicates, however, that a specific immunity was developed during the primary infection. In addition, a high degree of cutaneous DTH could be demonstrated from day 4 of the primary infection and persisted with little variation throughout the study (11).

Corresponding fluctuations in the total WBC and absolute monocyte counts during primary infection and reinfection are shown in Fig. 1. Counts of other major classes of white blood cells (not shown in Fig. 1) responded with qualitatively similar variations, except for a minor spike in blood granulocytes observed on day 1. The absolute monocyte count initially fell but eventually rose to over twice the preinfection levels and became stabilized with little variation between days 5–10, correlating with the developing state of equilibrium in the infection. After reinfection, the anticipated depression of absolute count was preceded by a sharp rise, in marked contrast to the response in the primary infection.

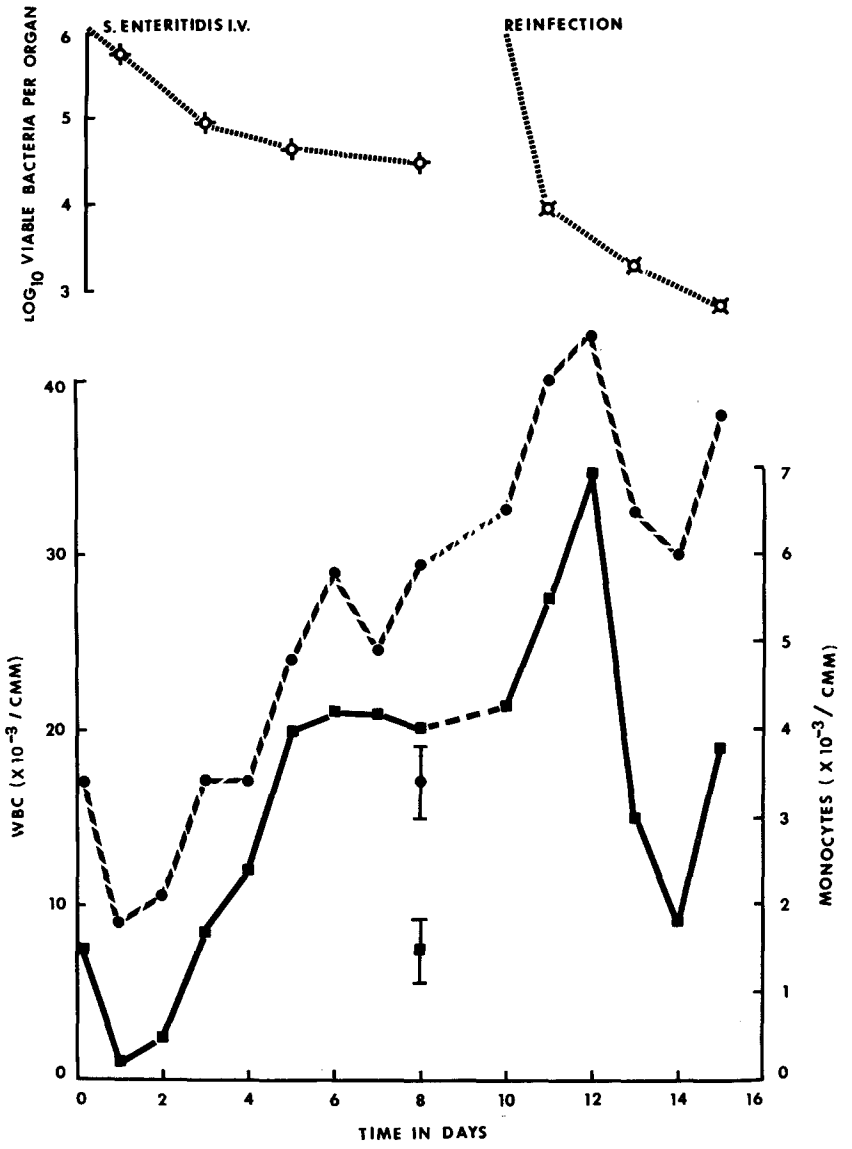

FIG. 1. (*Top*) Each point represents the sum of viable *S. enteritidis* in the livers and spleens of infected rats on successive days following primary infection and reinfection. (*Bottom*) Pooled total WBC (circles) and absolute monocyte counts (squares) on successive days after the infection of rats (day 0) or their reinfection (day 10) with *Salmonella enteritidis*. Mean WBC, absolute monocyte counts and 95% confidence intervals taken from 18 uninfected rats are indicated on the graph. The broken line linking days 8–10 indicates an extrapolation made in the absence of data.

The median labeling indices of blood monocytes on successive days after [^{3}H]TdR administration (day 0) are plotted in Fig. 2. The preinfection values in panel A were nearly identical with those in panel D (uninfected) until day 2. After infection, the rise in the labeling index in A (Fig. 2) continued for one more day despite the concurrent decline in the absolute number of monocytes (Fig. 1). This increase in the labeling index in A (Fig. 2) correlated with the presence of highly labeled cells (>25 grains) which constituted about 22%

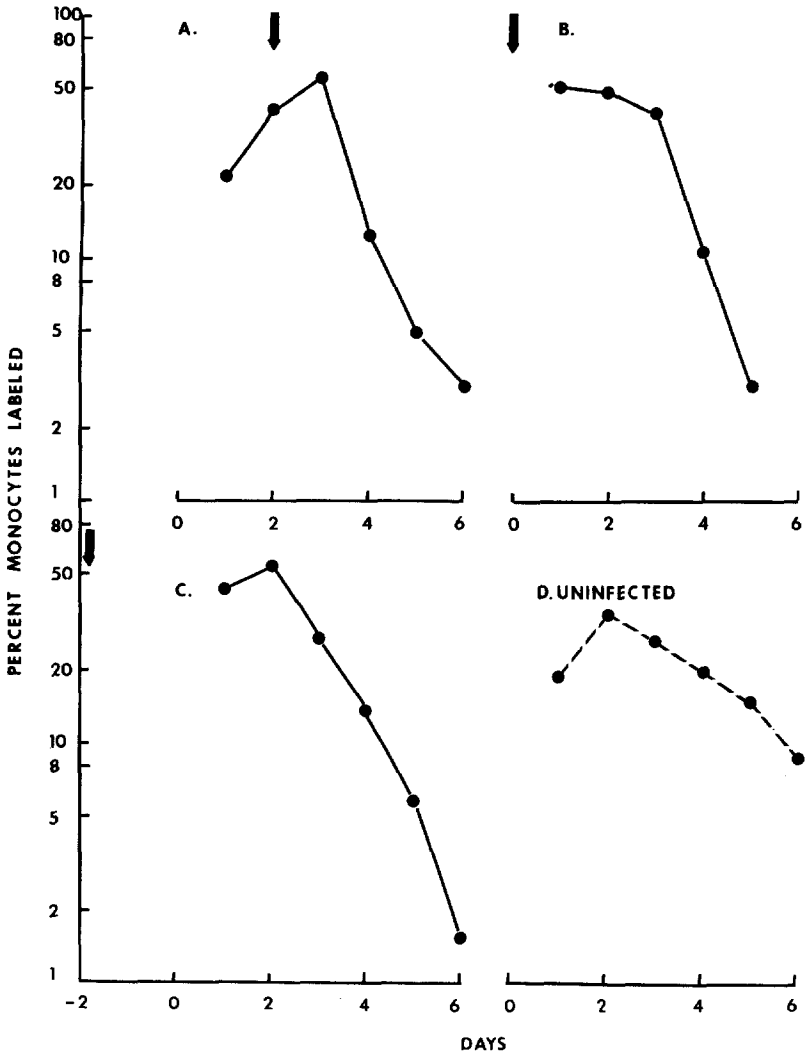

FIG. 2. The median percentage of labeled monocytes in panels of infected (A–C) and uninfected (D) rats. The ordinate indicates the number of days following pulse-label with [^{3}H]TdR on day 0. The time of intravenous inoculation with ca. 10^6 live *S. enteritidis* is indicated by the arrows (see text).

of the labeled monocyte population in that panel on day 3. These occurrences were unique to panel A, suggesting that they represented an immediate response of the monocyte system to infection. Actually, the presence of a corresponding cohort of monocytes in panel B ([^{3}H]TdR injected at the same time as *S. enteritidis*) should not have been directly detectable because such cells would have been unlabeled unless they themselves had been synthesizing DNA during the isotopic pulse. If unlabeled, their emergence into the blood should have flattened or eliminated the peak labeling index anticipated on day 2. This, in fact, is exactly what was observed in panel B (Fig. 2), thus reinforcing the earlier contention concerning the significance of the highly labeled cells.

The loss of labeled monocytes from the blood, shown in Fig. 2, is apparently exponential and thus random in all the panels tested. In addition, the rates of loss appear to be more rapid, as indicated by steeper slopes in the infected rats than in the controls. The half time (T) and average lifespan of monocytes in the blood were estimated as described earlier by fitting least squares regression lines to the data (Fig. 3). The results of this analysis are given in Table I

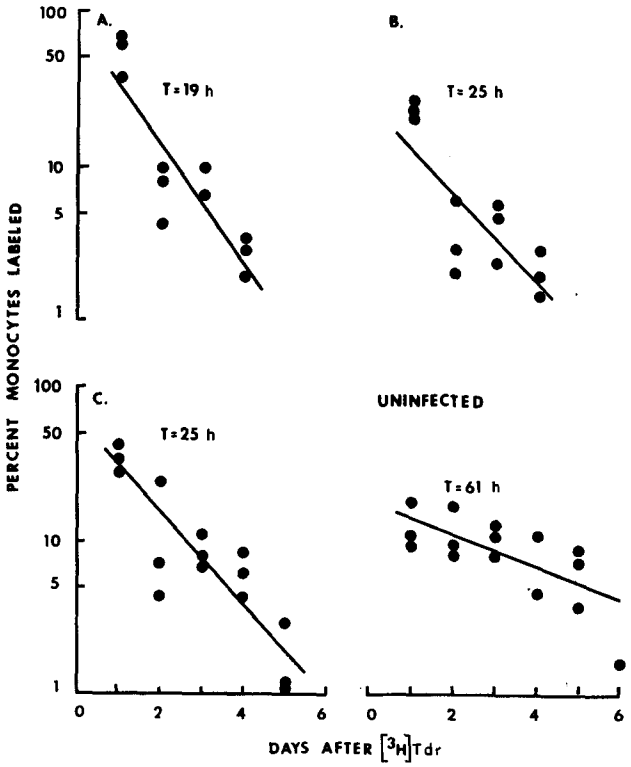

FIG. 3. Estimation of the half time (T) of monocytes in the blood of rats during progressive phases of primary infection. The regression lines were fitted by the method of least squares (see text and Table I).

TABLE I

Regression Analysis, Loss of [^3H]TdR-Labeled Monocytes from the Blood of Rats Infected with Salmonella Enteritidis

	Panel							
	A (11 samples)		B (11 samples)		C (15 samples)		D (uninfected controls)	
r	−0.89		−0.78		−0.87		−0.72	
b	0.38*	(0.24–0.52)	0.19	(0.12–0.46)	0.3	(0.2–0.4)	0.12	(0.06–0.18)
λ	0.88	(0.55–1.2)	0.67	(0.28–1.1)	0.68	(0.45–0.91)	0.27	(0.13–0.41)
T	18.9	(13.9–30.2)	24.9	(15.1–59.5)	24.5	(18.2–37.)	60.7	(40.5–127.9)
Average lifespan	27.4	(20.1–43.6)	35.9	(21.8–85.9)	35.4	(26.–53.4)	87.6	(58.4–184.6)

r, coefficient of correlation ($P < 0.01$ in all panels); b, coefficient of regression expressed as $\log_{10}$, and is negative; ($P < 0.01$ in all panels); λ, daily fractional loss constant = 2.3 × log b; and T, half time of loss from blood in hours = (ln 2/λ) × 24. Average lifespan in blood in hours = T/ln 2.

* Mean (95% confidence limits).

which shows that the coefficient of correlation, r, and the coefficient of regression, b, were invariably significant. Analysis of covariance showed that the regressions of panels A, B, and C did not differ significantly from each other; however, each varied significantly from the control, D ($P < 0.01$ for A and C; $P < 0.05$ for B). Additional estimates of T, based on more highly labeled (>15 grains), thus presumably more homogeneous, cohorts of blood monocytes yielded shorter half times: D = 35 h and C = 12 h. Whether the latter figures approximate the actual T values more closely is not known; what is more important for the present study, is that they stand in roughly the same ratio as the estimations recorded in Table I (D = 61 h and C = 25 h). Clearly, the halftime of monocytes in the blood of rats acutely infected with *S. enteritidis* is substantially shorter than normal.

The average generation time (T_G) for the dividing precursors of monocytes in each panel is shown in Fig. 4. The data for panel A were inhomogeneous with respect to the presence of infection and were dealt with as two subgroups: A′ representing the period before infection (days 1 and 2) and A″ (days 3, 4, and 5) representing the period of active infection. Data from the subgroup A″ were plotted to correspond to days 1, 2, and 3 for ease of comparison (Fig. 4). The T_G for dividing monocyte precursors in bone marrow was estimated at 31–32 h in uninfected rats and 14–16 h in early infection. This reduction was temporary as shown by the T_G for panel C (35 h), a figure comparable with the measurement in the uninfected rats. Numerically, the T_G values apparently fell into two "families" (Fig. 4). For further emphasis of this dichotomy the data were normalized to a first day mean of 100 and pooled according to "family" (Fig. 5). This procedure effectively increased the number of samples at most points and relatively good agreement of data is seen within each family (Table II). The T_G values of 15 and 34 h ($P < 0.01$) determined for the respective families were, as expected, virtually identical with the earlier estimates for their component panels.

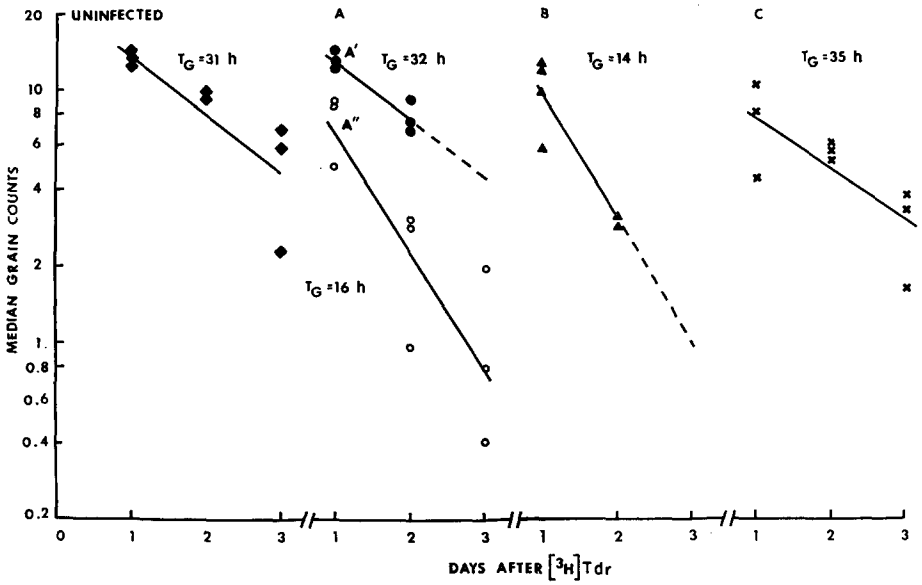

FIG. 4. Estimation of the average generation time (T_G) of proliferating monocyte precursors during progressive phases of infection, based on the halving time of the median grain count (see text). The regression lines were fitted by the method of least squares. The broken lines indicate extrapolations.

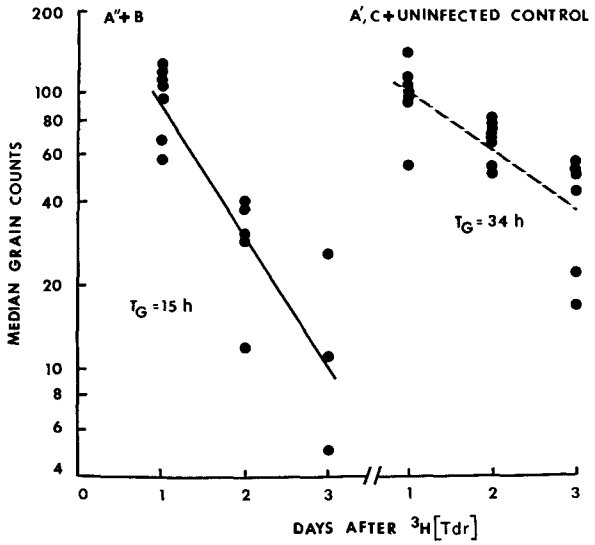

FIG. 5. Estimation of average T_G for proliferating monocyte precursors from normalized data pooled according to apparent family without respect to the presence of infection.

TABLE II

Regression Analysis for Determination of Generation Times by Halving of Median Grain Counts of Blood Monocytes in Rats Infected with Salmonella Enteritidis

	Family			
	A″, B (5 samples)		A′, C, D (23 samples)	
r	−0.88		−0.80	
b	0.48*	(0.34–0.54)	0.21	(0.14–0.28)
λ	1.1	(0.78–1.2)	0.49	(0.32–0.64)
T_G	15.1	(13.9–21.3)	33.9	(26.–52.)

r, coefficient of correlation ($P < 0.01$); b, coefficient of regression expressed as $\log_{10}$ and is negative ($P < 0.01$); λ, daily fractional loss constant; and T_G, average generation time of monocyte precursor pool = halving time of median grain counts.

* Mean (95% confidence limits).

Normally, blood monocytes rarely divide, but infection could have stimulated proliferative activity which, if appreciable, could have led to erroneous underestimations of the half time and generation time. To investigate this possibility, [^{3}H]TdR was injected i.v. 30 min before preparing blood smears from rats which had been infected for varying numbers of days. The highest values for direct incorporation of the isotopic marker (1–2%) was demonstrated by autoradiography to occur on days 4 and 5 of the infection. At other intervals, the proportion of labeled monocytes was comparable with the level in controls of about 0.1%. Intravascular division, therefore, could not have contributed significantly to the gross increase in the numbers of monocytes in the blood or to their substantially reduced half times (Table I). The length of the T_G estimated for this period also suggests that intravascular division exerted little influence on its calculation.

DISCUSSION

This study provides a profile of cytokinetic changes which characterize the monocyte response to acute salmonellosis in the rat. By way of background, the monocyte system can be simplified schematically into a dividing precursor compartment within the bone marrow, a nondividing intravascular pool, and extravascular accumulations of monocytes and their more mature forms, macrophages, within foci of inflammation (7). Considerable ignorance surrounds the identity of monocyte precursors but it has been known for some time that these cells are concentrated in the bone marrow (18) where they constitute a rapidly dividing pool with characteristic kinetics (7). According to recent evidence, not all intravascular monocytes are in circulation (19). That is, the total blood monocyte pool is comprised of a circulating blood monocyte pool (absolute monocyte count × blood volume) which is in equilibrium with a noncirculating or so-called marginal pool corresponding to the observed dynamics of granulocytes in blood (20). Normally, monocytes are lost from the

blood by a random process (7, 21, 22), but their usual fate is unknown. However, it is well established that the macrophages which accumulate within sites of inflammation are derived from circulating monocytes (18, 21, 23).

The earliest alteration in the output of monocytes indicated by autoradiographic analysis was found in the panel of rats which had been labeled 2 days before infection (panel A, Fig. 2). The labeling index for this panel reached its peak a day later than in the uninfected rats or in those panels labeled during subsequent stages of the infection. This occurrence, 24 h after infection, was attributable to the presence of highly labeled monocytes in excessive proportion for the 3rd day after isotopic labeling. This could have resulted from the selective loss from the blood of cells with low grain counts, or alternatively by the mobilization of monocytes with high grain counts from the marginal into the circulating pool. Both explanations appear unlikely because they invoke preferential mechanisms rather than the random behavior generally characteristic of monocyte kinetics. We favor a more plausible explanation, that the appearance of highly labeled monocytes was due to an influx from bone marrow of incompletely matured cells which had not yet undergone a complete series of mitoses. This interpretation implies that a proportion of the monocytes being formed in the precursor pool is normally retained in bone marrow in a noncycling state. In the strict sense, these cells should be considered a reserve. When mobilized, however, they proved insufficient to offset the initial monocytopenia (Fig. 1). The lack of substantial monocyte reserves was also suggested in another study by the precipitous fall in the blood monocyte count which occurred when the replication of precursor cells was interfered with by X-irradiation (24). Taken together these results indicate that the bone marrow reserve of monocytes is normally inadequate to meet an acute and large demand for the delivery of cells to the blood. Following reinfection with *S. enteritidis*, however, the rapid and impressive increase in the number of circulating monocytes (Fig. 1) is consistent with the notion that a greater than normal reserve had been built up in the bone marrow during the primary infection. Unfortunately, labeling data to substantiate this view were not obtained in the current study.

Transitory shifts of monocytes from the circulating to the marginal pool could have accounted for the phasic reductions in circulating monocytes after infection and reinfection. Such shifts have been ascribed to the action of endotoxin upon the distribution of granulocytes in the blood (25) and endotoxin would certainly have been liberated by Salmonellae during the present study. Indeed, the influences of endotoxin upon several aspects of monocytopoiesis must be considered in this study because of the leukokinetic effects attributable to this substance at the stem cell level (26) and upon the release of granulocytes from bone marrow (27).

The influence of infection upon the sojourn of monocytes in the blood was assessed by estimating the half time and average life span of labeled monocytes

(Table I). The half-time value in infected rats was found to be reduced to less than 50% of that in the uninfected controls. In the uninfected rats mean half times of 34 and 61 h were estimated. These two figures straddle the estimate of 42 h determined in an earlier study in which [^{3}H]TdR was continuously infused into normal rats (7). By way of further comparison, a half time of 3.1 days for blood monocytes in the rat was reported by Whitelaw, who employed repeated injections of [^{3}H]TdR (22). In pulse-labeling studies, however, Whitelaw and his colleagues have reported figures of 13 and 14 h (19, 28). This wide range of half-time values may be due to a number of factors some of which may be inherent in the methodology. Other sources contributing to these variations would include the strain and, obviously, the state of health of the subject animals. In the current study the approximation of the estimated to the actual half time is less important than the fact that the differences between the values in infected and control animals are consistent and significant (Table I).

The sparse literature dealing with the kinetics of monocytes in infection includes one report which indicates an increased half time in human infections (29). This finding agrees with demonstrations of increased half times of granulocytes in the blood during infection (30, 31). These results are in distinct contrast with the demonstration in the current study that the sojourn of monocytes in the blood of Salmonella-infected rats was consistently shorter than normal. A wholly satisfactory explanation for these differences is not apparent and is being sought in further investigations.

Early in infection the overall generation time of the monocyte precursors in bone marrow was found to be shortened to approximately half the value estimated in the uninfected rats. This reduction undoubtedly contributed substantially to the increased cellular output that rapidly resulted in a monocytosis. In the final phase of this study, however, the generation time reverted to normal, thus correlating with the stabilization of the blood monocyte count and, presumably, newly imposed steady state conditions. A stable absolute monocytosis was maintained during this period in the presence of a normal generation time among the precursors, a reduced half time of monocytes in the blood and in all probability, an increased monocyte turnover rate. In addition, monocyte reserves proved to be inconsequential, intravascular division was trivial and the half time and generation time data taken together are inconsistent with any notion of a large scale reentry of emigrated monocytes into the blood. Under these conditions the elevated blood monocyte level could only have been sustained by the increased output resulting from an enlarged precursor pool in the bone marrow. The data from the present study, however, provide no indication of the exact mechanisms by which this enlargement was effected. Our own bias favors the view that the expansion of the monocyte precursor pool was probably due to the induction of an undifferentiated stem cell into monocyte formation.

Clearly, the mode used in this study is highly complex and a number of direct and indirect influences upon monocyte formation are undoubtedly in-

herent within its framework. Although the resolution of these influences will require more refined systems, this model did provide a striking demonstration of the capabilities of the monocyte system to expand in response to the demands imposed by infection and also furnished considerable insight into the underlying cytokinetic mechanisms.

SUMMARY

The mechanisms responsible for monocytosis occurring in acute Salmonella infection were studied by means of isotopic labeling and autoradiography. Male (Lewis × BN)F_1 hybrid rats (160–180 g) were pulse-labeled with [^{3}H]TdR at varying intervals with respect to the time of i.v. injection of about 10^6 living *Salmonella enteritidis*. The half time for monocytes in the blood was estimated from the exponential decline in the percentage of labeled monocytes. The average generation time for dividing monocyte precursors in bone marrow was estimated by fitting a regression line to the decline in median grain counts (halving-time = T_G). After an initial fall, the absolute number of blood monocytes rose to a plateau about 2.5 × normal on day 5, suggesting the reimposition of steady state conditions. The half time of monocytes in the blood of infected rats was shortened to 25 h throughout the infection, compared with 61 h estimated in uninfected rats. T_G was reduced to 15 h (days 1–3) but later reverted to the preinfection level of 34 h (days 4–8). Another early response to infection was the release of immature monocytes into the blood. These cells, however, were too few to offset the initial monocytopenia. Under these conditions, with little or no division of blood monocytes, the sustained monocytosis (days 4–8) must have been due to enlargement of the dividing precursor pool. Excessive loss of monocytes from the blood thus appears to activate a feedback mechanism. However, a more direct stimulating effect on monocyte production by endotoxin could have contributed substantially to the monocytosis.

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