

## Increased Production of Endogenous Pyrogen and Lysozyme by Blood Monocytes in Sarcoidosis<sup>1</sup>

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Received October 19, 1978

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Blood monocytes from patients with sarcoidosis were incubated *in vitro*, and secretion of endogenous pyrogen (EP), the protein which mediates fever, and lysozyme (L) were measured. After incubation with endotoxin, monocytes from 5 patients with sarcoidosis released twice as much EP as did monocytes from normal individuals ( $p < .001$ ). Initial 24-hr secretion of L by monocytes from 6 of 11 additional patients with sarcoidosis exceeded the normal range of values for cells from 11 age- and sex-matched control individuals. Cells with initially augmented secretion rates continued to secrete increased amounts of L for 3 days. A correlation was noted between *in vitro* secretion of L by monocytes and serum levels of L in the same patient. These studies indicate that circulating mononuclear cells in some patients with sarcoidosis have an increased capacity to secrete EP and/or L prior to tissue localization.

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Granulomas in sarcoidosis are morphologically similar to "high-turnover" granulomas produced experimentally [1]. In such models of chronic inflammation, large numbers of monocytes newly released from bone marrow continuously enter tissues to participate in granuloma formation [2,3]. Patients with sarcoidosis exhibit a number of abnormalities which are presumably due to this pathologic stimulation of the mononuclear phagocyte system (MPS). Serum lysozyme levels are elevated in most patients with active disease [4]. This enzyme, which hydrolyzes N-acetyl glucosamine linkages of bacterial cell walls, is present in many secretory and phagocytic cells throughout the body [5]. High levels in serum or urine, however, are seen primarily in diseases where production of leukocytes is altered, including monocytic leukemia [6] and granulomatous diseases [4,7,8]. Monocytes and macrophages cultured *in vitro* secrete lysozyme steadily for many days [9,10].

Fever is a common symptom in certain forms of acute sarcoidosis [11]. Like other clinical fevers, it is probably due to the action of endogenous pyrogen (EP), a small protein which is released from stimulated phagocytes in blood and tissues. This protein in some way alters the function of certain hypothalamic neurons, resulting in elevation of body temperatures [12]. Both blood monocytes and tissue macrophages release EP during *in vitro* incubation, after the cells are stimulated by inflammatory agents such as endotoxin or phagocytosis [13-16].

Several abnormalities of blood monocyte structure and function in patients with sarcoidosis have been reported previously [17-20]. These reports raise the possibility that stimulation of the MPS in sarcoidosis alters the function of monocytes early in their development, even before they leave the blood to evolve into tissue macro-

<sup>1</sup>Supported by grants from the National Institute of Allergy and Infectious Diseases (AI 01564), National Cancer Institute (CA 14655) and National Institute of Health (HL 19237).

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phages. In order to further examine monocyte function in this disease, we measured release of EP and lysozyme from blood mononuclear cells incubated *in vitro*. The results of these studies indicate that monocytes from some patients with sarcoidosis have an increased capacity to secrete EP or lysozyme.

## MATERIALS AND METHODS

### *Pyrogen Studies*

All materials, glassware, and reagents were made sterile and pyrogen-free by standard techniques [14]. Ficoll solution was prepared with sterile pyrogen-free water and filtered before use through a  $.45\mu$  filter (Nalgene).

*Donors:* This study was approved by the Committee on Human Investigation at Yale Medical School. Blood samples were obtained from 5 patients with sarcoidosis, aged 30 to 48, and 5 healthy medical students. One individual in each group was studied on two separate occasions; the findings on the repeat study were similar to those of the first in each case. The diagnosis of sarcoidosis in all patients included here and below was made using standard clinical criteria and biopsy evidence of non-caseating granulomata in at least one organ, usually liver. All patients had enlarged hilar nodes or parenchymal lung disease. Three patients were studied within a few months of apparent disease onset; two were intermittently febrile, one of whom was being treated with prednisone. Two patients were considered to have chronic disease, one of whom was receiving prednisone.

*Preparation and incubation of leukocytes:* 50–100 ml heparinized blood, for leukocytes, and 10–20 ml without heparin, for serum, were drawn by venipuncture. Ficoll-Hypaque technique [21] was used to prepare mononuclear cells. Cell number was determined using a Coulter particle counter (Model Z<sub>F</sub>, Coulter Electronics, Inc., Hialeah, FL), differential smears were made, and cells were suspended in Eagle's minimum essential medium (MEM: Auto-POW, Flow Laboratories, Inc., Rockville, MD), with 15% fresh autologous serum, glutamine 2 mM, penicillin 50 U/ml, and streptomycin 50  $\mu$ g/ml. Aliquots of  $1.5\text{--}2.5 \times 10^7$  cells (20–35% monocytes, 65–80% lymphocytes, 0–4% granulocytes) in 5 ml were then incubated in 30 ml tissue culture flasks (Falcon Plastics, Div. of BioQuest, Oxnard, CA) at 37° C in 5% CO<sub>2</sub>-air. To two to four flasks in each experiment 0.1 ml endotoxin, a supernatant prepared from typhoid vaccine as described previously [13], was added to stimulate EP release. After 3–4 hours of incubation, supernatant was removed, and fresh media added for overnight incubation. Media change was repeated at subsequent time intervals, and all supernatants saved for pyrogen assay. Since initial experiments using cells from patients as well as control subjects confirmed previous observations [13] that non-adherent cells, primarily lymphocytes, did not produce EP and that their presence or absence did not influence EP release by adherent monocytes, in subsequent experiments lymphocytes were not returned to the flasks at the time media were changed. In all experiments, a control flask of cells incubated without endotoxin was included. In some experiments, a parallel flask was prepared with cells and heat-killed staphylococci, to measure pyrogen release after phagocytosis.

*Pyrogen assay:* Supernatants were prepared for injection by centrifugation at 1,000 g for 20 minutes. They were cultured in thioglycollate broth to confirm sterility, and stored at 4° C for 1–7 days before assay. Samples with positive cultures were discarded. In some experiments, at the end of an incubation period cells were disrupted by freeze-thawing, and cell-associated pyrogen assayed by techniques described previously [13].

Techniques for injection and temperature monitoring of rabbits and quantitative pyrogen assay were as described previously [13]. Briefly, serial twofold dilutions of each sample were injected into groups of 2-4 rabbits. Dilutions giving temperature responses which fell on the linear portion of the dose-response curve for monocyte EP were then used to calculate derived  $\Delta T$ , a measure of total pyrogen content of the supernatant, for each assay rabbit. Supernatants from different experimental flasks in any one experiment, such as those with and without endotoxin, but not from duplicate flasks, were usually injected into the same group of rabbits. Samples from different experiments were tested in different groups of animals, since development of hypersensitivity fevers made it necessary to use each group of rabbits for only one week. Assay results are expressed as EP release per  $10^7$  monocytes, estimated after differential counts of 300 cells on Wright's stained cover slips.

### *Lysozyme Studies*

*Donors:* 30 ml heparinized blood were drawn from 11 patients with sarcoidosis, and 11 normal donors, matched for age and sex with the patients. Since this study was conducted separately, only one of the patients from the first study was included in the second study. Ages ranged from 18 to 50; 5 men and 6 women were included in each group. Only one of the patients was receiving treatment with prednisone at the time of the study. Five patients had parenchymal lung disease only, and five patients had both parenchymal lung disease and hilar adenopathy. One had no evidence of lung disease at the time of the study. Apart from minor skin involvement, no patients showed clinical evidence of systemic disease, although almost all had liver granulomata on a prior diagnostic liver biopsy. Estimated duration of disease varied from three months to one year in 5 patients, to over one year in 6 patients. No patients were hypercalcemic or had elevations of BUN or creatinine. Blood was also obtained from one patient with a history of sarcoidosis who was hospitalized for lung abscess.

*Preparation and incubation of leukocytes:* Preparation of leukocytes was as described above for pyrogen studies, except that mononuclear cells were suspended in MEM-5% heat-inactivated fetal calf serum (MEM-FCS), and incubated overnight without medium change, and without added endotoxin. Supernatants were then removed, and fresh media added, consisting of 3 ml MEM with 0.25% w/v lactalbumin hydrolysate (MEM-lact). Cells were incubated for 24 hours, and the media change then repeated once more. All supernatants were centrifuged at 1,000 g for 10 minutes, cultured for sterility, and stored at  $-20^{\circ}\text{C}$  for 1-4 weeks.

*Lysozyme assay:* Supernatants were assayed for lysozyme content by the method of Gordon, Todd, and Cohn [9], using a cell wall preparation of *M. Lysodeikticus* (Difco) and a Gilford spectrophotometer with automatic recorder. 0.2% Triton (final concentration) was added to all supernatants before assay. Standards of egg white lysozyme were assayed before and after the experimental samples, and duplicate samples of supernatants from one patient and one control individual were always assayed on the same day. Lysozyme content of each supernatant was calculated from the standard curve, and results expressed as lysozyme release per  $10^7$  monocytes per 24 hours, based on a differential count of 300 cells. Assays of control fluids (MEM-FCS, MEM-lact) always measured less than  $0.2\ \mu\text{g/ml}$  lysozyme, the lowest level detected by this assay.

*Non-specific esterase stain:* Several cover slip smears, prepared for differential counts, from each of two control individuals and two patients with sarcoidosis were

fixed in paraformaldehyde-acetone, and stained by the procedure of Li et al. [22] for non-specific esterase, using the substrate  $\alpha$ -naphthyl butyrate, to provide a marker for monocytes. Parallel smears were stained as usual with Wright's stain.

## RESULTS

### *Pyrogen Studies*

Experiments were first done to compare the capacity for endogenous pyrogen (EP) release by blood monocytes from patients with sarcoidosis and from normal donors. Cells were obtained from five individuals in each category; two donors were studied twice. Endotoxin was added to mononuclear leukocytes, and after an initial 3–4 hours incubation to allow stimulation and adherence of monocytes to the flask surface, media were changed, and successive incubation supernatants collected at timed intervals for quantitative pyrogen assay.

The results are shown in Fig. 1. Monocytes from the patients with sarcoidosis produced twice as much EP as did those from normal individuals during the first 14-hr incubation ( $p < .001$ ). Less EP was released during a second 12-hr incubation by cells in both groups, and the amounts were not significantly different from each other. Cell-associated pyrogen, measured after both first and second incubation periods in a number of experiments, was similar to that reported previously [13], and was the same for cells in both groups. As documented previously [13], and confirmed in preliminary experiments here, significant EP was not produced by cells in either group during the first 3–4 hours after addition of endotoxin; non-adherent cells, predominantly lymphocytes, did not produce EP; and no endotoxin detectable by

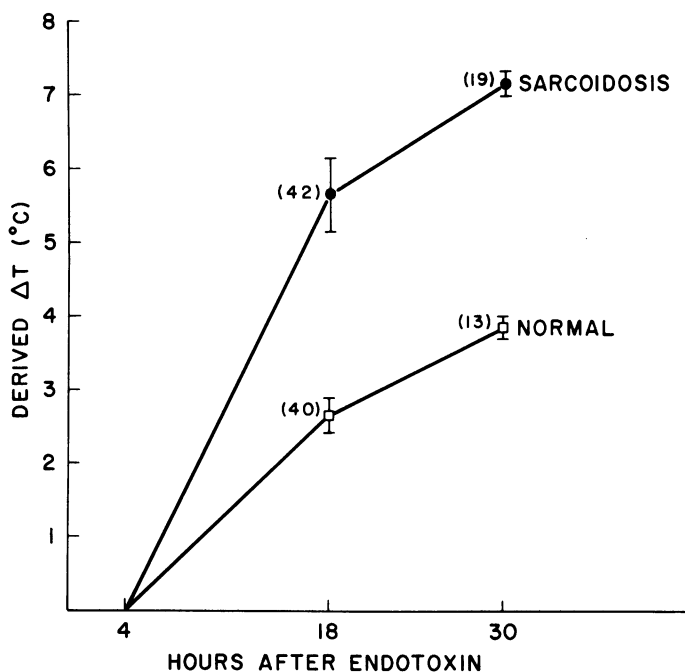


FIG. 1. Cumulative pyrogen release by blood monocytes from 5 patients with sarcoidosis (•—•) and 5 normal individuals (□—□), incubated with endotoxin. One individual in each group was studied twice. Average maximum height of fever,  $\pm$ S.E.M., is shown corrected for dose and cell number (see Methods) following injection of incubation medium into rabbits. Numbers of rabbits used for assay are shown in parentheses. Results of 6 experiments in each group are presented.

pyrogen testing was present in any supernatant. Also, cells from patients with sarcoidosis, like those from normal individuals or febrile patients with other diseases [23], did not release EP during incubation unless stimulated *in vitro* by an agent such as endotoxin. Our results, then, suggest that monocytes from these patients with sarcoidosis had an enhanced capacity for production and release of EP after stimulation with endotoxin.

Since it was necessary to use new groups of 4–6 rabbits for pyrogen assays in different experiments (see Methods), we could not accurately quantitate the EP produced by any one cell preparation in these studies. However, the measured values for 3 of the 5 sarcoidosis patients exceeded those for any control individual. Two of these three patients had had recent onset of disease, and were intermittently febrile; two were receiving prednisone.

#### *Lysozyme Studies*

We next examined the production and release of lysozyme by blood monocytes from 11 additional patients with sarcoidosis and 11 normal donors, matched for age and sex with each patient. Blood mononuclear cells from each individual were incubated in tissue culture flasks, and at 24-hr intervals, flask supernatants were removed for assay of lysozyme content. Lysozyme release is expressed per  $10^7$  monocytes, as estimated from differential counts of 300 cells.

The average values for lysozyme secretion by monocytes from patients with sarcoidosis and matched controls were 10.1 and 6.8  $\mu\text{g}$ , respectively, for the first 24 hours (see Fig. 2). It is apparent that there is a greater variation among the values in

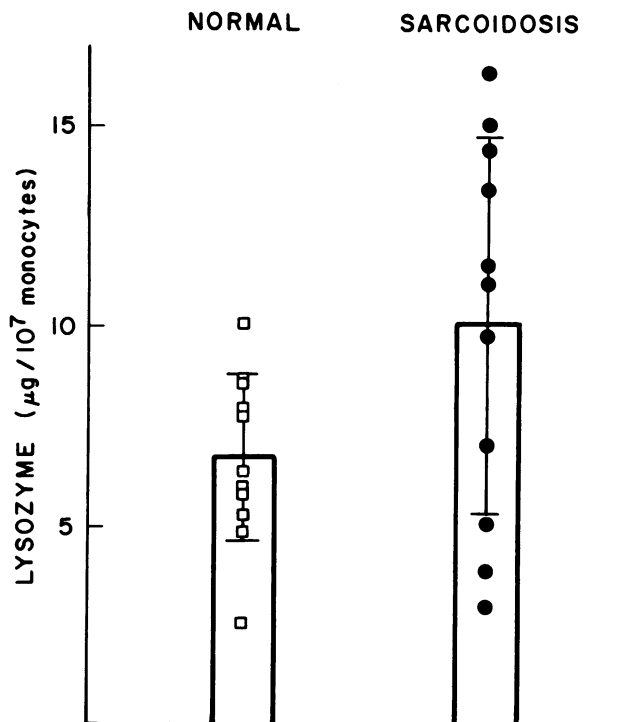


FIG. 2. Average lysozyme release  $\pm 1$  standard deviation during the first 24 hours in tissue culture by monocytes from 11 patients with sarcoidosis and 11 control individuals, matched for age and sex. Individual determinations are indicated by points.

the patient group. (The difference in variance, tested by a variance  $F$  test, is significant,  $F = 4.87$ ,  $p < .05$ ). The six highest values in the patient group exceeded the 95% confidence limits of the control group (mean plus  $2 \times$  S.D.), suggesting that they are abnormal values; the other five values fall within the range of normal.

In order to determine whether cell populations which demonstrate elevated initial lysozyme release *in vitro* continue to produce increased amounts of this enzyme, we examined values for lysozyme production by such abnormal mononuclear cells during their second and third days in culture. Data were available for cells from 5 of the 6 sarcoidosis patients with abnormally high initial values shown in Fig. 2 and for cells from one patient with tuberculosis (initial lysozyme release =  $12.9 \mu\text{g}/10^7$  monocytes). Average enzyme release by these cells, compared with that by cells from 11 normal individuals, is shown in Fig. 3. It is apparent that cells with elevated rates of release initially continued to produce enzyme at rates above those of normal cells. As expected, cells from patients exhibiting normal lysozyme release initially continued to release normal amounts during subsequent incubations (not shown).

The mononuclear cell preparations in these experiments consisted of both lymphocytes and monocytes; only monocytes, however, produce EP and lysozyme [9,13]. The significance of the data showing differences in production of these compounds by different mononuclear preparations, therefore, depends on the accuracy of our differential counts of monocytes. To evaluate this, we compared the percentage of monocytes estimated from routine Wright's stained differentials with the percentage of cells staining positively for  $\alpha$ -naphthyl butyrase, an esterase present in monocytes but almost entirely absent from lymphocytes [22]. Several differential smears from two patients with sarcoidosis and two normal controls were evaluated. The percentage of monocytes determined by the two methods agreed closely; average percentage by Wright's stain was 25 percent and from esterase stain 21 percent. Thus it is unlikely that inaccuracy in estimating numbers of monocytes from differential counts explains the differences in protein secretion by cells in the different groups.

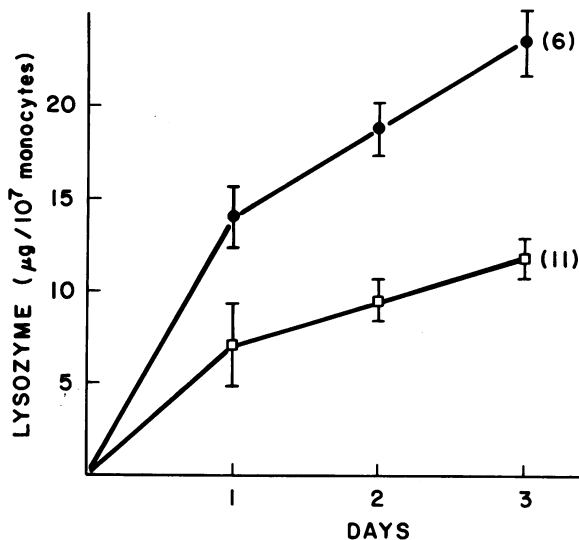


FIG. 3. Cumulative average lysozyme release  $\pm$  1 standard deviation by monocytes incubated *in vitro* for 72 hours. ( $\bullet - \bullet$ ) Data from monocytes exhibiting increased production during the first 24 hours from 6 patients. ( $\square - \square$ ) Data from monocytes of 11 normal subjects.

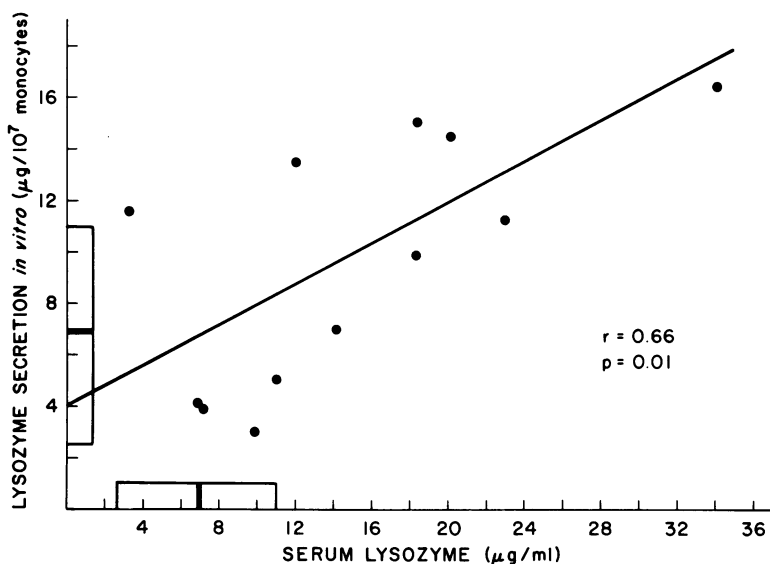


FIG. 4. Correlation between serum lysozyme level and production of lysozyme by monocytes *in vitro* for 12 patients with sarcoidosis. Values for the first 24-hr release of lysozyme by monocytes are shown. Bars indicate expected range of values for normal individuals (mean  $\pm$  2  $\times$  S.D.).

#### Correlation with Serum Lysozyme Levels

Serum lysozyme levels are frequently elevated in sarcoidosis [4]. We therefore examined the relation between the rates of lysozyme secretion by monocytes *in vitro* and the levels of serum lysozyme in the same patients. The latter were determined either at the time of the *in vitro* study or, in patients with stable disease, within a few weeks of the *in vitro* study. The results, shown in Fig. 4, show a significant correlation between values for monocyte lysozyme secretion during the first 24 hours in culture and the serum lysozyme level in twelve patients ( $R = 0.66$ ,  $p = 0.01$ ). It is apparent that 5 of 6 patients with elevated rates of lysozyme secretion *in vitro* also had elevated values of serum lysozyme.

#### DISCUSSION

Our studies indicate that monocytes from some patients with sarcoidosis have an increased capacity to secrete certain proteins *in vitro*. EP, the protein which mediates fever, is synthesized and secreted by monocytes after stimulation by agents such as endotoxin or phagocytosis [13-16]. The values for EP production by normal monocytes reported here agree closely with those reported previously [13]. As noted in Results, the increased production by patients' cells was apparently not due to increased levels of intracellular EP, or to abnormal lymphocyte function. A specifically increased responsiveness of the cells to endotoxin also does not appear to explain our findings, since in several experiments parallel studies were done with heat-killed staphylococci, and these gave similar results. Thus, the most likely explanation of our findings is that monocytes from some patients with sarcoidosis have an increased capacity to synthesize and secrete EP. However, since in all the experiments of pyrogen production, cells were incubated with autologous serum, it is possible that factors in serum from these patients may have been responsible for the augmented production of EP.

Lysozyme, a hydrolytic enzyme, is synthesized and secreted by blood monocytes and peritoneal macrophages for weeks [9,10] in culture. Intracellular levels of enzyme are very low, and lysozyme production is best measured by assessment of secreted enzyme [9,10]. These cells apparently require no *in vitro* stimulus for lysozyme secretion; enzyme release is not augmented after phagocytosis [9]. Our values for lysozyme production by normal human monocytes in culture agree well with those reported previously [9], when note is made of the different protein standards and numbers of cells used to report the assay results. Since autologous serum was not included in these incubations, it is unlikely that the observed differences in lysozyme production were due to serum factors. They were also not due to increased adhesiveness of monocytes from patients with sarcoidosis, since for the first 24 hours non-adherent cells remained in all flasks. Also, when examined on the second and third days of incubation, microscopic observations showed no consistent differences in numbers of healthy, adherent cells in flasks of cells from patients compared with control individuals. Since cells with elevated initial rates of release continued to produce enzyme at increased rates, our data are most consistent with certain cell populations that have an increased capacity for synthesis and secretion of this protein. Because the studies of pyrogen and lysozyme production reported here were conducted separately, we have no information on the production of both proteins by cells from a single patient or control individual.

Although abnormalities of lymphocyte function have been repeatedly demonstrated in sarcoidosis [24], there have been few studies of mononuclear phagocyte function. Two investigators have reported that increased numbers of monocytes migrate into sites of skin abrasions and attach to coverslips within a few hours [17,18]; typical giant cells were also reported to develop subsequently [18]. Monocytes from patients with sarcoidosis showed enhanced phagocytic capacity for latex as well as IgG and complement-coated erythrocytes [19]. Increased levels of  $\beta$ -glucuronidase, as determined by cytochemical staining, have also been noted in cells from such patients [20]. It is of interest that similarly enhanced migratory, phagocytic, and enzymatic function of monocytes characterize "activated" mononuclear phagocytes [25] studied in many experimental models. As originally described, these cells develop altered function as a result of specific immunologic stimulation. Such phagocytes also exhibit enhanced bactericidal capacity. Although blood monocytes from patients with tuberculosis show enhanced killing of staphylococci [26], to our knowledge similar studies have not been done with monocytes from patients with sarcoidosis. We did not include patients with other diseases in our studies, so we do not know whether the secretory abnormalities we noted are specific for sarcoidosis.

In a recent study [27], increased monocytopoiesis and high tissue turnover of macrophages was described in cases of active tuberculosis, but not in 4 of 6 patients with sarcoidosis. The variable clinical syndromes in this latter disease, however, make comparisons between small numbers of patients difficult. Other authors, in fact, have compared sarcoidosis with experimental models of "high-turnover" granulomas [1], situations in which monocyte production and entry into tissues are accelerated [2]. Such enhanced production would probably alter the state of maturation of blood monocytes, or lead to recirculation of tissue macrophages. There is no reliable information about the relative capacity for EP or lysozyme production by mononuclear phagocytes at different stages of maturation. Although mouse macrophages produce larger amounts of lysozyme *in vitro* than do human monocytes [9], no such comparisons have been made between these two cell types in the same species.



Preliminary ultrastructural examination of cells from three patients in our studies failed to identify monocytes with definite morphologic abnormalities, or recirculating macrophages or promonocytes (Bodel, P. and Bainton, D.F., unpublished observations).

Finally, we observed a correlation between *in vitro* secretion of lysozyme by monocytes and serum lysozyme levels (Fig. 4). However, the relation of these values to the clinical status of the patients was uncertain, since the "activity" as well as extent of sarcoidosis was usually difficult to determine. When renal disease and hypercalcemia are absent, earlier work indicated that levels of serum lysozyme correlate fairly well with the extent of tissue involvement by granulomas [4]. This aspect of the biochemical assessment of sarcoidosis has been reviewed elsewhere [28]. Our findings thus suggest that monocytes from patients with multiple granulomata show enhanced secretory activity even before they localize in tissues as macrophages. Such altered function of blood monocytes may be related to the rapid production and turnover of mononuclear phagocytes which is postulated to occur in sarcoidosis [1].

#### ACKNOWLEDGEMENTS

The authors thank Ms. Jean Vuitier and Ms. Heather Miller for technical assistance.

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