Clin. exp. Immunol. (1982) **47**, 169–175.

Kinetic studies on phagocytosis

IV. CELLULAR DEFECTS AND HUMORAL INHIBITION AS CAUSES OF IMPAIRED NEUTROPHIL PHAGOCYTOSIS IN SARCOIDOSIS

R. HÄLLGREN,* LENA HÅKANSSON,† BIRGITTA SCHMEKEL,‡ G. STÅLENHEIM§ & P. VENGE† *Departments of * Internal Medicine, † Clinical Chemistry, ‡ Clinical Physiology and § Lung Medicine, University Hospital, Uppsala, Sweden*

(Accepted for publication 17 July 1981)

SUMMARY

Kinetic measurements of the serum-independent uptake of IgG-coated or complement-opsonized latex particles have been performed in 58 patients with sarcoidosis. The mean rate for phagocytic uptake of IgG particles was 0·56 min^{-1} which was not different from that of the controls (0·59 min^{-1}). The phagocytosis of complement-opsonized particles was in the patient group 0·53 min^{-1} and significantly ($P < 0{\cdot}001$) reduced compared to the rate of the controls (mean rate 0·94 min^{-1}), indicating neutrophil C3b-receptor dysfunction in sarcoidosis. PMNs from patients with sarcoidosis were not stimulated by the presence of autologous serum in contrast to PMNs from normals and in individual cases even a reduced uptake was found. More than one-third of the sarcoid sera also inhibited the phagocytosis of normal PMNs indicating the presence of a phagocytosis-inhibitory activity in sarcoid sera. Patients with more severe lung affection as estimated by measurements of total lung capacity, central airway obstruction, small airway function and pulmonary X-ray changes had a more reduced PMN phagocytosis in the presence of autologous serum than those with minor signs of lung affection ($P < 0{\cdot}05$). The phagocytosis-inhibitory activity of sarcoid serum was also more pronounced in those individuals who had high pulmonary score ($P < 0{\cdot}05$) or radiographic stage II–IV sarcoidosis ($P < 0{\cdot}01$). No correlation was found between serum levels of lactoferrin or lysozyme and any of the phagocytic variables while elevated β_2-microglobulin levels were associated with more pronounced serum-mediated inhibition of PMN phagocytosis ($P < 0{\cdot}05$). The relevance of these findings to the pathogenesis of granuloma formation in sarcoidosis is discussed.

INTRODUCTION

In certain chronic granulomatous inflammatory disorders such as sarcoidosis and Crohn's disease migration of neutrophils into skin windows is grossly defective (Gange *et al.*, 1977; Segal & Loewi, 1976), possibly due to the presence of a serum factor(s) inhibiting chemotaxis (Gange, Carrington & Black, 1980; Maderazo *et al.*, 1976). The neutrophil dysfunction has been suggested as fundamental for the characteristic granuloma formation observed in such diseases (Segal & Loewi, 1976). In the present study we have explored further the neutrophil function in sarcoidosis using a kinetic technique to measure the phagocytic performance of isolated neutrophils (Hällgren, Jansson & Venge, 1977). Since neutrophils bear two main receptors, the Fc receptor and the C3 receptor, to enhance the attachment and ultimately the engulfment of immune complexes (Schribner &

Correspondence: Roger Hällgren, MD, Department of Internal Medicine, University Hospital, S-750 14 Uppsala, Sweden.

0009-9104/82/0100-0169$02.00 © 1982 Blackwell Scientific Publications

Fahrney, 1973) we have tested the phagocytic uptake of particles coated with IgG as well as with IgG and activated complement components. Earlier studies on chemotaxis having suggested the presence of cell-directed serum inhibitors in sarcoidosis (Gange *et al.*, Carrington & Black, 1980), we also investigated the influence of sarcoid serum on the phagocytic activity of autologous and normal neutrophils. Data on phagocytic performance were correlated to pulmonary function tests, radiographic pulmonary changes and serological variables reflecting activity/turnover of lymphomyeloid cells.

MATERIALS AND METHODS

Subjects. Fifty-eight patients with sarcoidosis were included in the study. There were 25 females and 33 males. The mean age was 40·1 years, range 25–74 years. In 28 patients the morphological diagnosis was settled by biopsy showing characteristic epitheloid granulomata without necrosis. The other patients had no subjective symptoms and their disorder was detected by miniature chest radiographs at a general health survey in the county of Uppsala. These patients had bilateral hilar lymph glandular enlargement, characteristic for sarcoidosis. Due to the mildness of the disease and since extensive investigations and follow-ups excluded disorders other than sarcoidosis, mediastinoscopy was not considered justified.

Donors of normal serum and blood leucocytes were either apparently healthy laboratory workers or blood donors at the Blood Centre, University Hospital, Uppsala.

Preparation of granulocytes. Granulocytes were prepared from heparinized blood as described in detail in a previous work (Hällgren *et al.*, 1977). The purity of the polymorphonuclear neutrophils (PMNs) was $90 \pm 5\%$ (s.d.) on average.

Protein coating of latex particles. Polyvinyltoluene latex particles with a diameter of 2·03 μm and a volume of $4{\cdot}38 \times 10^{-15}$ l were obtained from Coulter Electronics Ltd, Dunstable, Bedfordshire, England. The particles were coated with human IgG (AB Kabi, Stockholm, Sweden) as previously described (Hällgren *et al.*, 1977) and designated IgG-coated particles. In order to obtain a complement opsonization, washed IgG-coated particles were incubated in 20% fresh pooled normal serum diluted in Ringerdex (Pharmacia, Uppsala, Sweden) at 37°C for 10 min. The serum was washed away by two subsequent washes in Ringerdex immediately before the particles were used in the phagocytosis assay. To such particles, which were designated complement-coated particles, the complement components C3 and C4 were bound (Håkansson, Hällgren & Venge, 1980).

Phagocytosis assay. The assay for measuring the initial rate of phagocytosis has been described in detail previously (Hällgren *et al.*, 1977). Briefly, 2×10^6 isolated PMNs in Ringerdex with glucose (6·94 mmol/l) were mixed with 20×10^6 IgG- or complement-coated particles, likewise in Ringerdex–glucose solution, in a final volume of 1 ml in a siliconized glass tube. Incubation was made at 37°C during constant magnetic stirring (1,600 r.p.m.). Aliquots of 100 μl were taken out from the mixture every minute for 6 min and particles not cell-associated were counted in an electronic counter (Trombocounter, Coulter Electronics Ltd). The initial rate of disappearance of the latex particles per minute was used as a measure of the initial rate of phagocytosis.

In some experiments, when the effect of serum on phagocytosis was evaluated, IgG-coated particles were incubated for 10 min in 20% fresh serum at 37°C before mixing with PMNs. The final cell and particle concentrations were the same as above and the final serum concentration was 10%. In these experiments we used a serum standard of pooled normal sera, freshly frozen in small portions at −80°C. The particle uptake rate of isolated normal PMNs in the presence of this serum standard was given the value 100%; the uptake rate of PMNs from the same cell preparation in the presence of sarcoid or normal serum was expressed as a percentage of the standard uptake. The coefficient of the intraday variation of the assays was 8% and of the intra- and interday variation, 14%.

Serum analyses. The serum samples were handled as previously described (Lundin, Hällgren & Venge, 1980). All sera were subjected to the following analyses [reference ranges (95% confidence intervals) are given in parentheses]. Radioimmunoassays of lysozyme (985–2,990 μg/l) and lactoferrin (190–645 μg/l) were performed as previously described (Venge *et al.*, 1979). Beta-2-

microglobulin (1·2–2·4 mg/l) was measured by means of a commercial kit (Phadebas β_2-microtest, Pharmacia AB, Uppsala, Sweden). The coefficient of variation of the methods was $\leqslant 7\%$.

Scoring of pulmonary function and X-ray. On the same day as blood was collected all patients went through chest radiogram and 32 of them pulmonary function tests. Total lung capacity (TLC) and volume airway conductance were measured with a constant volume body plethysmograph (Siemens Siregnost FD 40 + FD 91 S). Spirometry with measurement of forced expiratory volume in 1 sec and forced vital capacity was performed with a rolling seal spirometer. Closing volume and gas distribution were measured using the single-breath nitrogen technique. Diffusing capacity for carbon monoxide was measured by the single-breath method (CPI Pulmolab 5300). The static elastic recoil pressures of the lung during slow interrupted exhalation were measured with a thin-walled balloon in mid-oesophagus, with recording of the maximal elastic recoil pressure and static compliance between 50 and 80% of TLC. The details of the methods have been described elsewhere (Fridriksson *et al.*, 1981). These tests independently gave an estimate of total lung capacity, central airway obstruction, small airway function and lung stiffness. Each of these functions was scored from 0 to 2 points according to the principles described elsewhere (Schmekel *et al.*, 1981) and the higher the score the more severely affected were the tested pulmonary functions. The maximum overall functional score was thus 8 points. To this score up to 2 points from the X-ray score were added; radiological stage I gave 1 point and radiological stages II–IV, 2 points. The pulmonary score means the overall functional plus the X-ray score. Staging of chest radiogram was done according to the principles of Miller *et al.* (1980) by one chest physician who had no clinical information about the patients. The duration of disease, defined as duration of symptoms, was not estimated in patients who had attended the health survey without symptoms.

Student's *t*-test was used in the statistical evaluations.

RESULTS

Fig. 1 shows the phagocytic rate in buffer medium of PMNs isolated from normal controls and patients with sarcoidosis. The mean rate (±s.d.) for phagocytic uptake of IgG-coated particles was

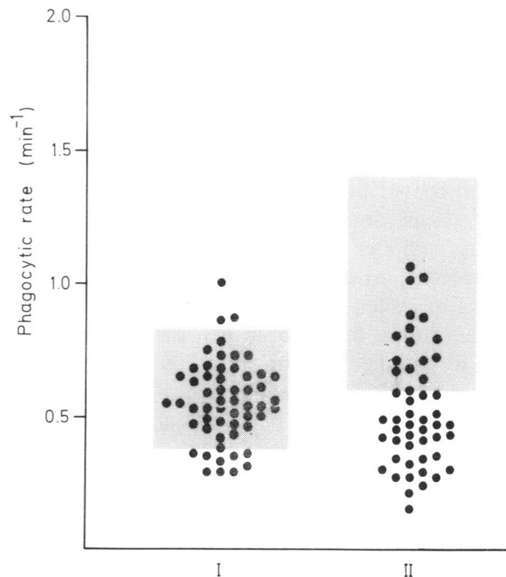

Fig. 1. The phagocytosis of IgG-coated (I) and of complement-coated particles (II) in buffer medium by PMNs isolated from patients with sarcoidosis. The phagocytosis of such particles by normal PMNs is indicated by the shaded areas (95% confidence intervals).

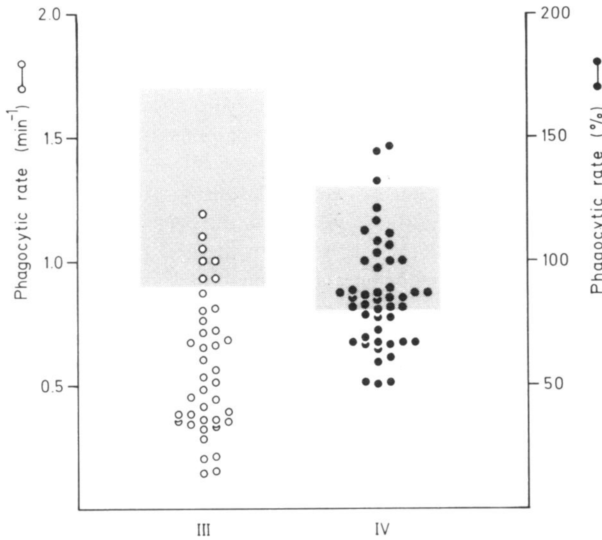

Fig. 2. The phagocytosis of IgG-coated particles in the presence of sarcoidosis serum by PMNs isolated from patients with sarcoidosis (III) and by PMNs isolated from healthy controls (IV). The uptake of such particles by normal PMNs in the presence of normal fresh serum is indicated by the shaded areas (95% confidence intervals).

for the patient group $0{\cdot}56 \pm 0{\cdot}15$ min^{-1} which was not different ($P > 0{\cdot}05$) from the rate observed in the controls ($n = 65$; mean phagocytic rate $0{\cdot}59 \pm 0{\cdot}15$ min^{-1}). PMNs of the controls ($n = 21$) phagocytosed complement-coated particles at an enhanced rate (mean phagocytic rate $0{\cdot}94 \pm 0{\cdot}29$ min^{-1}) compared to their uptake of IgG-coated particles ($P < 0{\cdot}01$). Corresponding opsonin-dependent enhancement of phagocytosis was not observed with PMNs from patients with sarcoidosis ($P > 0{\cdot}05$). The mean phagocytic uptake of complement-coated particles in the patient group was $0{\cdot}53 \pm 0{\cdot}22$ min^{-1}, which was a significantly decreased ($P < 0{\cdot}001$) uptake compared to the PMN uptake of such particles in the controls.

Isolated PMNs from patients with sarcoidosis had a mean phagocytic rate of $0{\cdot}59 \pm 0{\cdot}28$ min^{-1} in the presence of autologous serum (Fig. 2) which was not significantly different from their uptake rate in buffer medium of IgG-coated or complement-coated particles ($P > 0{\cdot}05$). This was in contrast to the normal situation where the serum milieu further enhanced the phagocytosis. The phagocytosis of complement-coated particles by normal PMNs was $1{\cdot}22 \pm 0{\cdot}32$ min^{-1} in the presence of fresh normal serum and significantly enhanced ($P < 0{\cdot}01$) compared to their phagocytosis of such particles in buffer medium (Figs. 1 & 2). Analysing individual cases, PMNs from 38% patients had a depressed particle uptake in autologous serum compared to their uptake of complement-coated particles in buffer medium. This observation indicated that sarcoid serum inhibited PMN performance. To test this idea further, the uptake by normal PMNs of particles incubated in sarcoid serum was studied. As shown in Fig. 2, 17 of 47 of the sarcoid sera studied inhibited particle uptake by normal PMNs (mean phagocytic uptake was 86 ± 22%). There was no correlation between the uptake rates of normal and sarcoid PMNs ($r = 0{\cdot}01$, $P > 0{\cdot}05$) when phagocytosis was studied in the presence of sarcoid sera.

None of the variables reflecting phagocytic performance in sarcoidosis correlated to the duration of the disease. No correlation was found between X-ray stage or pulmonary score and PMN phagocytic uptake of IgG-coated particles while an inverse correlation ($P < 0{\cdot}05$) was obtained between pulmonary score and phagocytic uptake of complement-coated particles when phagocytosis was performed in a serum-free medium. In the presence of autologous serum, isolated PMNs from the patients with none or low-grade pulmonary affection had a tendency ($P < 0{\cdot}05$) to a better phagocytic performance than those isolated from patients with higher pulmonary score.

There was a significant inverse correlation between X-ray stage ($P<0.01$) or pulmonary score ($P<0.05$) and phagocytic rate of normal PMNs incubated in sarcoid sera.

In the patients with sarcoidosis, the serum levels of lysozyme, β_2-microglobulin and lactoferrin were on average 2,492 μg/l (range 863–7,202), 1·89 mg/l (range 0·97–3·51) and 528 μg/l (range 126–1,936) respectively. We found no relationship between these serum variables and the various phagocytic tests, except for an inverse correlation ($P<0.05$) between the serum β_2-microglobulin levels and the phagocytic rate of normal PMNs incubated in sarcoid sera (data not shown).

DISCUSSION

Earlier reports have stated that particle ingestion by phagocytes is promoted by cell surface recognition and binding of fragments of C3 but that the very engulfment requires the interaction between particle-associated IgG and the Fc receptor of the cell membrane (Schribner & Fahrney, 1973; Mantovani, 1975; Ehlenberger & Nussenzweig, 1977). In accordance with this concept we have earlier reported (Håkansson, Hällgren & Venge, 1981) and also shown in this study that normal neutrophils phagocytose particles opsonized with IgG as well as C3 at a higher rate than particles coated with IgG only. Isolated neutrophils from patients with sarcoidosis internalized IgG-coated particles at the same rate as normal neutrophils but did not show an enhanced particle uptake of complement-opsonized particles. These data suggest that the internalization mediated by the Fc receptor is normal in sarcoidosis but that the C3 receptor function in neutrophil phagocytes is functionally defective. This notion of a selective receptor dysfunction in phagocytes is not unique. Kupffer cells from patients with primary biliary cirrhosis, a disease also characterized by chronic granuloma formation, are thus reported to suffer from a C3 receptor dysfunction (Jaffe *et al.*, 1978). The mechanism behind the impaired isolated neutrophil function in sarcoidosis is unknown but might be associated with the severity of the disease, since an inverse relationship was found between the pulmonary score and the PMN uptake of complement-coated particles.

Normal fresh serum provides, as shown in this report, a milieu which further enhances the particle uptake by normal neutrophils. However, such an enhancement was not observed when PMNs from patients with sarcoidosis were incubated in autologous serum throughout the phagocytic performance. The suspicion that sera from patients with sarcoidosis might inhibit phagocytosis was confirmed by the study of normal isolated PMNs, which exhibited a reduced particle uptake in the presence of more than one-third of the sarcoid sera. This serum-mediated inhibition seems to be associated with the degree of the pulmonary affection. The presence in sarcoid sera of a cell-directed inhibition of PMN migration and monocyte activity has earlier been reported (Gange *et al.*, 1980; Umbert, Belcher & Winkelmann, 1976); whether these activities could play a role in the inhibition of neutrophil phagocytosis has not been determined. The lack of correlation between the phagocytic performance of sarcoid PMNs in autologous serum and the sarcoid-serum-mediated inhibition of normal PMN phagocytosis indicates that the cellular PMN defect in sarcoidosis is not a consequence of the sarcoid serum component inhibiting phagocytosis.

The majority of the patients with sarcoidosis had either an isolated neutrophil defect with reduced uptake of complement-opsonized particles or a serum-mediated inhibition of autologous or normal neutrophils. About one-third of the patients had a combination of both serum-dependent inhibition of phagocytosis and a cellular defect. Whether the neutrophil defect and the serum-inhibitory activity in sarcoidosis are primary or secondary to the granuloma formation is at present unsettled. Segal & Loewi (1976) have stressed the possibility that the characteristic granulomata in inflammatory diseases could result from impaired neutrophil function and an overwhelming phagocytic burden for macrophages. Another possibility to consider is that the granuloma lesions may release factors influencing the neutrophil activity. Increased circulating levels of lysozyme, a monocyte secretory product, have been reported in sarcoidosis (Pascual, Gee & Finch, 1973; Selroos & Klockars, 1977) and such elevated levels may correlate with the total mass of fresh sarcoid granulomas (Selroos *et al.*, 1980). However, we have no support for the idea that the granuloma mass might influence the PMN phagocytic performance indirectly, since no correlation was found between the lysozyme levels in sarcoid sera and the phagocytic activity of PMNs isolated

from patients with sarcoidosis. *In vitro*, lysozyme modulates neutrophil function by inhibiting chemotaxis (Gordon *et al.*, 1980). However, we found no correlation between serum lysozyme levels and phagocytosis-inhibitory activity of sarcoid sera. Based on experimental studies (Gordon *et al.*, 1980), it also seems unlikely that increased serum concentrations of lysozyme might reduce the phagocytic activity of neutrophils directly. We found no correlation between any phagocytic variable and the circulating levels of lactoferrin, a marker of neutrophil turnover or activation (Hansen, Malmquist & Thorell, 1975). Beta-2-microglobulin is another serum variable which has been reported to be elevated in sarcoidosis and supposed to reflect an increased activity of lymphoid cells (Mornex *et al.*, 1979). The relationship observed between the serum-mediated inhibition of PMN phagocytosis and circulating β_2-microglobulin may suggest that the inhibitory serum component originates from activated lymphoid cells.

Although sarcoidosis has been considered a syndrome caused by various infectious organisms, the attempts to isolate infectious agents from sarcoid tissue have met with failure. However, favouring the hypothesis that the granuloma formation is a consequence of a defective neutrophil clearance of antigenic material, the predominantly macrophage response observed in sarcoidosis could be caused by a variety of inflammatory infective or non-infective agents, making the search for a single aetiologic factor fruitless. Regarding the presence in sarcoid serum of inhibitors of PMN migration and phagocytosis, it may be justified to propose plasmapheresis as an alternative treatment in selected patients with severe manifestations of their sarcoidosis.

The authors wish to thank Dr E. Nou, Department of Lung Medicine, for the staging of chest radiograms. Mrs Kerstin Lindblad, Ms IngBritt Persson and Mrs Margit Tjernberg are gratefully acknowledged for skilful technical assistance. This study was supported by the Swedish Medical Research Council and Gustaf V's 80-Year-Foundation and the Swedish National Association against Heart and Chest diseases.

REFERENCES

EHLENBERGER, A.G. & NUSSENZWEIG, V. (1977) The role of membrane receptors for C3b and C3d in phagocytosis. *J. exp. Med.* **145,** 357.

FRIDRIKSSON, H.F., MALMBERG, P., HEDENSTRÖM, H. & HILLDERDAL, G. (1981) Reference values for respiratory function tests in males. Prediction formula with tobacco smoking parameters. *Clin. Physiol.* (In press.)

GANGE, R.W., BLACK, M.M., CARRINGTON, P. & MCKERRON, R.A. (1977) Defective neutrophil migration in sarcoidosis. *Lancet*, **ii,** 379.

GANGE, R.W., CARRINGTON, P. & BLACK, M.M. (1980) Altered neutrophil behaviour in granulomatous disorders: possible role of a serum factor. In *Sarcoidosis and Other Granulomatous Diseases* (ed. by W. J. Williams and B. H. Davies). Alpha Omega Publications Ltd, Cardiff.

GORDON, L.I., DOUGLAS, S.D., KAY, N.E., OSSERMAN, E.F. & JACOB, H.S. (1980) Modulation of neutrophil function by lysozyme, a macrophage secretory product. In *Sarcoidosis and Other Granulomatous Disorders* (ed. by W. J. Williams and B.D. Davies). Alpha Omega Publications Ltd, Cardiff.

HÅKANSSON, L., HÄLLGREN, R. & VENGE, P. (1980) Effect of hyaluronic acid on phagocytosis of latex particles. *Scand. J. Immunol.* **11,** 649.

HÅKANSSON, L., HÄLLGREN, R. & VENGE, P. (1981) Kinetic studies on phagocytosis. III. The complement-dependent opsonic and anti-opsonic effects of normal and SLE sera. (Submitted for publication.)

HÄLLGREN, R., JANSSON, L. & VENGE, P. (1977) Kinetic studies of phagocytosis of IgG coated latex particles using a thrombocyte counter. *J. Lab. clin. Med.* **90,** 786.

HANSEN, N.E., MALMQUIST, J. & THORELL, J. (1975) Plasma myeloperoxidase and lactoferrin measured by radioimmunoassay: relations to neutrophil kinetics. *Acta Med. Scand.* **198,** 437.

JAFFE, C.J., VIERLING, J.M., JONES, E.A., LAWLEY, T. & FRANK, M.M. (1978) Receptor specific clearance by the reticuloendothelial system in chronic liver diseases: demonstration of defective C3b specific clearance in primary biliary cirrhosis. *J. clin. Invest.* **62,** 1069.

LUNDIN, L., HÄLLGREN, R. & VENGE, P. (1980) Sequential studies on serum levels of lysozyme, lactoferrin and eosinophil cationic protein in alcoholics after alcohol withdrawal. *Scand. J. Haematol.* **25,** 431.

MADERAZO, E.G., WARD, P.A., WORONICK, C.L., KUBIK, J. & GRAFF, A.C. (1976) Leukotactic dysfunction in sarcoidosis. *Ann. Intern. Med.* **84,** 414.

MANTOVANI, B. (1975) Different roles of IgG and complement receptors in phagocytosis by polymorphonuclear leukocytes. *J. Immunol.* **115,** 15.

MILLER, A., EINSTEIN, K., THORNTON, J., TEIRSTEIN, A.S. & SILTZBACH, L.E. (1980) Physiologic classification and staging of intrathoracic sarcoidosis. In *Sarcoidosis and Other Granulomatous Disorders* (ed. by W. J. Williams and B. H. Davies). Alpha Omega Publications Ltd, Cardiff.

MORNEX, J.F., REVILLARD, J.P. VINCENT, C., DETEIX, P. & BRUNE, J. (1979) Elevated serum β_2-microglo-

bulin levels and C1q-binding immune complexes in sarcoidosis. *Biomedicine*, **31**, 310.

PASCUAL, R.S., GEE, J.B.L. & FINCH, S.G. (1973) Usefulness of serum lysozyme measurements in diagnosis and evaluation of sarcoidosis. *N. Engl. J. Med.* **289**, 1074.

SCHMEKEL, B., HÅLLGREN, R., STÅLENHEIM, G. & VENGE, P. (1981) Serum levels of lysozyme, lactoferrin and β_2-microglobulin—differential indices of inflammatory activity and lung affection in sarcoidosis. (Submitted for publication.)

SCHRIBNER, D.J. & FAHRNEY, D. (1976) Neutrophil receptors for IgG and complement: their roles in the attachment and ingestion phase of phagocytosis. *J. Immunol.* **116**, 892.

SEGAL, A.W. & LOEWI, G. (1976) Neutrophil dysfunction in Crohn's disease. *Lancet*, **ii**, 219.

SELROOS, O. & KLOCKARS, M. (1977) Serum lysozyme in sarcoidosis. Evaluation of its usefulness in determination of disease activity. *Scand. J. Respir. Dis.* **58**, 110.

SELROOS, O., TIITINEN, H., GRÖNHAGEN-RISKA, C., FYHRQVIST, A. & KLOCKARS, M. (1980) Angiotensin-converting enzyme and lysozyme in sarcoidosis. In *Sarcoidosis and Other Granulomatous Disorders* (ed. by W. J. Williams and B. H. Davies). Alpha Omega Publications Ltd, Cardiff.

UMBERT, P., BELCHER, R.W. & WINKELMANN, R.K. (1976) Lymphokines (MIF) in the serum of patients with sarcoidosis and cutaneous granuloma annulare. *Br. J. Dermatol.* **95**, 481.

VENGE, P., HÄLLGREN, R., STÅLENHEIM, G. & OLSSON, I. (1979) Effects of serum and cations on the selective release of granular proteins from human neutrophils during phagocytosis. *Scand. J. Haematol.* **22**, 317.