Biochemical Pharmacology, Vol. 29, pp. 2273–2278.

0006-2952/80/0901–2273 $02.00/0

EFFECT OF D-PENICILLAMINE *IN VITRO* AND *IN VIVO* ON MACROPHAGE PHAGOCYTOSIS

L. BINDERUP, E. BRAMM and E. ARRIGONI-MARTELLI
Department of Pharmacology, Leo Pharmaceutical Products, DK-2750 Ballerup, Denmark

(*Received* 8 *February* 1980; *accepted* 14 *April* 1980)

Abstract—Preincubation of rat peritoneal macrophages with D-penicillamine increased their uptake of labelled aggregated human gamma globulin ([^{125}I]AHG) without affecting the rate of degradation of the aggregates. Administration of D-penicillamine (50 mg.kg^{-1}.day^{-1} p.o.) to normal rats resulted in increased uptake of [^{125}I]AHG by peritoneal macrophages after 4 days of treatment, but not after 14 and 28 days of treatment. In contrast, macrophages from rats with adjuvant arthritis treated with D-penicillamine (50 mg.kg^{-1}.day^{-1} p.o.) exhibited an increased uptake of [^{125}I]AHG throughout the course of the disease. Administration of D-penicillamine *in vivo* had no effect on the rate of degradation of [^{125}I]AHG by freshly prepared macrophages. Culture for 24 hr *in vitro* prior to incubation with [^{125}I]AHG led to a decrease in the rate of degradation of the labelled aggregates by macrophages from untreated or D-penicillamine-treated rats and from rats with adjuvant arthritis, but not by macrophages from D-penicillamine-treated adjuvant arthritic rats. It is suggested that D-penicillamine may exert a stimulatory effect on the reticuloendothelial function during chronic inflammatory disease, and that this effect may be mediated via an interaction with the macrophage plasma membrane.

D-Penicillamine has been used for more than twenty years in the treatment of rheumatoid arthritis, but its mechanism of action still remains obscure. Treatment with D-penicillamine has been reported to influence a number of immunological parameters during the course of rheumatoid arthritis [1–4]. A consistent feature is the reduction in the level of circulating immune complexes which has been observed both in patients with rheumatoid arthritis [5–7] and with primary biliary cirrhosis [8]. It has been suggested that D-penicillamine may alter the antigenic stimulus to immunoglobulin production [5], interfere with cellular immunological reactions leading to immunoglobulin synthesis [9] or interact with the *de novo* formation of complexes [10]. Another possibility is that treatment with D-penicillamine may increase the removal of immune complexes by a stimulatory effect on the reticuloendothelial system. D-Penicillamine has previously been shown to influence macrophage functions in a number of animal studies. Rat peritoneal macrophages preincubated with D-penicillamine were found to modulate the response of purified lymphocytes to Concanavalin A [11]. *In vivo* administration of D-penicillamine to rats has been shown to increase the response of spleen and lymph node cells to Concanavalin A, but only in the presence of intact macrophages [12]. *In vitro* [^{14}C]D-penicillamine binds to the plasma membrane of macrophages, but not to lymphocytes [13], and D-penicillamine enhances the uptake of [^{14}C]D-glucosamine by rat peritoneal macrophages [11].

In the present study the effect of D-penicillamine *in vitro* and *in vivo* on macrophage phagocytosis was investigated. The uptake and rate of degradation of ^{125}I-labelled human heat-aggregated gamma globulin by rat peritoneal macrophages were studied using macrophages preincubated with D-penicillamine and macrophages from D-penicillamine-treated rats. Experiments were also performed with macrophages from rats with adjuvant arthritis in order to study the effect of D-penicillamine on the phagocytic process during chronic inflammatory disease.

MATERIALS AND METHODS

Preparation of macrophages. Macrophages were obtained from the peritoneal cavity of inbred, female Lewis rats (body wt 180–200 g) by injection of 10 ml sterile saline. The abdomen was gently massaged and the cells were aspirated, counted, washed, and resuspended in culture medium at 0.5×10^6 cells/ml. The culture medium consisted of RPMI 1640 supplemented with 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Macrophages were collected from normal rats and from rats with adjuvant arthritis. Adjuvant arthritis was induced by injecting 0.3 mg of heat-killed *Mycobacterium butyricum* (Difco) suspended in 0.1 ml of mineral oil into the footpad of the right hind paw. Animals treated with D-penicillamine were dosed daily with 50 mg/kg orally (D-penicillamine, free base, pyrogen-free, Sigma Chem. Co.) and macrophages were collected 24 hr after the last dose.

Preparation of labelled aggregated human gamma globulin [^{125}I]*AHG.* Human IgG (Sigma Chem. Co., Cohn fraction V, 4 mg/ml) was aggregated at 63° for 30 min and centrifuged at 1500 *g* for 30 min to remove any insoluble aggregates. Iodination was performed by the chloramine-T method [14], using 2 mCi of carrier free [^{125}I] (100 mCi/ml, The Radiochemical Centre, Amersham, U.K.) per ml of aggregated IgG. [^{125}I]AHG was dialysed against phosphate buffered saline (PBS), pH 7.4, for 24 hr, with four changes

of buffer, and centrifuged at 2000 g for 30 min. The preparation was adjusted to 2 mg/ml of protein, with a specific activity of 3.3×10^5 cpm/μg protein, and stored at 4° for a maximum of 3 weeks.

Assay of phagocytosis. The test was performed in flat bottomed tissue-culture-treated multidishes (Nunc No. 145-68357, Denmark) with 24 wells. The wells were pretreated for 1 hr with culture medium containing 10% heat-inactivated Lewis rat serum to reduce nonspecific adherence of [^{125}I]AHG to the plastic surface [15]. Aliquots of 0.5 ml macrophage suspension (0.5×10^6 cells/ml) were added to each well and the macrophages were allowed to adhere for 1 hr at 37° in 5% CO_2 in air. Nonadherent cells were removed and counted. Adherent cell monolayers were found to consist of 99 per cent pure macrophages, as assessed by staining for membrane-associated nonspecific esterase [16]. A 0.5 ml portion of medium containing 10 μg/ml [^{125}I]AHG was added to each well. After incubation for 60 min at 37°, the cultures were washed with 4 × 1 ml medium and the adherent cells were solubilized in 0.5 N NaOH overnight and counted. In some experiments the cultures were preincubated with D-penicillamine (0–500 μg/ml) for 2 hr prior to incubation with [^{125}I]AHG.

All samples were performed in triplicate and the results were calculated as mean cpm/10^6 macrophages, corrected for nonspecific adherence of [^{125}I]AHG. Nonspecific adherence was assessed both in cultures with [^{125}I]AHG, but without macrophages, incubated for the same period of time as the cultures with macrophages, and in cultures where [^{125}I]AHG was added to the macrophages just prior to the washings. The two methods gave comparable results, nonspecific adherence being about 10 per cent of the total amount of [^{125}I] incorporated. Macrophages were collected and assayed individually from three rats from each experimental group, with the exception of the experiments with D-penicillamine *in vitro* where pooled macrophages from three rats were used. All experiments were repeated three times and the results were expressed as cpm/10^6 macrophages ± S.E.M. Statistical analysis was performed using Student's t-test. The viability of the cells was determined before culture and at the end of incubation by the eosin Y exclusion method.

Degradation of [^{125}I]*AHG.* Macrophages (0.5×10^6 cells/ml) were incubated with 10 μg/ml of [^{125}I]AHG for 60 min, washed with 4 × 1 ml medium and reincubated in 1 ml medium. At various times aliquots of 100 μl medium were removed and precipitated with 1 ml 6% trichloroacetic acid (TCA); 25 μl of serum was added as carrier protein. The amount of nonprecipitable [^{125}I] was determined in the supernatant fraction. Calculation of the half-life of [^{125}I]AHG was performed as described by Tew and Mandel [17].

RESULTS

Effects of D-*penicillamine* in vitro *on macrophage phagocytosis.* Uptake of [^{125}I]-labelled aggregated human IgG ([^{125}I]AHG, 10 μg/ml) by rat peritoneal macrophages (0.5×10^6 cells/ml) was found to be maximal after 60 min of incubation. Longer periods of incubation resulted in degradation of the aggregates taken up, as measured by the appearance of nonprecipitable [^{125}I]AHG in the culture medium (results not shown). An incubation time of 60 min was therefore adopted throughout the experiments. Preincubation of rat peritoneal macrophages with D-penicillamine (0–500 μg/ml) for 2 hr prior to the addition of [^{125}I]AHG resulted in a dose-dependent increase in the uptake of labelled aggregates (Fig. 1), with a 57 per cent increase for the highest concentration of D-penicillamine. No decrease in cell viability or in the number of adherent cells was found

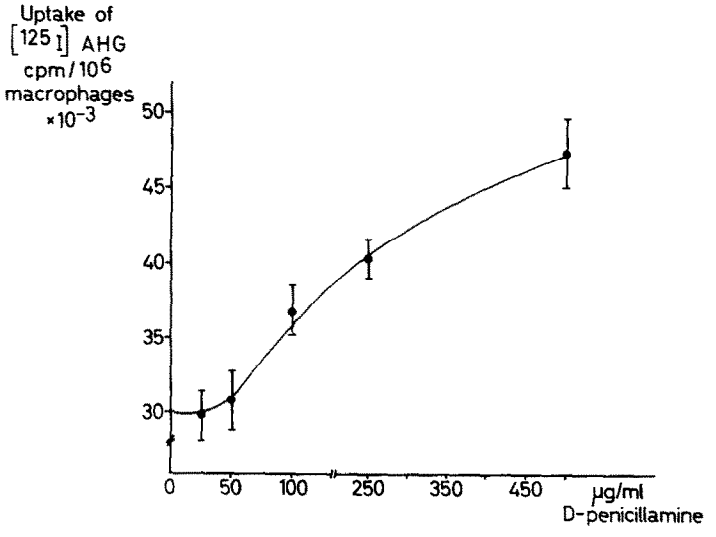

Fig. 1. Uptake of [^{125}I]AHG by D-penicillamine-preincubated macrophages. Peritoneal macrophages (0.5×10^6 cells/ml) pooled from three Lewis rats were preincubated with D-penicillamine (0–500 μg/ml) for 2 hr, washed and reincubated with 10 μg/ml [^{125}I]AHG for 60 min. Uptake of radioactivity was determined after digestion of the macrophages with 0.5 N NaOH. Each point represents the mean of three separate experiments, expressed as cpm/10^6 macrophages (±S.E.M.).

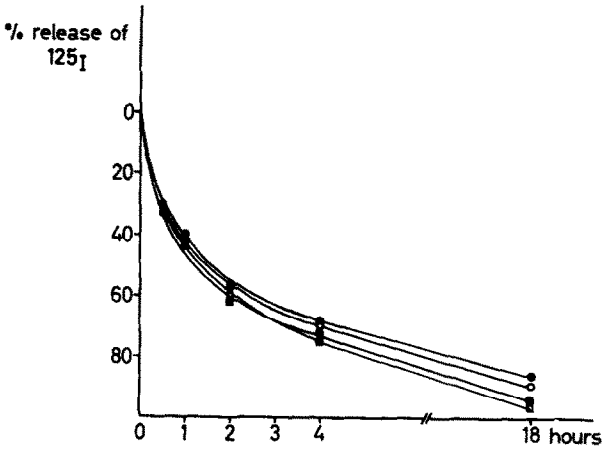

Fig. 2. Degradation of [^{125}I]AHG by D-penicillamine-preincubated macrophages. Peritoneal macrophages (0.5×10^6 cells/ml) pooled from three Lewis rats were preincubated with D-penicillamine (○—○ 0 μg/ml; ●—● 100 μg/ml; ■—■ 250 μg/ml; □—□ 500 μg/ml) for 2 hr, washed and reincubated with 10 μg/ml [^{125}I]AHG for 60 min. The cells were washed and the release of nonprecipitable [^{125}I] was measured after 0.5, 1.0, 2.0, 4.0 and 18.0 hr. Each point represents the mean of three separate cultures, expressed as per cent release of [^{125}I] from the [^{125}I]AHG taken up.

under these conditions. The rate of degradation of the [^{125}I]AHG taken up was next measured. Figure 2 shows the release of nonprecipitable [^{125}I] from macrophages preincubated with D-penicillamine (100, 250 and 500 μg/ml). The half-life of the labelled aggregates was found to be 1.5 hr in untreated macrophages, while no significant differences were observed with macrophages preincubated with D-penicillamine.

Uptake of [^{125}I]*AHG by macrophages from rats treated with* D-*penicillamine.* In order to study the effect of D-penicillamine *in vivo* on the uptake of [^{125}I]AHG, peritoneal macrophages were obtained from Lewis rats treated orally with D-penicillamine (50 mg.kg^{-1}.day^{-1}) for various periods of time. Maximum uptake of [^{125}I]AHG by macrophages occurred after 60 min of incubation of cultures from both untreated and D-penicillamine-treated rats (results not shown). Table 1 shows that the recovery of macrophages from the peritoneal cavity was unaffected by treatment with D-penicillamine. After four days of treatment with D-penicillamine, the uptake of [^{125}I]AHG was significantly increased (by 158 per cent), compared to the uptake by macrophages from untreated rats. After 14 and 28 days of treatment with D-penicillamine, however, increased uptake of [^{125}I]AHG was no longer evident (Table 1). No differences in cell viability or in the number of adherent cells were observed throughout the experiment.

Uptake of [^{125}I]*AHG by macrophages from rats with chronic inflammation.* To study the effect of treatment with D-penicillamine on the uptake of

Table 1. Uptake of [^{125}I]AHG by macrophages from rats treated with D-penicillamine*

Treatment	Recovery of macrophages (10^6 cells/rat ± S.D.)	Uptake of [^{125}I]AHG (cpm/10^6 macrophages ± S.E.M.)	Increase in uptake (%)	P
Control rats (N = 15)	10.19 ± 1.07	30,744 ± 2625	—	
D-penicillamine 50 mg.kg^{-1}.day^{-1} (4×) (N = 15)	9.15 ± 1.92	79,242 ± 8820	158	<0.001
D-penicillamine 50 mg.kg^{-1}.day^{-1} (14×) (N = 9)	11.10 ± 1.54	31,772 ±6448	3	n.s.
D-penicillamine 50 mg.kg^{-1}.day^{-1} (28×) (N = 9)	10.30 ± 1.31	30,246 ± 2929	−2	n.s.

* Peritoneal macrophages (0.5×10^6 cells/ml) were collected from Lewis rats, treated with D-penicillamine (50 mg.kg^{-1}.day^{-1} p.o.) for 4, 14, and 28 days. The macrophages were incubated with 10 μg/ml [^{125}I]AHG for 60 min and the uptake of radioactivity was expressed as cpm/10^6 macrophages (±S.E.M.). N = number of rats in each experimental group, n.s. = not significant.

[^{125}I]AHG by macrophages during chronic inflammatory disease, adjuvant arthritis was induced in Lewis rats. Macrophages were collected from the peritoneal cavity of normal and adjuvant arthritic rats, untreated or treated with D-penicillamine (50 mg.kg^{-1}.day^{-1} orally) for various periods of time. Figure 3 shows that the uptake of [^{125}I]AHG by macrophages from untreated adjuvant arthritic rats was not significantly different from that of the corresponding cultures of macrophages from normal rats, with the possible exception of day 8 after induction of adjuvant arthritis where the uptake was inhibited by 46 per cent. This value, however, did not reach statistical significance. On the other hand, macrophages from adjuvant arthritic rats treated with D-penicillamine showed a significantly increased uptake of [^{125}I]AHG at all times of assay, with a mean increase of 140 per cent, compared to the cultures from untreated adjuvant arthritic rats. No differences in cell viability, recovery of cells or in the number of adherent cells were observed between any of the experimental groups. Maximum incorporation of [^{125}I]AHG was always observed after 60 min of incubation.

Degradation of [^{125}I]AHG by macrophages from rats with chronic inflammation. Figure 4, panel a, shows the release of nonprecipitable [^{125}I] from freshly prepared macrophages from normal and adjuvant arthritic rats, untreated or treated with D-penicillamine for 14 days. Macrophages from all experimental groups released [^{125}I] at a comparable rate; the half-life of the [^{125}I]AHG being 1.0–1.5 hr. In contrast, when the macrophages were cultured for 24 hr before incubation with [^{125}I]AHG, the rate of degradation was found to be much slower. Only 50 per cent of the label was released after 6 hr of incubation from macrophages from normal rats, untreated or treated with D-penicillamine and from rats with adjuvant arthritis (Figure 4, panel b). Macrophages from D-penicillamine-treated adjuvant arthritic rats, however, released [^{125}I] at a rate comparable to that observed with freshly prepared macrophages, and all the [^{125}I]AHG taken up was degraded after 6 hr of incubation. No differences were observed in the uptake of [^{125}I]AHG by freshly prepared macrophages and macrophages cultured for 24 hr prior to incubation with [^{125}I]AHG (results not shown). All cultures were found to contain more than 90 per cent viable cells after 24 hr of culture.

DISCUSSION

D-penicillamine was found to increase the uptake of labelled aggregated human gamma globulin ([^{125}I]AHG) by rat peritoneal macrophages when added *in vitro* in concentrations from 100 to 500 μg/ml. [^{125}I]AHG was degraded with a half-life of 1.5 hr both by untreated and D-penicillamine-preincubated macrophages, in accordance with the reported value of 2 hr for clearance of human immune complexes by mouse peritoneal macrophages [17]. D-penicillamine has previously been shown to bind to the plasma membrane of rat peritoneal macrophages [13] and this interaction may result in an increased access to or exposure of receptors for immune complexes and IgG aggregates. Alternatively, binding of D-penicillamine to the macrophage membrane may increase membrane turnover, thus increasing the rate of internalization of bound aggregates. Evidence in favour of this possibility may be provided by the findings of increased [^{14}C]-glucosamine uptake in macrophages incubated with D-penicillamine [11].

When D-penicillamine was administered *in vivo* to rats an increased uptake of [^{125}I]AHG was found after four days of treatment. Despite continuous

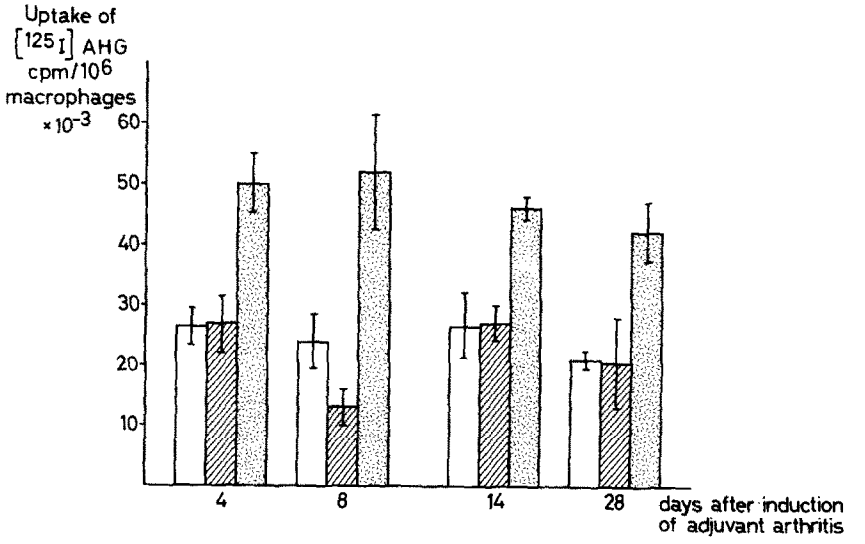

Fig. 3. Uptake of [^{125}I]AHG by macrophages from rats with adjuvant arthritis. Peritoneal macrophages were collected from normal rats (□), rats with adjuvant arthritis (▨) and rats with adjuvant arthritis treated with 50 mg.kg^{-1}.day^{-1}, p.o. of D-penicillamine (▩). The macrophages (0.5 × 10^6 cells/ml) were incubated with 10 μg/ml [^{125}I]AHG for 60 min and the uptake of radioactivity was expressed as cpm/10^6 macrophages. Each bar represents the mean (±S.E.M.) of three separate experiments, each experimental group consisting of three rats.

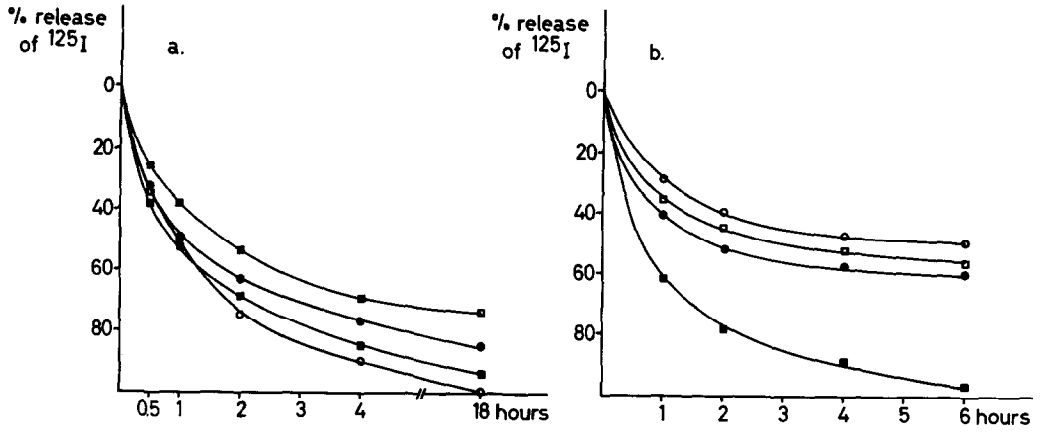

Fig. 4. Degradation of [^{125}I]AHG by macrophages from rats with adjuvant arthritis. Release of nonprecipitable [^{125}I] from peritoneal macrophages (0.5×10^6 cells/ml) was measured at selected intervals after incubation with 10 μg/ml [^{125}I]AHG for 60 min. (a) Freshly prepared macrophages. (b) Macrophages cultured for 24 hr prior to incubation with [^{125}I]AHG. Macrophages were collected 14 days after the induction of adjuvant arthritis from rats receiving no treatment (●——●) or from rats treated with 50 mg.kg^{-1}.day^{-1} of D-penicillamine (■——■). Control macrophages were collected from nonarthritic rats receiving no treatment (○——○) or treated with 50 mg.kg^{-1}.day^{-1} of D-penicillamine for 14 days (□——□). Each point represents the mean release of [^{125}I] from three separate cultures prepared individually from three rats from each experimental group.

administration of D-penicillamine, this effect was not found at later times of assay. Treatment with D-penicillamine has previously been shown to increase the response of rat lymphocytes to the T cell mitogen Concanavalin A [12]. This effect was self-limiting and vanished after two to three weeks, despite continued administration of the drug. These observations suggest that the organism may respond by a feedback mechanism to control the stimulatory effects of D-penicillamine. In contrast, treatment with D-penicillamine of rats with adjuvant arthritis resulted in an increased uptake of [^{125}I]AHG by the peritoneal macrophages throughout the course of the disease, thus giving rise to a longlasting effect of D-penicillamine on phagocytic functions during experimental chronic inflammatory disease. These results are consistent with previous reports describing an increased clearance of carbon particles in New Zealand White rabbits treated with D-penicillamine [18] and an increase in the phagocytic rate of polymorphonuclear leucocytes from patients with rheumatoid arthritis after treatment with D-penicillamine [19].

Treatment with D-penicillamine *in vivo* had no effect on the rate of degradation of [^{125}I]AHG by freshly prepared macrophages. Macrophages collected from normal untreated and D-penicillamine-treated rats and from rats with adjuvant arthritis and maintained in culture for 24 hr prior to incubation with [^{125}I]AHG exhibited a decreased rate of degradation of the labelled aggregates, despite an unchanged uptake of [^{125}I]AHG. This may be due to membrane alterations induced by culture of macrophages in serum free medium [20]. It is possible to speculate that binding of [^{125}I]AHG to membrane receptors may proceed normally, but that membrane internalization or phago-lysosome formation may be impaired. In contrast, macrophages collected from rats with adjuvant arthritis treated with D-penicillamine displayed a rate of degradation of [^{125}I]AHG similar to that found with freshly prepared macrophages. These findings provide additional evidence for an effect of D-penicillamine at the level of the macrophage membrane but require further investigations in order to clarify the exact mechanism of action.

The reported observations may contribute to an understanding of the clinical effects of D-penicillamine. A stimulatory effect of D-penicillamine on the reticuloendothelial system resulting in increased clearance of circulating immune complexes may be of special benefit in view of the recently described deficient function of the splenic reticuloendothelial system in patients with rheumatoid arthritis [21].

Acknowledgements—The authors wish to thank Ms. Elisabeth Greve Petersen, Ms. Bente Hasselriis and Ms. Anne-Mette Heie Jørgensen for their excellent technical assistance.

REFERENCES

1. I. A. Jaffe, *Arthritis Rheum.* **8**, 1064 (1965).
2. I. A. Jaffe, *Arthritis Rheum.* **13**, 436 (1970).
3. E. C. Huskisson, *Proc. R. Soc. Med.* **70**, Suppl. 3, 142 (1977).
4. A. G. Mowat, *Ann. rheum. Dis.* **37**, 1 (1978).
5. I. A. Jaffe, *Ann. N.Y. Acad. Sci.* **256**, 330 (1975).
6. R. Norberg and P. O. Gedda, in *Pencillamine Research in Rheumatoid Disease* (Ed. E. Munthe), p. 146. Fabritius, Oslo (1976).
7. I. Mohammed, D. Barraclough, E. J. Holborow and B. A. Ansell, *Ann. rheum. Dis.* **35**, 458 (1976).
8. O. Epstein, D. De Villiers, S. Jain, B. J. Potter, H. C. Thomas and S. Sherlock, *New Engl. J. Med.* **300**, 274 (1979).
9. R. Bluestone and L. S. Goldberg, *Ann. rheum. Dis.* **32**, 50 (1973).

10. F. A. Wollheim, J. O. Jeppsson and C. B. Laurell, *Scand. J. Rheum.* Suppl. **28**, 21 (1979).
11. L. Binderup, E. Bramm and E. Arrigoni-Martelli, *Scand. J. Immun.* **7**, 259 (1978).
12. L. Binderup, E. Bramm and E. Arrigoni-Martelli, *Scand. J. Immun.* **11**, 23 (1980).
13. L. Binderup and E. Arrigoni-Martelli, *Biochem. Pharmac.* **28**, 189 (1979).
14. P. J. McConahey and F. J. Dixon, *Int. Archs. Allergy appl. Immun.* **29**, 185 (1966).
15. I. Mohammed, B. Thompson and E. J. Holborow, *Ann. rheum. Dis.* **36**, 49 (1977).
16. L. T. Yam, C. Y. Li and W. H. Crosby, *Am. J. clin. Path.* **55**, 283 (1971).
17. J. G. Tew and T. E. Mandel, *Immunology* **37**, 69 (1979).
18. I. M. Hunneyball, G. A. Stewart and D. R. Stanworth, *Immunology* **35**, 159 (1978).
19. R. Hällgren, L. Håkansson and P. Venge, *Arthritis Rheum.* **21**, 107 (1978).
20. P. J. Edelson and Z. A. Cohn, *J. exp. Med.* **144**, 1581 (1976).
21. B. D. Williams, C. M. Lockwood, B. A. Pussell and C. Cotton, *Lancet* **i**, 1311 (1979).