

## **Altered antigen-presentation in the induction of the in-vitro antigen-specific T helper cell function in patients with chronic granulomatous disease**

COBI J. HEIJNEN, J. W. M. VAN DER MEER\* & B. J. M. ZEGERS *Department of Immunology, University Hospital for Children and Youth 'Het Wilhelmina Kinderziekenhuis', Utrecht, The Netherlands and \*Department of Infectious Disease, University Hospital, Leiden, The Netherlands*

*(Accepted for publication 21 May 1986)*

### SUMMARY

Phagocytic cells of patients with chronic granulomatous disease (CGD) are severely impaired in the killing process, on the basis of defective oxygen metabolism. In this study we investigated the antigen-presenting function of monocytes (i.e. adherent cells) of CGD patients. Adherent cells of CGD patients were investigated for their capacity to present ovalbumin (OA) in such a way that T helper cells are activated and OA-specific IgM-plaque forming cells (PFC) are generated. The results showed that the dose of OA required for optimal PFC responses was 20-25 times higher than the dose of antigen which is necessary for induction of PFCs from peripheral blood mononuclear cells of normal donors. We could show that the CGD monocytes were responsible for the observed shift which indicates peculiar antigen-presenting capacities. The finding could be mimicked in normal donors by treating adherent cells with phenylbutazone, a drug known to interfere with oxygen-dependent degradation of micro-organisms. Moreover, our results suggested that CGD monocytes and phenylbutazone-treated monocytes of normal donors absorb OA but do not re-express the processed antigen on the membrane. We conclude that our system used to study antigen presentation allowed the discovery of altered antigen presentation of CGD monocytes most probably on the basis of defective antigen processing. However, antigen presentation is not entirely absent, since relatively high doses of OA induced normal PFC responses in CGD mononuclear cells.

**Keywords** chronic granulomatous disease antigen presentation

### INTRODUCTION

An essential step in the activation of T helper cells by complex antigens is the presentation of the antigen by accessory cells in conjunction with Class II molecules of the MHC complex (Rosental & Shevach, 1973; Benacerraf, 1981). T helper cells do not recognize antigens in their native form but they only respond to 'processed' antigens presented on the surface of the accessory cells (Benacerraf, 1981; Ziegler & Unanue, 1981).

Processing of antigen by accessory cells involves a sequence of events comprising recognition and ingestion of the antigen, and degradation and re-expression of the antigen on the membrane

Correspondence: Dr Cobi J. Heijnen, Dept. of Immunology, University Hospital for Children and Youth, 'Het Wilhelmina Kinderziekenhuis', Nieuwe Gracht 137, 3512 LK Utrecht, The Netherlands.

(Ziegler & Unanue, 1981; Ziegler & Unanue, 1982). However, in more recent years the necessity of processing of antigen for effective presentation to T helper cells has been disputed.

In previous studies we have reported on the regulatory role of T cells and monocytes (i.e. adherent cells) in the in-vitro activation of human peripheral blood B lymphocytes with antigens like ovalbumin (OA) and sheep red blood cells (Ballieux *et al.*, 1979; Heijnen *et al.*, 1981). In this system antigen-presenting monocytes and antigen-specific T helper and T suppressor lymphocytes interact in the activation of antigen-specific resting B cells into the stage of a small IgM-secreting plaque forming cell (PFC) (Ballieux & Heijnen, 1983; Van Tol *et al.*, 1984; 't Hart *et al.*, 1985). In the present study it was investigated whether peripheral blood monocytes from patients with chronic granulomatous disease (CGD) can present OA in such a way that T helper cells are activated and OA-specific IgM-PFCs are generated. In this way monocytes defective in oxygen metabolism (Gallin & Fauci, 1983) are used as a model to study antigen presentation.

## MATERIALS AND METHODS

*Mononuclear cell isolation.* Peripheral blood mononuclear cells were isolated by density gradient centrifugation as described by Heijnen *et al.* (1979).

*Monocyte depletion and isolation.* Monocytes were isolated from the mononuclear cell suspension ( $1 \times 10^6$  cells/ml) by allowing them to adhere to plastic Falcon flasks at 37°C in RPMI-1640 medium (Gibco) supplemented with 20% fetal calf serum (FCS). After 60 min of incubation at 37°C the supernatant containing the lymphocytes was decanted and the adherent cells were harvested by gently scraping with a rubber policeman and then collected in Earle's balanced salt solution without calcium and magnesium (Gibco) supplemented with 0.002 M EDTA.

*Cell cultures.* This method has been described extensively (Heijnen *et al.*, 1979). In brief,  $5 \times 10^6$  peripheral blood lymphocytes (PBL) were cultured in the presence of the antigen ovalbumin (OA; Grade VI, Sigma, St Louis) and 10% adherent cells. Cultures were performed in roundbottom tubes (Falcon) in 10 ml medium which consisted of RPMI-1640, supplemented with 2 mM L-glutamine, antibiotics and 10% human AB serum. Before use the serum was heat-inactivated and extensively absorbed with sheep red blood cells (SE) to prevent pseudoplaque formation (Ballieux *et al.*, 1979).

*Pulsing of adherent cells with OA.* Monolayers of adherent cells were incubated with 1000 µg OA/ml RPMI-1640 supplemented with 10% heat-inactivated SE-absorbed AB serum for 2 h. After the incubation period the cells were harvested and washed three times with RPMI-1640 supplemented with 5% pre-treated AB serum.

*Enzyme treatment of the adherent cells.* Adherent cells ( $10 \times 10^6$  cells/ml RPMI-1640) were incubated with pronase (8 mg/ml, Sigma, St Louis) and DNase (10 µg/ml) for 30 min at 37°C. After the incubation 1 ml FCS was added to the cells which were spun down (4°C) and washed twice with RPMI-1640 supplemented with 10% FCS.

*Phenylbutazone treatment.* Monolayers of adherent cells were incubated with phenylbutazone (100 µg/ml) for 30 min at 37°C. After this procedure the cells were washed twice.

*Plaque forming cell assay.* PFC were determined as described in detail earlier (Ballieux *et al.*, 1979; Heijnen *et al.*, 1979). In brief, OA-coated SE (Heijnen *et al.*, 1979; Goding, 1969) were centrifuged for 5 min at 1000 g on the bottom of Falcon microtitre plates, coated by incubation with poly-L-lysine (Sigma, St Louis, M,  $\leq 100,000$ ) in a concentration of 100 µg/ml distilled water. OA-stimulated cells in various dilutions were incubated for 60–90 min in the presence of SE-absorbed guinea pig complement (C). Controls comprised the addition of cultured cells to the monolayers without C and vice versa. Only IgM-PFCs are detected in this system and antigen-specificity has been investigated extensively as described earlier (Ballieux *et al.*, 1979; Heijnen *et al.*, 1979).

## RESULTS

*PFC-response of PBL from normal donors and of CGD patients.* PBL from normal donors and from four CGD patients were cultured with various doses of the antigen ovalbumin (OA) for 6 days

at 37°C. After the incubation period, the cells were harvested and tested for their capacity to secrete IgM anti-OA antibodies *in vitro* by measuring the number of anti-OA PFC (Heijnen *et al.*, 1979).

It was shown that in PBL from normal donors formation of PFC can be generated when the dose of antigen in the culture is in between 1 and 3 µg OA/ml (Fig. 1). When normal PBL are stimulated with 100 µg OA or more, the PFC-response decreases to baseline levels. We have shown previously that the decline in the PFC-response of lymphocytes cultured in the presence of the high concentration of antigen is due to the activation of T suppressor effector cells (UytdeHaag, Heijnen & Ballieux, 1978). However, when PBL of CGD patients are cultured with various doses of antigen, a shift of the PFC-response towards the right side of the curve was observed in all patients, with an optimal PFC-response at 100 µg OA/ml (Fig. 1).

*PFC-response of lymphocytes of normal donors and of CGD patients after stimulation with antigen-pulsed adherent cells.* To study the process of antigen-presentation by accessory cells of CGD patients more precisely, adherent cells of both CGD patients and normal donors were pulsed with ovalbumin (1000 µg OA/ml) for 2 h at 37°C. After the incubation period, the non-adhered antigen was washed away and the pulsed cells were cultured for 6 days with PBL that were depleted of adherent cells under standard conditions. The results depicted in the upper part of Fig. 2 show

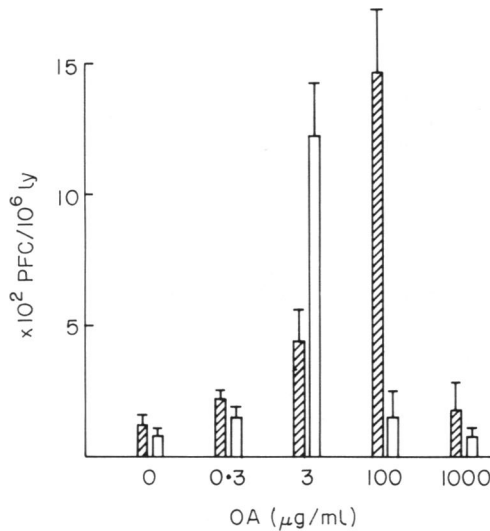


Fig. 1. PFC response of CGD patients (■, n = 4) and normal control donors (□, n = 4), generated in the presence of various doses of the antigen OA. Lymphocytes (5 × 10<sup>6</sup>) (T/B ratio: about 7/1) were cultured with OA and 10% adherent cells. The T/B ratio in CGD patients and normal donors did not differ significantly. OA-specific IgM-PFCs were measured after 6 days of culture.

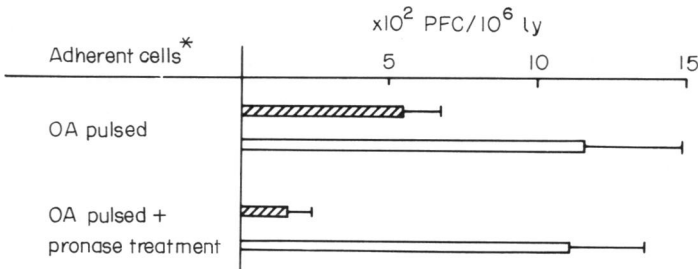


Fig. 2. PFC response of PBL (5 × 10<sup>6</sup>) from CGD patients (■, n = 5) and of normal control donors (□, n = 5) in the presence of 10% OA-pulsed adherent cells. \*Assay system: PBL depleted of adherent cells and reconstituted with the indicated adherent cell suspensions. Background values (PBL cultured with 10% non-pulsed adherent cells) are less than 200 PFC.

that CGD lymphocytes cultured with antigen-pulsed adherent cells mount a PFC-response which indicates that CGD adherent cells are able to present antigen. However, the PFC response obtained is much lower than that of cultures of antigen-pulsed adherent cells and lymphocytes of control donors.

*PFC-response of normal donors and of CGD patients after stimulation with antigen-pulsed, pronase-treated adherent cells.* Next, it was investigated whether the PFC-response observed in cultures of antigen-pulsed monocytes and lymphocytes of CGD patients is actually the result of antigen-processing and re-expression of the antigen on the membrane of the adherent cells.

To that end, antigen-pulsed adherent cells were 'stripped' by pronase-treatment to remove cell-bound antigen that had not yet been ingested. After several washing procedures the adherent cells were added to the lymphocyte suspension that was depleted of adherent cells and cultured for 6 days.

These experiments show that when the adherent cells of CGD patients are cleared from cell-bound antigen, the antigen-pulsed adherent cell population is incapable of inducing a PFC-response (lower part of Fig. 2). This finding suggests that surface-bound OA is responsible for the low PFC response obtained when antigen-pulsed adherent cells, not treated with pronase, were used for induction of PFCs. Unlike CGD adherent cells, antigen-pulsed pronase-treated adherent cells from normal donors can activate the lymphocytes, leading to the differentiation of resting B cells into IgM-secreting PFCs (Fig. 2).

*PFC-response of PBL from normal donors in the presence of adherent cells treated with phenylbutazone.* In the next series of experiments, we tried to mimic the defect of monocytes of CGD patients, by pre-incubating normal adherent cells with phenylbutazone (100  $\mu\text{g}/\text{ml}$ ). This agent is known to inhibit intracellular killing of phagocytes, probably by interference with the hexose monophosphate shunt (Solbey, 1975). When normal adherent cells are pre-treated with phenylbutazone for 30 min and cultured with PBL and various doses of OA for 6 days, a shift towards the right of the PFC-response curve is observed, resembling the PFC-antigen dose relationship of CGD patients (Figs 1 and 3).

*PFC-response of PBL of normal donors in the presence of phenylbutazone-treated, antigen-pulsed and pronase-treated adherent cells.* Normal adherent cells, pre-treated either with medium or with phenylbutazone, were pulsed with antigen for 2 h. After the pulse-period, one part of the cells was subsequently treated with pronase to remove cell-bound antigen. After several washing procedures the adherent cell preparations were added separately to lymphocyte suspensions which were carefully depleted of adherent cells, and cultured for 6 days at 37°C. Figure 4 demonstrates that

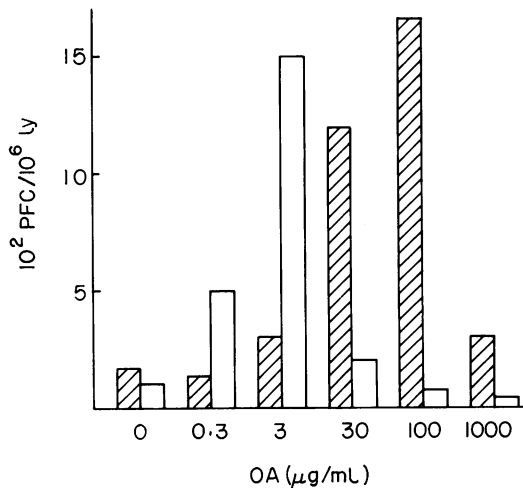


Fig. 3. PFC response of PBL from normal donors with either phenylbutazone treated adherent cells ( $\blacksquare$ ,  $n=4$ ) or untreated cells ( $\square$ ,  $n=4$ ) in the presence of various doses of OA.

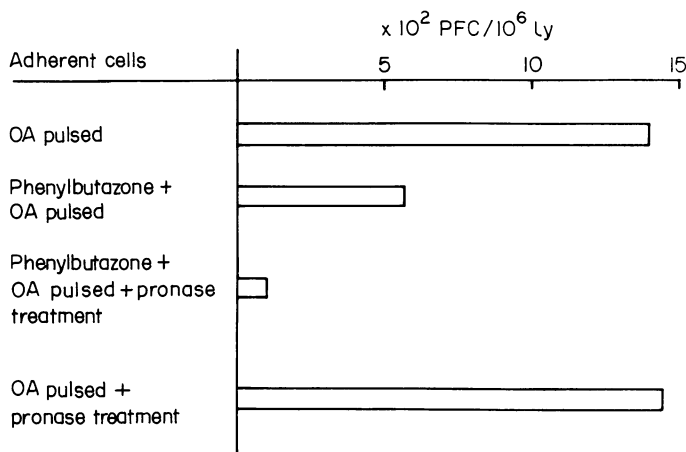


Fig. 4. PFC response of PBL from normal donors in the presence of adherent cells treated as indicated.

indeed phenylbutazone treatment mimics the results obtained with the adherent cells of CGD patients (Fig. 2), i.e. PFC are generated in cultures with phenylbutazone-treated adherent cells but just as in CGD patients the results are lower than with untreated adherent cells. When phenylbutazone-treated, antigen-pulsed adherent cells are cleared of cell-bound antigen, the adherent cells are no longer capable of presenting the antigen in such a way that it leads to PFC formation.

## DISCUSSION

The question whether the antigen has to be processed by an accessory cell and re-expressed on the membrane before it can activate T helper cells is a subject of intense investigation (Unanue *et al.*, 1984). In contrast to T helper cells, T suppressor ( $T_s$ ) cells recognize the antigen in its soluble form, since  $T_s$  cells can be stimulated efficiently in the absence of monocytes with low doses of antigen (0.003  $\mu\text{g}$  OA/ml; UytdeHaag *et al.*, 1982). However, when the number of adherent cells in the culture is increased, more antigen is required (30–100  $\mu\text{g}$  OA/ml at 10% adherent cells) to activate T suppressor cells (UytdeHaag *et al.*, 1982). The bell-shaped PFC-response curve is therefore a reflection of the net result of the balance between T helper and T suppressor cell activities induced by the various doses of antigen (Ballieux *et al.*, 1979).

When mononuclear cells from CGD patients are cultured with various doses of OA, the PFC-response curve has an optimum at 100  $\mu\text{g}$  OA/ml, indicating that unlike normal donors relatively high doses of antigen are required to let Th cell activity prevail over  $T_s$  activity. In separate experiments it was shown that CGD mononuclear cells produced neither OA-specific T helper cell factors nor OA-specific T suppressor cell factors (Ballieux *et al.*, 1979; Heijnen *et al.*, 1981) at a concentration of 3  $\mu\text{g}$  OA/ml (data not shown). The latter finding indicates that with this dose of OA no free antigen is available to activate  $T_s$  cells which suggests that OA is internalized in the CGD monocytes without induction of T helper function. When subsequently adherent cells are pulsed with antigen, extensively washed and treated with pronase to remove non-ingested cell-bound antigen, the PFC-response is abolished (Fig. 2). We therefore conclude that the adherent cells of CGD patients apparently do not process the antigen in a way it is re-expressed on the membrane for the activation of T helper cells. From the data presented it appeared that adherent cells of CGD patients that have been pulsed with OA but not stripped for non-ingested OA can present antigen to T helper cells, although the PFC-responses are much lower than those of normal donors (Fig. 2). These results imply that there are T cells present in the peripheral circulation that can recognize antigen that has not been processed by monocytes. We do not yet know whether the antigen is

partially processed at the level of the membrane of the adherent cells or whether enzymes present in the AB serum pool can partially degrade the antigen. It might also be possible that a specialized subset of antigen-presenting cells, e.g. dendritic cells which are known to be capable of stimulating T helper cells without ingesting and processing the antigen (Van Voorhis *et al.*, 1982), is responsible for the presentation of the antigen to the T helper cells.

The present result, i.e. a shift of the OA-dose response towards high concentrations in mononuclear cells of CGD patients, is reminiscent of earlier observations using cord blood mononuclear cells. In this respect van Tol *et al.* (1984) showed that neonatal and adult adherent cells differ in antigen-handling which is reflected in a shift of the OA dose needed to induce an optimal PFC response in cord blood cells to low (i.e. 0.03  $\mu\text{g}$ ) concentrations. In addition it was shown that neonatal adherent cells secrete more PGE<sub>2</sub> than adherent cells from adult controls. The difference between the adult and cord blood dose response curves could be abolished by treatment with indomethacine, an inhibitor of cyclooxygenase. These results suggest a relation to exist between antigen-handling and PGE<sub>2</sub> secretion and/or sensitivity. We did not measure PGE<sub>2</sub> secretion by CGD adherent cells but, unlike our results, one would expect that if PGE<sub>2</sub> secretion by CGD adherent cells is increased, a shift of the antigen-dose/PFC relationship towards low concentrations would be obtained. It should be mentioned that the CGD patients had no overt infections when investigated. Furthermore several patients with infections were investigated for PFC responses in the last years in our laboratory. However a shift in the antigen-dose response relationship towards high antigen concentrations was never observed.

In order to demonstrate more directly that the aberrant PFC-responses are due to a defect in the antigen-presenting cells, we tried to mimic the defect of monocytes of CGD patients by treating adherent cells from normal donors with phenylbutazone. The latter drug interferes with oxygen-dependent degradation by inhibiting H<sub>2</sub>O<sub>2</sub> production through an effect on the hexose monophosphate shunt pathway (Soleby, 1975). The PFC dose response using phenylbutazone-treated adherent cells of normal donors completely mimics those of CGD patients. Furthermore, results using antigen-pulsed adherent cells pretreated with phenylbutazone and stripped with pronase show unequivocally that the adherent cells are no longer capable of presenting the antigen in a way that it can activate T helper cells. These results indicate also that oxygen-dependent biochemical pathways play an important role in antigen-processing and presentation of OA. The importance of lysosomal degradation is suggested by data from Allen & Unanue (1984), who showed that agents like chloroquine inhibit the subsequent activation of T helper cells.

Taking these data together we can conclude that under the particular circumstances of our in-vitro system adherent cells of CGD patients can internalize the antigen, but are defective in the presentation of the antigen in a processed form to human peripheral blood T helper cells. Consequently a defect in oxygen metabolism may interfere at some stage with antigen-processing of OA, possibly since oxidants needed to denature sulfhydryl-containing proteins like OA before processing are lacking (Weiss, Lampert & Test, 1983).

This study was supported in part by the Foundation for Medical Research (Fungo, grant No. 13-40-91), which is subsidized by the Netherlands Organisation for the Advancement of Pure Research (Z.W.O.) The authors are indebted to Dr J. C. Kluin-Nelemans for the clinical assessment of one of the patients studied and to Dr H. C. W. van Velzen-Blad for the co-operation shown in the course of this study. They also thank Mrs Hélène van Moorsel for her secretarial assistance.

## REFERENCES

- ALLEN, P.M. & UNANUE, E.R. (1984) Differential requirements for antigen-processing by macrophages for lysosome-specific T cell hybridomas. *J. Immunol.* **132**, 1077.
- BALLIEUX, R.E., HEIJNEN, C.J., UYTDEHAAG F. & ZEGERS, B.J.M. (1979) Regulation of B cell activity in man: role of T cells. *Immunol. Rev.* **45**, 3.
- BALLIEUX, R.E. & HEIJNEN, C.J. (1983) Immunoregulatory T cell subpopulations in man: dissection by monoclonal antibodies and Fc-receptors. *Immunol. Rev.* **74**, 5.
- BENACERRAF, B. (1981) Role of MHC gene products in immune regulation. *Science*, **212**, 1229.
- GALLIN, J.I. & FAUCI, A.S. (1983) Chronic granulo-

- matous disease. In: *Advances in Host Defence Mechanisms* Vol. 3 (ed by J. I. Gallin & A. S. Fauci). p. 1. Raven Press, New York.
- GODING, J.W. (1969) The chromic chloride method of coupling antigens to erythrocytes: definition of some important parameters. *J. Immunol. Methods* **10**, 61.
- 'T HART, L.A., HEIJNEN, C.J., ZIJLSTRA, J. & BALLIEUX, R.E. (1985) Analysis of the antigen-induced in vitro differentiation of human peripheral blood B cells: stimulation with ovalbumin induces the transition from the resting state into the excited state. *Clin. exp. Immunol.* **61**, 648.
- HEIJNEN, C.J., UYTDEHAAG, F., GMELIG MEYLING, F.H.J. & BALLIEUX, R.E. (1979) Localization of human antigen-specific helper and suppressor function in distinct T cells subpopulations. *Cell. Immunol.* **43**, 282.
- HEIJNEN, C.J., UYTDEHAAG, F., POT, K.H. & BALLIEUX, R.E. (1981) Antigen-specific human T cell factors. I. T cell helper factor: biologic properties. *J. Immunol.* **126**, 497.
- ROSENTHAL, A.S. & SHEVACH, E.M. (1973) Function of macrophages in antigen recognition by guinea pig T lymphocytes. I. Requirement for histocompatible macrophages and lymphocytes. *J. exp. Med.* **138**, 1194.
- SOLBEY, C.O. (1975) Influence of therapeutic concentrations of phenylbutazone on granulocyte function. *Acta Path. Microbiol. Scand. Sect. B* **83**, 100.
- VAN TOL, M.J.D., ZIJLSTRA, J., THOMAS, C.M.G., ZEGERS, B.J.M. & BALLIEUX, R.E. (1984) Distinct role of neonatal and adult monocytes in the regulation of the in vitro antigen-induced plaque forming cell response in man. *J. Immunol.* **134**, 1902.
- UNANUE, E.R., BELLER, D.I., LU, C.Y. & ALLEN, P.M. (1984) Antigen presentation: comments on its regulation and mechanism. *J. Immunol.* **132**, 1.
- UYTDEHAAG, F., HEIJNEN, C.J. & BALLIEUX, R.E. (1978) Induction of antigen-specific human suppressor T lymphocytes in vitro. *Nature* **271**, 556.
- UYTDEHAAG, F., HEIJNEN, C.J., POT, K.H. & BALLIEUX, R.E. (1982) Antigen-specific human T cell factors. V. Interference of adherent cells in the generation of T cell suppressor factor by T cells. In: *Human B Cell Function. Activation and Immunoregulation* (ed. by A. S. Fauci & R. E. Ballieux). p. 253. Raven Press, New York.
- VAN VOORHIS, W.S., HAIR, L.S., STEINMAN, R.M. & KAPLAN, G. (1982) Human dendritic cells: enrichment and characterization from peripheral blood. *J. exp. Med.* **155**, 1172.
- WEISS, S.J., LAMPERT, M.B. & TEST, S.T. (1983) Long-lived oxidants generated by human neutrophils: characterization and bioactivity. *Science* **222**, 626.
- ZIEGLER, K. & UNANUE, E.R. (1981) Identification of a macrophage antigen-processing event required for I-region-restricted antigen presentation to T lymphocytes. *J. Immunol.* **127**, 1869.
- ZIEGLER, K. & UNANUE, E.R. (1982) Decrease in macrophage antigen catabolism by ammonia and chloroquine is associated with inhibition of antigen-presentation to T cells. *Proc. Natn. Acad. Sci. USA.* **79**, 175.