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Human neutrophils require activation by mononuclear leucocyte conditioned medium to kill the pathogenic free-living amoeba, *Naegleria fowleri*

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SUMMARY

Naegleria fowleri is a free-living amoeba which causes a fulminant and rapidly fatal meningoencephalitis in man. Human neutrophils fail to kill the amoeba *in vitro*, but can do so if they are exposed to conditioned medium (CM) from PHA stimulated mononuclear leucocytes (MNLs). Specific antibody or complement was required to effect amoeba killing by CM modified neutrophils. Only short time exposure of the leucocytes to CM was required to endow them with amoebicidal properties. The CM was also shown to contain neutrophil migration inhibition activity and an activity(ies) which induced a respiratory burst in neutrophils. The results highlight the importance of MNL products other than specific antibody in neutrophil anti-microbial activity.

Keywords *Naegleria fowleri* killing neutrophil activation antibodies complement

INTRODUCTION

Recently, lymphokines have been shown to influence neutrophils (Pick, 1979) in a manner similar to their effects on the macrophage. Potentiation of neutrophil activity by lymphokines and/or monokines could be of major significance when micro-organisms evade anti-microbial activities of neutrophils (Densen & Mandell, 1980). In one particular example, human neutrophils failed to kill virulent strains of *Entamoeba histolytica* (Guerrant *et al.*, 1981). Similarly, we have observed that human neutrophils fail to kill the pathogenic free-living amoebae *Naegleria fowleri* and *Acanthamoeba culbertsoni*. Now we present evidence that neutrophils exposed to lymphokine containing supernatants from lymphocyte cultures are able to kill the virulent free-living amoeba, *N. fowleri*, by an antibody or complement-dependent mechanism.

MATERIALS AND METHODS

Amoeba. Naegleria fowleri, Northcott strain (Thong *et al.*, 1978) originally isolated from a patient with amoebic meningoencephalitis was maintained axenically in tissue culture flasks using Chandler Fulton A (CFA) medium (Fulton, 1970) as described previously (Ferrante, 1982).

Preparation of leucocytes. Mononuclear leucocytes (MNL) and neutrophils were prepared from healthy volunteers' blood by a rapid single step technique involving centrifugation of blood on

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Hypaque-Ficoll of density 1·114 g/ml (Ferrante & Thong 1980a, 1982). Neutrophils were recovered in high yields and at purity of >96%. Viability of both leucocyte populations was >99% (by the trypan blue exclusion technique).

Preparation of anti-amoeba antisera. Rabbits were immunized with an amoeba culture supernatant antigen. This has previously been shown to induce marked resistance in mice to naegleria meningoencephalitis (Thong *et al.*, 1979, 1980, 1983). The degree of protection displayed by immunized mice correlated well with the level of specific anti-naegleria antibody, determined by an enzyme linked immunosorbant assay (Ferrante & Rowan-Kelly, unpublished observations). Rabbits were injected subcutaneously with 0·5 ml of the antigen at intervals of 2 weeks; four immunizations were made. Serum was prepared from rabbits 1 week after the last immunization and it contained an agglutination titre of 64.

Agglutination assay. The agglutination test was performed in microtitre plates as described previously (Thong *et al.*, 1979) except that *in lieu* of formalin fixed amoebae, live amoebae were used. The titre was the reciprocal of the highest serum dilution at which agglutination could be observed.

Adsorption of antiserum. A total of 1×10^8 amoebae were pelleted by centrifugation, the pellet resuspended in 1 ml of heat-inactivated antiserum and incubated for 1 h/2–4°C. After incubation the amoebae were removed and the serum subjected to two additional adsorption steps. The serum was finally filtered through a millipore membrane (0·22 μm). After adsorption the serum contained an agglutination titre of <2.

Partial purification of IgG antibodies in the antiserum. Ten millilitres of the antiserum were chromatographed on a 90 × 5 cm Sephacryl S-300 column (Pharmacia, Uppsala, Sweden) equilibrated with 0·01 M Tris buffer (pH 8·0). The elution rate was 20 ml/h. Fractions of 5 ml were collected and those corresponding to the serum IgG antibody fraction (column was calibrated with anti-sheep red blood cell IgG antibodies prior to running the above purification step) were pooled and concentrated to the original serum volume. The IgG fraction contained an anti-amoeba agglutination titre of 16–32.

Preparation of MNL conditioned medium. The MNL, at a concentration of 2×10^6 cells/ml, were incubated in RPMI 1640 medium containing 10% heat-inactivated FCS in either the presence or absence of 1 μg/ml of phytohaemagglutinin (PHA). The cells were incubated at 37°C for 24 h in an atmosphere of 5% CO_2–air mixture and high humidity. The MNL were washed three times with medium resuspended in fresh medium containing 10% FCS and reincubated for 48 h. The cell free supernatants were prepared by centrifugation and millipore filtration (0·22 μm membrane). The supernatants from MNL incubated with or without PHA were retained as conditioned media (CM) and non-conditioned media (NCM) respectively.

Treatment of neutrophils with CM. To 0·25 ml containing 1×10^7 neutrophils was added 0·75 ml of CM or NCM and these were incubated at 37°C/1 h. After incubation, the leucocytes were washed three times in medium 199 and used immediately in the amoebicidal assay.

Amoebicidal assay. Two and a half million neutrophils were added to $2{\cdot}5 \times 10^4$ amoebae in a total volume of 0·5 ml, using medium 199, in tissue culture tubes (16 × 125 mm, LUX Scientific Corporation). The sera, FCS, normal rabbit serum (NRS), rabbit anti-amoeba antisera or the IgG fraction were added to a final concentration of 4%. All sera were heated at 56°C/30 min except where the effects of serum complement were examined. The interaction was allowed to proceed at 37°C/4 h with regular mixing. After 4 h incubation, 10 ml of amoeba culture medium (CFA) was added to the tubes. The neutrophils are killed in this medium. The amoebae were washed in CFA, counted and finally resuspended in CFA and added to tissue culture Petri dishes to test for growth (Ferrante, 1982). After 24 h of incubation at 37°C, the amoeba numbers were counted in an inverted microscope. Approximately 20 fields were randomly counted (×200 magnification). The % amoebae killed was estimated as follows: $\%\ \text{killed} = (C - T) \times 100/C$ where C = number of amoebae in the presence of FCS only and T = number of amoebae in cultures subjected to other conditions.

Measurement of neutrophil migration inhibitory factor (N–MIF). N-MIF activity was measured by examining the ability of CM or NCM to inhibit random migration of neutrophils under agarose. The migration technique used was essentially that of Nelson, Quie & Simons (1975) with some modification (Ferrante, Beard & Thong, 1980). In these experiments, neutrophils at a concentration

of 4×10^7/ml were incubated for 1 h with an equal volume of fresh culture medium (+10% FCS), CM or NCM and then tested for migration.

Nitroblue tetrazolium (NBT) reduction assay. A microassay method for measuring reduction of NBT by neutrophils was used (Hofstaetter & Brammsen, 1981). To 100 μl of 5×10^6/ml of neutrophils in wells (flat bottomed) microtitre plates, was added 1 mg/ml NBT solution and either fresh medium (+10% FCS), NCM or CM. The cells were incubated at 37°C for 60 min. After incubation the optical density was measured at 540 nm using a Titertek Multiskan Spectrophotometer (Flow Laboratories).

Hexose monophosphate (HMP) shunt assay. Neutrophils were treated for 1 h with fresh medium (+10% FCS), NCM or CM and washed prior to HMP shunt measurement. The HMP shunt activity of neutrophils was measured as described previously (Paton & Ferrante, 1983). The amount of $^{14}CO_2$ evolved from (1-^{14}C)-L-glucose (3·96 mCi/nmol) was measured.

Hydrogen peroxide assay. H_2O_2 production by neutrophils was measured by a microassay technique as described previously (Paton & Ferrante, 1983). The assay was carried out in 0·2 ml volume per well, 40 μl of which was either fresh medium (+10% FCS), CM or NCM.

Statistical analysis. The data were analysed by the unpaired *t*-test. In the amoebicidal assays all reactions were compared to the control (amoeba incubated in the presence of FCS only). In all other assays the effects of NCM and CM were compared with the controls (cells incubated with medium +10% FCS).

RESULTS

Killing of naegleria by CM treated neutrophils

Neutrophils pre-incubated with CM or NCM were examined for amoebicidal activity against *N. fowleri*. The results showed that NCM treated neutrophils failed to cause significant killing of the amoeba, in the presence of FCS, NRS or anti-naegleria antiserum (Fig. 1). Neutrophils treated with CM also failed to kill the amoeba in the presence of either FCS or NRS but did so in the presence of antiserum ($P < 0{\cdot}001$). The results also showed that significant enhancement of amoeba growth

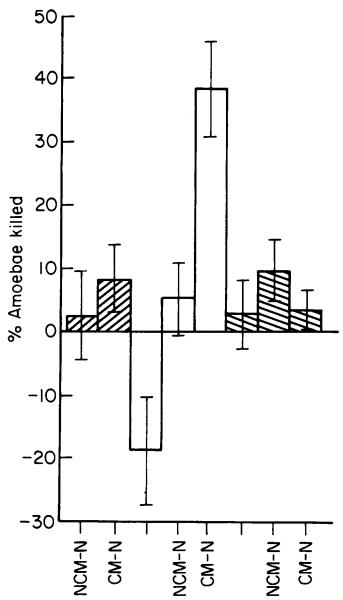

Fig. 1. The killing of *N. fowleri* by CM-activated human neutrophils (N). Results are expressed as mean ± standard error of 11 experiments. Assays were conducted in the presence of either FCS (■), IRS (□),or NRS (▩) and in the presence or absence of neutrophils treated with either CM or NCM.

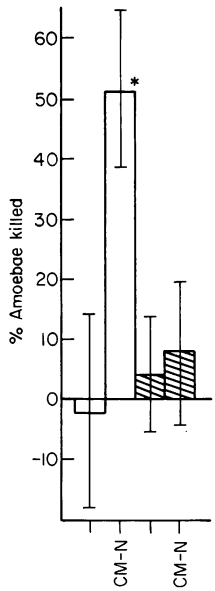

Fig. 2. The ability of amoeba adsorbed antiserum to aid CM treated neutrophils (N) in the killing of *N. fowleri*. Results are expressed as the mean ±s.e. of five experiments. Assays were conducted in the presence of either non-adsorbed (□) or adsorbed (▨) antiserum and in the presence of CM treated neutrophils. *$0{\cdot}01 > P > 0{\cdot}001$.

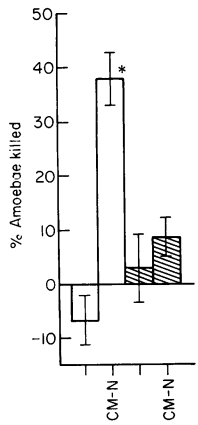

Fig. 3. Synergistic action of CM treated neutrophils (N) and partially purified IgG in the killing of naegleria. Results are expressed as mean ±s.e. of four experiments. Assays were conducted in either the presence or absence of CM treated neutrophils and either IgG (□) or NRS (▨). *$P < 0{\cdot}001$.

occurred in the presence of antibody alone ($0{\cdot}01 > P > 0{\cdot}001$). Neutrophils exposed to a range of concentrations of PHA (0·001–1·0 μg/ml) in the same manner as the cells exposed to CM, failed to mediate amoeba killing in the presence of antiserum (data not presented). Adsorbed antiserum failed to aid CM treated neutrophils in the killing of naegleria (Fig. 2). These neutrophils were shown to be effective in killing naegleria in the presence of IgG separated from the antiserum (Fig. 3).

The role of complement in neutrophil-mediated killing

Naegleria was incubated with CM treated neutrophils and native or heat-inactivated C6 deficient rabbit serum (from rabbits genetically deficient in the sixth component of complement). C6

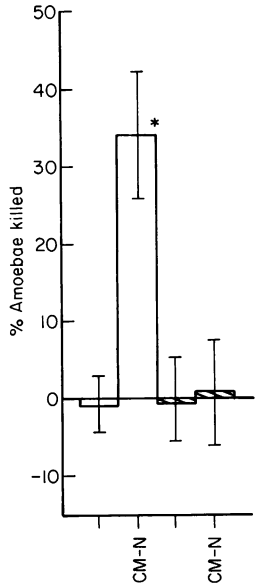

Fig. 4. Complement-dependent neutrophil-mediated killing of *N. fowleri*. Results are expressed as mean ± s.e. of 10 experiments. Assays were conducted in the presence of 40% of either C6 deficient rabbit serum (□) or heat-inactivated (56°C/30 min) C6 deficient rabbit serum (■) and in the presence or absence of CM treated neutrophils (N). *$P < 0.001$.

deficient rabbit serum was used to avoid lysis, since the amoeba is known to activate complement via the alternative pathway (Rowan-Kelly, Ferrante & Thong, 1980; Holbrook *et al.*, 1980).

The neutrophils were capable of killing naegleria in the presence of 40% C6 deficient rabbit serum but failed to do so in the presence of heat-inactivated C6 deficient serum (Fig. 4).

Neutrophil inhibition of random migration and stimulation of a respiratory burst by CM

The results presented in Table 1 show that CM contained neutrophil migration inhibitory factor (N-MIF) activity. This activity was absent in NCM. CM treated neutrophils, in contrast to NCM treated leucocytes, exhibited increased NBT reduction, elevated HMP shunt activity and increased H_2O_2 production (Table 2).

Table 1. Presence of neutrophil migration inhibition activity in CM

Treatment	% inhibition of random migration*
NCM	0·04 ± 2·41
CM	51·27 ± 8·28†

* Results are expressed as mean ± s.e. of 10 experiments and presented as % inhibition of random migration (when compared to random movement in controls in which the neutrophils were incubated with RPMI 1640 + 10% FCS).

† $P < 0.001$.

Table 2. Stimulation of neutrophil NBT reduction, HMP shunt activity and H_2O_2 production by CM

	Stimulation index*		
Treatment	NBT reduction	HMP shunt	H_2O_2 production
NCM	1·0±0·17 (5)	1·0±0·09 (4)	1·2±0·19 (5)
CM	3·1±0·20 (5)†	2·0±0·15 (4)†	1·9±0·32 (5)‡

* Results are expressed as mean±s.e. of the number of experiments indicated in parentheses.

† $0{\cdot}01 > P > 0{\cdot}001$.

‡ $0{\cdot}02 > P > 0{\cdot}01$.

DISCUSSION

Our previous studies demonstrated that immunized mice mounted a rapid inflammatory cellular reaction at the site of *N. fowleri* challenge (Ferrante & Thong, 1980b; Thong *et al.*, 1983); neutrophils reach the site relatively early and in large numbers. Accordingly, these effector cells could play a significant role in resistance to *N. fowleri* but we have been unable to demonstrate that human neutrophils, in the presence of specific antibody, can kill the amoeba.

Interestingly, the data obtained in the present study show that human neutrophils require modification(s) to demonstrate amoebicidal properties. These studies suggest that another MNL product, possibly a lymphokine, and specific antibody act synergistically in the neutrophil-mediated killing of *N. fowleri*. Neutrophils treated with CM (from PHA stimulated MNL) but not NCM (from MNL cultured in the absence of PHA) were capable of killing *N. fowleri* in the presence of antiserum. The augmented neutrophil-mediated killing is unlikely to be due to any residual PHA that may have been contaminating the CM since neutrophils treated with a range of concentrations of PHA failed to demonstrate amoebicidal activity.

The CM treated leucocytes were washed prior to the addition of amoeba. Thus it is evident that the continuous presence of CM is not required. The antiserum used with the CM treated neutrophils had been heated at 56°C/30 min, implicating a complement-independent mechanism, most likely involving specific antibody. Antiserum adsorbed with *N. fowleri* could not aid the neutrophils in amoeba killing and the IgG fraction of the antiserum was effective in aiding the neutrophils supporting a role for specific antibody.

Attachment of micro-organisms to neutrophils is generally mediated via the Fc receptor or C3b receptor on these leucocytes (Ehlenberger & Nussenzweig, 1977). In the present study we showed that activated neutrophils killed *N. fowleri* in either the presence of specific IgG or complement which is most likely activated by the alternative pathway. Thus amoeba killing (cytotoxicity) can occur via two types of mechanisms, antibody-dependent neutrophil-mediated killing (ADNK) or complement-dependent neutrophil-mediated killing (CDNK). ADNK has been demonstrated with respect to a number of other targets, including tumour cells and HSV-1 infected cells (Clark & Klebanoff, 1975; Russell & Miller, 1978; Hokland & Berg, 1981; Vadas, Nicola & Metcalf, 1983). In addition a CDNK mechanism has been described for bovine neutrophils in the killing of HSV-1 infected cells (Grewal, Rouse & Babiuk, 1980).

Unexpectedly, antibody alone enhanced the growth of *N. fowleri*, arguing that this perhaps should be considered as the control for the reactions which include specific antibody. Thus it can be interpreted that NCM treated neutrophils do cause significant killing. However, we are against this view, since it is likely that the enhanced growth is related to redistribution, capping and internalization of surface bound antibody by the amoeba (Ferrante & Thong, 1979). This phenomenon is unlikely to occur if neutrophils are binding to the amoeba via the Fc receptor.

Lomnitzer, Glover & Rabson (1977) reported that neutrophils exposed to PHA activated MNL supernatants have increased negative surface charge, enhanced phagocytic activity, greater ability

to reduce NBT and elevated HMP shunt activity. We found increased NBT reduction ability and enhanced HMP shunt activity and, in addition, increased production of H_2O_2 in neutrophils treated with CM. It has been reported that lymphokine preparations induce aggregation (Badenoch-Jones, 1982) and migration inhibition (Chess *et al.*, 1975; Weisbart & Billing, 1980; Badenoch-Jones, 1982; Klempner & Rocklin, 1982). Neutrophil migration inhibition activity was also observed in our CM preparation. However, it remains to be established whether these altered characteristics are responsible for, or are merely associated with, the neutrophil augmented killing.

Virulent *E. histolytica* strains, in contrast to those of low virulence, are not killed by human neutrophils (Guerrant *et al.*, 1981). Lymphokines could play the important role of activating neutrophils to kill these amoebae in an analogous manner to the pathogenic free-living amoeba. Both *E. histolytica* (Diamantstein *et al.*, 1981) and *N. fowleri* (Ferrante & Smyth, 1984) contain mitogenic factors for T lymphocytes. Thus it is most probable that during an infection, lymphokines are produced and these modify neutrophils in the manner described. Mouse peritoneal neutrophils harvested from the peritoneal cavity following the injection of thioglycollate, also failed to kill *N. fowleri in vitro* but did so when activated with CM (unpublished observations). Previously we have shown that mouse neutrophils were able to kill in a semi-*in vitro* system (Ferrante & Thong, 1980b). This involved the elicitation of a neutrophil inflammation in the mouse peritoneal cavity, injection of live *N. fowleri*, intraperitoneally, and after 2–3 h withdrawal and examination of the peritoneal cellular exudate for the interaction of neutrophils with amoebae by optical microscopy. Killing was evident under these experimental conditions. We believe that under these conditions the amoeba mitogenic factor was capable of stimulating the release of lymphokines which may have activated the neutrophils and thus being responsible for the observed amoeba killing.

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REFERENCES

BADENOCH-JONES, P. (1982) Lymphokine-induced neutrophil aggregation. *Immunology*, **47**, 169.

CHESS, L., ROCKLIN, R.E., MACDERMOTT, R.P. & SCHLOSSMAN, S.F. (1975) Leukocyte inhibitory factor (LIF): production by purified human T and B lymphocytes. *J. Immunol.* **115**, 315.

CLARK, R.A. & KLEBANOFF, S.J. (1975) Neutrophil-mediated tumour cell cytotoxicity: role of the peroxidase system. *J. exp. Med.* **141**, 1442.

DENSEN, P. & MANDELL, G.L. (1980) Phagocyte strategy vs. microbial tactics. *Rev. Infect. Dis.* **2**, 817.

DIAMANTSTEIN, T., KLOS, M., GOLD, D. & HAHN, H. (1981) Interaction between *Entamoeba histolytica* and the immune system. I. Mitogenicity of *Entamoeba histolytica* extracts for human peripheral T lymphocytes. *J. Immunol.* **126**, 2084.

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

FERRANTE, A. (1982) Comparative sensitivity of *Naegleria fowleri* to amphotericin B and amphotericin B methyl ester. *Trans. R. Soc. Trop. Med. Hyg.* **76**, 476.

FERRANTE, A., BEARD, L.J. & THONG, Y.H. (1980) Early decay of human neutrophil chemotactic responsiveness following isolation from peripheral blood. *Clin. exp. Immunol.* **39**, 532.

FERRANTE, A. & SMYTH, C. (1984) Mitogenicity of *Naegleria fowleri* extract for murine T lymphocytes. *Immunology*, (In press.)

FERRANTE, A. & THONG, Y.H. (1979) Antibody induced capping and endocytosis of surface antigens in *Naegleria fowleri*. *Int. J. Parasitol.* **9**, 599.

FERRANTE, A. & THONG, Y.H. (1980a) Optimal conditions for simultaneous purification of mononuclear and polymorphonuclear leucocytes from human blood by the Hypaque-Ficoll method. *J. Immunol. Meth.* **36**, 109.

FERRANTE, A. & THONG, Y.H. (1980b) Unique phagocytic process in neutrophil-mediated killing of *Naegleria fowleri*. *Immunol. Lett.* **2**, 37.

FERRANTE, A. & THONG, Y.H. (1982) Separation of mononuclear and polymorphonuclear leucocytes from human blood by the one-step Hypaque-Ficoll method is dependent on blood column height. *J. Immunol. Meth.* **48**, 81.

FULTON, C. (1970) Amoeba-flagellates as research partners. In *The Laboratory Biology of* Naegleria *and* Tetramitus: *Methods in Cell Physiology* (ed by D.M. Prescott) Vol 4. pp. 341–376. Academic Press, New York.

GREWAL, A.S., ROUSE, B.T. & BABIUK, L.A. (1980) Mechanisms of recovery from viral infections: destruction of infected cells by neutrophils and complement. *J. Immunol.* **124**, 312.

GUERRANT, R.L., BRUSH, J., RAVDIN, J.I., SULLIVAN, J.A. & MANDELL, G.L. (1981) Interaction between *Entamoeba histolytica* and human polymorphonuclear neutrophils. *J. Infect. Dis.* **143**, 83.

HOKLAND, P. & BERG, K. (1981) Interferon enhances

the antibody-dependent cellular cytotoxicity (ADCC) of human polymorphonuclear leukocytes. *J. Immunol.* **127,** 1585.

HOFSTAETTER, T. & BRAMMSEN, H. (1981) A microassay for nitroblue tetrazolium reduction by human neutrophils. *Immunobiol.* **159,** 283.

HOLBROOK, T.W., BOACKLE, R.J., PARKER, B.W. & VESELEY, J. (1980) Activation of the alternative complement pathway by *Naegleria fowleri*. *Infect. Immunol.* **30,** 58.

KLEMPNER, M.S. & ROCKLIN, R.E. (1982) Specific binding of leukocyte inhibitory factor to neutrophil plasma membranes. *J. Immunol.* **128,** 2040.

LOMNITZER, R., GLOVER, A. & RABSON, A.R. (1977) The effect of PHA activated MN cell supernatants on polymorphonuclear leucocyte function. *Clin. exp. Immun.* **29,** 501.

NELSON, R.D., QUIE, P.G. & SIMMONS, R.L. (1975) Chemotaxis under agarose: a new and simple method for measuring chemotaxis and spontaneous migration of human polymorphonuclear leukocytes and monocytes. *J. Immunol.* **115,** 1650.

PATON, J.C. & FERRANTE, A. (1983) Inhibition of human polymorphonuclear leukocyte respiratory burst, bactericidal activity, and migration by pneumolysin. *Infect. Immun.* **41,** 1212.

PICK, E. (1979) Mechanism of action of migration inhibitory lymphokines. In *Biology of the Lymphokines* (ed. by S. Cohen, E. Pick & J.J. Oppenheim) pp. 59–119. Academic Press, New York.

ROWAN-KELLY, B., FERRANTE, A. & THONG, Y.H. (1980) Activation of complement by Naegleria. *Trans. R. Soc. Trop. Med. Hyg.* **73,** 684.

RUSSELL, A.S. & MILLER, C. (1978) A possible role for polymorphonuclear leucocytes in defence against recrudescent herpes simplex virus infection in man. *Immunology*, **34,** 371.

THONG, Y.H., CARTER, R.F., FERRANTE, A. & ROWAN-KELLY, B. (1983) Site of expression of immunity to *Naegleria fowleri* in immunized mice. *Parasite Immunol.* **5,** 67.

THONG, Y.H., FERRANTE, A., ROWAN-KELLY, B. & O'KEEFE, D.E. (1979) Immunization with culture supernatant in experimental *Naegleria* meningoencephalitis. *Trans. R. Soc. Trop. Med. Hyg.* **73,** 684.

THONG, Y.H., FERRANTE, A. ROWAN-KELLY, B. & O'KEEFE, D.E. (1980) Immunization with live amoeba, amoebic lysate and culture supernatant in experimental *Naegleria* meningoencephalitis. *Trans. R. Soc. Trop. Med. Hyg.* **74,** 570.

THONG, Y.H., SHEPHERD, C., FERRANTE, A. & ROWAN-KELLY, B. (1978) Protective immunity to *Naegleria fowleri* in experimental amebic meningoencephalitis. *Am. J. Trop. Med. Hyg.* **27,** 238.

VADAS, M.A., NICOLA, N.A. & METCALF, D. (1983) Activation of antibody-dependent cell-mediated cytotoxicity of human neutrophils and eosinophils by separate colony-stimulating factors. *J. Immunol.* **130,** 795.

WIESBART, R.H. & BILLING, R. (1980) Requirement for B-lymphocyte alloantigen in the production of neutrophil migration inhibition factor from T lymphocytes (NIF-T). *Immunology*, **39,** 577.