Clin. exp. Immunol. (1978) 34, 52–58.

Effect of cyclophosphamide treatment on the course of *Mycobacterium lepraemurium* infection and development of delayed-type hypersensitivity reactions in C57Bl and BALB/c mice

J. ALEXANDER *National Institute for Medical Research, Mill Hill, London*

(*Received* 5 *April* 1978)

SUMMARY

Pre-treatment of *Mycobacterium lepraemurium* susceptible, BALB/c, and resistant, C57Bl, mice with cyclophosphamide markedly altered the development of delayed hypersensitivity during foot-pad infections with this organism. A tuberculin-type response demonstrated by untreated C57Bl mice was significantly intensified after week 3 in cyclophosphamide-pre-treated mice although this response had returned to normal levels by week 8. A Jones-Mote-type response demonstrated throughout experiments by untreated BALB/c mice was considerably increased in magnitude by week 3 in cyclophosphamide-pre-treated mice. By week 6 this response had become considerably protracted and was of the tuberculin-type. By week 8 however this response had started to diminish and by week 12 cyclophosphamide-treated and untreated BALB/c mice produced similar Jones-Mote-type responses when skin-tested. Cyclophosphamide pre-treatment had no effect on the growth of *M. lepraemurium* in C57Bl mice over 12 weeks. In BALB/c mice however cyclophosphamide-pre-treated mice demonstrated considerable resistance to infection at weeks 8 and 10 after infection but not thereafter. Whereas the magnitude of the delayed hypersensitivity response in C57Bl mice could not be correlated with resistance such a relationship could be demonstrated in BALB/c mice.

INTRODUCTION

The protective immune response against *Mycobacterium leprae* in man is considered to be entirely cell-mediated whereas the humoral response is considered to be unimportant or even detrimental (Myrvang, Feek & Godal, 1974). In human leprosy there is a wide range of clinical manifestations of disease ranging from the highly resistant tuberculoid, to the low resistant lepromatous, disease types. Whereas tuberculoid patients demonstrate high levels of delayed hypersensitivity, lepromatous patients demonstrate no or only low levels of delayed hypersensitivity.

Inbred strains of mice demonstrate similar wide ranges of resistance to *M. lepraemurium* (Kawaguchi, 1959; Closs & Haugen, 1974). Recent studies have also indicated that a strong correlation exists between the development of delayed hypersensitivity to *M. lepraemurium* infections and the acquisition of protective immunity (Alexander & Curtis, 1978). C57Bl, mice which are relatively resistant to infection, rapidly develop a protracted delayed hypersensitivity response, when skin-tested with soluble *M. lepraemurium*-derived antigen, which reaches maximum intensity between 24 and 48 hours, persists up to and beyond 72 hours and is similar to the classical tuberculin reaction. BALB/c mice, which are relatively susceptible, develop a response which reaches maximum intensity at 24 hours drops dramatically at 48 hours, is imperceptible at 72 hours and is reminiscent of a Jones-Mote reaction.

Correspondence: Dr J. Alexander, National Institute for Medical Research, Mill Hill, London NW7 1AA.

0099-9104/78/0100-0052$02.00

Treatment of animals with cyclophosphamide depletes thymus-independent areas of lymphoid tissue (Turk, Polak & Parker, 1976). Although T lymphocytes are affected they are less affected than B lymphocytes (Willers & Sluis, 1975). On stimulation with antigen T lymphocytes recover quicker and proliferate at an accelerated rate, leading to increased delayed hypersensitivity and depression of humoral responses to certain antigens. Pre-treatment of guinea pigs with cyclophosphamide has also been shown to change the delayed hypersensitivity response to ovalbumin (OA) in animals immunized with OA in Freund's incomplete adjuvant (FIA) from a Jones-Mote-type response to a tuberculin-type response (Turk & Parker, 1973).

As there would appear to be an association between Jones-Mote and tuberculin-type delayed hypersensitivity responses and susceptibility and resistance to *M. lepraemurium* in BALB/c and C57Bl mice, the effect of cyclophosphamide pre-treatment on the development of delayed hypersensitivity and the growth of *M. lepraemurium* in these mice was studied.

MATERIALS AND METHODS

Mice. Ten-week-old inbred BALB/c and C57Bl mice of either sex bred at the National Institute for Medical Research were used.

Mycobacterium lepraemurium. The Douglas strain of *M. lepraemurium* was maintained by serial passage of 10^9 bacilli intravenously in Parkes outbred mice.

Preparation of M. lepraemurium for infecting animals. M. lepraemurium was harvested from infected livers and purified by the method of Draper (1971). Acid-fast bacilli stained by the Ziehl-Neelsen technique were counted by the method described by Hart & Rees (1960).

Infection of mice with M. lepraemurium. Mice were infected in the right hind footpad with 10^7 *M. lepraemurium* in 0·05 ml sterile saline.

Injection of cyclophosphamide. Cyclophosphamide was kindly provided by Ward Blenkinsop (Wembley, Middlesex). Mice were inoculated intraperitoneally with 250 mg/kg cyclophosphamide 2 days before infection with 10^7 *M. lepraemurium.*

Preparation of M. lepraemurium skin-test antigen. Suspensions of purified bacteria in saline were broken by exposure to ultra-sound. Cell walls and unbroken bacteria were removed by centrifugation (35,000 *g*, 30 min) and the protein content of the supernatant was estimated by the method of Lowry *et al.* (1965). This soluble protein moiety constituted the skin-test antigen and the stock solution adjusted to 200 μg protein/ml was kept at $-20°C$.

Skin-testing. Mice were inoculated in the left hind footpad with 5 μg antigen in 0·025 ml sterile saline. Increase in footpad thickness was measured 4, 24, 48 and usually 72 hours thereafter using a screw-gauge micrometer (Moore & Wright, Sheffield).

Assessment of M. lepraemurium growth. Mice were killed by rapid dislocation of the neck. Right hind footpads were excised and homogenized in 2 ml of 0·1% albumin in saline and diluted 1:1 in 0·1% albumin in water for counting. Mycobacteria were counted by the spot-slide method of Hart & Rees (1960).

Experimental design. In each experiment groups of 40 mice of each strain were injected intraperitoneally with 250 mg/kg cyclophosphamide. These mice and equal numbers of untreated mice were infected 2 days later with 10^7 *M. lepraemurium* in the right hind footpad. At weekly or two-weekly intervals groups of at least six mice from each group were skin-tested with 5 μg antigen in the contralateral footpad. After skin-testing some mice were killed and the growth of *M. lepraemurium* in the right hind footpad measured.

RESULTS

Effect of cyclophosphamide pre-treatment (250 mg/kg) on the development of delayed hypersensitivity reactions in Mycobacterium lepraemurium infected BALB/c and C57Bl mice

Three weeks after infection with *M. lepraemurium* in the right hind footpad untreated BALB/c mice demonstrated a Jones-Mote-type response when skin-tested in the contralateral footpad (Fig. 1a). This response was characterized by reaching a peak of intensity at 24 hours and falling dramatically by 48 hours. At this time cyclophosphamide pre-treated BALB/c mice produce a response of similar kinetics to untreated mice but of significantly greater magnitude. The intensity and duration of the delayed hypersensitivity response in both cyclophosphamide-pre-treated and untreated C57Bl mice was similar at 3 weeks and was of the tuberculin-type persisting up to and beyond 48 hours (Fig. 1b).

Between weeks 3 and 8 after infection untreated BALB/c mice continue to produce a Jones-Mote-type response when skin-tested. Cyclophosphamide pre-treated BALB/c mice not only maintain a greater magnitude of response at this time but the intensity of the response is maintained after 24 hours. This

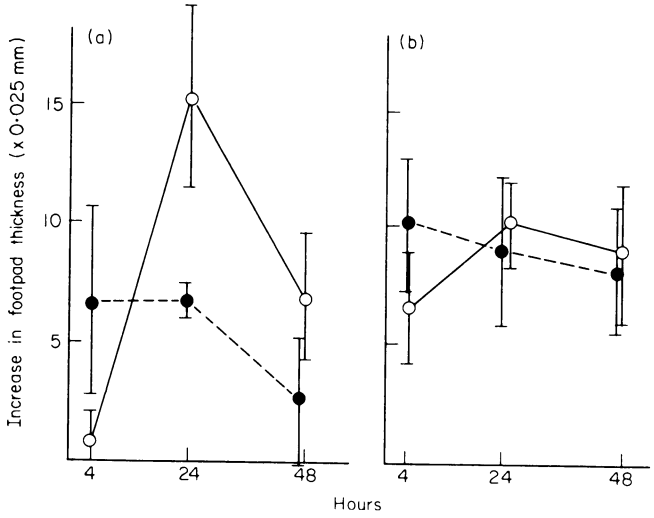

FIG. 1. Kinetics of the footpad swelling in cyclophosphamide pre-treated (○) and untreated (●) BALB/c (a) and C57Bl (b) mice inoculated subcutaneously in the left hind footpad with 5 μg soluble antigen. Mice had been infected in the right hind footpad with 10^7 *Mycobacterium lepraemurium* 3 weeks previously. Means of six animals ± s.d.

prolonged response reaches maximum duration at week 6 (Fig. 2a) when it is very similar to that demonstrated by C57Bl mice. The tuberculin-type response of cyclophosphamide pre-treated C57Bl mice is of greater intensity over this period than in untreated mice (Fig. 2b).

Over the next few weeks delayed hypersensitivity responses in cyclophosphamide-treated and untreated mice become increasingly alike and by week 12 no difference in the magnitude or kinetics of the response could be measured between mice in either strain. At this stage BALB/c mice had a pronounced Jones-Mote-type response (Fig. 3a) while C57Bl mice had a more protracted tuberculin-type response (Fig. 3b).

Growth of M. lepraemurium in cyclophosphamide pre-treated and untreated BALB/c and C57Bl mice

In several experiments mice which had been skin-tested were killed and the number of *M. leprae-*

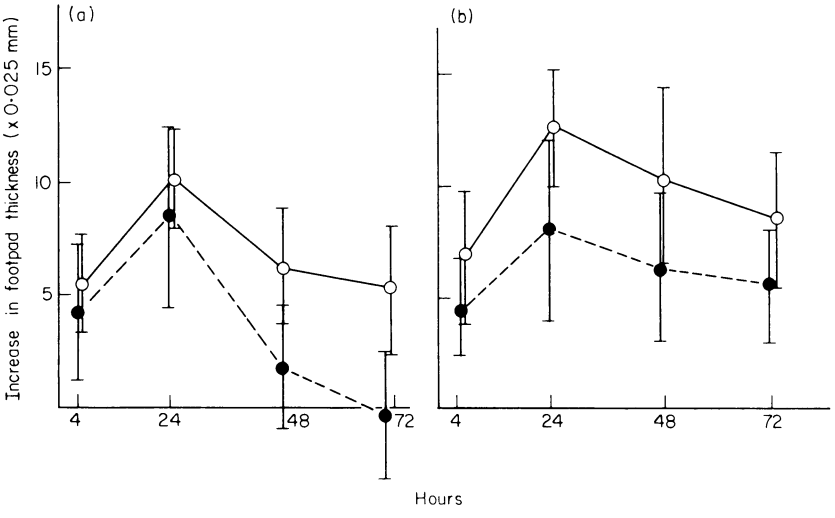

FIG. 2. As Fig. 1a & b, but kinetics of footpad swelling measured 6 weeks after infection with 10^7 *Mycobacterium lepraemurium*. Means of ten animals ± s.d.

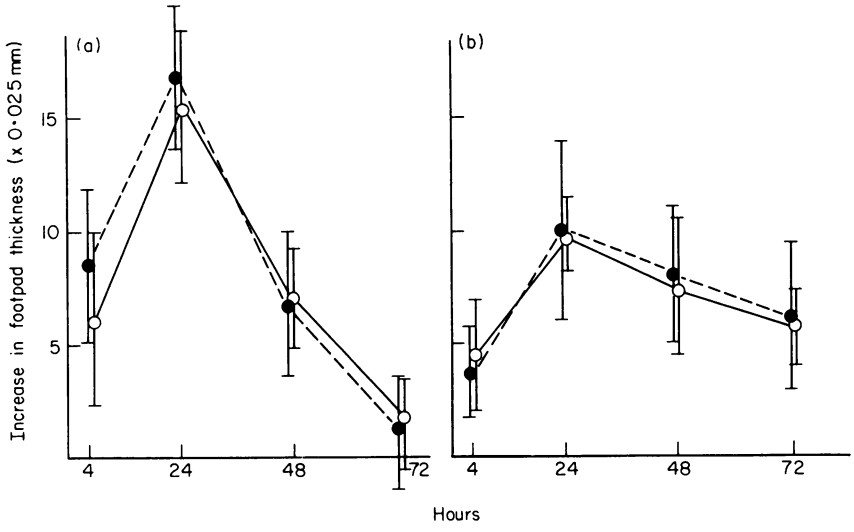

FIG. 3. As Fig. 1a & b, but kinetics of footpad swelling measured 12 weeks after infection with 10^7 *Mycobacterium lepraemurium*. Means of 6 animals ± s.d.

murium in the right hind footpad counted. The results of 2 experiments are summarized in Fig. 4a and b. The growth of *M. lepraemurium* in untreated BALB/c mice (Fig. 4a) was much greater than in untreated C57Bl mice (Fig. 4b). Whereas cyclophosphamide had no apparent effect on the growth of *M. lepraemurium* in C57Bl mice (Fig. 4b), growth in cyclophosphamide pre-treated BALB/c mice was significantly less between weeks 8–10. Resistance to *M. lepraemurium* had not become apparent in these cyclophosphamide-pre-treated BALB/c mice by week 6 and was gone by week 12.

Relationship between the magnitude of the 24-hour-delayed hypersensitivity response and resistance to M. lepraemurium

Twelve-week *M. lepraemurium* infected but otherwise untreated BALB/c and C57Bl mice were used. The mice were skin-tested in the normal way. After testing mice were killed and the magnitude of the 24-hour skin reaction compared with the number of organisms recovered from the right hand footpad for each mouse.

While no correlation existed between the increase in C57Bl footpad thickness at 24 hours and the number of recoverable *M. lepraemurium* (Fig. 5b) a highly significant correlation between these two parameters was found in BALB/c mice (Fig. 5a): the increase in footpad thickness at 24 hours in these mice was inversely proportional to the bacillary load.

DISCUSSION

Diseases such as leprosy, leishmaniasis, syphilis and tuberculosis produce a wide spectrum of clinical disease (Turk & Bryceson, 1971; WHO Scientific Group Report, 1973; Lenzini, Rotolli & Rotolli, 1977). It is now becoming increasingly apparent that such diseases also produce a wide spectrum of delayed hypersensitivity responses. What remains to be ascertained is the relationship between these cell-mediated responses as measured by delayed hypersensitivity and the acquisition of a protective immune response.

In a recent publication Rook (1978) has described the development of at least 3 distinct types of delayed hypersensitivity during the early stages of certain mycobacterial infections in BALB/c mice. Rook (1978) suggests that protective immunity is associated with a peak of delayed hypersensitivity 10 days after infection as this peak occurs when the infecting organism is non-pathogenic but not when it is

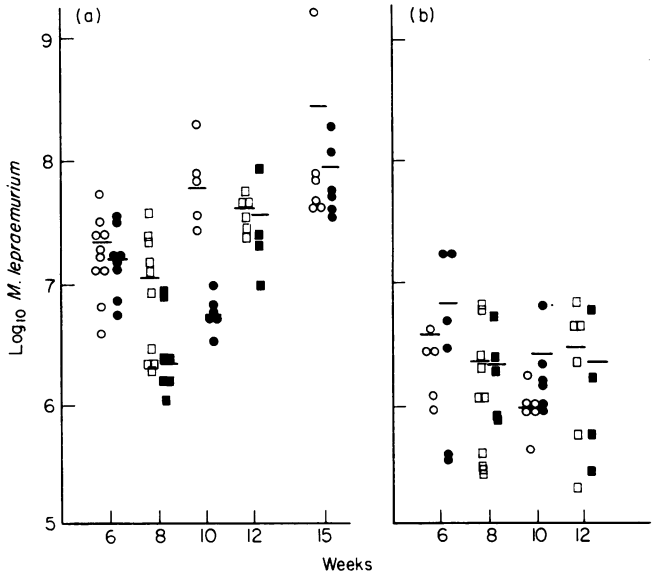

FIG. 4. Growth of 10^7 *Mycobacterium lepraemurium* in the right hind footpad of BALB/c (Fig. 4a) and C57Bl (Fig. 4b) mice. Results are from 2 experiments (Expt. 1 (●) (○): Expt. 2 (■) (□)) and mice were either pre-treated with 250 mg/kg cyclophosphamide (●) (■) or left untreated (○) (□). Points represent number of *M. lepraemurium* recovered from each animal.

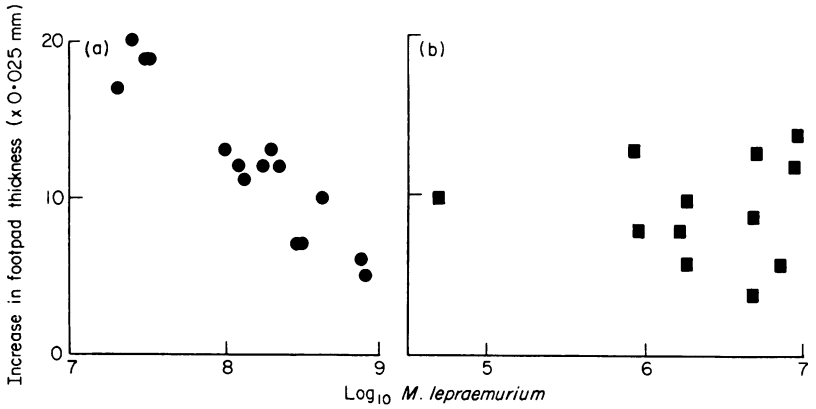

FIG. 5. Relationship between increase in footpad thickness at 24 hours in animals inoculated subcutaneously with 5 μg antigen in the left hind footpad and the number of *Mycobacterium lepraemurium* recovered from the right hind footpad. Mice had been infected in the right hind footpad with 10^7 *Mycobacterium lepraemurium* 12 weeks previously (Fig. 5a, BALB/c mice: Fig. 5b C57Bl mice).

pathogenic. Alexander & Curtis (1978) have also demonstrated a variety of delayed hypersensitivity responses during the early phases of *Mycobacterium lepraemurium* infections in BALB/c and C57Bl mice which are described as being due to the interaction of various effector and regulatory mechanisms. These workers, however, have suggested that the stabilized delayed hypersensitivity condition established some 3 weeks after infection is the more important indicator of resistance to infection. At this stage of *M. lepraemurium* infection C57Bl mice, which are comparatively resistant to infection, demonstrate tuberculin-type hypersensitivity while the comparatively susceptible BALB/c strain demonstrates a Jones-Mote-type response. That there is a relationship between the development of a stable tuberculin-type delayed hypersensitivity response after 3 weeks in C57Bl mice and protective immunity is suggested by

the histological studies of Closs & Haugen (1975). These workers demonstrated inflammatory cell infiltration of infected footpads with subsequent killing of *M. lepraemurium* in C57Bl mice around 4 weeks after infection.

The close relationship between susceptibility and resistance and Jones-Mote and tuberculin-type reactions in murine leprosy has previously been compared with the similar relationship between delayed hypersensitivity and resistance in human tuberculosis (Alexander & Curtis 1978). Whereas in both murine leprosy and human tuberculosis individuals with Jones-Mote-type hypersensitivity were at the more susceptible end of the disease spectrum it would appear that the Jones-Mote reaction in itself indicates a limited degree of resistance. This is suggested firstly because tuberculosis patients with Jones-Mote reactions, unlike those with negative skin-test reactions, respond favourably to chemotherapy (Lenzini *et al.*, 1977) and secondly because there is a direct correlation between the intensity of the Jones-Mote-type response and resistance to *M. lepraemurium* infection in BALB/c mice.

Whereas these experiments showed a direct correlation between the intensity of the Jones-Mote-type hypersensitivity and the level of resistance to *M. lepraemurium* in susceptible BALB/c mice no correlation between the intensity of the tuberculin-type response and resistance could be measured in the resistant C57Bl strain. While cyclophosphamide pre-treatment markedly intensified the delayed hypersensitivity response between weeks 3–8 in C57Bl mice this was not reflected in any increase or decrease in resistance. This would indicate a definite dissociation between tuberculin-type hypersensitivity and a protective immune response. Such a situation has also been described in *Listeria monocytogenes* infections of mice where there is also a clear dichotomy between the development of delayed hypersensitivity and acquired resistance (Kerckhaert, Hofhuis & Willers, 1977). Acquired resistance however in *L. monocytogenes* and *Mycobacterium lepraemurium* infections was always accompanied by positive delayed hypersensitivity. Tuberculin-type hypersensitivity and protective immunity in *M. lepraemurium* infected C57Bl mice would therefore appear to be quite independent manifestations of a related phenomenon.

Cyclophosphamide treatment of BALB/c mice before *M. lepraemurium* infection not only increased the intensity of the early Jones-Mote reaction but also promoted the development of a tuberculin-type response which reached maximum at week 6. This alteration in the development of delayed hypersensitivity was accompanied by a temporary increase in resistance to *M. lepraemurium* growth at weeks 8–10. We therefore have the possibility that either this increased resistance is related to a more intense Jones-Mote response or, as is more likely, the short-term and concurrent acquisition of the tuberculin-type hypersensitivity, similar in type to that displayed by resistant C57Bl mice.

Cyclophosphamide pre-treatment has been shown to enhance delayed hypersensitivity responses in a number of experimental systems by removing suppressor cells which may be either B lymphocytes (Katz, Parker & Turk, 1974; Neta & Salvin, 1976) or T lymphocytes (Askenase, Hayden & Gershon, 1975; Rollinghoff *et al.*, 1977). Experiments are now in progress to identify possible suppressor mechanisms in *M. lepraemurium* infected BALB/c mice. As the development of tuberculin-type hypersensitivity in cyclophosphamide pretreated BALB/c mice is protracted compared with C57Bl mice this would suggest that there is a pool of cyclophosphamide in sensitive short-lived suppressor cells. As these cells diminish in numbers a tuberculin-type response would develop with a subsequent increase in resistance. The regeneration of cyclophosphamide-sensitive precursor cells would result in a restored Jones-Mote-type response after 10 weeks with subsequent increased susceptibility to infection.

I wish to thank Dr R. J. W. Rees and Dr Jill Curtis for invaluable discussion and help in preparation of this manuscript. The excellent technical assistance of Mr. C. Smith is also gratefully acknowledged.

REFERENCES

Alexander, J. & Curtis, Jill (1978) Development of delayed hypersensitivity responses in *Mycobacterium lepraemurium* infections in resistant and susceptible strains of mice. *Immunology*, (in press).

Askenase, P.W., Hayden, B.J. & Gershon, R.K. (1975) Augmentation of delayed-type hypersensitivity by doses of cyclophosphamide which do not affect antibody responses. *J. exp. Med.* **141**, 697.

Closs, O. & Haugen, O.A. (1973) Experimental Murine Leprosy I Clinical and histological evidence for varying

susceptibility of mice to infection with *Mycobacterium lepraemurium*. *Acta. path. microbiol. scand.* Sect. A **81**, 401.

CLOSS, O. & HAUGEN, O.A. (1975) Experimental murine leprosy 3. Early local reaction to *Mycobacterium lepraemurium* in C3H and C57Bl mice. *Acta path. microbiol. scand.* **83**, 51.

DRAPER, P. (1971) The walls of *Mycobacterium lepraemurium*; chemistry and ultrastructure. *J. Gen. Microbiol.* **69**, 313.

HART, P.D. & REES, R.J.W. (1960) Effect of macrocyclon in acute and chronic pulmonary tuberculosis infection in mice as shown by viable and total bacterial counts. *Br. J. exp. Path.* **41**, 414.

KATZ, S.I., PARKER, D. & TURK, J.L. (1974) B-cell suppression of delayed hypersensitivity reactions. *Nature*, **251**, 550.

KAWAGUCHI, Y. (1959) Classification of murine leprosy. *Jap. J. exp. Med.* **29**, 651.

KERKHAERT, J.A.M., HOFHUIS, F.M.A. & WILLERS, J.M.N. (1977) Influence of cyclophosphamide and acquired cellular resistance to *Listeria monocytogenes* in the mouse. *Immunology*, **32**, 1027.

LENZINI, L., ROTTOLI, P. & ROTTOLI, L. (1977) The spectrum of human tuberculosis. *Clin. exp. Immunol.* **27**, 230.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, J. (1965) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265.

MYRVANG, B., FEEK, C.M. & GODAL, T. (1974) Antimicrobial antibodies in sera from patients throughout the clinico-pathological disease spectrum of leprosy. *Acta. path. microbiol scand.* **82**, 701.

NETA, R. & SALVIN, S.B. (1976) T and B lymphocytes in the regulation of delayed hypersensitivity. *J. Immunol.* **117**, 2014.

ROLLINGHOFF, M., STARZINSKI-POWITZ, A., PFIZENMAIER, K. & WAGNER, H. (1977) Cyclophosphamide-sensitive T lymphocytes suppress the *in vivo* generation of antigen-specific cytotoxic T lymphocytes. *J. exp. Med.* **145**, 455.

ROOK, G.A.W. (1978) Three forms of delayed skin-test response evoked by mycobacteria. *Nature* (*Lond.*), **271**, 64.

TURK, J.L. & PARKER, D. (1973) Further studies on B lymphocyte suppression in delayed hypersensitivity indicating a possible mechanism for Jones-Mote hypersensitivity. *Immunology*, **24**, 751.

TURK, J.L., POLAK, L. & PARKER, D. (1976) Control mechanisms in delayed-type hypersensitivity. *Br. Med. Bull.* **32**, 165.

TURK, J.L. & BRYCESON, A.D.M. (1971) Immunological phenomena in leprosy and related diseases. *Advanc. Immunol.* **13**, 209.

WILLERS, J.M.N. & SLUIS, E. (1975) The influence of cyclophosphamide on antibody formation in the mouse. *Ann. Immunol.* (*Inst. Pasteur*).

WORLD HEALTH ORGANIZATION (1973) Technical report series No. 59. Cell-mediated immunity and resistance to infection. *World Health Organization*, Geneva.