Clin. exp. Immunol. (1980) **39**, 442–448.

Cellular hypersensitivity to tuberculin in BCG-revaccinated persons studied by skin reactivity, leucocyte migration inhibition and lymphocyte proliferation

H. REPO, ANJA A. I. KOSTIALA & T. U. KOSUNEN *Department of Bacteriology and Immunology, University of Helsinki, Helsinki, Finland*

(*Accepted for publication* 24 *July* 1979)

SUMMARY

Some persons seem to lose long-standing peripheral hypersensitivity to tuberculin earlier than others. This was seen in a group of five healthy student nurses who had been BCG-vaccinated as children and were found, in routine skin testing, to be negative to 100 TU of tuberculin purified protein derivative (PPD). They were revaccinated, which resulted in conversion to 10 TU skin test positivity. In agreement with this, their buffy coat cells achieved reactivity to 100 μg/ml of PPD in a leucocyte migration inhibitory factor (LIF) assay. However, the LIF response, being maximal at 4 weeks, faded away earlier than skin reactivity. Peripheral blood lymphocyte proliferation was studied with several PPD concentrations, 10 μg/ml always inducing the maximum ^{3}H-thymidine uptake. This was still high at 6 months after vaccination, when the skin reactions tended to be smaller than earlier.

The reason why the various parameters of cellular hypersensitivity followed different courses is not known, but it may involve different subpopulations of lymphocytes, activity of suppressor cells or influence by serum factors such as mycobacterial antigen–antibody complexes.

INTRODUCTION

The frequency of positive delayed-type skin reactions to tuberculin is high in Finland. According to Selroos (1969), only 10% of the population below the age of 60 fails to react to the intermediate strength (10 TU) of tuberculin purified protein derivative (PPD). The obvious reason for this is that 95–99% of the newborn are BCG-vaccinated, and they are revaccinated at school if skin test negativity is found (Härö, 1977). In addition, natural infection may have contributed to the skin test positivity (Härö, 1977; Selroos, Pasternack & Virolainen, 1973).

On the basis of both animal experiments (Collins, 1972; Kostiala, Lefford & McGregor, 1978) and human studies (Collins, 1972; Härö, 1977; Tuberculosis program, USPHS, 1955), the development of post-vaccination skin sensitivity is thought to correlate roughly with the presence of immunity, although opposite views have also been expressed (Hart, Sutherland & Thomas, 1967). Customarily, persons subject to high infection risk, e.g. medical personnel, are skin-tested with tuberculin before entering hospital duty, and if negative to 10 TU, they are BCG-vaccinated. Testing is repeated after 6 weeks, and if the result is still negative, the subject is vaccinated once more to rule out technical pitfalls in the previous procedure.

The present study describes five healthy student nurses who had been BCG-vaccinated as children but were found at present to show no skin reactivity, leucocyte migration inhibitory factor (LIF) release or lymphocyte proliferation in response to PPD. After revaccination they developed delayed-type skin

Correspondence: Heikki Repo, M.D., Department of Bacteriology and Immunology, University of Helsinki, Haartmaninkatu 3, SF-00290 Helsinki 29, Finland.

0099-9104/80/0200-0442 $02.00

reactivity that peaked at 6 weeks, as well as a short-lived LIF response to a high dose of PPD, whereas their lymphocytes were found to respond *in vitro* by proliferation to PPD over at least 6 months.

MATERIALS AND METHODS

Subjects of study. Five healthy student nurses (four women and one man) from Helsinki College of Nursing, Helsinki, aged 18 to 23 years, were studied. They had been BCG-vaccinated as children, as evidenced by a vaccination scar. They showed negative skin reactions to 10 TU of PPD in the routine skin testing programme in which all student nurses participate upon entering training.

Skin testing. Intradermal skin tests were performed in the dorsal surface of the forearm with 1, 10 or 100 TU (0·02, 0·2 and 2 μg, respectively) of tuberculin purified protein derivative (PPD, batch RT 23, State Serum Institute, Copenhagen, Denmark). The tests were performed serially with an injection volume of 0·1 ml, starting from 1 TU and, if negative, applying a higher dose. The results were recorded at 72 hr by measuring the maximum diameter of the erythema with a ruler. Similarly, the extent of induration was determined after palpation. According to the PPD manufacturer's specifications, a value higher than 6 mm in palpable induration was defined as a positive reaction.

BCG-vaccination. Freeze-dried BCG-vaccine prepared by the State Serum Institute, Copenhagen, was used. The vaccination was performed intradermally in the left hip.

Cells. Immediately prior to, as well as 2, 4, 6 and 24 weeks after, the BCG-vaccination, 90 ml of peripheral blood was drawn from the antecubital vein into a heparinized tube. One of the five subjects could not be reached for re-study at 24 weeks. Buffy coat cells were separated by the Böyum two phase method (Böyum, 1974), as described previously (Repo, 1977). One portion of buffy coat cells was washed three times with Hanks' balanced salt solution (HBSS) and used for the LIF assay. The other portion was applied to the Ficoll–Isopaque density gradient, and the separated mononuclear (MN) cells were washed three times with HBSS and used for the proliferation assay.

Antigen. Preservative-free PPD (1 mg/ml, batch RT 23, State Serum Institute, Copenhagen) was used for both LIF and proliferation assays.

Leucocyte migration inhibitory factor assay. The agarose method of Clausen (1971) with minor modifications was used. The medium consisted of 1% agarose (Biomedical Division of Marine Colloids Incorporated, Rockland, Maine, USA), 10% inactivated (30 min, 56°C) horse serum (Helsinki City Slaughter House, Helsinki, Finland), 10% ten-fold-concentrated Medium 199 with Hanks' salts (Orion Company, Helsinki), 350 μg/ml $NaHCO_3$ (Orion Company), 80 units/ml penicillin and 40 μg/ml streptomycin (Hoechst AG, Frankfurt, Germany). Five-millilitre portions of the fresh agarose medium were applied to disposable tissue culture dishes 60 × 15 mm (Falcon Plastics, Oxnard, California, USA) and allowed to turn solid. Wells were made in the agarose gel with a punch (diameter 2·3 mm).

The components of the cell medium were the same as above, with the exception of agarose. The final PPD concentrations used were 0, 1, 10 and 100 μg/ml. Buffy coat cells at the density of 2×10^8/ml were incubated in their media for 60 min at 37°C and thereafter applied in aliquots of 5 μl to the agarose wells. Eight wells were made in parallel for each PPD concentration. The dishes were incubated for 20 hr in 5% CO_2 in air. Thereafter, a 3·5% formaldehyde solution was applied for 12–24 hr as a fixative. The gel was then removed, and the migration areas were magnified under a projection microscope and measured by planimetry. Per cent migration was calculated as follows: (mean migration with PPD/mean migration without PPD) × 100.

Lymphocyte proliferation assay. In the MN cell culture assays, the components of the cell medium were the same as those in the LIF assay, with the exception of inactivated, pooled human serum (The Finnish Red Cross Blood Transfusion Service, Helsinki), which was used instead of horse serum. The final concentrations of PPD in the culture media were 0, 0·01, 0·1, 1, 10 and 100 μg/ml. The response of 2×10^5 MN cells to PPD was tested in triplicate in 0·2 ml of medium in a flat-bottomed microtitre tray (Falcon Plastics). After incubation for 3, 4, 5 or 6 days as in the LIF assay, 2 μCi of ^{3}H-thymidine (5 Ci/mmol, New England Nuclear, Boston, Massachusetts, USA) was added to each well. After additional incubation for 20 hr, the cells were collected on a glass fibre filter by using an automatic cell harvester (Scatron, Lierbyen, Norway). The dried filters were placed in a scintillation fluid consisting of 4·05 g Permablend III (Packard, Downers Grove, Illinois, USA) in one litre of toluene, and radioactivity was measured in a liquid scintillation counter (Wallac 81000). The results were expressed in decays per minute (d.p.m.) obtained by the channel ratio method. The proliferation index is the ratio of radioactivity in PPD-stimulated culture to that in PPD-unstimulated culture.

Statistical analysis. Differences between mean per cent migrations and mean d.p.m. values were evaluated by Student's *t*-test. Comparisons between proliferation indices were made by the paired sample *t*-test.

RESULTS

Skin reactions

The delayed-type skin reactions before vaccination of the five persons studied are shown in Table 1. It can be seen that 10 TU of PPD provoked in them no skin reactions whatsoever, and significant induration did not occur even with 100 TU. This situation was the basis for BCG-vaccination. According to

TABLE 1. Skin reactions at 72 hr to PPD before and after BCG vaccination

		Person									
		1		2		3		4		5	
Time*	Dose†	Er‡	Ind§	Er‡	Ind§	Er‡	Ind§	Er‡	Ind§	Er‡	Ind§
Before	10	0	0	0	0	0	0	0	0	0	0
	100	14	0	0	0	16	0	0	0	23	5
6 weeks	1	20	2	12	0	0	0	17	0	40	17
	10	48	17	20	6	20	16	22	8	70	25
24 weeks	1	15	0	0	0	0	0	5	0	n.d.	n.d.
	10	20	12	20	5	18	10	16	6	n.d.	n.d.

* Time in respect to BCG-vaccination.

† Intracutaneous skin tests were performed with the indicated amount of PPD in TU/ml.

‡ Maximum diameter of skin test erythema in mm.

§ Maximum diameter of skin test palpable induration in mm. Positive reaction ≥6 mm.

n.d. = Not determined.

local practice, the subjects of study were again skin-tested at 6 weeks. It was found that only one of them reacted with significant induration to 1 TU, whereas all five exhibited positive reactions to 10 TU of PPD. Skin testing of four persons was repeated at 24 weeks. At this time all reactions to 1 TU were negative, and those to 10 TU tended to be smaller than earlier. However, three out of the four tested still showed significant induration.

Leucocyte migration inhibition

The LIF responses of peripheral blood cells were studied prior to BCG-vaccination, and at 2, 4, 6 and 24 weeks thereafter, using the direct leucocyte migration agarose test. After vaccination, the cells for this assay were always obtained before skin tests were performed. The results are illustrated in Fig. 1 and

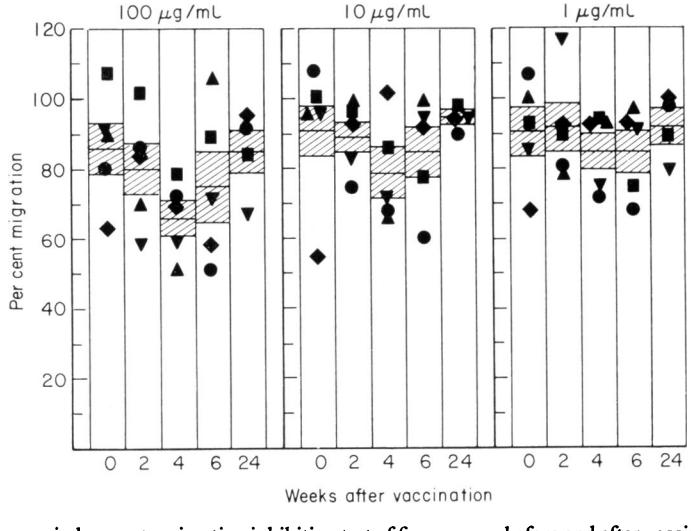

FIG. 1. The response in leucocyte migration inhibition test of five persons before and after vaccination. The cell migration was studied in the presence of 100, 10 and 1 μg/ml of PPD. At 4 weeks after vaccination significant LIF responses of leucocytes of subject 1 (●), 2 (■), 3 (◆), 4 (▼) and 5 (▲) were seen with the highest PPD concentration. Hatched areas denote means ± s.e.m.

show that 100 μg/ml of PPD provoked significant ($P<0·05$) inhibition of mean cell migration at 4 weeks, whereas the mean responses to 10 and 1 μg/ml were not significantly different from values before vaccination at any time during the observation period. Subject number 5, who at 6 weeks reacted strongly in the skin test (Table 1), showed a strong LIF response at 4 weeks but none at 6 weeks. Before vaccination the cells of subject number 2 were strongly inhibited with all the PPD concentrations used and at 4 and 6 weeks after vaccination only with 100 μg/ml.

Lymphocyte proliferation

The *in vitro* ^{3}H-thymidine incorporation by MN cells in response to PPD was determined simultaneously with the LIF assays. Since differences have been observed in the optimum dose of PPD and the time of culture for the cells of different persons (Nilsson, 1972), lymphocytes of each donor were cultured for 3 to 6 days in the presence of several PPD concentrations. The results are illustrated in Fig. 2, which shows that before BCG-vaccination none of the antigen doses used (0·01 to 100 μg/ml) induced significant proliferation on any day of culture. After vaccination 10 μg/ml of PPD was found to give consistently the maximum d.p.m. values. At 6 and 24 weeks the responses to 10 μg/ml were always significant when compared with unstimulated control cultures ($P<0·05$), and they were frequently significant even earlier after BCG. The optimum culture period with 10 μg/ml of PPD was 5 days. At 6 and 24 weeks, even 1 μg/ml was stimulatory in cultures incubated for 4 to 6 days, and it was always more effective than 100 μg/ml of PPD. The rate of ^{3}H-thymidine incorporation in control cultures was not markedly affected by BCG-vaccination or by changes in the cell culture time.

The maximum proliferation indices for each individual are shown in Table 2. It can be seen that the optimal time of culture before BCG varied from 4 to 6 days. After vaccination, one person reacted best at 3 days; in others, however, 5 to 6 days were required. In general, the indices were highest at 6 weeks or more after BCG. The mean maximum proliferation index after vaccination was 33·9, and it differed significantly ($P = 0·05$) from that prior to BCG.

DISCUSSION

In the present experiments, healthy student nurses were revaccinated with BCG because in routine skin testing they were found to be negative to 10 and 100 TU of PPD. Vaccination induced skin reactivity to

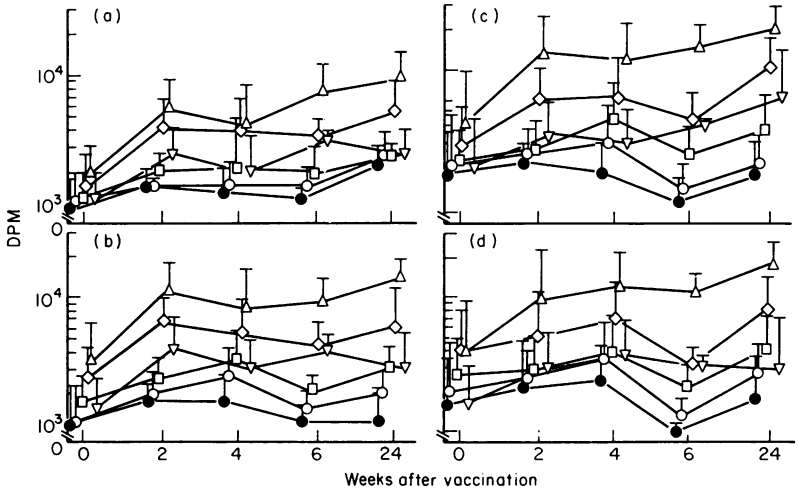

FIG. 2. The response in lymphocyte proliferation test of five persons before and after BCG vaccination. The culture periods were 3 (a), 4 (b), 5 (c) and 6 (d) days and the PPD concentrations used were 0·01 (○—○), 0·1 (□—□), 1 (◇—◇), 10 (△—△) and 100 (▽—▽) μg/ml. Maximum responses were obtained with 10 μg/ml of PPD in a 5-day culture. Closed symbols denote ^{3}H-thymidine incorporation without PPD. Geometric means ± s.e.m.

TABLE 2. Maximum lymphocyte proliferation indices before and after BCG vaccination

	Before BCG			After BCG			
Person	Index*	Day of culture†	PPD/ml‡	Index*	Day of culture†	PPD/ml‡	Weeks after BCG§
1	5·2	4	10	57·0	5	10	4
2	8·9	4	100	21·8	6	10	24
3	5·3	5	10	15·9	3	10	6
4	4·7	4	1	27·8	5	10	24
5	2·7	6	10	46·8	5	10	6
Means ± s.e.m.	5·4 ± 1·0	—	—	33·9 ± 17·4¶	—	—	—

* Triplicate wells containing 2×10^5 mononuclear cells were incubated for 3, 4, 5 or 6 days with or without PPD. The ratio of mean ^{3}H-thymidine incorporation in wells with PPD to that without PPD was expressed as a proliferation index. The highest index for each individual is given.

† The day of maximum lymphocyte proliferation is indicated.

‡ PPD concentration in wells varied from 0·01 to 100 μg/ml. The optimum concentration is indicated.

§ The week of maximum lymphocyte proliferation is indicated.

¶ Significant ($P < 0·05$) when compared with the value before BCG.

10 TU but not to lower doses of PPD, as well as a LIF-response by buffy coat cells confronted with 100 μg/ml of PPD (Table 1, Fig. 1). The latter was most pronounced at 4 weeks after vaccination. At 6 weeks, the cells of only two persons were clearly inhibited and, when comparing individual subjects, no correlation could be found between the size of the skin reaction and the LIF response. The cells of one person were inhibited by PPD even prior to vaccination in spite of cutaneous anergy. This is in accordance with the findings of Astor *et al.* (1973) concerning the unexpected significant inhibition of leucocytes of some newborns, as well as those of Fleer *et al.* (1976) in healthy volunteers negative to 10 TU PPD. However, it has been show in several studies both in man (Astor *et al.*, 1973; Clausen, 1971; Senyk & Hadley, 1973) and in animals (Hoffman, Spitler & Hsu, 1976) that LIF production in response to PPD assayed by the leucocyte migration agarose test generally correlates with delayed-type skin reactivity. In agreement with this, the subjects of the present study as a group responded to vaccination by the development of skin reactivity and an ability to release LIF. The latter, however, faded away earlier than skin reactivity. LIF has been shown to be distinct from the macrophage migration inhibitory factor (Hoffman *et al.*, 1977), to possess enzymatic activity (Rocklin & Rosenthal, 1977) and to be elaborated after stimulation with PPD by both T and B lymphocytes (Räsänen, 1979).

As to lymphocyte proliferation, it has been demonstrated by employing human cells separated either on the basis of B cell (Chess, MacDermott & Schlossman, 1974; Jensen, Kurpisz & Rubin, 1977) or T cell (Blomgren, 1975; Littman, David & Rocklin, 1976) surface markers that purified T cells, but not B cells, from PPD-sensitive donors respond by proliferation to a PPD challenge *in vitro*. In fact, it has been claimed that B cells suppress the proliferation response, particularly in the case of cells from BCG-vaccinated subjects with negative skin reactions (Bona & Chedid, 1976). However, synergism with T cells has also been found, presumably based on B cell mitogenic factors released by T cells (Blomgren, 1975). The present results show that reactivity in a lymphocyte transformation assay developed after BCG-revaccination and was maximal when 10 μg/ml of PPD was used (Fig. 2, Table 2). No significant change in this proliferation response to any of the antigen concentrations used could be seen during the observation period from 2 to 24 weeks after vaccination. According to Nilsson (1972), lower sensitivity was best detected with a high (100 μg/ml) concentration of PPD and with a long culture period (6 days). This could not be found in the present vaccinated individuals. The results are in agreement with those of Miller & Jones (1973) and demonstrate a good correlation between skin reactivity and lymphocyte transformation.

Since BCG vaccination of the newborn is a local custom, it was virtually impossible to find a group of previously unvaccinated adult controls for the persons studied. Thomas, Clements & Grzybowski (1971)

have reported on responses of healthy Canadian student nurses after primary vaccination. Their findings show increasing magnitude in the skin reactions to 5 TU PPD up to one year, as well as more than ten-fold increases in cpm of lymphocytes cultured with PPD at 3 months. This is in agreement with the common experience that tuberculin hypersensitivity is usually long-lasting (Collins, 1972). In our nurses the LIF response was short-lived and the skin reactions tended to be smaller at 6 months than at 6 weeks after revaccination. Lymphocyte proliferation responses, however, were still good at that time. The reason why the healthy persons of the present study repeatedly seem to lose at least some of their peripheral reactivity to PPD earlier than others is not known. It might be that suppressor cells (Bona & Chedid, 1976) or serum factors such as mycobacterial antigens and their antibodies (Jensen *et al.*, 1977) are involved. It is noteworthy, however, that the parameters of cellular hypersensitivity studied in the present report turned positive after vaccination. The initial intensity of the reactions after vaccination may be more important with regard to resistance to re-infection than the length of the period over which tuberculin reactivity persists, since any tuberculous infection induces an immediate anamnestic response (Collins, 1972). In addition, it has been shown that absence of peripheral sensitivity does not imply that immunologically committed cells are lacking in the tissues (Collins, 1972).

The different courses followed by skin reactivity and lymphocyte responses *in vitro* in the present study may be due to the production of various lymphokines by different subpopulations of lymphocytes (Blomgren, 1975; Chess *et al.*, 1974; Doroszczak, Yoshida & Cohen, 1977; Jensen *et al.*, 1977; Littman *et al.*, 1976; Räsänen, 1979). The present results also imply that at different times after sensitization the results concerning correlations between the three reactivities may be ambiguous.

This study was supported by grants from the Finnish Anti-Tuberculosis Association, Helsinki, and the Sigrid Jusélius Foundation, Helsinki. The skilful technical assistance of Mrs Eine Virolainen and Mrs Maire Laakso is gratefully acknowledged.

REFERENCES

Astor, S.H., Spitler, L.E., Frick, O.L. & Fudenberg, H.H. (1973) Human leukocyte migration inhibition in agarose using four antigens: correlation with skin reactivity. *J. Immunol.* **110**, 1174.

Blomgren, H. (1975) Role of B cells in the expression of the PPD response of human lymphocytes *in vitro*. *Scand. J. Immunol.* **4**, 499.

Bona, C. & Chedid, L. (1976) Stimulation of lymphocytes by purified protein derivative: suppression by cells from humans vaccinated with Bacille Calmette-Guérin. *J. Infect. Dis.* **133**, 465.

Böyum, A. (1974) Separation of blood leukocytes, granulocytes and lymphocytes. *Tissue Antigens*, **4**, 269.

Chess, L., MacDermott, R.P. & Schlossman, S.F. (1974) Immunological functions of isolated human lymphocyte subpopulations. II. Antigen triggering of T and B cells *in vitro*. *J. Immunol.* **113**, 1122.

Clausen, J.E. (1971) Tuberculin-induced migration inhibition of human peripheral leukocytes in agarose medium. *Acta allergol.* (*Kbh*), **26**, 56.

Collins, F.M. (1972) Acquired resistance to mycobacterial infections. *Adv. Tuberc. Res.* **18**, 1.

Doroszczak, N., Yoshida, T. & Cohen, S. (1977) Subpopulations of lymphocytes to produce various lymphokines. I. Function of subpopulations separated by velocity sedimentation. *J. Immunol.* **119**, 1617.

Fleer, A., Van der Hart, M., Blok-Schut, B.J.Th. & Schellekens, P.Th.A. (1976) Correlation of PPD and BCG-induced leukocyte migration inhibition, delayed cutaneous hypersensitivity, lymphocyte transformation *in vitro* and humoral antibodies to PPD in man. *Eur. J. Immunol.* **6**, 163.

Härö, A.S. (1977) Long-term evaluation of mass-BCG-vaccination campaign: a study of thirty years of experience in Finland. *Tuberculosis and Respiratory Diseases*, Vol. 6, p. 1. Yearbook of the Finnish Anti-Tuberculosis Association.

Hart, P.D'A., Sutherland, I. & Thomas, J. (1967) The immunity conferred by effective BCG and vole bacillus vaccines, in relation to individual variations in induced tuberculin sensitivity and to technical variations in the vaccines. *Tubercle*, **48**, 201.

Hoffman, P.M., Hanes, D.M., Horsmanheimo, M. & Fudenberg, H.H. (1977) Leukocyte migration in guinea pigs. II. Partial characterization of a leukocyte migration inhibitory factor distinct from macrophage migration inhibitory factor. *Cell. Immunol.* **30**, 92.

Hoffman, P.M., Spitler, L.E. & Hsu, M. (1976) Leukocyte-migration inhibition in guinea pigs. I. Correlation with skin test reactivity and macrophage-migration inhibition. *Cell. Immunol.* **21**, 358.

Jensen, B., Kurpisz, M. & Rubin, B. (1977) Antigen specific lymphocyte activity *in vitro* by peripheral blood leucocytes from Mantoux-positive and negative human beings. I. Comparison of quantitative and qualitative differences in the PPD-specific lymphoproliferative response of lymphocytes from two kinds of donors. *Clin. exp. Immunol.* **27**, 303.

Kostiala, A.A.I., Lefford, M.J. & McGregor, D.D. (1978) Immunological memory in tuberculosis. II. Mediators of protective immunity, delayed hypersensitivity and macrophage migration inhibition in central lymph. *Cell. Immunol.* **41**, 9.

Littman, B.H., David, J.R. & Rocklin, R.E. (1976) Migration inhibitory factor (MIF) and proliferative

responses by human lymphocyte subpopulations separated by sheep erythrocyte rosette formation. *Cell. Immunol.* **24**, 241.

MILLER, S.D. & JONES, H.E. (1973) Correlation of lymphocyte transformation with tuberculin skin-test sensitivity. *Am. Rev. respir. Dis.* **107**, 530.

NILSSON, B.S. (1972) The response of lymphocytes from tuberculin-positive or negative humans to various doses of PPD-tuberculin *in vitro*. *Cell. Immunol.* **3**, 493.

RÄSÄNEN, L. (1979) Cellular cooperation in the production of human leukocyte inhibitor factor by lymphocyte subpopulations stimulated with antigens and allogeneic cells. *Immunology*, **37**, 715.

REPO, H. (1977) Leukocyte migration agarose test for the assessment of human neutrophil chemotaxis. I. Effects of environmental factors on neutrophil migration under agarose. *Scand. J. Immunol.* **6**, 203.

ROCKLIN, R.E. & ROSENTHAL, A.S. (1977) Evidence that human leukocyte inhibitory factor (LIF) is an esterase. *J. Immunol.* **119**, 249.

SELROOS, O. (1969) The frequency, clinical picture and prognosis of pulmonary sarcoidosis in Finland. *Acta med. Scand.* (*Suppl.*), **503**, 1.

SELROOS, O., PASTERNACK, A. & VIROLAINEN, M. (1973) Skin test sensitivity and antigen-induced lymphocyte transformation in uraemia. *Clin. exp. Immunol.* **14**, 365.

SENYK, G. & HADLEY, W.K. (1973) *In vitro* correlates of delayed hypersensitivity in man: ambiguity of polymorphonuclear neutrophils as indicator cells in leukocyte migration test. *Infect. Immun.* **8**, 370.

THOMAS, J.W., CLEMENTS, D. & GRZYBOWSKI, S. (1971) *In vitro* lymphocyte responses and skin test reactivity following BCG vaccination. *Clin. exp. Immunol.* **9**, 611.

TUBERCULOSIS PROGRAM, USPHS. (1955) Experimental studies of vaccination, allergy and immunity in tuberculosis. V. Relation between degree of tuberculin sensitivity and resistance in the vaccinated individual. *American Journal of Hygiene*, **62**, 185.