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Prostaglandin-dependent regulation of the *in vitro* proliferative response to mycobacterial antigens of peripheral blood lymphocytes from normal donors and from patients with tuberculosis or leprosy

G. M. BAHR, G. A. W. ROOK & J. L. STANFORD *Department of Microbiology, Middlesex Hospital Medical School, London*

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SUMMARY

The response to soluble mycobacterial antigens of peripheral blood mononuclear cells, from most normal donors, tuberculosis patients or cases of tuberculoid leprosy (TT/BT) was enhanced by the addition of indomethacin. In contrast, indomethacin caused no enhancement of the response of cells from lepromatous leprosy (BL/LL) cases. Moreover the addition of 10^{-5} M prostaglandin E_2 (PGE_2) failed to inhibit the proliferative responses of cells from the BL/LL patients, although it markedly inhibited the responses of peripheral blood mononuclear cells from the other groups. The addition of PGE_2 or indomethacin to cells which had been precultured for 48 hr had no significant effect on the proliferative responses of cells from any of the groups of donors. These results suggest that a *normal*, prostaglandin-dependent, indomethacin-sensitive regulatory mechanism is absent from the peripheral blood mononuclear cells of BL/LL patients.

INTRODUCTION

Peripheral blood mononuclear cells from patients with severe tuberculosis or lepromatous leprosy (BL/LL) frequently fail to proliferate *in vitro* in response to the antigens of the infecting organism (Ellner, 1978; Myrvang *et al.*, 1973). Regulation of *in vitro* lymphocyte proliferation involves interactions of several cell types and at least three pathways have been described using cells from normal individuals (Rice, Laughter & Twomey, 1979). Moreover, suppression of responses to antigens or mitogens has been attributed to T lymphocytes (Shou, Schwartz & Good, 1976), B lymphocytes (Bona *et al.*, 1976) and to cells of the macrophage/monocyte series (Laughter & Twomey, 1977). Thus in theory the lack of responsiveness of some patients with mycobacterioses could be attributed to absence or sequestration of antigen-recognizing T cells (Rook, Carswell & Stanford, 1976), to lack of appropriate helper function, or to any of several kinds of suppressor mechanism.

There is growing evidence for the importance of suppressor mechanisms in these infections, though it is not clear whether they are the cause or consequence of severe disease. Thus the response of cells from some tuberculosis patients is restored following depletion of a population of adherent cells (Ellner, 1978), and removal of a subset of T lymphocytes from the peripheral blood mononuclear cells of leprosy patients eliminates a lepromin-induced suppressor activity (Mehra *et al.*, 1980). Two other lines of evidence also suggest that potentially antigen-responsive cells are

Correspondence: Dr G. M. Bahr, Department of Experimental Immunotherapy, Institut Pasteur, 28 rue du Dr Roux, 75724 Paris, Cedex 15, France.

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present in peripheral blood of these individuals. First, although unresponsive to *M. leprae* their cells may proliferate in the presence of other strongly cross-reactive mycobacterial antigens (Turk & Bryceson, 1971). Secondly, it has been claimed that apparently anergic cell populations can respond if supplemented with normal allogeneic adherent cells (Hirschberg, 1978; Nath *et al.*, 1980). We have therefore investigated the possibility that prostaglandin-dependent or indomethacin-sensitive mechanisms may be involved in these regulatory mechanisms.

Suppression of *in vitro* lymphoproliferative responses by prostaglandins has been well documented in man (Lomnitzer, Rabson & Koornhof, 1976; Smith, Steiner & Parker, 1971; Ellner & Spagnuolo, 1979). The inhibitory effect of prostaglandins of the E series on cell-mediated immunity, immediate hypersensitivity and acute inflammatory reactions has been reviewed by Bourne *et al.* (1974) and suggests that prostaglandin E can act as an endogenous regulator of human immune reactions. Particularly striking prostaglandin-dependent effects have been observed in studies of patients with Hodgkin's disease, and of their peripheral blood mononuclear cells *in vitro* (Goodwin *et al.*, 1977b).

In contrast, the studies reported here demonstrate that cells from lepromatous leprosy patients show a decrease rather than an increase in prostaglandin-dependent regulation.

MATERIALS AND METHODS

Test subjects. The normal donors were staff at the School of Pathology, Middlesex Hospital Medical School. The leprosy patients were individuals attending Dr A. Bryceson's clinic at St John's Hospital, Lisle Street. They were classified clinically and histologically (Ridley & Jopling, 1966) and had been on treatment for at least 1 year. None of them were on steroids or had reversal reactions at the time blood samples were taken. Patients with pulmonary tuberculosis admitted to the Middlesex Hospital and Charing Cross Hospital, London, were included in this study. They were all diagnosed clinically and bacteriologically and had been on multiple drug therapy for a few weeks.

Antigens. The antigens were all sonicates which had been centrifuged and filtered to remove macromolecular debris. All except the *M. leprae* were prepared as described by Paul, Stanford & Carswell (1975) from fresh cultures on Sauton's medium. The *M. leprae* was a gift from Dr R. J. W. Rees, and had been prepared in a similar manner by Dr P. Draper from organisms derived from an infected armadillo. The antigens were used at final concentrations of 2, 20 and 40 μg/ml. Only the data for the 20 μg/ml dose are presented, this concentration giving maximal response in most individuals.

Drugs. Indomethacin was purchased from Sigma, London (Cat. No. I-7378) in crystalline form. It was dissolved in ethyl alcohol at a concentration of 10 mg/ml, diluted to 11 μg/ml in RPMI and added in 20 μl aliquots to cultures to give a final concentration of 1 μg/ml.

PGE was kindly provided by May & Baker, London. It was synthetically prepared with very high purity, diluted in RPMI and added to cultures in 20 μl aliquots to give final concentrations of PGE_2 ranging between 10^{-4} and 10^{-7} M.

Lymphocyte transformation test. Defibrinated venous blood, diluted 1/2 in RPMI 1640 (GIBCO–BIOCULT), was centrifuged over Ficoll–Paque at 400 ***g*** for 35 min. The cells from the interface, referred to as peripheral blood mononuclear cells, were washed twice in RPMI containing 20% fetal calf serum and resuspended in 5×10^5 cells/ml in RPMI 1640 containing standard concentrations of L-glutamine, penicillin, streptomycin and 20% unheated human serum. For patients, AB serum was always used because of the risk of inhibitory factors and anti-lymphocyte antibodies. For normal individuals, experiments performed in duplicate showed that results were comparable in autologous or AB serum; therefore, autologous serum was used.

Cultures were set up in triplicate in flat-bottomed microtitre trays (ISFB96, Linbro). Each well contained 0·1 ml of cell suspension (5×10^4 lymphocytes/well) and antigen was added immediately, or after 48 hr in a further 0·1 ml of RPMI 1640.

To some cultures, 20 μl of indomethacin, PGE_2 or RPMI (as a control) were added either immediately or after 48 hr, together with the antigen. In each case cells were harvested 5 days after addition of the antigen. Tritiated thymidine (Amersham TRK 120), 0·1 μCi/well, was added for the

last 5 hr in culture. The cells were harvested onto Whatman's No. 1 (cellulose) filter paper, and counted in a liquid scintillation counter using standard techniques.

This technique, originally devised for field work and for economy, employs a low cell number, a short pulse with a small dose of tritiated thymidine, and cellulose filter paper which quenches the counts by approximately 70%. The counts per minute (c.p.m.) per culture are therefore less than 1/10 of those obtained by more conventional protocols. Backgrounds are 10–50 c.p.m./culture (proliferation of cells without antigen), and strong responses are in the range 1,000–3,000 c.p.m./culture.

Cell separation. T cell-enriched populations were prepared by rosetting with neuraminidase-treated sheep erythrocytes (Moretta *et al.*, 1977). The purity of these preparations varied in different individuals, from 88 to 95% E rosette-forming cells.

Statistical analysis. Student's *t*-test was used for statistical analysis, except where indicated otherwise.

RESULTS

Effect of indomethacin on the lymphoproliferative response to mycobacterial and fungal antigens

Peripheral blood mononuclear cells from normal individuals and from patients with leprosy or tuberculosis were stimulated with 20 μg/ml of soluble antigen from *M. tuberculosis* (Tuberculin), *M. vaccae* (Vaccin), *M. leprae* (Leprosin), or *Candida albicans* (Candidin), with or without 1 μg/ml indomethacin. The mean counts per minute of triplicate cultures with indomethacin were divided by the mean counts per minute of triplicate cultures without indomethacin to give an enhancement index and the results were plotted on a linear scale (Fig. 1). No enhancement index below 1·4 was found to be significant ($P < 0{\cdot}05$) when the mean of triplicate cultures without indomethacin was compared with the mean of triplicate cultures with indomethacin.

Indomethacin caused significant, often striking enhancement of the response to Tuberculin of

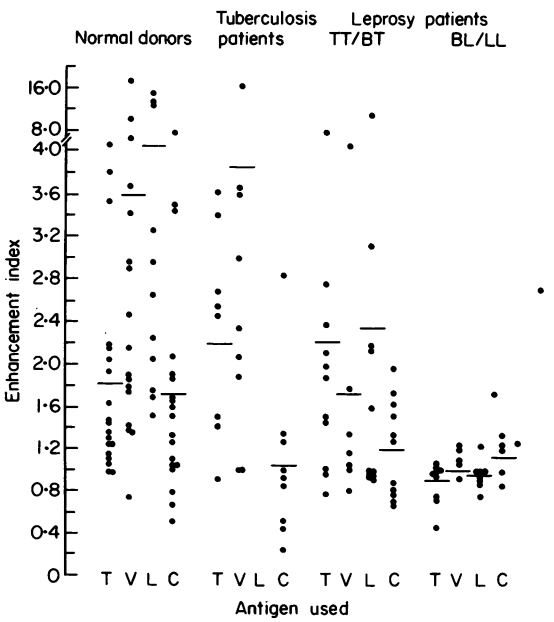

Fig. 1. The effect of indomethacin (1 μg/ml) on the proliferative responses of peripheral blood mononuclear cells to soluble antigen from *M. tuberculosis* (T), *M. vaccae* (V), *M. leprae* (L), or *Candida albicans* (C). Donors were normal individuals, or patients with tuberculosis, tuberculoid leprosy (TT/BT) or lepromatous leprosy (BL/LL). Results are expressed as *enhancement index* (see Results).

peripheral blood mononuclear cells from 10 out of 19 normals, eight out of nine tuberculosis patients and eight out of 11 TT/BT patients, whereas the response of cells from BL/LL patients was never enhanced ($P < 0{\cdot}005$; χ^2 test). This was not due to total unreactivity of the cells from the BL/LL group, which responded well to Candidin and Tuberculin. An appropriate dilution of ethanol (used to dissolve the indomethacin) had no effect.

A similar significant difference between the BL/LL patients and the other groups was seen when the other mycobacterial antigens were used (Fig. 1).

When the antigens were used at 2 and 40 μg/ml the same pattern of enhancement was observed, indicating that the phenomenon is not dependent on antigen concentration within this range.

Effect of 48-hr delay in addition of antigen plus indomethacin on the proliferative response of peripheral blood mononuclear cells

Since it has been reported that lymphocytes lose their binding sites for PGE after 24 hr of preculture without stimulation (Goodwin *et al.*, 1979), we studied the effect of addition of indomethacin plus antigen to cells that were precultured for 48 hr and compared the results with immediate stimulation of fresh cells. As can be seen in Fig. 2, enhancement by indomethacin of the lymphoproliferative response to 20 μg/ml of Tuberculin was totally lost when addition of antigen and indomethacin was delayed for 48 hr.

Effect of indomethacin on the proliferative responses of E rosette-forming cells

PGE has been found to be synthesized and released in *in vitro* cultures by adherent cells of the monocyte/macrophage series (Goodwin *et al.*, 1977b, 1979). We therefore studied the effect of indomethacin on the proliferative responses of cell populations, enriched for T lymphocytes and relatively depleted of macrophages by E rosetting. The effect of indomethacin on the proliferative responses to 20 μg/ml of antigen is presented in Table 1 as percentage change relative to the response without indomethacin. All changes of 20% or more were significant ($P < 0{\cdot}01$) when the mean c.p.m./culture obtained with indomethacin were compared to the mean value without it. Indomethacin generally failed to cause a significant enhancement of the proliferative responses of E rosette-forming cells from six normal individuals stimulated with Tuberculin, Vaccin or Candidin (Table 1).

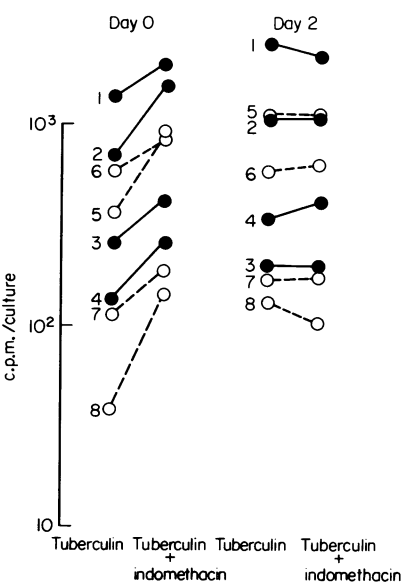

Fig. 2. The effect of indomethacin (1 μg/ml) on the response to tuberculin of peripheral blood mononuclear cells from normal donors (●——●) and tuberculosis patients (o- - -o). Antigen, with or without indomethacin, was added at the time of culture initiation (day 0) or after 48 hr (day 2). Each number refers to a separate individual.

Table 1. The effect of indomethacin (1 μg/ml) on the responses of peripheral blood mononuclear cells or E rosette-forming cells to soluble antigens (20 μg/ml), expressed as per cent enhancement or inhibition (−) of the response without indomethacin

Individual number	Tuberculin		Vaccin		Candidin	
	PBMNC	ERFC	PBMNC	ERFC	PBMNC	ERFC
1	15	−9	195	5	69	−53
2	421	−5	928	0	242	−1
3	117	10	n.d.	n.d.	4	−43
4	104	11	52	13	n.d.	n.d.
5	59	−15	111	−16	−5	−11
6	93	5	41	0	85	0

PBMNC = peripheral blood mononuclear cells, ERFC = E rosette-forming cells.

However, indomethacin caused highly significant ($P<0{\cdot}01$) enhancement of proliferative responses of unseparated peripheral blood mononuclear cells from the same individuals. This indicates that by E rosetting we are either depleting the cells that produce PGE, or the cells which are needed for the effect of PGE to be expressed.

Effect of the addition of exogenous PGE_2 on the proliferative responses of peripheral blood mononuclear cells

Preliminary experiments using different concentrations of exogenous PGE_2 ranging from 10^{-4} to 10^{-7} M indicated that 10^{-4} M was toxic and abolished completely the proliferative response to any antigen. There was no significant effect with 10^{-7} M, and in many cases 10^{-6} M, whereas 10^{-5} M was capable of inhibiting by about 50% the responses of normal individuals to various antigens. This concentration of PGE_2 was added to cell cultures from five normal individuals and from five leprosy patients who did not show enhancement of the lymphoproliferative response in the presence of indomethacin. PGE_2 caused significant suppression of the lymphoproliferative response of the five normal individuals to all the antigens tested, but failed to inhibit the responses of the leprosy patients to the three mycobacterial antigens (Table 2).

Table 2. The effect of 10^{-5} M PGE_2 on the response of peripheral blood mononuclear cells to soluble antigen (20 μg/ml), expressed as per cent enhancement or inhibition (−) of the response without PGE_2

Individual	Tuberculin	Vaccin	Leprosin	Candidin
Normal				
1	−44	−36	−58	−62
2	−35	−46	−52	−54
3	−50	−59	−7	−33
4	−29	−42	n.d.	−41
5	−35	−61	n.d.	−50
TT/BT				
1	+6	+13	+1	+3
2	−10	0	−11	−37
3	−4	−1	−6	−38
BL/LL				
1	−13	−9	+18	+1
2	+73	−2	−1	−57

Table 3. The effect of 1 μg/ml of indomethacin (I), PGE_2 (10^{-5} M) or both on the response to soluble antigens (20 μg/ml) of peripheral blood mononuclear cells from normal donors. Drugs and antigens were added simultaneously either at the time of culture initiation or after a delay of 48 hr

Time of antigen and drug addition	Individual number	Tuberculin			Vaccin			Candidin		
		I	PGE_2	I+PGE_2	I	PGE_2	I+PGE_2	I	PGE_2	I+PGE_2
Immediate (0 hr)	1	+30	−45	−47	+55	−68	−70	+11	−73	−72
	2	+15	−18	+11	+22	−75	−85	−10	−91	−91
	3	+54	−44	−47	+70	−65	−68	+4	−81	−76
Delayed (48 hr)	1	+4	+1	−8	n.d.	n.d.	n.d.	+12	0	+5
	2	+14	+2	+6	n.d.	n.d.	n.d.	−9	−14	−16
	3	+11	+9	+7	n.d.	n.d.	n.d.	+5	+12	+2

This difference between patients and normals was not related to their relative responsiveness to the antigens used, since stimulation ratios were comparable in the two groups, except for poor responses when leprosy antigen was used with peripheral blood mononuclear cells from lepromatous donors.

Effect of immediate or delayed addition of PGE_2 with or without indomethacin on the lymphoproliferative responses to mycobacterial and fungal antigens

Indomethacin alone (1 μg/ml), PGE_2 alone (10^{-5} M) or both were added to peripheral blood mononuclear cells from three normal individuals stimulated with 20 μg/ml of Tuberculin, Vaccin or Candidin. Prostaglandin caused significant inhibition ($P < 0{\cdot}01$ compared to response in absence of drugs) of the response to all three antigens (with the exception of the response to Tuberculin of donor 2), whether or not indomethacin was present (Table 3). Moreover, indomethacin failed to reverse the inhibition caused by PGE_2 even when indomethacin used alone caused enhancement. These results confirm that indomethacin does not block the function of PGE_2, but only its synthesis.

When PGE_2 or PGE_2+indomethacin were added with antigen 48 hr after initiation of the culture, no significant inhibitory or stimulatory effects were noted (Table 3).

DISCUSSION

The addition of indomethacin to cultures of peripheral blood mononuclear cells from more than 50% of the normal individuals, tuberculosis patients, or cases of tuberculoid leprosy, caused increased responses to mycobacterial antigens. In striking contrast, indomethacin caused no enhancement of the responses of cells from lepromatous (BL/LL) cases. This difference between the groups was not related to the size of the response, since many lepromatous cases responded strongly to *M. tuberculosis* and Candida. The absence of this normal regulatory mechanism from the peripheral blood mononuclear cells of BL/LL cases could be due either to failure of the antigens to induce the synthesis or release of PG, or to absence or refractoriness of the target cells upon which the PG usually acts. The failure of added PGE_2 to inhibit significantly the proliferation of cells from those patients whose cultures were not enhanced by indomethacin points to the latter mechanism.

Addition of the same concentration (10^{-5} M) of PGE_2 to cells from normal donors caused a significantly decreased response, even in cases where addition of indomethacin had caused no enhancement of proliferation.

The concentration of PGE_2 that was found to cause significant inhibition of the responses to antigens tested was similar to that employed by Smith *et al.* (1971) and by Lomnitzer *et al.* (1976). It is at least 2 orders of magnitude higher than either the concentration employed by Berenbaum, Cope & Bundick (1976) or by Goodwin, Bankhurst & Messner (1977a) to inhibit the PHA responses

of human leucocytes. Moreover, it is higher than the concentration of endogenous PGE released into the culture by 10^6 human leucocytes stimulated with an optimal concentration of PHA (Ferraris & Derubertis, 1974). The reason for such discrepancies is not quite clear, but could be related to differences in the techniques employed and to the nature or purity of PGE used.

Indomethacin caused no enhancement of responses of peripheral blood mononuclear cells from any of the groups if the cells were precultured for 48 hr before the antigen and drug were added. This agrees with previous findings by Ellner & Spagnuolo (1979) who showed that the period of maximal vulnerability of lymphocytes to prostaglandins in culture is during the first 24 hr. Studies by Goodwin *et al.* (1979) suggest that the maintenance of mononuclear cells in culture for 2 days results in loss of 'receptors' for prostaglandin. This observation could also explain the finding that addition of 10^{-5} M PGE_2 caused inhibition when added on day 0, but not when added at 48 hr.

The failure of indomethacin to cause enhancement of responses of E rosette-forming cells is consistent with the view that cells of the monocyte/macrophage series are the main source of PGE_2 *in vitro* (Goodwin *et al.*, 1977a; Ellner & Spagnuolo, 1979), since E rosette-forming cells are relatively depleted of such cells. [A contrary view has been expressed by Webb & Nowowiejski (1978) who regarded lymphocytes as the important source of prostaglandin in murine spleen cell cultures.]

In conclusion, the hyporesponsiveness of cells from BL/LL patients is not due to excessive prostaglandin synthesis *in vitro*. There is prostaglandin-dependent regulation of proliferative responses of peripheral blood mononuclear cells from normals, tuberculosis patients and tuberculoid leprosy cases, but it is absent from the peripheral blood mononuclear cells of the lepromatous group.

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