Indomethacin-mediated Enhancement of Lymphocyte Response to Mitogens in Healthy Subjects and Lung Cancer Patients

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Indomethacin (prostaglandin synthetase inhibitor) was found to be capable of enhancing the mitogen-induced lymphocyte proliferative responses of healthy subjects and patients with lung cancer. A whole-blood culture technique was used. Indomethacin had no mitogenic activity. We observed a greater enhancement of lymphocyte response by indomethacin in weak responders as compared with strong responders in healthy subjects and lung cancer patients. A greater enhancement was also noted in lung cancer patients with active disease as compared with lung cancer patients in remission. In a separated cell culture system, the indomethacin exerted no effect on purified T cells in the absence of monocytes, while this agent exerted its enhancement effect on T lymphocyte response in the presence of autologous monocytes of lung cancer patients. This suggests that monocytes (suppressor cells) may secrete prostaglandins, which are responsible for the impairment of T lymphocyte response in lung cancer patients.

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IN VITRO T LYMPHOCYTE proliferative responses to mitogens, antigens, or allogeneic cells are depressed in patients with lung cancer.[3,8,9,13,18] This impairment of T-cell response in lung cancer patients has previously been demonstrated either in a whole-blood culture system or in an unseparated mononuclear cell culture system. Thus, it was not possible to determine whether the T lymphocytes are intrinsically defective or these T cells are functionally depressed secondary to the presence of serum factors and/or so-called suppressor cells. In a recently completed study, we observed that the purified T lymphocyte response to phytohemagglutinin (PHA) is normal and that this T-cell response is depressed in the presence of irradiated non-T suppressor cells in a majority of lung cancer patients.[12] The presence of non-T suppressor cells in patients with other neoplastic diseases has recently been reported by us and other investigators.[2,6,10–12,14,17,21]

Prostaglandins of the E series have been shown to inhibit mitogen-induced lymphocyte proliferative response in experimental animals and in man.[7,16] Goodwin *et al.* recently demonstrated that the prostaglandins, produced by glass-adherent suppressor cells, are responsible for the hyporesponsiveness of T lymphocytes to PHA and that the blockade of prostaglandin production with indomethacin results in a significant increase in the PHA-induced T lymphocyte proliferative response.[5,6]

The present study describes the indomethacin (prostaglandin synthetase inhibitor)-mediated enhancement of lymphocyte response to mitogens in healthy subjects and in lung cancer patients. This enhancement suggests that the prostaglandins may play a role in suppressing cell-mediated immune responsiveness.

Materials and Methods

Patients

Twenty-six patients with lung cancer were studied. There were 16 males and 10 females. Their age ranged from 48 to 72 years. There were 18 patients with Stage I, five with Stage II, and three with Stage III disease. Of these 26 patients, 11 had squamous cell, eight had adenocarcinoma, four had large cell, and three had bronchioalveolar cell type. At the time of the study, eight patients were newly diagnosed and untreated, whereas 18 patients were previously treated with either five-drug combination chemotherapy (cis-diamminedichloroplatinum (II), Adriamycin, cyclophosphamide,

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CCNU, and vincristine, given every four to six weeks for four to six doses) or immunotherapy (complete Freund adjuvant alone or tumor vaccine including complete Freund adjuvant, given every four weeks for three doses) following the resection of the tumor. All 18 treated patients were clinically free of disease; the interval between the therapy and testing period in these patients ranged from six months to two years.

Control Subjects

Seventeen healthy subjects aged somewhat younger than lung cancer patients, ranging from 22 to 55 years old were also studied.

Lymphocyte Stimulation Test

In almost all experiments, short-term lymphocyte cultures were performed by a whole blood culture technique.[15] In brief, heparinized blood was washed twice and resuspended in RPMI 1640 culture medium containing 10% fetal calf serum and antibiotics (100 units of penicillin and 50 μg of streptomycin/ml) at a ratio of 1:20 of blood: culture medium. Two milliliters of cell suspension were transferred to 16 × 125 mm Falcon disposable plastic tubes. One μg of purified PHA (Burroughs Wellcome Company, Research Triangle Park, North Carolina), 10 μg of concanavalin-A (Con-A, Cal-Biochem., Los Angeles, California), and 0.05 ml of reconstituted pokeweed mitogen (PWM, Grand Island Biological, Grand Island, New York) were added to each tube. Control culture received no mitogen. To half of control cultures and cultures with mitogens, one μg or 0.1 μg of indomethacin per ml of culture was added. Indomethacin (Indocin, Merck, Sharp and Dohme, West Point, Pennsylvania) was dissolved in 95% ethyl alcohol at 10 mg per ml and further diluted with phosphate-buffered saline, resulting in a final concentration of 0.01% ethyl alcohol in the solution. Experiments were carried out in duplicate.

In a few experiments, short-term lymphocyte cul-

TABLE 1. Mitogen-induced Lymphocyte Proliferative Response

	Mitogen	No. of experiments	^{3}H-thymidine incorporation (cpm × 10^3) mean ± SE
Healthy subjects	PHA	17	114.7 ± 16.7
Lung cancer patients	PHA	23	69.1 ± 10.7
Healthy subjects	PWM	13	39.7 ± 5.3
Lung cancer patients	PWM	16	27.1 ± 6.2
Healthy subjects	Con-A	12	11.8 ± 2.3
Lung cancer patients	Con-A	15	6.6 ± 2.2

PHA = phytohemagglutinin; PWM = pokeweed mitogen; Con-A = concanavalin-A.

TABLE 2. Effect of Indomethacin on *in vitro* Lymphocyte Responses in Healthy Subjects

	^{3}H-thymidine incorporation (cpm × 10^3)								
	PHA response			Con-A response			PWM response		
	No indomethacin	Indomethacin (1.0 μg/ml)	Indomethacin (0.1 μg/ml)	No indomethacin	Indomethacin (1.0 μg/ml)	Indomethacin (0.1 μg/ml)	No indomethacin	Indomethacin (1.0 μg/ml)	Indomethacin (0.1 μg/ml)
Experiment									
1	32.3	104.2	89.0	2.1	2.3	1.9	2.8	4.6	4.8
2	34.3	39.7	52.2	2.9	4.4	4.9	6.0	5.8	11.5
3	35.6	32.9	37.3	3.3	6.4	4.5	31.8	34.7	36.4
4	55.1	95.0	70.3	4.9	10.1	10.9	37.8	68.2	56.1
5	63.4	75.0	66.6	7.6	8.1	8.1	39.3	52.5	54.8
6	79.1	81.1	54.5	10.0	24.4	28.8	41.6	50.7	54.7
7	84.6	81.3	101.1	10.8	19.3	14.0	43.9	40.8	39.2
8	91.6	139.7	142.9	14.0	11.1	12.8	44.6	63.0	63.5
9	100.1	125.0	147.2	18.0	18.2	15.8	50.1	55.3	47.1
10	106.4	124.7	130.7	20.5	25.0	27.9	51.9	67.7	68.2
11	110.3	139.5	136.8	23.0	28.1	25.7	62.9	115.7	113.2
12	143.7	129.4	169.7	24.9	30.2	28.8	63.6	53.9	69.4
13	166.6	203.2	196.4				67.9	81.9	84.4
14	178.2	133.4	163.4						
15	185.1	197.3	221.3						
16	207.2	170.6	192.8						
17	276.8	326.1	309.3						
Mean	114.7	129.3	134.2†	11.8	15.6*	15.3	39.7	53.4*	54.0†
SE	16.7	16.9	17.4	2.3	2.8	2.9	5.3	8.1	7.9

* $P < 0.05$; † $P < 0.01$ by the paired t test.

TABLE 3. Effect of Indomethacin on *in vitro* Lymphocyte Responses in Lung Cancer Patients

	^{3}H-thymidine incorporation (cpm × 10^3)								
	PHA response			Con-A response			PWM response		
	No indomethacin	Indomethacin (1.0 μg/ml)	Indomethacin (0.1 μg/ml)	No indomethacin	Indomethacin (1.0 μg/ml)	Indomethacin (0.1 μg/ml)	No indomethacin	Indomethacin (1.0 μg/ml)	Indomethacin (0.1 μg/ml)
Experiment									
1	0.1	3.3	3.0	0.1	1.0	1.4	0.1	22.0	3.3
2	0.1	16.3	16.6	0.1	0.2	0.2	0.2	7.2	3.7
3	2.6	41.5	36.1	0.2	0.2	0.2	0.3	6.9	4.5
4	4.7	5.0	19.8	0.2	0.3	0.5	2.6	7.5	5.3
5	19.5	47.3	37.0	1.3	8.5	7.8	4.0	13.2	7.5
6	20.0	50.9	45.4	1.4	1.0	1.1	10.0	19.6	24.7
7	29.7	24.4	39.7	1.7	4.9	4.7	13.4	19.1	16.2
8	41.7	53.2	78.1	3.1	5.9	8.1	16.1	23.6	22.5
9	42.8	62.4	66.4	3.7	5.2	3.8	27.8	59.1	61.0
10	57.6	69.3	106.1	4.1	3.6	3.5	28.9	48.2	36.3
11	71.1	59.5	66.0	4.5	6.2	4.7	39.5	39.5	43.7
12	72.3	68.0	71.7	11.8	10.8	11.9	48.1	47.1	44.4
13	73.4	133.1	148.1	21.5	28.8	29.9	57.3	73.2	69.7
14	75.6	113.8	132.2	21.8	27.2	28.8	57.5	60.2	72.8
15	81.5	94.6	106.7	23.0	48.3	42.5	60.6	70.9	71.1
16	86.8	115.6	103.6				67.4	83.2	84.5
17	101.6	112.8	94.3						
18	103.5	100.4	109.7						
19	103.7	84.6	107.1						
20	125.4	149.7	128.7						
21	140.0	140.8	142.0						
22	166.5	122.5	147.2						
23	168.1	181.1	172.9						
Mean	69.1	80.4*	86.0†	6.6	10.1	9.9*	27.1	37.6‡	35.7†
SE	10.7	10.1	10.0	2.2	3.6	3.4	6.2	6.5	7.2

* $P < 0.05$; †$P < 0.01$; ‡$P < 0.001$ by the paired t test.

tures were performed by a separated cell culture technique.[12] In brief, T lymphocytes were separated from non-T cells (B lymphocytes and monocytes) by neuraminidase-treated sheep erythrocyte rosetting technique. Monocytes were isolated from non-T cell fraction by carbonyl iron ingestion technique. T-enriched fraction and monocyte-enriched fraction contained more than 95% purity of respective cells. T cells and monocytes were washed twice and resuspended in RPMI 1640 culture medium at a concentration of 1×10^5 cells and 2×10^4 cells per ml. Monocytes were irradiated with 6000 rads to get rid of helper cell activity.[12]

TABLE 4. Effect of Indomethacin on PHA Responses in Lung Cancer Patients in Remission or with Active Disease

	No. of experiments	^{3}H-thymidine incorporation (cpm × 10^3) mean ± SE		
		No indomethacin	Indomethacin (1.0 μg/ml)	Indomethacin (0.1 μg/ml)
Active disease	17	78.0 ± 12.7	84.5 ± 11.5	91.5 ± 10.8
Remission	6	43.7 ± 16.9	68.9 ± 21.9	70.6 ± 23.5

One ml of T-cell suspension was transferred to 16 × 125 mm Falcon disposable plastic tubes. To some culture tubes, containing T-cell suspension, 1 ml of irradiated (6000 rads) monocyte suspension was added. One μg of purified PHA was then added to each tube. Control culture received no PHA. One tenth microgram indomethacin per ml of culture was added to some cultures containing PHA. Experiments were carried out in duplicate. Culture tubes with loose-fitting caps were incubated at 37 C in a humidified atmosphere of 5% CO_2 in air for seven days (maximum PHA response with low concentration of T cells in whole-blood culture or separated cell culture technique is usually seen on day 6–7 of incubation in our laboratories). One microcurie of ^{3}H-thymidine (specific activity, 2.0 Ci/mMole) was added to each tube 24 hours before harvesting the cells. Incorporation of ^{3}H-thymidine into DNA was measured according to the method previously published.[15]

Results

Table 1 shows the mitogen-induced lymphocyte proliferative responses in healthy subjects and lung

cancer patients. As expected, a mean lymphocyte response to PHA, PWM, or Con-A was depressed in lung cancer patients as compared with that in healthy subjects. Although *in vitro* lymphocyte response in lung cancer patients in remission or with Stage I disease, on the average, was higher than that in patients with active disease or with Stage II and III, the difference was not statistically significant. The avergae lymphocyte responses in remission patients treated with chemotherapy and immunotherapy were quite similar. Table 2 shows the enhancement of indomethacin on mitogen-induced lymphocyte proliferative response in each of 17 healthy subjects. Indomethacin at a concentration of 1.0 μg/ml or 0.1 μg/ml significantly enhanced the PHA, Con-A, or PWM response in a majority of these donors ($P < 0.05$ or $P < 0.01$). Table 3 shows the enhancement effect of indomethacin on lymphocyte response in each of 23 lung patients. Indomethacin at both concentrations significantly enhanced the lymphocyte response in a majority of these patients ($P < 0.05$ or $P < 0.01$). On the average, a higher degree of enhancement of lymphocyte response by indomethacin in healthy subjects or lung cancer patients with weaker responses was observed as compared to those with stronger responses.

Table 4 shows the correlation of indomethacin-induced enhancement of PHA responses between lung cancer patients in remission and those with active disease. Indomethacin only slightly enhanced the PHA response in remission patients, whereas this agent markedly enhanced the PHA response in active disease patients. Of interest is the fact that the PHA response seen in cultures with indomethacin of active disease patients is only slightly lower on the average than that seen in cultures without indomethacin of remission patients.

Indomethacin had no enhancement effect on ^{3}H-thymidine incorporation of unstimulated lymphocytes, indicating the lack of mitogenic activity (Table 5).

In three experiments, the effect of indomethacin was investigated in cultures containing purified T cells only or purified T cells plus irradiated autologous monocytes from patients with lung cancer. The results are presented in Table 6. The indomethacin had no effect on purified T cells in the absence of monocytes, whereas this agent exerted its enhancement effect on T-cell response in the presence of autologous monocytes in each experiment.

TABLE 5. Lack of Mitogenic Activity of Indomethacin

	No. of experiments	^{3}H-thymidine incorporation (cpm) mean ± SE		
		No indomethacin	Indomethacin (1.0 μg/ml)	Indomethacin (0.1 μg/ml)
Healthy subjects	17	1027 ± 279	799 ± 233	856 ± 208
Lung cancer patients	23	640 ± 188	409 ± 81	514 ± 119

Discussion

The present study shows that the mitogen responses of T lymphocytes in a whole-blood culture system are markedly depressed in a majority of lung cancer patients. This observation agrees with our previously published report.[9]

The mechanisms for depression of T lymphocyte response in cancer patients are not fully understood. Several factors such as intrinsic defect of T lymphocytes, serum factors and suppressor cells have been suggested.[1,10,11,19] In a recently completed study using a separated cell culture system, we observed that the depression of T lymphocyte response to PHA is attributed to the presence of non-T suppressor cells rather than to an intrinsic defect of T lymphocytes in lung cancer patients.[12] The presence of suppressor cells in patients with solid tumors including lung cancer as well as in patients with Hodgkin's disease has recently been reported by us and other investigators.[2,6,10–12,14,17,21] There is no disagreement among published studies that the monocytes (adherent cells or phagocytic cells) are

TABLE 6. Effect of Indomethacin on T Cell Response to PHA in the Presence or Absence of Monocytes in Lung Cancer Patients

	^{3}H-thymidine incorporation (cpm × 10^3)			
	T cells	T cells + indomethacin (0.1 μg/ml)	T cells + monocytes	T cells + monocytes + indomethacin (0.1 μg/ml)
Patient 1, active disease	27.7	14.6	19.1	32.7
Patient 2, active disease	62.5	58.5	29.9	62.2
Patient 3, remission	98.8	96.3	91.3	180.4
Mean	63.0	56.5	46.8	91.8

suppressor cells for the T-lymphocyte response in cancer patients.[2,12,14,17,21] However, there are some conflicting observations regarding the suppressor T and B cells.[14,21]

The exact mechanism of action of suppressor cells on T lymphocyte response is not clear. One possibility is that the suppressor cells might have simply signalled the T lymphocytes to attenuate their blastogenic response by cell-to-cell contact. Another possibility, which should be considered, is that the suppressor cells might have produced soluble factor that is responsible for depression of T-lymphocyte response. We carried out several experiments to investigate the suppressor cell activity of cell-free media collected from non-T cells of lung cancer patients as well as Hodgkin's disease patients and found no consistent suppressor activity (unpublished observation). Recently, Goodwin *et al.* described the existence of a prostaglandin-producing glass-adherent suppressor cell in the peripheral blood of normal subjects,[4] of patients with Hodgkin's disease,[6] and sarcoidosis.[5]

In the present study, we observed that the addition of indomethacin to mitogen-stimulated cultures (whole-blood system) of lung cancer patients and healthy subjects results in an increase in the ^{3}H-thymidine incorporation of T lymphocytes. We also noted that the degree of indomethacin-mediated enhancement of T lymphocyte response in healthy subjects as well as in lung cancer patients is inversely proportional to the original lymphocyte response of indomethacin-free cultures. Similar observations have been described in patients with Hodgkin's disease and sarcoidosis.[5,6] Goodwin *et al.*[6] observed that the removal of glass-adherent cells markedly decreases the enhancement seen with indomethacin, that it reduces prostaglandin production by more than 80%, and that it eliminates the differences in PHA response between Hodgkin's disease patients and healthy subjects.

We also observed in the present study that the addition of indomethacin to PHA cultures of purified T lymohocytes of lung cancer patients does not enhance PHA response, whereas the addition of this agent to PHA cultures of purified T lymphocytes in the presence of monocytes of lung cancer patients does enhance PHA response. These observations suggest that iron-ingesting suppressor cells (monocytes) may secrete prostaglandins, which are responsible for the hyporesponsiveness of T-cell response in lung cancer patients.

Indomethacin can enhance the lymphocyte response *in vitro* at low concentrations (0.1 to 1.0 μg/ml) in the present study. Indomethacin has been shown to be effective as an antiarthritic agent; the recommended dose of this agent in the management of arthritis can achieve serum concentrations, greater than 1.0 μg/ml.[20] Further studies of the *in vivo* effect of indomethacin on cell-mediated immunity in man are in progress.

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