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Leukocyte Activation in Advanced Cancer as an Explanation for Absent Leukocyte Adherence Inhibition to Cancer Extracts and Chemoattractant*

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Abstract—*Tube leukocyte adherence inhibition (LAI) measures human immunity by antigen-binding leukocytes releasing chemoattractant-like mediators that are the ultimate inhibitors of adherence by bystander leukocytes. We determined whether the absent LAI responses to cancer extracts for patients with large body burdens of bladder cancer was related to a defect in antigen binding or chemoattractant responsiveness. Leukocytes from patients with small body burdens of bladder cancer gave positive LAI responses of a similar extent to either bladder cancer extracts or chemoattractant* [n-*formyl*-L-*methionylleucylphenylalanine* (*FMLP*)]. *Of the adherent leukocytes, about* 20–30% *became non-adherent with a positive LAI response: monocytes, neutrophils and lymphocytes responded. In the control tubes, leukocytes from patients with large body burdens of bladder cancer were more non-adherent and about* 15% *less adherent than leukocytes from controls or patients with early cancer. They showed no further decrease in adherence, or conversely increase in non-adherence, with either extracts of bladder cancer or FMLP. The leukocytes also failed to transduce transmembrane signals to the same stimuli. The defect was reversible since* PGE_2 *restored the adherence of leukocytes to normal, and subsequently they exhibited membrane potential changes and about* 34% *non-adherence to either bladder cancer extracts or FMLP. From these results we conclude that chemoattractant LAI-responsive leukocytes from patients with large body burdens of bladder tumor are activated* in vivo, *probably by mediators from inflammatory cells.*

INTRODUCTION

THE LEUKOCYTE adherence inhibition (LAI) assay has been successfully used as a method of detecting cell-mediated immunity against various antigens, including those of tumors [1–11; see reviews 12, 13], the authenticity of which has been verified with coded samples of blood [14, 15], tumor [16] and tumor isolates [17]. Analysis of the biochemical events underlying LAI [18–20] led to appreciation that the reaction can also be mediated by authentic chemoattractants [21]. Using genetically uniform (inbred) mouse strains which express quantitative variations in the magnitude of chemotaxis *in vitro* and of the inflammatory response *in vivo*, we showed that the degree of chemoattractant LAI reactivity is positively correlated with the macrophage chemotactic reactivity *in vitro* and local inflammatory response *in vivo* [22].

The mechanism of tumor antigen-induced LAI has been studied in both animals and humans bearing cancers and is mediated by both T cells and antibody-dependent monocytes [4, 23–39; see reviews 12, 13]. Pharmacologic studies of antigen-induced tube LAI suggest that oxidative metabolites of arachidonic acid such as leukotriene B_4 (LTB_4) are ultimately responsible for LAI [18, 19, 38, 40]. Mediators from the antigen-binding leukocytes or authentic LTB_4 in the tube assay inhibit the adherence of about 30% of bystander cells, which include mononuclear cells, T cells and neutrophils [21, 41].

There is a correlation between positive LAIs in tube assays and body burden of cancer: patients with small burdens often have positive LAIs, whereas those with heavy burdens less often have positive LAIs [3, 12, 13, 42–45]. Transiently increasing the leukocytes' intracellular cAMP reverses negative LAIs to positive [19, 43, 44, 46, 47]. Positive LAI responses require recognition of tumor antigen and production of and responsive-

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Abbreviations: $\Delta\Psi$, membrane potential change; [^{3}H]TPP, [phenyl-^{3}H]tetraphenylphosphonium ion; cAMP, 3′, 5′-cyclic adenosine monophosphate; FMLP, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; HEPES, 4(2-hydrodxyethyl)-1-piperazine ethansulfonate; LAI, leukocyte adherence inhibition; LTB_4, leukotriene B_4 (isomer 111:5(s),12(R)-dihydroxyl-6, 14-*cis*-8,10-*trans*-eicosatetraenoic acid); NAI, non-adherence index; OSN, organ-specific cancer neoantigens; PBL, peripheral blood leukocytes; PGE_2, prostaglandin E_2.

ness to mediators. Accordingly, we studied whether leukocytes from patients with advanced bladder cancer had a defect in antigen recognition or responsiveness to chemoattractants. The results suggested that leukocytes from patients with advanced cancer, having been activated *in vivo*, probably by chemoattractants, were unable to react to bladder cancer extracts or to chemoattractant *in vitro* until first they were 'turned-off', which was effected by PGE_2 pretreatment.

MATERIALS AND METHODS

Reagents

Medium 199 concentrated × 10, 1.0 M HEPES, 7.5% Na_2HCO_3 (Flow Laboratories, Mississauga, Ontario), prostaglandin E_2 (PGE_2), aminophylline, dibutyryl cAMP, FMLP (Sigma Chemical Co., St. Louis, MO), [phenyl-^{3}H]tetraphenylphosphonium bromide (sp. act. 4.3 Ci/mmol) (New England Nuclear, Montreal, Quebec), Vacutainer® tubes (Becton & Dickinson, Montreal, Quebec), glass culture tubes 12 × 75 mm, liquid scintillation vials (Fisher Scientific Co., Montreal, Quebec), Prosil^{TM}–28 (Can Lab, Montreal, Quebec), 0.5 μm-pore-diameter Celotate filters, 30 place vacuum filtration manifold, vacuum pumps (Millipore, Mississauga, Ontario) and Ready-Solv^{TM} HP liquid scintillation fluid (Beckman, Montreal, Quebec) were purchased.

Medium 199 was prepared from medium 199 concentrated ×10 by adding 10 ml to 90 ml sterile distilled water to which was added 0.5 ml 1.0 M HEPES and 1.75 ml 7.5% Na_2HCO_3 per 100 ml of medium.

A stock solution of 10^{-2} M FMLP in ethanol was prepared and kept at −70°C; a sample was diluted serially in phosphate-buffered saline (PBS) (0.14 M NaCl, 0.01 M Na_2PO_4, pH 7.3), and the final dilution in 10 dilutions was made with medium 199 to reach the working concentration.

Leukocyte preparation

Before surgery, venous blood from patients with bladder cancer, benign diseases, chronic inflammatory bladder disease and other malignancies unrelated to bladder cancer was drawn into 10 ml heparinized Vacutainer tubes. The tubes were incubated nearly vertical at 37°C for 45 min. The leukocyte-rich fraction was aspirated with a Pasteur pipette, red blood cells were lysed with ice-cold, isotonic Tris-buffered NH_4Cl solution and leukocytes were washed and suspended in medium at a concentration of 1×10^7/ml as previously described [3]. Neutrophils, mononuclear cells and T cells were enriched as previously described [18, 19, 36, 38]. Cell smears were prepared with the use of a Shandon Southern cytospin, stained with Diff-Quick and differentially counted under oil immersion (×400).

Tumor extracts

Bladder carcinoma extract was prepared from malignant bladder tissue that was removed from metastatic lesions of the liver of fresh autopsy material, and the non-specific cancer extracts were prepared similarly from metastases of pancreatic carcinoma to the liver as previously described [3]. The crude extracts in PBS were stored in 0.3 ml aliquots at −70°C till used. For the LAI assay, the stock extracts were diluted in medium 199 to ≃ 100 μg/0.1 ml and added to the tubes.

Tube LAI assay

Cancer-extract-induced LAI. The assay was performed in 20 ml, 16 × 150 mm glass test tubes (Kimax) in triplicate as described previously [3]. We added to each set of three tubes 0.3 ml medium 199, 0.1 ml (≃100 μg) of either the bladder cancer or non-specific pancreatic cancer extract, and 0.1 ml of the suspended PBL. The tubes were incubated horizontally at 37°C in a humidified atmosphere of 5% CO_2. After 2 hr, the tubes were placed vertically and a sample of the non-adherent cells was taken by Pasteur pipette, placed on a hemocytometer and counted by image analysis (Bauch and Lomb, Rochester, NY). The computer-linked instruments calculated the mean number of non-adherent cells in the presence of the bladder cancer and pancreatic cancer extracts, and the difference in the non-adherent cells was expressed as a percent change [non-adherence index (NAI)]:

$$\text{NAI} = \frac{A - B}{B} \times 100,$$

where A equals the number of non-adherent cells in a sample after incubation with the bladder cancer extract and B equals the number of non-adherent cells in the sample after incubation with the pancreatic cancer extract. From previous studies an NAI > 30 is positive and < 30 is negative [43].

Chemoattractant-induced LAI

The ability of FMLP to inhibit the adherence of leukocytes to glass was assessed by the LAI assay [21]. We chose the chemoattractant FMLP because it is well characterized, commercially available as a pure synthetic substance and reasonably stable at −70°C. We added to one set of three tubes 0.2 ml medium 199, 0.1 ml of the control pancreatic cancer extract (≃ 100 μg protein) and 0.1 ml FMLP appropriately diluted in medium 199, and 0.1 ml suspended leukocytes. To another three tubes we added 0.3 ml medium 199, 0.1 ml of the same

tumor extract and 0.1 ml suspended leukocytes. For example, 10^{-7} M FMLP in 0.1 ml medium was added to tubes already containing 0.2 ml medium 199 and 0.1 ml tumor extract in medium 199; then 0.1 ml of the leukocyte preparation was added, which resulted in the FMLP having a final concentration of 2×10^{-8} M. Dose–response curves showed that this concentration was optimum for giving the greatest LAI [21]. As a control source of protein for the LAI assay, crude human tumor extracts unrelated to the cancer of the leukocyte donor was used because in our experience, they contained no chemoattractant or sensitizing factor which produced non-specific changes in leukocyte adherence [21]. The NAI was calculated as described above, where *A* equals number of non-adherent cells in the sample after incubation with the chemoattractant FMLP and *B* equals the number of non-adherent cells in the sample after incubation without chemoattractant.

LAI after elevating intracellular cAMP of leukocytes

The PBL were divided into two aliquots, one being added to the tube assay without treatment while the other was preincubated at 2×10^7/0.5 ml of medium 199 containing 2.5×10^{-6} M PGE_2 and then further diluted to 1×10^7 cells/ml in medium 199. Aminophylline (10^{-5} M) or dibutyryl cyclic AMP (10^{-8} M) can be substituted for PGE_2 with similar results [19, 47]. The cells were plated in the tubes, and the assay was conducted as described above.

Assay for membrane potential change ($\Delta\Psi$)

A modification of the procedure of Schuldiner and Kaback [48] was used as previously described [38, 49, 50]. PBL (1.5×10^6) were incubated in 300 µl medium 199 with 10 ml of 6 µM [^{3}H]TPP$^+$ (4.3 Ci/mmol) for 30 min at 37°C to allow the [^{3}H]TPP$^+$ to reach equilibrium inside and outside the cells. Then the PBL were challenged separately and simultaneously with 100 µl of the extracts of bladder cancer and pancreatic carcinoma [38, 49, 50]. Incubation was carried out in 12 × 75 mm glass culture tubes siliconized with Prosil^{TM}–28 to prevent leukocyte adherence. At appropriate intervals, the [^{3}H]TPP$^+$ uptake by the leukocytes was stopped at 0.5, 1, 2, 5, 10 and 15 min by the rapid addition of 3 ml of ice-cold saline (0.09% NaCl) to each tube and immediate filtration through a 0.5 mm-pore-diameter Celotate® filter, using a 30-place Millipore® multiple-sampling manifold. The filters were rinsed with a further 3 ml ice-cold saline. The whole process of filtering and washing was completed in less than 15 sec. Then the filters were removed from the manifold and placed in liquid scintillation vials containing 10 ml of Ready-Solv^{TM} HP. The radioactivity trapped on the membrane was counted in an IsoCap/300 Beta Counter (Nuclear Chicago). A zero time control was obtained by the addition of ice-cold saline to PBL just after adding the [^{3}H]TPP$^+$, followed by immediate filtering and washing. The radioactivity of the zero time control was subtracted from all other values. To study the effect of FMLP, FMLP was added to one set of tubes and medium to the other. An index of [^{3}H]TPP$^+$ uptake was calculated:

$$\text{index of } [^3\text{H}]\text{TPP}^+ = \frac{A - B}{B} \times 100,$$

where *A* is cpm in the presence of the specific cancer extract or FMLP and *B* is cpm in the presence of the nonspecific cancer extract.

The maximum change in [^{3}H]TPP$^+$ uptake was calculated as follows:

(highest index of [^{3}H]TPP$^+$ uptake (representing hyperpolarization)) − (lowest index of [^{3}H]TPP$^+$ uptake, (representing depolarization))

provided they were not separated by more than 5 min. The maximum change in the index of [^{3}H]TPP$^+$ uptake was used to quantitate the $\Delta\Psi$ changes in leukocytes from different patients or to determine the effect of raising leukocyte intracellular cAMP [38, 49, 50].

RESULTS

Response in LAI to bladder cancer extracts and to FMLP

Table 1 shows that leukocytes from most patients with small, localized bladder cancer had positive LAIs (NAI > 30) to the bladder cancer extract or to FMLP, whereas leukocytes from patients with metastasis seldom had positive LAIs to either. The negative LAIs to either bladder cancer extract or FMLP for leukocytes from patients with metastatic bladder cancer were reversed by transiently raising with PGE_2 the leukocytes' intracellular cAMP before the assay (Table 1). By comparison, leukocytes from patients with inflammatory bladder disease or controls with benign disease or early unrelated cancer seldom showed a positive LAI to the bladder cancer extract but had positive LAIs to FMLP; the LAI responses were unchanged by pretreating the leukocytes with PGE_2 (Table 1). A different pattern of LAI response was observed for leukocytes from patients with advanced cancer unrelated to bladder cancer: they had negative LAIs to both the bladder cancer extract and FMLP; but when the leukocytes' cAMP was raised, the subsequent LAI response to FMLP turned positive whereas the

LAI response to bladder cancer extract remained negative (Table 1).

Analysis of adherent and non-adherent leukocytes to bladder cancer extract

Immediately after plating 1×10^6 leukocytes in the tubes, there were 509 ± 19 non-adherent cells in a counted sample. Using 509 ± 19 as representing the 1×10^6 cells plated (input), we calculated the percentage of cells at the end of the assay that were adherent or non-adherent in the control tubes (B) and to either bladder cancer extract or FMLP in the specific tubes (A). For the control subjects, about 178–190 leukocytes in a sample were non-adherent in tubes B after 2 hr or about 331 cells (509–178) or 65% were adherent (Table 2). About the same number of leukocytes were adherent to the extract of bladder cancer in tubes A ($P > 0.1$) (Table 2). Similarly, leukocytes from bladder cancer patients showed about 65% (509–178) adherent in the presence of the control extract of pancreatic cancer extract (B). However, in the presence of the bladder cancer extract, the adherent cells fell to 52% (A). Conversely, the non-adherent cells increased to 245 ± 25 leukocytes, which represented a 20% increase in non-

Table 1. Summary of LAI response of leukocytes to bladder cancer extracts or FLMP (chemoattractant) in the tube LAI assay

Leukocyte donors	Preincubation with PGE_2 before assay*	No. of patients tested	Percent LAI positive† to: Bladder cancer extract	FMLP
Bladder cancer				
Stage I	−	10	90‡	90§
	+	10	100	100
Stage III or IV	−	18	6	12
	+	18	100	100
Chronic inflam. bladder diseases	−	14	7	86
	+	14	0	93
Controls				
Benign diseases	−	35	0	100
	+	35	6	97
Unrelated malignancies				
Early	−	16	6	100
	+	16	0	100
Late	−	12	0	0
	+	12	0	100

* +, Leukocytes preincubated with 2.5×10^{-6} M PGE_2. −, Leukocytes not preincubated with PGE_2.

† NAI > 30 is positive to 100 μg/ml bladder cancer extract or 2×10^{-8} M FMLP (chemoattractant).

‡Chi square analyses of positive and negative LAIs for bladder cancer extract: early bladder cancer vs late bladder cancer, $P < 0.001$; early bladder cancer vs chronic inflam. cancer, $P < 0.001$; early bladder cancer vs benign controls, $P < 0.001$; early bladder cancer vs early or late unrelated cancer, $P < 0.001$. Same comparison with PGE_2: early bladder cancer vs each group, $P < 0.001$; except late bladder cancer, $P > 0.8$; late bladder cancer vs chronic inflam., $P < 0.9$; vs late unrelated cancer, $P < 0.8$; vs control subjects, $P < 0.7$. Same comparison with PGE_2-treated leukocytes: late bladder cancer vs same groups, $P < 0.001$.

§, Chi square analysis of positive and negative NAIs for FMLP without PGE_2: early bladder cancer vs late bladder cancer, $P < 0.001$; controls vs late bladder cancer, $P < 0.001$.

Table 2 Non-adherence to bladder cancer extracts of leukocytes from patients with early and late stages of bladder cancer and from patients with other diseases

Leukocyte donors	Preincubation with PGE_2 before assay*	No. of non-adherent leukocytes after incubation with extracts of: Bladder cancer (A)	Pancreatic cancer (B)	% change in non-adherence (NAI)†
Bladder cancer				
Stage I	−	245 ± 25‡	179 ± 16§‖	36 ± 7
	+	240 ± 40	168 ± 31‖	44 ± 5
Stage III or IV	−	275 ± 12	265 ± 15	6 ± 4
	+	290 ± 21	180 ± 15‖	58 ± 5
Chronic inflam. bladder diseases	−	200 ± 15	190 ± 15	6 ± 4
	+	200 ± 20	185 ± 15	3 ± 4
Controls				
Benign disease	−	200 ± 10	178 ± 11	11 ± 2
	+	190 ± 10	189 ± 9	1 ± 3
Unrelated malignancies				
Early	−	220 ± 16	205 ± 17	6 ± 3
	+	204 ± 20	200 ± 21 8	3 ± 4
Late	−	285 ± 15	260 ± 11	10 ± 6
	+	195 ± 26	187 ± 32	5 ± 4

* +, Leukocytes preincubated with 2.5×10^{-6} M PGE_2. −, Leukocytes not preincubated with PGE_2.

† $NAI = \frac{A - B}{B} \times 100$; > 30 is positive.

‡ Mean ± S.E. of patients tested as shown in Table 1.

§ Student's dependent t test of non-adherent leukocytes in tubes A & B; ‖ $P < 0.005$.

adherence ($P < 0.005$) (Table 2). Pretreating the leukocytes with PGE_2 or dibutyryl cAMP did not affect the adherence of leukocytes from either control subjects with benign disease or patients with early bladder cancer, respectively (Table 2). A similar analysis of leukocytes from patients with late bladder cancer showed only 50% of the leukocytes adherent in the control tubes and 46% in the tubes with the bladder cancer extract (Table 2). Unlike the control subjects or patients with early bladder cancer, PGE_2 increased the adherence of leukocytes from 50 to 64%. After pretreatment with PGE_2, there was 64% adherence to the control extract (B) and 43% adherence with bladder cancer extract (A), which represented a 33% increase in non-adherence ($P < 0.005$) (Table 2).

Analysis of adherent and nonadherent leukocytes to FMLP

A similar analysis of adherent and non-adherent leukocytes was performed for the LAI response to FMLP to determine whether the defect was in the response to mediators. Table 3 shows the FMLP-induced non-adherence for leukocytes from either patients with early bladder cancer or control patients, which was not potentiated by pretreating leukocytes with PGE_2. However, leukocytes from patients with either advanced bladder cancer or other advanced cancers showed a minimal increase in non-adherence to FMLP (Table 3). Thus the leukocytes from patients with advanced bladder cancer had a defect in the ability to respond to FMLP as well as to bladder cancer extracts. Nonetheless, the defect was reversible since leukocytes pretreated with PGE_2 first became significantly more adherent and on exposure to FMLP showed even slightly higher non-adherence than leukocytes from either control subjects or patients with early cancer (Table 3).

Table 3 Non-adherence to FMLP (chemoattractant) of leukocytes from patients with early and late stages of bladder cancer and from patients with other diseases

Leukocyte donors	Preincubation with PGE_2 before assay*	No. of non-adherent leukocytes after incubation with FMLP (2×10^{-8} M) (A)	Without FMLP (B)	% change in non-adherence (NAI)†
Bladder cancer				
Stage I	–			
	+	275 ± 30‡	177 ± 21§‖	55 ± 7
		265 ± 35	178 ± 29‖	47 ± 7
Stage III or IV	–	270 ± 15	235 ± 14	14 ± 5
	+	290 ± 25	173 ± 14¶	68 ± 6
Chronic inflam. bladder diseases	–	300 ± 15	200 ± 15‖	50 ± 8
	+	280 ± 20	185 ± 15‖	52 ± 10
Controls				
Benign diseases	–	300 ± 15	204 ± 15‖	48 ± 6
	+	280 ± 10	188 ± 6‖	54 ± 3
Unrelated malignancies				
Early	–	265 ± 25	178 ± 16‖	48 ± 7
	+	260 ± 20	175 ± 15‖	47 ± 6
Late	–	295 ± 10	265 ± 16	11 ± 5
	+	310 ± 25	185 ± 21‖	68 ± 10

* +, Leukocytes preincubated with 2.5×10^{-6} M PGE_2. –, Leukocytes not preincubated with PGE_2.

† NAL = $\frac{A - B}{B} \times 100$; > 30 is positive to chemoattractant FMLP.

‡ Mean ± S.E. of patients tested as shown in Table 1.

§ Student's dependent *t* test of non-adherent leukocytes in tubes A & B; ‖ $P < 0.005$; ¶ $P < 0.01$.

Evidence for in vivo *alteration of leukocyte adherence in advanced cancer*

To show that leukocyte nonadherence was altered *in vivo*, the results of non-adherence in the control tubes before and after incubation of the leukocytes with PGE_2 was analyzed (Table 4). PGE_2 pretreatment did not affect non-adherence to glass of leukocytes from either control subjects or patients with early cancer (Table 4). In contrast, PGE_2 pretreatment did affect non-adherence of leukocytes from patients with either advanced bladder cancer or other advanced cancers. Table 4 shows PGE_2 pretreatment decreasing non-adherent leukocytes in the control tubes. Thus leukocytes from advanced bladder cancer patients were less adherent than leukocytes from either control subjects or patients with early cancer, and this state was initiated *in vivo*. The results are shown for 2.5×10^{-6} M PGE_2, but similar results were observed with 10^{-5} M aminophylline and 10^{-8} M dibutyryl cAMP.

Characteristics of non-adherent leukocytes

The viability of non-adherent cells was greater than 95% at the end of the assay. The morphology of the non-adherent leukocytes in the control tubes and in the tubes with bladder cancer extract or FMLP were examined for patients with early bladder cancer, and the percentage of monocytes, neutrophils and lymphocytes were similar in both tubes: about 9% monocytes, 51% neutrophils and 40% lymphocytes. The results indicated that all cell populations participated in the LAI response to either bladder cancer extract or FMLP. The

Table 4. Effect of raising leukocytes' intracellular cAMP with PGE_2 on change in leukocyte non-adherence in control tubes

Leukocyte donors	Assay	No. of non-adherent leukocytes after incubation with control extracts: Untreated with PGE_2 (B)	Treated with PGE_2 (B_1)	% change in non-adherence*
Bladder cancer				
Stage I	bladder tumor†	179 ± 16§	168 ± 31	7
	FMLP‡	177 ± 21	178 ± 29	−1
Stage III or IV	bladder tumor	265 ± 15	180 ± 15‖	42
	FMLP	235 ± 14	173 ± 16‖	62
Chronic inflam. bladder diseases	bladder tumor	190 ± 15	185 ± 15	3
	FMLP	200 ± 15	185 ± 15	8
Controls				
Benign disease	bladder tumor	178 ± 11	189 ± 9	−6
	FMLP	204 ± 15	188 ± 6	9
Unrelated malignancies				
early	bladder tumor	205 ± 17	200 ± 20	2
	FMLP	178 ± 16	175 ± 15	2
Late	bladder tumor	260 ± 11	187 ± 30‖	39
	FMLP	265 ± 16	185 ± 21‖	43

* % change in nonadherence $= \frac{B - B_1}{B_1} \times 100.$

† Results in control tubes from Table 2.
‡ Results in control tubes from Table 3.
§ Mean ± S.E. of patients tested as shown in Table 1.
‖ Student's dependent t test of non-adherent leukocytes in tubes B & B_1; $p < 0.005$.

morphology of non-adherent leukocytes from patients with advanced cancer in the control tubes or in the tubes with bladder cancer extract or FMLP was similar to the patients with early bladder cancer and was not detectably changed by PGE_2 pretreatment. Enriched populations of neutrophils, mononuclear cells and T cells from advanced cancer patients showed no LAI response to FMLP, but after PGE_2 pretreatment the different enriched populations subsequently showed positive LAIs to FMLP. Thus all leukocyte populations had their adherence properties altered in advanced cancer.

Transmembrane signal for LAI response

Alterations in the adherence properties of LAI-responsive population of leukocytes from patients with advanced cancer seemed to be responsible for the negative responses to both chemoattractant and tumor antigen. However, leukocyte function was possibly entirely normal if measured in another manner. Consequently, we measured the ability of the leukocytes to show $\Delta\Psi$ changes after a membrane stimulus for two reasons: $\Delta\Psi$ changes do not depend on the adherent properties on the cells, and $\Delta\Psi$ changes also reflect whether the cells transmit a signal after a membrane stimulus. Leukocytes from 11 control subjects had a maximum change in $[^3H]TPP^+$ uptake of 32 ± 4 to bladder cancer extracts, whereas leukocytes from five patients with early bladder cancer had a maximum change of 62 ± 9, which was not changed by preincubating the leukocytes with PGE_2 (Table 5). The difference was statistically significant ($P < 0.05$). The transmembrane signal response of leukocytes from seven patients with advanced cancer was similar to control subjects, with a mean maximum uptake of 32 ± 5 (Table 5). The response, while not significantly different from control subjects, was statistically different from patients with early cancer ($P < 0.05$). When the leukocytes were pretreated with PGE_2, the response subsequently increased to mean maximum uptake of 47 ± 4, which was significantly different from the results without PGE_2 treatment (Table 5).

Table 5. Membrane signal transmission (ΔΨ change) induced by bladder cancer extracts or FMLP chemoattractant

Diagnosis of leukocyte donor	Preincubation with PGE_2 before assay*	Maximum change in $[^3H]TPP^+$ uptake induced by:† Bladder cancer extract (mean ± S.E.)‡	FMLP (mean ± S.E.)‡
Bladder cancer			
Stage I ($n = 5$)	−	62 ± 9	63 ± 9
	+	52 ± 5	58 ± 10
Stage III or IV ($n = 7$)	−	32 ± 5§‖	35 ± 3 §¶
	+	47 ± 4*	66 ± 9 ¶
Control subjects ($n = 9$)	−	32 ± 4	53 ± 6
	+	29 ± 6	66 ± 5

* +, Leukocytes preincubated with 2.5×10^{-6} M PGE_2. −, Leukocytes not preincubated with PGE_2.
† Maximum change in $[^3H]TPP^+ > 44$ is positive.
‡ Student's *t* dependent test of means: to bladder cancer extract without pretreating with PGE_2: early bladder cancer vs controls, $P < 0.05$; late bladder cancer vs controls, $P > 0.2$; early bladder cancer vs late bladder cancer, $P < 0.05$; with pretreating with PGE_2; early bladder cancer vs controls, $P < 0.05$; late bladder cancer vs controls, $P < 0.05$; early bladder cancer vs late bladder cancer, $P > 0.2$. To FMLP without pretreating with PGE_2: early bladder cancer vs controls, $P > 0.2$; late bladder cancer vs controls, $P < 0.05$; early bladder cancer vs late bladder cancer, $P < 0.05$; with pretreating with PGE_2: no significant differences between any group.
§Student's *t* dependent test of means before and after raising cAMP;‖ $p < 0.05$; ¶ $p < 0.005$.

The difference in mean maximum uptake between controls and late bladder cancer also became statistically significant ($P < 0.05$), while between early and late bladder cancer patients the response was no longer statistically different. Thus the tumor antigen stimulated no transmembrane signal for leukocytes from patients with advanced cancer until the cyclic AMP was raised with PGE_2.

FMLP induced a mean maximum change in $[^3H]TPP^+$ uptake of 53 ± 6 and 63 ± 9 in leukocytes from control subjects and from patients with early bladder cancer, respectively, and was not significantly changed by pretreating the leukocytes with PGE_2 (Table 5). By comparison, FMLP induced only a 35 ± 3 maximum change in $[^3H]TPP^+$ uptake for leukocytes from patients with advanced cancer, and this was statistically different from the other groups of patients. When the leukocytes were pretreated with PGE_2, FMLP subsequently produced a mean maximum uptake of 66 ± 5, a statistically significant change (Table 5).

DISCUSSION

The change in adherence initiated by the bladder cancer extract on leukocytes from patients with bladder cancer was not great. Nevertheless, the change is sufficient even in individual patients to be able to use the LAI assay to routinely screen patients attending our cytoscopy clinic for primary or recurrent bladder cancer with a high degree of sensitivity and specificity [51]. However, the present study was concerned with understanding why the LAI-responsive population of leukocytes from patients with advanced bladder cancer did not respond positively unless they were pretreated briefly with PGE_2. Not only were leukocytes from patients with advanced bladder cancer unresponsive to bladder cancer extracts, but they also failed to respond to the chemoattractant FMLP. The observed loss of normal adherence of leukocytes in advanced bladder cancer suggested that they had been activated *in vivo*. The LAI-responsive population was already non-adherent at the start of the assay. PGE_2 or dibutyryl cAMP restored normal adherence or 'turned off' the LAI-responsive population of leukocytes; subsequently, the leukocytes were able to exhibit LAI to either bladder cancer extract or FMLP.

We also measured membrane potential ($\Delta\Psi$) change of the leukocytes as part of the transmembrane signal induced by the surface stimulus of either bladder cancer extract or FMLP since previous studies indicated that transmembrane signals induced by either tumor antigen or chemoattractants correlated well with LAI [49, 50]. Also, FMLP-induced $\Delta\Psi$ change in neutrophils correlate with chemotaxis [52]. Leukocytes from patients with advanced bladder cancer did not transmit a transmembrane signal when incubated with either bladder cancer extracts or FMLP. Like the LAI response, the refractory state to $\Delta\Psi$ change was reversible by pretreatment with PGE_2.

Leukocytes from patients with metastatic bladder cancer were more non-adherent than leukocytes from patients with early bladder cancer or control subjects. The adherence of leukocytes from patients with metastatic bladder cancer was restored to control levels by PGE_2 pretreatment. The inhibitory effect was reversible since subsequently the leukocytes transmitted signals and exhibited LAI to both bladder cancer extracts and FMLP. Prostaglandins and cAMP are known to modulate many cellular functions. PGE_2 and prostacyclin rapidly and reversibly inhibit FMLP-induced superoxide production by human neutrophils [53], and modulation of the neutrophils' biologic response is a result of increased cAMP levels [53]. Agents that increase cAMP levels inhibit the function of a variety of leukocytes involved in in-

flammation and immunity [54, 55], including chemotactic responses [56, 57]. Several macrophage functions, including tumoricidal activity [58] and chemiluminescence [59], are sensitive to inhibition by PGE_2 by its effect of stimulating cAMP formation [60]. In addition, the activity of cytotoxic T lymphocytes are inhibited by agents that increase cAMP [61]. How increased cAMP modulates leukocyte functions is not known; however, in platelets cAMP control is exerted before the formation of 1, 2-diacylglycerol, possibly by inhibition of phospholipase C [62, 63].

The antigen-binding leukocytes in the tube assay [18, 19, 38] synthesize and release oxidative products of arachidonic acid metabolism such as LTB_4 and thromboxanes, which inhibit the adherence of bystander leukocytes [18, 19, 38, 40]. An identical percentage of about 30% of adherent monocytes, neutrophils and T cells, either $T8^+$ cytotoxic or $T4^+$ helper, have their adherence inhibited by antigen-generated mediators or authentic LTB_4 [21, 41, 64]. Because the tube LAI response depends on measuring adherence changes predominately in the bystander population, it is difficult to know how many leukocytes are actually binding tumor antigen. Thus the limiting factor in the LAI response is the number of cells that are responsive to chemoattractants. Mature blood leukocytes are heterogeneous with respect to responsiveness towards chemoattractants [65]. Seligmann *et al.* [65] suggest that among neutrophils, at least, differential binding of chemoattractants and its modulation may account for seemingly identical neutrophils responding and not responding in chemotaxis and chemoattractant-induced membrane potential changes.

The results indicated that the chemoattractant-LAI-responsive leukocytes from patients with advanced bladder cancer were 'turned on' *in vivo*. All leukocyte subtypes failed to respond *in vitro* to either authentic or immunologically generated chemoattractants. One explanation for finding leukocytes 'turned on' in advanced cancer is that blood leukocytes bind circulating tumor antigen and release chemoattractants which act on bystander leukocytes in the same fashion as occurs in LAI. It is known that once neutrophils respond to chemoattractants, they adapt to the stimulus with a decrease in chemotactic responsiveness to the same concentration of stimulus [52, 66]. Moreover, antigen-induced release of leukotrienes from macrophages is rapid in onset, and once activated, macrophages are capable of prolonged and continuous release of leukotrienes [67]. Moreover, immune complexes containing as little as 2 ng of antigen elicit the release of leukotrienes [67]. Serum from patients with advanced cancer specifically reacts in LAI with leukocytes from patients with the same cancer, suggesting that the serum contains circulating tumor antigen able to trigger chemoattractant or leukotriene release [18, 19, 68]. Release of chemoattractants locally by leukocytes infiltrating small tumors may act to attract other leukocytes; however, systemic generation of chemoattractants as observed in patients with large tumor burdens may act to confuse leukocyte accumulation at tumor foci.

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