Europ. J. Cancer Vol. 15, pp. 1095–1106.

0014-2964/79/0901-1095 $02.00/0

Abrogation of the Phenomenon of Leukocyte Adherence Inhibition by Excess Circulating Tumour Antigen*

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Abstract—*Leukocytes from control subjects show some non-adherence to glass when incubated with extracts of cancer, however, when the cancer extracts are of similar protein concentrations, the leukocyte non-adherence is also similar. Likewise, leukocytes from patients with limited cancer when incubated with extracts of unrelated cancer show non-adherence that is equal to control subjects. When the leukocytes from patients with limited cancer are incubated with extracts of the sensitizing cancer, then the mean leukocyte non-adherence is significantly higher than control subjects. By contrast, the leukocytes from patients with metastatic cancer exhibit a mean leukocyte non-adherence similar to leukocytes from patients with limited cancer when incubated with the sensitizing or unrelated cancer extract. Hence, LAI-negative patients with metastatic cancer have no residual population of LAI-reactive cells. The LAI-reactivity of leukocytes was abrogated by preincubation with the sensitizing TSA either from solid tumour or isolated from serum. Moreover, the tumour antigen coat on the leukocytes from patients with metastatic cancer was removed by gentle trypsinization of the leukocytes surface which restored the monocyte's capacity to react with the sensitizing tumour antigen. Hence, the increased non-adherence to glass of leukocytes from patients with metastatic cancer results from the LAI reactive cells (monocytes) being coated* in vivo *with TSA. The non-adherence of leukocytes from patients with limited and metastatic cancer is induced by the binding of TSA to the monocyte's cell surface; in the former instance binding occurs* in vitro *and in the latter* in vivo. *The results indicate that in advanced cancer there is no decrease in the number of host cells responding to the cancer antigens; however, the TSA shed from the tumour and present in excess in the circulation abrogates the tumour-specific responses* in vitro *and perhaps* in vivo.

INTRODUCTION

THE PHENOMENON of antigen-induced leukocyte adherence inhibition was discovered by Halliday and Miller [1]. A tube leukocyte adherence inhibition (LAI) assay was adopted and modified from Holan *et al.* [2] and with this *in vitro* assay our laboratory studied the immune response to cancers of human breast, pancreas, colon, stomach and melanomas [3–6].

The results of our studies indicated that antitumour immunity was present, in general, with small tumour burdens and usually absent and not detectable in patients with widespread metastases [3, 4, 6]. The biological significance of the LAI response in the tube assay was studied [7–9]. In patients with limited cancer, cytophilic antitumour antibody in the circulation "armed" the monocytes [8] and these armed monocytes were inhibited *in vitro* from adhering to glass by the sensitizing antigen. Most leukocytes from patients with advanced cancer did not display LAI activity. An excess of free circulating

Accepted 15 February 1979.

*This work was supported by the Medical Research Council of Canada, Ottawa, Canada, the Cancer Research Society Inc. of Montreal and the National Cancer Institute of Canada.

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Abbreviations used: HDL, high density lipoprotein; LAI, leukocyte adherence inhibition; NAI, non-adherence index; PBL, peripheral blood leukocytes; PBS, phosphate buffered saline; TSA, tumour-specific antigen.

tumour-specific antigen (TSA) shed from the tumour was present in the circulation of patients with metastatic cancer [9]. The TSA coated the cell surface of the monocytes and abrogated their response to antigen added to the *in vitro* assay [9]. When the tumour antigen coat of the monocyte was removed by gentle trypsinization of its surface, the monocyte's capacity to react with the sensitizing tumour antigen and to bind free cytophilic antitumour antibody from the microenvironment was restored [9]. The TSA in the circulation exists as a lipoprotein molecule, some of which undergoes enzymatic cleavage or degradation and is removed by ultrafiltration in the kidney [10].

The monocytes of patients with limited cancer bind *in vitro* to TSA in the supernatant and are inhibited from adhering to glass. The monocytes of advanced cancer patients if they are responding immunologically to the tumour will be coated *in vivo* with TSA and should also show the same phenomenon of antigen-induced leukocyte adherence inhibition to glass without the necessity of exposure *in vitro* to the sensitizing antigen. If the aforesaid hypothesis is correct, leukocytes from patients with advanced cancer should be expected to show greater mean non-adherence to glass when incubated with specific and nonspecific tumour extracts than leukocytes derived from control subjects. Hence, the present study was designed to examine the patterns of non-adherence of a group of control subjects, of a group of patients with colon and breast cancer whose leukocytes showed antigen-induced LAI reactivity to their respective tumours, and of a group of patients with metastatic colon and breast cancer whose leukocytes showed no LAI activity.

MATERIALS AND METHODS

Subjects

Heparinized blood samples were withdrawn from patients with adenocarcinoma of the breast and colon, who had either localized cancer or widespread metastatic cancer. Control subjects had non-malignant disease. All patients with early cancer or control subjects were tested before any treatment. All patients with advanced cancer were tested at least one month after any chemotherapy and before irradiation or palliative surgery.

Tumour extracts

Tumour tissues were from metastatic deposits in the liver, obtained at autopsy. The tumour tissue was finely minced and approximately 20 g was placed in ice-cold phosphate buffered saline (0.01 M phosphate and 0.14 M saline) (PBS) pH 7.3 and homogenized as previously described [3]. The homogenate was centrifuged at 20,000 ***g*** for 30 min and the supernatants pooled and stored at −40°C in 0.3 ml aliquots. Each sample was used once and discarded. The protein concentration of the tumour extract was determined by the method of Lowry *et al.* [11] with bovine albumin as the standard. The optimum protein concentration of the PBS tumour extracts was determined by titration curves against PBL from patients with limited cancer and control subjects as previously described [4, 12]. In addition, the titration of the extracts was tested daily on leukocytes from control subjects.

Automated tube leukocyte adherence inhibition assay (tube LAI assay)

The tube LAI assay was performed as described previously [3]. Peripheral blood leukocytes (PBL) derived from patients with breast cancer and control subjects were tested against a phosphate buffered saline (PBS) pH 7.3 extract of breast cancer as the specific antigen and an extract of melanoma as the nonspecific antigen. PBL from patients with colon cancer and control subjects were tested against an extract of colon cancer as the specific antigen and an extract of lung cancer as the nonspecific antigen. The assays on the 2 groups of patients were done independently by 2 individuals.

Antigen-induced LAI was performed in 20 ml (16 × 150 mm) glass Kimax test tubes (Fisher Scientific, Montreal, Canada). Aliquots of 0.1 ml of a PBL suspension (1×10^7/ml) were placed in the glass tubes, then 0.3 ml of Medium 199 and 0.1 ml of specific tumour extract (~100 μg) or unrelated tumour extract (~100 μg) was added to each tube. The suspension in each tube was agitated and the tubes were then placed horizontally so that the contents covered 4/5 of the lower surface of each tube. The tubes were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. After 2 hr of incubation, the tubes were placed vertically and their contents agitated with a Pasteur pipette and a sample was placed on the haemocytometer (Albert Sass, Berlin 41, Filandastrasse B2, W. Germany) designed for counting by image analysis. All samples were done in triplicate and all blood samples were coded.

The non-adherent cells were counted electronically by image analysis on a haemocytometer with a Leitz microscope with 10× phase-contrast objective [5] with the focus of the microscope, stage movement, and calculation and expression of the results programmed on a Data General Nova 3 16K computer. The results were expressed as a Non-adherence Index (NAI):

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

where A is the number of non-adherent cells in the presence of specific antigen, and B is the number of non-adherent cells in the presence of nonspecific antigen.

NAI's of $\geqq 30$ were positive and those of <30 were negative. This value was chosen (arbitrarily) on the basis that in previous studies more than 95% of control subjects had values less than 30 [3–6].

Blocking tube LAI assay

The blocking tube LAI was previously described [9, 10, 13, 14]. In brief, PBL were derived from patients with localized breast or colon cancer who reacted in the tube LAI against their respective cancer extracts. The samples used to block LAI-reactivity were previously described PBS tumour extracts and TSA's in the serum of advanced cancer patients that were partially isolated by precipitation of the high density lipoprotein fraction of serum by the polyanions sodium phosphotungstate (NaPhT) and magnesium chloride ($MgCl_2$) as previously described [10]. The PBL (1.3×10^7) were incubated with samples of either the tumour extracts or the serum isolate in separate tubes in a total volume of 1 ml in a 5% CO atmosphere with frequent agitation of the tubes. After 30 min, the cells were spun down, washed twice with Medium 199 and plated separately in glass test tubes with Medium 199 alone and with the specific and nonspecific cancer extracts. After 2 hr, the non-adherent cells were counted by image analysis. NAI's of $\geqq 30$ were positive and indicated that the sample had not negated LAI-reactivity, whereas those of <30 were negative and indicated that the sample had negated LAI-reactivity.

Conversion of non-reactive cells to LAI-reactive cells by trypsinization of their cell surfaces

PBL from 10 patients with metastatic colon or breast cancer who had had no recent surgery, irradiation or chemotherapy that were unreactive in the assay, were either treated with trypsin or washed and then retested in the assay. Briefly, 10^8 cells were suspended in an Erlenmyer flask in 25 ml of Medium 199 containing 10% fetal calf serum. and the resulting mixture was incubated at room temperature, with constant slow stirring by a magnetic bar and stirrer. At the end of 1 hr, 3 ml of calf serum were added to the suspension of PBL to inactivate the trypsin. The cells were then washed twice with Medium 199 containing 10% fetal calf serum. The cells were resuspended in Medium 199 containing 10% fetal calf serum at a concentration of 1×10^7 cells/ml and incubated for 1 hr at 37°C in 5% CO_2 atmosphere. The cells were then washed twice in Medium 199 and 1×10^6 cells/0.1 ml were plated in the tube LAI assay. As a control, PBL from the same patients were treated in an identical fashion with the exception that trypsin was omitted.

RESULTS

Figures 1 and 2 show the LAI results of patients tested during a 6–7 month period. The NAI values of PBL from patients with colon cancer and control subjects incubated with the specific extract of colon cancer and the nonspecific extract of lung cancer are shown (Fig. 1). The NAI value of the patients with Dukes' A and B colon cancer, all of whom were tested before surgery, was significantly different (Student's t-test $P<0.001$) from the NAI value of the control subjects. Moreover, the NAI value of the patients with Stage A and B colorectal cancer was significantly different ($P<0.001$) from the NAI value of patients with Stage D widespread colorectal cancer.

The NAI values of PBL from patients with breast cancer and control subjects incubated with the specific extract of breast cancer and the nonspecific extract of lung cancer are shown (Fig. 2). The NAI value of the patients with Stage I breast cancer, all of whom were tested before surgery, was significantly different ($P<0.001$) from the NAI value of control subjects. Moreover, the NAI value of Stage I breast cancer compared to Stage IV, widespread breast cancer was significantly different ($P<0.001$).

NAI's of $\geqq 30$ were chosen (arbitrarily) as positive since in this and previous studies [12] more than 95% of control subjects had NAI's of <30. NAI's of $\geqq 30$ were positive whether or

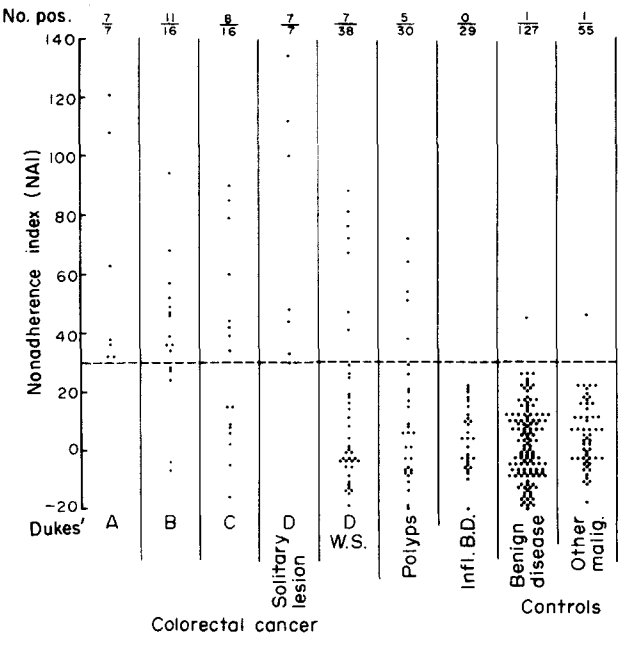

Fig. 1. Distribution of NAI values in patients with colorectal cancer, polyps and control subjects. Patients suspected of having colorectal cancer and control subjects were tested with an extract of colon cancer as the specific antigen and an extract of lung cancer as the nonspecific antigen before surgery or any treatment.

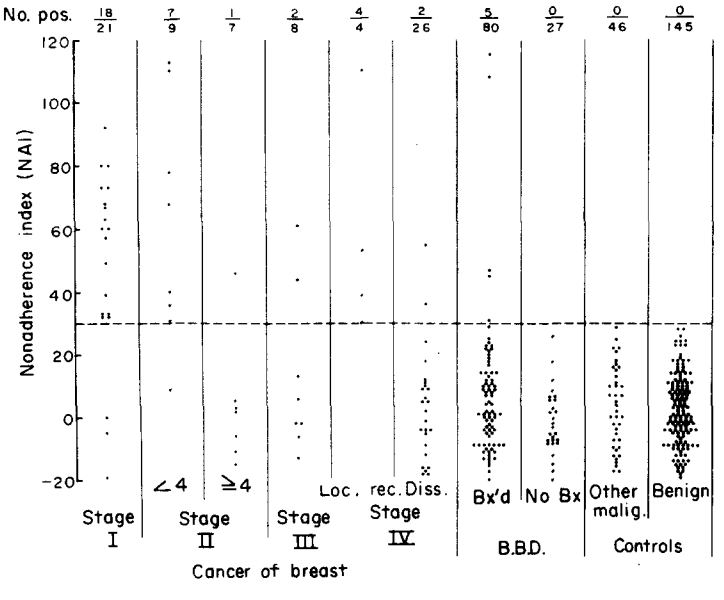

Fig. 2. Distribution of NAI values in patients with breast cancer, benign breast disease and control subjects. Patients suspected of having breast cancer and control subjects were tested with an extract of breast cancer as the specific antigen and an extract of malignant melanoma as the nonspecific antigen before surgery or any treatment.

not the difference in non-adherence of leukocytes from that patient to the two unrelated cancer extracts was significantly different by Student's *t*-test. In fact, the difference in leukocyte non-adherence to 2 unrelated cancer extracts in about one-half of the NAI's that are positive, are not significantly different by Student's *t*-test.

From the patients shown in Figs. 1 and 2, the 3 groups were chosen. Control subjects who had benign surgical conditions were group 1. Group 2 consisted of

patients with either localized colon or breast cancer who showed LAI reactivity. Group 3 was composed of patients with metastatic colon or breast cancer who showed no LAI reactivity (Tables 1 and 3). Whenever cancer patients were assayed, control subjects were also tested. Hence, one representative from each of the 3 groups was usually tested on the same day. Patients with limited cancer or metastatic cancer who had a positive and negative LAI, respectively, were chosen consecutively until each group had 10 patients. The patients were allocated in a random manner to the groups to eliminate any element of bias in patient selection for this study.

Antigen-induced non-adherence of leukocytes from colon cancer patients

Table 1 shows the number of non-adherent cells in each of the replicate tubes when leukocytes from group 1 and from the patients with limited (group 2) and metastatic colon (group 3) cancer were incubated with 2 different tumour extracts. The difference in non-adherence to the 2 tumour extracts is expressed as an NAI (Table 1). The difference in non-adherence to the 2 tumour

Table 1. *Number of non-adherent leukocytes from control subjects and patients with limited and metastatic colon cancer to PBS extracts of colon and lung cancer*

Groups	Patient No.	Number and mean ± S.D. of non-adherent leukocytes to: colon cancer extract (No.*)	colon cancer extract (Mean ± S.D.)	lung cancer extract (No.*)	lung cancer extract (Mean ± S.D.)	NAI†	$P^{\ddagger}$
1							
Control	1	150,261,251	221 ± 59	199,227,249	225 ± 25	−2	N.S.
subjects	2	235,299,269	268 ± 28	379,296,167	281 ± 106	−5	N.S.
	3	245,213,266	241 ± 27	246,217,254	239 ± 20	1	N.S.
	4	297,267,222	262 ± 38	216,200,254	223 ± 28	17	N.S.
	5	211,249,284	248 ± 37	276,306,207	263 ± 51	−6	N.S.
	6	180,185,187	184 ± 4	151,163,162	159 ± 7	13	N.S.
	7	294,242,270	269 ± 20	276,272,278	275 ± 17	−2	N.S.
	8	233,246,170	216 ± 43	265,237,221	221 ± 16	−2	N.S.
	9	178,176,185	180 ± 13	249,189,168	202 ± 42	−11	N.S.
	10	327,378,403	369 ± 43	312,260,363	312 ± 48	18	N.S.
2							
Patients	1	311,256,343	303 ± 47	134,171,133	146 ± 22	108	<0.005
with	2	343,378,375	365 ± 27	209,243,181	214 ± 30	71	<0.001
limited	3	611,317,387	438 ± 155	349,300,336	328 ± 31	34	N.S.
colon	4	316,413,332	354 ± 49	249,217,223	230 ± 8	54	<0.02
cancer	5	283,238,234	252 ± 22	172,111,168	150 ± 36	68	<0.02
	6	467,425,408	433 ± 37	247,248,265	253 ± 19	71	<0.001
	7	301,278,288	289 ± 12	248,168,247	221 ± 46	31	N.S.
	8	318,287,318	310 ± 9	194,231,266	230 ± 39	35	<0.025
	9	428,574,516	506 ± 74	262,442,431	378 ± 103	34	N.S.
	10	204,305,227	245 ± 55	144,153,201	166 ± 31	48	N.S.
3							
Patients	1	792,733,786	770 ± 43	705,616,630	650 ± 54	19	N.S.
with	2	446,484,419	450 ± 25	539,526,498	521 ± 21	−14	N.S.
metastatic	3	331,290,284	302 ± 26	277,350,235	287 ± 61	5	N.S.
colon	4	242,198,146	195 ± 50	160,246,196	201 ± 41	−3	N.S.
cancer	5	347,386,384	372 ± 29	362,309,390	354 ± 37	5	N.S.
	6	515,509,418	481 ± 50	444,516,618	526 ± 87	9	N.S.
	7	351,254,427	344 ± 87	316,355,318	330 ± 12	4	N.S.
	8	289,315,322	308 ± 17	347,310,296	317 ± 26	2	N.S.
	9	267,371,361	333 ± 57	306,351,392	350 ± 39	−5	N.S.
	10	184,163,240	196 ± 40	220,210,144	191 ± 41	2	N.S.

*The number of non-adherent cells in the samples from each of the 3 replicate tubes when incubated with colon and lung cancer extracts.

†The differences in leukocyte response to the 2 tumour extracts is calculated by the formula $\frac{A-B}{B} \times 100$ where A = mean number of non-adherent cells to colon cancer extract and B = mean number of non-adherent cells to the lung cancer extracts.

‡P derived from a comparison of the difference in non-adherent cells in columns 3 and 5 and calculated by Student's t-test. N.S., not significant at $P<0.05$.

extracts in some patients with limited cancer was statistically significant by Student's *t*-test. Leukocyte adherence inhibition shows marked variability from patient to patient; for this reason, it would be difficult to know whether PBL from metastatic cancer patients show greater non-adherence than from control subjects (Table 1). Table 2 shows the mean leukocyte non-adherence of the 3 groups. Leukocytes from group 1 showed similar non-adherence when incubated with the 2 cancer extracts (Table 2).

Leukocytes from groups 1 and 2 when incubated with the control extract of lung non-adherence of the leukocytes derived from group 3 to the specific colon cancer and the nonspecific lung cancer extracts was similar to the mean leukocyte non-adherence of group 2 incubated with the specific extract of colon cancer (Table 2).

Antigen-induced non-adherence of leukocytes from breast cancer patients

A different experimenter independently tested leukocytes from control subjects (group 1) and breast cancer patients with localized

Table 4. Mean number of non-adherent leukocytes from control subjects and patients with limited and metastatic breast cancer to extracts of breast and melanoma cancer

Groups	Non-adherent cells to breast cancer Mean ± S.D.*	Coeff. of variation†	$P^{\pm}_{+}$	Non-adherent cells to melanoma Mean ± S.D.	Coeff. of variation†	$P^{\pm}_{+}$	NAI§
1 Control subjects	225 ± 83	37	1 vs 3 <0.001	225 ± 80	36	1 vs 3 <0.001	0
2 Patients with early breast cancer	418 ± 199	48	2 vs 1¶ <0.001	257 ± 128	50	2 vs 1¶ N.S.	63
3 Patients with metastatic breast cancer	391 ± 216	55	3 vs 2 N.S.	368 ± 205	56	3 vs 2 <0.001	6

*The mean of non-adherent cells from the 30 test tubes in the 10 patients.

†Coefficient of variation is the $\frac{\text{S.D.}}{\text{Mean}} \times 100$.

‡Student's *t* test was used to determine if the means of the groups were significantly different. 1, 2 or 3 represents the groups.

§The NAI was calculated from the means.

¶In group 2, mean non-adherence of leukocytes incubated with extracts of breast and melanoma cancer was also significantly different ($P < 0.001$).

that coats the surface of the LAI-reactive monocyte, 2 different experiments were performed.

If LAI-reactive leukocytes are preincubated with the sensitizing PBS tumour extract, the LAI reactivity to that extract is abrogated (Table 5). The abrogation of LAI reactivity is specific since unrelated tumour extracts have no effect (Table 5). A similar result is observed when the sensitizing TSA in whole serum or isolated from serum of patients with metastatic cancer is preincubated with the LAI-reactive leukocytes (Table 5). The TSA present in the HDL fraction of serum has minimal amounts of other serum proteins, in particular, there is only trace contamination with IgG. Although the table shows the re-

were not LAI-reactive had their specific activity restored to the sensitizing tumour antigen by trypsinization. The repeated washing of the leukocytes involved in the trypsinization was not responsible for the recovery of activity since the same PBL treated in an identical manner but without the addition of trypsin remained non-reactive (Table 6). This experimental result was reproduced in 8 LAI-negative patients with metastatic cancer. The PBL were from LAI-negative patients with metastatic cancer who had never received chemotherapy, irradiation or had not had recent surgery. It appears that the experimental conditions for removal of the tumour antigen coat are critical since LAI-reactive cells were not generated with different con-

Table 3. Mean number of non-adherent leukocytes from control subjects and patients with limited and metastatic breast cancer to extracts of breast and melanoma cancer

Groups	Patient no.	Number and mean ± S.D. of non-adherent leukocytes to: breast cancer extract No.*	Mean ± S.D.	melanoma cancer extract No.*	Mean ± S.D.	NAI†	$P^{\pm}_{\pm}$
1							
Control	1	250,289,302	280 ± 27	238,258,269	255 ± 16	10	N.S.
subjects	2	231,140,238	203 ± 55	206,176,160	181 ± 23	12	N.S.
	3	197,234,270	234 ± 36	303,259,257	273 ± 26	−14	N.S.
	4	249,194,135	193 ± 57	198,118,205	174 ± 48	11	N.S.
	5	114,163,123	133 ± 26	112,136,157	135 ± 22	−1	N.S.
	6	292,221,451,188	288 ± 117	296,391,352,317	339 ± 42	−15	N.S.
	7	309,397,272	326 ± 64	315,327,253	298 ± 40	9	N.S.
	8	161,255,124,140	170 ± 59	138,158,178,141	154 ± 18	11	N.S.
	9	255,116,70,245,	171 ± 93	73,184,169,168	148 ± 51	15	N.S.
	10	259,292,240	264 ± 26	303,291,306	302 ± 10	−13	N.S.
2							
Patients	1	411,583,228	407 ± 177	247,180,146	191 ± 51	113	N.S.
with early	2	604,401,507	504 ± 101	158,246,262	222 ± 56	127	<0.01
breast	3	810,678,794	761 ± 72	388,408,395	397 ± 10	92	<0.001
cancer	4	317,414,382	371 ± 49	323,291,197	270 ± 65	37	N.S.
	5	581,512,581	556 ± 40	385,273,410	356 ± 73	57	<0.01
	6	599,474,424,499	499 ± 74	446,325,523,268	388 ± 112	30	N.S.
	7	697,500,392	530 ± 155	357,280,476	371 ± 99	43	N.S.
	8	347,222,262	277 ± 64	131,162,189	161 ± 29	73	<0.05
	9	313,241,204	253 ± 55	235,103,169	169 ± 66	49	N.S.
	10	98,90,117,98	101 ± 11	72,62,67,71	68 ± 4	44	<0.005
3							
Patients	1	221,165,338	241 ± 88	242,115,300	219 ± 95	10	N.S.
with	2	235,276,283	265 ± 26	243,264,268	258 ± 13	2	N.S.
metastatic	3	193,213,134	180 ± 41	225,189,180	198 ± 24	−9	N.S.
breast	4	328,649,405,538	480 ± 142	400,274,547,407	407 ± 112	18	N.S.
cancer	5	228,427,390,269	341 ± 87	437,341,218,257	313 ± 97	5	N.S.
	6	653,811,601	688 ± 09	921,402,629	651 ± 260	6	N.S.
	7	648,694,840	727 ± 100	582,604,718	635 ± 73	14	N.S.
	8	173,220,139	177 ± 41	172,151,159	161 ± 11	−7	N.S.
	9	215,200,198	204 ± 9	210,220,235	222 ± 13	−7	N.S.
	10	686,538,595	606 ± 75	739,512,621	624 ± 113	−3	N.S.

*The number of non-adherent cells in the samples from each of the 3 triplicate tubes when incubated with breast and melanoma extracts.

†The difference in leukocyte response to the 2 tumour extracts is calculated by the formula $\frac{A-B}{B} \times 100$ where A = mean number of non-adherent cells to breast cancer extract and B = mean number of non-adherent cells to the melanoma cancer extract.

‡P derived from a comparison of the difference in non-adherent cells in columns 3 and 5 by Student's t test.

N.S., not significant at $P<0.05$.

leukocytes from group 1 with the extracts of melanoma and breast cancer (Table 4). By contrast, when the leukocytes from group 2 are incubated with the extract of breast cancer, the sensitizing cancer, the mean leukocyte non-adherence is significantly higher than group 1 ($P<0.001$). Moreover, the mean non-adherence of leukocytes from group 2 incubated with extracts of breast cancer and melanoma was also significantly different ($P<0.001$). Leukocytes from the group 3 show a significantly higher mean leukocyte non-adherence compared to group 1 when incubated with the breast cancer and melanoma extracts (Table 4). Moreover, the leukocytes of group 3 show almost the same mean number of non-adherent leukocytes when incubated with the breast cancer or melanoma extracts as the leukocytes from group 2 show to the extract of breast cancer (Table 4).

Abrogation of LAI reactivity by exposure to tumour antigen

A number of different possibilities might explain the increased non-adherence and lack of specific activity of leukocytes from patients with advanced cancer. To show that the effect is mediated by excess TSA in the circulation

Table 4. Mean number of non-adherent leukocytes from control subjects and patients with limited and metastatic breast cancer to extracts of breast and melanoma cancer

Groups	Non-adherent cells to breast cancer Mean ± S.D.*	Coeff. of variation†	P^{+}_{+}	Non-adherent cells to melanoma Mean ± S.D.	Coeff. of variation†	P^{+}_{+}	NAI§
1 Control subjects	225 ± 83	37	1 vs 3 <0.001	225 ± 80	36	1 vs 3 <0.001	0
2 Patients with early breast cancer	418 ± 199	48	2 vs 1¶ <0.001	257 ± 128	50	2 vs 1¶ N.S.	63
3 Patients with metastatic breast cancer	391 ± 216	55	3 vs 2 N.S.	368 ± 205	56	3 vs 2 <0.001	6

*The mean of non-adherent cells from the 30 test tubes in the 10 patients.

†Coefficient of variation is the $\frac{\text{S.D.}}{\text{Mean}} \times 100$.

‡Student's *t* test was used to determine if the means of the groups were significantly different. 1, 2 or 3 represents the groups.

§The NAI was calculated from the means.

¶In group 2, mean non-adherence of leukocytes incubated with extracts of breast and melanoma cancer was also significantly different ($P<0.001$).

that coats the surface of the LAI-reactive monocyte, 2 different experiments were performed.

If LAI-reactive leukocytes are preincubated with the sensitizing PBS tumour extract, the LAI reactivity to that extract is abrogated (Table 5). The abrogation of LAI reactivity is specific since unrelated tumour extracts have no effect (Table 5). A similar result is observed when the sensitizing TSA in whole serum or isolated from serum of patients with metastatic cancer is preincubated with the LAI-reactive leukocytes (Table 5). The TSA present in the HDL fraction of serum has minimal amounts of other serum proteins, in particular, there is only trace contamination with IgG. Although the table shows the results from a few patients, these results have been reproduced consistently in about 20 patients.

Removal of tumour antigen coat

If leukocytes from patients with metastatic cancer are non-reactive because they are coated with tumour antigen, removal of the tumour antigen should restore the ability of the leukocytes to react specifically in the assay. Non-reactive leukocytes from patients with metastatic breast and colon cancer were gently trypsinized and then plated in the tube LAI assay. Table 6 shows that leukocytes that were not LAI-reactive had their specific activity restored to the sensitizing tumour antigen by trypsinization. The repeated washing of the leukocytes involved in the trypsinization was not responsible for the recovery of activity since the same PBL treated in an identical manner but without the addition of trypsin remained non-reactive (Table 6). This experimental result was reproduced in 8 LAI-negative patients with metastatic cancer. The PBL were from LAI-negative patients with metastatic cancer who had never received chemotherapy, irradiation or had not had recent surgery. It appears that the experimental conditions for removal of the tumour antigen coat are critical since LAI-reactive cells were not generated with different concentrations of trypsin or incubation times.

DISCUSSION

The results of the present study indicate that patients with metastatic cancer have a population of PBL that exhibits a greater mean non-adherence to glass than PBL from control subjects whether incubated with the specific (sensitizing cancer) or nonspecific tumour extracts. Moreover, PBL from patients with metastatic cancer show a mean non-adherence to both specific and nonspecific antigens similar to leukocytes from patients

Table 5. Effect on leukocyte adherence inhibition reactivity of preincubation of leukocytes with phosphate buffered saline tumour extracts or high density liporotein isolated from serum of advanced cancer patients

Donor of leukocytes	NAI of leukocyte donor	Donor leukocytes preincubated with:	NAI after preincubation
Breast cancer	54	PBS Extract of:*	
		breast cancer	−10
		colon cancer	44
Colon cancer	46	breast cancer	49
		colon cancer	0
Colon cancer	68	Whole serum from:	
		normal	55
		metastatic breast cancer	60
		metastatic colon cancer	−7
Breast cancer	45	normal	78
		metastatic breast cancer	9
		metastatic colon cancer	56
Breast cancer	71	HDL of:†	
		normal	74
		metastatic breast cancer serum	3
		metastatic colon cancer serum	66
Colon cancer	62	normal serum	
		metastatic breast cancer serum	52
		metastatic colon cancer serum	−1

*More purified preparations of the TSA has also been used with identical results [13, 14, 29].
†More purified preparations of the TSA has also been used with identical results.

Table 6. Conversion of non-reactive to LAI-reactive cells by trypsinization of their cell surfaces

	NAI before treatment to*			NAI after treatment to¶	
Donor of leukocytes	breast cancer†	colon cancer‡	Treatment of leukocytes	breast cancer†	colon cancer‡
Metastatic breast cancer	−16		Wash§	−18	
			Trypsin§	40	
Metastatic colon cancer		−18	Wash		−9
			Trypsin		61
Metastatic breast cancer	7		Wash	−2	
			Trypsin	48	
Metastatic colon cancer		−5	Wash		10
			Trypsin		38

*The leukocytes were processed in the usual manner and tested in the tube LAI assay.
†The NAI was calculated as described in the Materials and Methods with an extract of breast cancer as the specific antigen and an extract of malignant melanoma as the nonspecific antigen.
‡The extract of colon cancer was the specific antigen and the extract of lung cancer was the nonspecific antigen.
§The leukocytes were incubated with or without trypsin as described in Materials and Methods and then tested in the tube LAI.
¶The LAI-reactivity of the leukocytes after incubation with or without trypsin.

with limited cancer incubated with the sensitizing tumour antigen.

Exposure *in vitro* of LAI-reactive PBL to the sensitizing TSA derived either directly from the tumour or from the serum of metastatic cancer patients, before the addition of the PBL to the tube LAI assay, negated their activity in an immunologically specific manner. The serum blocking factor (TSA) was present in the HDL fraction of serum which rules out the possibility that blocking was mediated by immune complexes of antigen and antibody, or antibodies to either a common tumour antigen or autoantigen. Moreover, mild trypsinization of the non-reactive PBL resulted in recovery of the immunologic specific LAI reactivity. Since specific LAI-reactivity was restored by trypsinization of cells from patients with metastatic cancer, this indicates that the population of potentially LAI-reactive cells is present in equal numbers in patients with early and metastatic cancer.

Hence, in metastatic cancer the peripheral blood monocytes have bound TSA *in vivo*. As a result, the monocytes coated *in vivo* with TSA do not adhere to glass in the presence of either the specific or non-specific antigens. In patients with limited cancer, the circulating monocytes, by and large, have not yet encountered TSA *in vivo* and hence they retain their glass adherence properties when incubated with nonspecific antigens. When the monocytes of the patient with limited cancer are incubated with the sensitizing antigen, the TSA is specifically bound to the monocyte's cell surface by the cytophilic antitumour antibody which alters their ability to adhere to glass. Thus, the non-adherence of leukocytes from patients with limited or metastatic cancer is induced by the immunologically specific binding of TSA to the monocyte's cell surface; however, in the patient with limited cancer this reaction takes place *in vitro*, whereas in metastatic cancer binding of TSA occurs *in vivo*. In addition, since the mean number of non-adherent leukocytes to the specific and nonspecific cancer extracts from metastatic cancer patients is identical, this indicates that there is no residual population of monocytes in the LAI-negative patient with metastatic cancer that is able to show specific LAI reactivity.

In a previous study, enrichment and depletion of PBL populations indicated that the LAI-reactive cell was phagocytic, had Fc cell surface receptors, and adhered to glass and nylon wool [7]. Monocytes freed from glass wool or separated from other leukocytes by velocity sedimentation in a continuous Ficoll-gradient proved to be the LAI-reactive cell [7]. The mechanism whereby the specific tumour antigen was recognized was through the binding of cytophilic antitumour antibodies and not mediators released from T-cells [8]. In an animal tumour model with a microplate LAI assay, Russo and Goldrosen [15] have shown in detail that monocytes through tumour-specific antibody mediate the LAI response to tumour antigens.

In the tube LAI assay, Shani *et al.* [16] also observed similar differences in the mean leukocyte non-adherence in control subjects and patients with early or metastatic colon cancer. In an experimental animal model, Leveson *et al.* [17] with an automated micro-LAI assay showed that LAI activity correlated with concomitant immunity [18]. By contrast, Hellström *et al.* [19] found no differences in the LAI response of patients with early or metastatic malignant melanoma in the tube LAI assay.

In the hemocytometer LAI, LAI-reactivity remains detectable in patients with large tumour burdens [20–23]. The results of the present study also indicate that in patients with metastatic cancer that the tumour-specific immune response remains active; however, excess circulating TSA specifically binds to the reactive monocytes *in vivo;* as a result, the LAI reactivity of these cells is not detectable. In an experimental animal model, we also observed that tumour-specific antibodies were not detected by *in vitro* assays in animals bearing large tumours because the tumour-specific antibodies were complexed with excess tumour antigen [24–26].

Although the differences between the tube and the hemocytometer LAI assay appear to be trivial, the technical changes appear to result in the measurement of a different reactive cell population [27] which leads to different results and conclusions. The results of the present study indicate that the difference in response to two tumour extracts must be examined if the LAI-response of monocytes is being measured since leukocytes from patients with metastatic cancer will appear to show LAI-reactivity to a single extract because of an alteration of their glass adherence properties that has already taken place *in vitro*.

A number of observations made in this and previous studies may be relevant in explaining, in part, the paradox of cancer progression in spite of the host's immune response. In an animal tumour model, the ability of the tumour-bearing animals to reject tumour cells

in vivo was inversely correlated with the presence of circulating TSA [26]. It was concluded that the primary tumour escaped destruction because the ability of the immune cells to reject tumour was impaired by TSA shed into the local microenvironment of the tumour [26]. In human cancer, a tumour-specific immune response is measurable by LAI when the cancer exists as a microfocus or is even *in situ* [6]. In spite of the tumour-directed response, the cancer is not rejected but invades and eventually metastasis occurs. Although it cannot be ruled out that the cancer escapes destruction because the tumour-specific response is never sufficiently potent, an equally likely possibility is that the human cancer cell also avoids rejection because the TSA shed from the tumour cell surface into the microenvironment inhibits the local cytotoxic response of the effector arm of the immune response.

Acknowledgement—We thank Mrs. Mary Bergin for the typing of this manuscript.

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