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Fluorescent Biomarker in Colorectal Cancer

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1. Introduction

Patients with gastrointestinal cancer exhibit a poorly functioning immune system that is characterized, in part, by decreases in T-lymphocyte proliferation (Greenstein, et al., 1991; Milasiene et al., 2007) and reduced $CD4^+$:$CD8^+$ ratios (Arista et al., 1994; Franciosi et al., 2002). In different pathologies (including these and other types of cancers), membrane damage in immune cells (and other cell types) often evolves as a consequence of alterations that are induced in cell-associated lipids and proteins in the affected patients (Gryzunov and Dobretsov, 1994, 1998; Rolinsky et al., 2007). It is now widely accepted that the dynamics (actual rate of occurrence - not only incidence) of these changes, along with the types of alterations in structure(s) of the immune system cells' lipids/proteins themselves, play a critical role in the maintenance of the immune status of any given organism (Lakowicz, 2000).
As a result of the potential importance of changes in the structural integrity of cells of the immune system, it is important for clinicians to receive information on the biophysical status of these cells via quick, reliable, reproducible methods. In this regard, fluorescent probes have shown to be excellent tools for use in such protocols (Lakowicz, 2000, 2006). The work reported here, which built upon earlier findings reported by our laboratories, investigated the possibility of using the fluorescent probe ABM (an amine derivative of benzanthrone) for the detection of structural/functional alterations in blood plasma albumin and among immunocompetent cells in patients with select types of pathologies, i.e,. cancers. Such an analysis has a great potential for use not only for helping to comprehend mechanisms of immunomodulation associated with the induction/progression of malignancies, but might also have the potential to serve as a very important prognostic indicator of long-term survival among patients with such pathologies.
In the work reported here, ABM fluorescence intensity in blood plasma and following combination with cell suspensions from colorectal cancer patients was examined in the context of the host's immunological parameters and state of cancer progression. For study patients with colorectal cancer were examined: 1) 1 day before and 10 days after their surgical treatment (Stages II-III) (Kalnina et al., 2009); 2) as disease worsened (Stages IIa, IIIb, IV) (Kalnina et al., 2010b); 3) advanced cancer patients, they were divided into two groups in accordance of its survival rate (0-6 months and > 24 months) (Kalnina et al., 2011). Apart from the aforementioned potential benefits from these types of studies to clinicians in general, this type of research is very important in Latvia itself. This is because, in the context

of oncological diseases seen among the Latvian population (as recently as in 2006), colorectal cancers rank third in incidence, only surpassed by lung cancers and urogenital tumors.

2. ABM: Distribution and spectral characteristics in cells and blood plasma

A new fluorescent probe, a derivative of 3-aminobenzanthrone (ABM) at the Daugavpils University, Daugavpils, Latvia.

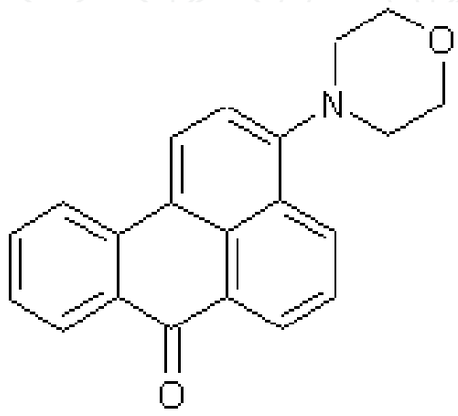

Fig. 1.Chemical Structure of probe ABM

Synthesis and properties of probe ABM were described in (Kalnina et al., 2004, 2007, 2009, 2010a; Kirilova et al., 2008).

3. ABM binding with blood plasma albumin

3.1 ABM binding with blood plasma albumin before and after surgical treatment

In the colorectal cancer patients, the ABM emission spectra maximum (i.e., at 650 nm)—after combination with the patients' blood plasma—was not altered in comparison to that seen with the plasma from the healthy control volunteers. In contrast, with respect to fluorescence intensity, before their individual surgical treatments, the average ABM intensity in the patients' blood plasma was decreased compared to that seen with the samples from the healthy donors. Specifically, the fluorescence associated with samples from the colorectal cancer group (Figure 2) were decreased by 23.0%. At 10 days after their operations, the average ABM fluorescence intensity in the samples from the colorectal cancer group were decreased further by 13.9%.

The average intensity values of the plasma samples from the cancer patients were significantly ($P < 0.05$) different from the control volunteers' average value. Whether these observations tracked actual changes in the levels of plasma albumin were also investigated. The results (data not shown) indicate that plasma albumin concentrations (μg/μL) pre-surgery in the patient group (71.73 ± 1.34) were below those in the plasma of the health controls (83.41 ± 1.16). This meant that the pre-surgery values for albumin only indicated levels in colorectal group samples that were ≈ 14% below control, while the samples' fluorescence intensity was correspondingly lower than the control value by 23.0% . The plasma albumin concentrations after surgery seemed to be insignificantly impacted. Specifically, these value was in colorectal cancer patients samples 68.48 ± 1.78 (in μg/μL, mean ± SD).

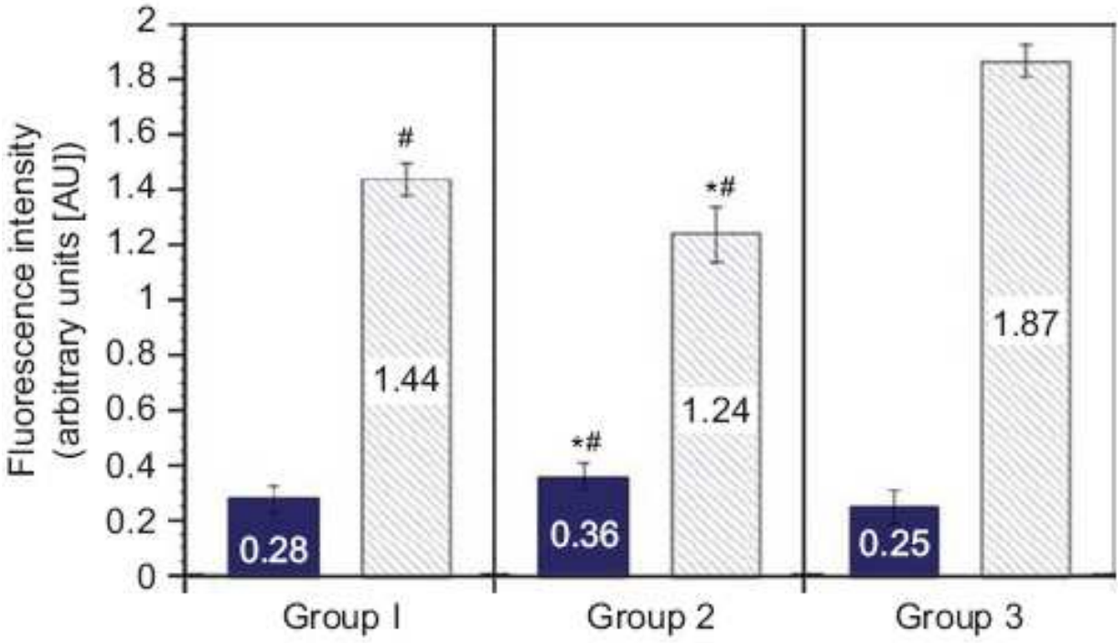

Fig. 2. ABM fluorescence intensity in lymphocytes and in plasma from "Colorectal group" patients. Colorectal cancer patients only (Stage II–III) (n = 10). Group number in figure is used to reflect from whom/when samples were isolated [i.e., Group 1: pre-surgery; Group 2: post-surgery; and Group 3: from healthy donors (control group; n = 14)]. Solid bar in each set: ABM fluorescence in lymphocytes; hatched bar in each set: ABM fluorescence in plasma. All intensity values are shown in AU (arbitrary units; mean ± SE). At $P < 0.05$, *value significantly different from pre-surgical value and/or #significantly different from control group value.

It is of interest to note that while the post-surgery albumin values in these patients samples were ≈18% below control levels, their corresponding fluorescence intensity were now even more depressed relative to that of the controls by ≈ 34%. These results strongly suggest that the noted changes in ABM fluorescence post-surgery in the cancer patients were most likely attributable to some change in the protein(s) themselves rather than due to post-surgery complications (i.e., bleeding or other mechanisms affecting blood volume/composition). To ascertain whether these results (different ABM spectral characteristics) could be explained, in part, by altered structural characteristics of the plasma albumin in the cancer patients, the average binding constant (Ka) values were determined using the Klotz graphical method. These analyses revealed that the average Ka values for the pre-surgery samples from the colorectal patient group decreased strongly from the respective constant associated with the control group (i.e., 1.0 X 10^5 M−1, colorectal cancer group; 1.8 X 10^5 M−1, healthy control group). These values represent decrements of from 28% to 45% in the binding of ABM to albumin in the plasma of these patients; the reason for the decrement remains to be fully determined. Interestingly, even though the fluorescence intensity values decreased further for the patients following their surgical procedures, the average Ka values for the samples from the patients increased relative to corresponding preoperative values (i.e., 1.3 X 10^5 M−1).

3.2 ABM binding with blood plasma albumin as a function of stage

The average ABM fluorescence intensity in patient's blood plasma was decreased compared to that seen with healthy donors. Specifically, the fluorescence intensity associated with samples from colorectal cancer patients (Table 1) was decreased by 23% (average value of 1.44 fluorescence units for those at Stage IIA-IIIB) and 42% (average value of 1.09 units for those at Stage IV) relative for the healthy controls(an average value of 1.87 units).

Group	Stage	F (PI)[a]
1	IIA-IIIB	#*1.44±0.12
2	IV	*1.09±0.11
3	Controls	1.87±0.13

[a]F (PI) = fluorescence intensity in blood plasma; Values shown are in mean (± SE).
Value ($p<0.05$) significantly different from that of * control or # Group 2 patients.

Table 1. Spectral characteristics of ABM in blood plasma of colorectal cancer patients

3.3 ABM spectral characteristics in blood plasma of advanced cancer patients

The average ABM fluorescence intensity in the patients (Group 1 and Group 2) blood plasma was decreased (i.e., by 37.4% and 24.1%, respectively) as compared to that seen in healthy donors (Table 2). In Group 2 average intensity significantly ($p<0.05$) differs from Group 1 value and also control group value by 10.6 % and 31.7 %, respectively. Total albumin (TA) concentration in Group 1 and Group 2 patients was decreased (relative to control value) by 23.5% and 16.1%, respectively. Effective albumin (EA) concentration was decreased by 40.0% and 27.7%, respectively. The lowest value EA/TA in these patients plasma reached 0.61 (Group 1), and 0.66 (Group 2); (donor group 0.79-0.81).

Group	Survival rate (months)	F (Pl)	EA	TA	EA/TA
1	0-6	1.17 ± 0.14	39.0 + 1.1	63,80 + 1.02	0,61
2	> 24	1.42 ± 0.09	47.0 + 1.2	70,80 + 1.14	0,66
Control		1.87 ± 0.13	65.0 + 1.3	83,4 + 1.16	0,78
P<0.05 between groups		1-2, 1-3, 2-3	1-2, 1-3, 2-3	1-2, 1-3, 2-3	

F (Pl) = fluorescence intensity in blood plasma;. Values shown are in mean (± SE).
EA- "healthy" albumin equivalent in patients plasma, g/L
TA- total albumin concentration, g/L
EA/TA- reserve of albumin binding capacity

Table 2. Spectral parameters and binding sites čaracteristics of ABM in blood plasma of advanced cancer patients

4. ABM binding with lymphocytes

4.1 ABM binding with lymphocytes before and after surgical treatment

In the colorectal cancer patients, the ABM emission spectra maximum (i.e., at 650 nm) after combination with the patients' lymphocytes (as with their plasma) was not altered in comparison to that seen with the cells from the healthy control volunteers. Surprisingly, the average ABM fluorescence intensity value noted from colorectal patients group was actually 12.0% greater than the control level (Figure 3); however, even with this increase, the value was not significantly different from the average control value. In contrast to what was observed with the plasma samples, the average ABM fluorescence intensity values noted with the cells from patients at 10 days after their operations were greater than the values seen with the control volunteers' cells by 44%. In comparison to the pre-operative values, these average ABM fluorescence intensity values at 10 days after the patients' operations had increased by 28.6%.

COLORECTAL CANCER PATIENTS (Stage II-III) (n=10)					
	$CD16^+$%	[a]$CD16^+$	$CD4^+$:$CD8^+$	$CD38^+$%	Lymphocytes (%)
[b]1	‡15.95±2.18	‡314.18±39.27	‡1.27±0.10	‡3.40±1.20	28.00±1.30
2	*‡7.93±1.43	*‡144.75±22.44	*‡1.50±0.13	*‡12.70±3.40	*‡23.50±2.20
3(Controls)	12.50±1.10	389.00±24.11	1.88±0.16	24.60±1.60	28.00±1.30

[a] Values shown are in terms of absolute numbers (mean ± SE).
[b] Indicates when or from whom samples were isolated: (1) before surgical treatment; (2) after surgical treatment; and (3) healthy donors (control group; n=14).
* Value significantly different from pre-surgical value ($P<0.05$); ‡significantly different from control group value ($P<0.05$).

Table 3. Peripheral blood lymphocyte subpopulation counts in the distinct subsets of the study's cancer patients.

4.2 ABM binding with lymphocytes as a function of stage

In general, among the gastrointestinal cancer patients examined here, the ABM emission spectra maximum (i.e., at 650 nm) after combination of the probe with the patients' lymphocytes was not altered in comparison to that seen with the cells from the healthy control volunteers (spectral data not shown). The ABM fluorescence intensity in the samples from colorectal patient group in Stages IIA-IIIB was not significantly different from the average control value (0.28 vs. 0.25 fluorescence units, respectively; Fig.3). In contrast, there was a significant reduction in ths parameter among the cells from the cancer patients in Stage IV (a decrease of 44%; (0.14 vs. 0.25 units).

4.3 ABM spectral characteristics in lymphocytes of advanced cancer patients

The ABM fluorescence intensity in the samples from advanced cancer patients (Group 1) was not different from the average value (0.25 vs. 0.25) fluorescence units, respectively: (Table 4). In contrast, there was a significant increase in this parameter among the cells from the cancer patients Group 2 as compared with Group 1 (by 80.8%) and healthy donors (by 112%) (0.25 vs. 0.52 units).

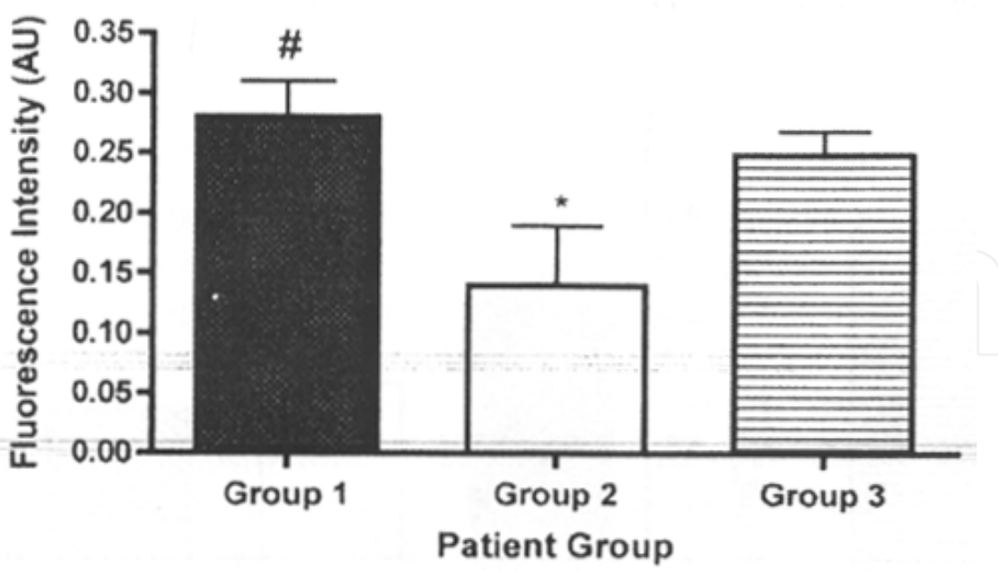

Fig. 3. ABM fluorescence intensity in lymphocytes from colorectal cancer patients as a function of stage. 1) Stage IIA-IIIB (black fill); 20 Stage IV (clear fill); 3) healthy donors (control group; stripped fill). All values are shown as mean (-+SE). At $p < 0.05$, values significantly different from that of "control patients, Group 2 patients.

5. Lymphocyte count and subpopulations

5.1 Lymphocytes count and subpopulations before and after surgical treatment

The absolute number of CD3+ cells and CD16+ natural killer cells; the relative percentages of all lymphocytes, CD16+ and CD38+ cells; as well as the CD4+:CD8+ ratio in the blood samples of the healthy volunteers and of the cancer patients, before and after each underwent their operations, were determined. The results among the patients in the "colorectal" group (Table 3) indicate that, before surgery, the numbers of CD16+ cells, the relative percentage of CD38+ cells, and the CD4+:CD8+ ratio were each significantly decreased (i.e., by 19.2%, 86.2%, and 32.4%, respectively) as compared to corresponding control subject values. Somewhat unexpectedly, relative percentages of CD16+ cells in this group were actually significantly greater (by 27.6%)—and the relative percentage of lymphocytes no different—than in the blood of the control volunteers. Within this same group, after surgery, the number and percentages of CD16+ cells, as well as the percentages of all lymphocytes, were each significantly reduced (i.e., by 53.9%, 50.3%, and 16.1%, respectively) relative to corresponding pre-surgery levels. Again, the relative percentage of CD38+ cells and the CD4+:CD8+ ratio increased (by 27.4% and 18.1%, respectively) compared to pre-surgical values, but again did not reach control levels (i.e., still were 48.4 and 20.2% lower, respectively).

5.2 Lymphocytes count and subpopulations as a function of stage

On the other hand, there were significant changes in the relative percentages of $CD4^+$ cells at almost every stage in both cancer groups (Fig. 4). Among colorectal cancer patients, the percentages of $CD4^+$ cells decreased 25.5 and 38.3% from control levels as the stages

progressed (actual values: 28.6%, Stage IIA-IIIB; 23.7%, Stage IV; and, 38.4%, controls). Patients with Stage IV colorectal cancer yielded any statistically significant shift from control subject levels of CD8+ cells (an increase of ≈38%, i.e., shift from 19.5% to 26.9%).
The levels of lymphocytes (both total and the $CD4^+$ and $CD8^+$ sub-populations) in the blood samples of the cancer patients and healthy volunteers were also assessed here. The results show that among the patients in both cancer groups, the relative percentages of lymphocytes were moreover not significantly altered relative to the control levels irrespective of disease stage (Figures 4 and 5). Of all the patients, only those in Stage IV had lower blood lymphocyte levels that approached or reached statistical significance. Among the Stage IV colorectal cancer patients, levels were decreased 18.5% (a shift from 28.0% [control] down to ≈22.8%);

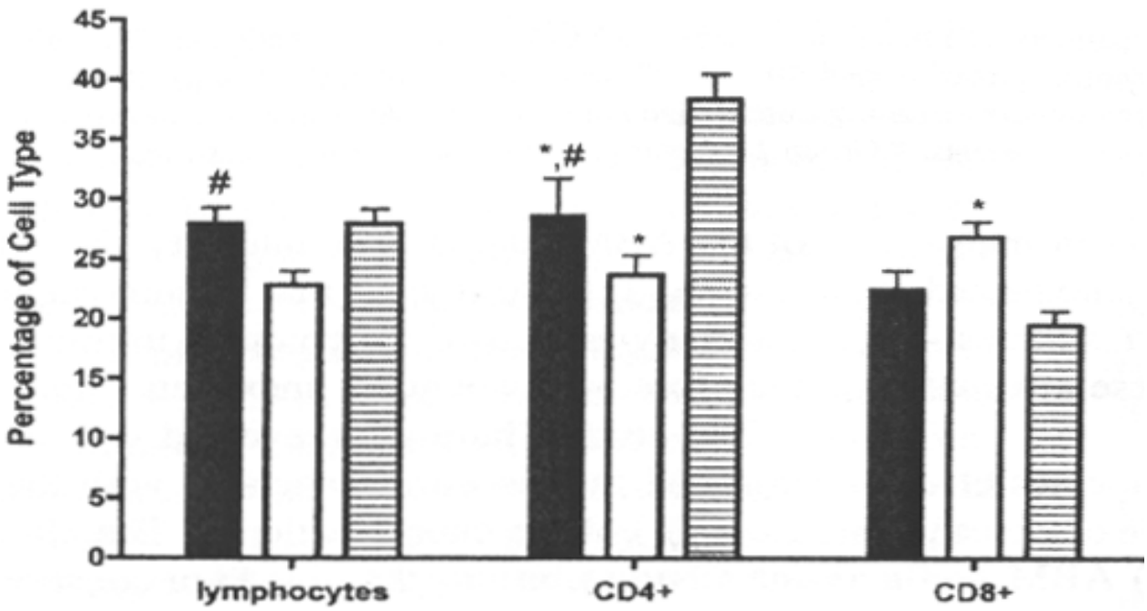

Fig. 4. Lymphocyte counts (as percentage %) and T-lymphocyte subpopulation
Levels (as % of all lymphocytes present) in cancer patients as a function of stage.
1) Stage IIA-IIIB (black fill); 2) Stage IV (clear fill); 3) healthy donors (control group; striped fill).

Because shifts in $CD4^+$:$CD8^+$ ratios are often used as indices of altered host immune status, these values were also calculated from the patients' blood samples. The results show that among the colorectal cancer patients (Fig 5.), the $CD4^+$:$CD8^+$ ratios were all significantly lower than those for the healthy control subjects and became significantly further lower as the stage worsened. Specifically, the ratios dropped to 1.27 and 0.88 (shifts of 32.4% and 53.2%) at Stages IIA-IIIB and IV, respectively, from the 1.88 value for the controls.

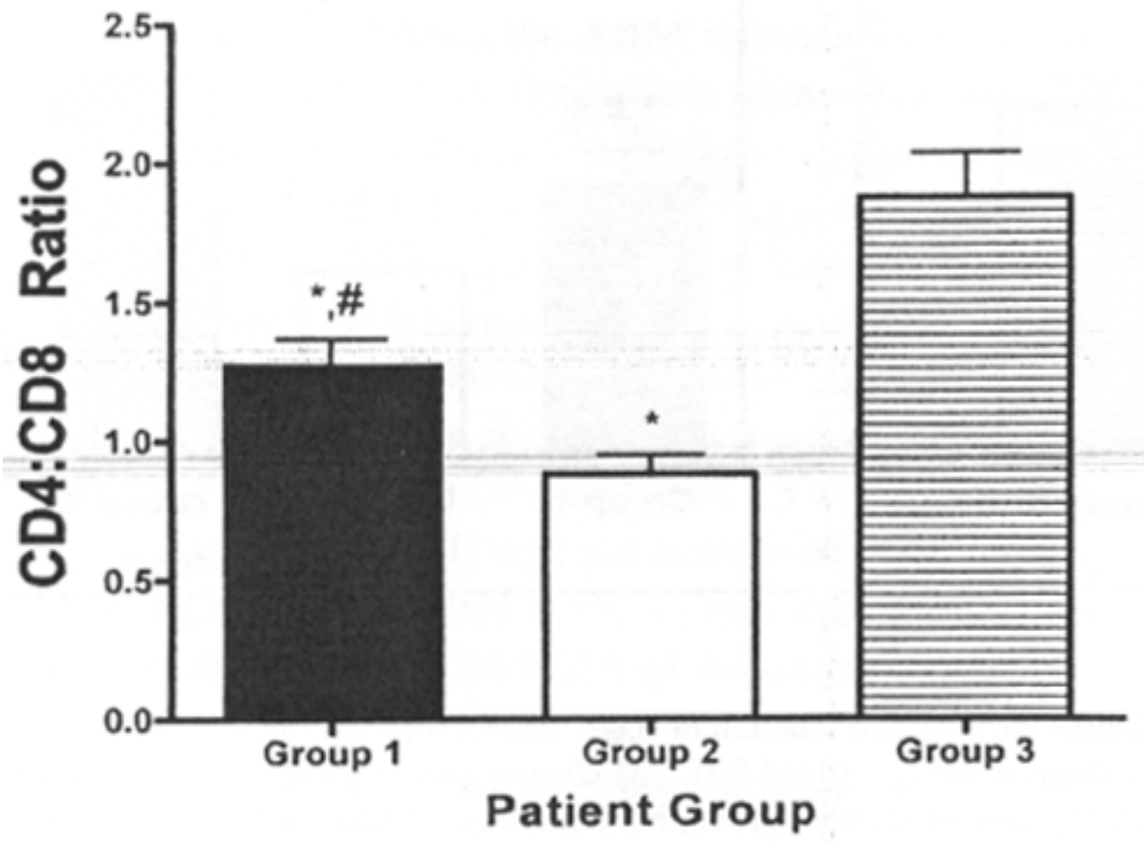

Fig. 5. T-lymphocyte subpopulation ratios in cancer patients as a function of stage.
1) Stage IIA- IIIB (black fill); 2) Stage IV (clear fill); 3) healthy donors (control group; striped fill).

5.3 Lymphocytes count and subpopulations in advanced cancer patients

Among the patients in both advanced cancer groups, the relative number of lymphocytes were significantly decreased (i.e.by 52.6% and 39.6%, respectively) as compared to corresponding control values. It is necessary to note that relative number of lymphocytes in Group 2 is significantly higher than in the Group 1 patients, but lower than the control value (see Table 4). In patients with advanced cancer and metastases there is reduction in both numbers of lymphocytes and proportions of CD4+/CD8+ T-lymphocytes which are thought to play an important role in cell-mediated immunity. The results indicate that in Group 1 and Group 2 patients the ratio CD4+/CD8+ was significantly reduced (i.e. by 58.5% and 47.9%, respectively) relative to corresponding control level. Actual values of Group 1 and Group 2 – 0.78, 0.98, respectively from the 1.88 control value. It is of interest to note that in Group 2 this parameter stay significantly higher as compared with results in Group 1, but did not reached control value.

Group	Survival rate, months	F(Ly), a.u.	Ly (%)	CD4+(%)	CD8+(%)	CD4+/CD8+
1	0-6	0,25±0,03	13,31±1,16	20,93±1,13	27,00±1,39	0,78±0,09
2	>24	0,53±0,11	16,95±1,18	26,14±1,32	26,70±1,31	0,98±0,08
3 Control		0,25±0,03	28,00±1,30	38,40±2,10	19,50±1,20	1,88±0,16
$p<0.05$, between groups		1-2;2-3	1-2;1-3;2-3	1-2;1-3;2-3	1-3;2-3	1-2;1-3;2-3

F(Ly) - fluorescence intensity in lymphocytes
Values shown are in mean (±SE)

Table 4. ABM fluorescence intensity in lymphocytes. Peripheral blood lymphocytes subpopulation counts in advanced cancer patients

6. Examination of relationship between ABM fluorescence and blood lymphocyte profiles

Pre-operation, in the colorectal patient group, the ABM fluorescence intensity was found to correlate with the relative number of CD38+ cells (r = +0.956). After the operations, both the CD4+:CD8+ ratio and relative number of CD38+ cells in patients blood was observed to be increased.

In colorectal cancer group, the ABM fluorescence intensity in blood plasma and the lymphocytes was found to correlate with $CD4^+$:$CD8^+$ ratios (in all stages of cancer) and the percentage (%) of lymphocytes/ subtypes in their blood. The degree of any relationship between cancer progression/staging, lymphocyte levels, and fluorescence among the lymphocytes was less obvious. Specifically, in terms of disease progression, cell levels and fluorescence intensity weakly tracked together (i.e., r = +0.512) for the colorectal patients. . With respect to associations between the plasma albumin fluorescence measurements and each of the individual lymphocyte-associated endpoints measured (except for cell fluorescence itself), many of the same patterns as noted above were evident. Once again, in terms of disease progression, although cell levels and albumin fluorescence intensity weakly tracked together (i.e., r = +0.513) among the colorectal patients.

In both groups of advanced cancer the ABM fluorescence intensity in blood plasma and lymphocytes was found to correlate to with CD4+/CD8+ ratios. In advanced cancer groups (in contrast to other groups) there is direct (not inverse) correlation between lymphocytes count and ABM fluorescence intensity. There seemed to be a good relationship between total lymphocyte (and subpopulation) levels and ABM fluorescence in both groups of patients. There is also good associations between the plasma albumin fluorescence measurements and each of the individual lymphocyte/albumin associated endpoints measured "effective" and total albumin concentration, reserve of albumin binding capacity.

7. Discussion

The novel fluorescent probe ABM (an amino derivative of benzanthrone) localizes deep within the phospholipids bilayer of lymphocytes membrane. Thus, in studies with lymphocytes, it can be concluded that changes in the spectral parameters of ABM (i.e., shifts in magnitude of fluorescence or actual wavelength associated with normal maximal fluorescence [i.e., *F*max]) could reflect modifications in one/ more interdependent (i.e., inter-related) properties of the cells. These could include the lymphocytes' (1) outer membrane physicochemical state, (2) membrane microviscosity,
(3) proliferative activity, (4) lipid metabolism, and/or (5) phenotypical profile. As seen in the studies mentioned here, while the noted changes in the studied parameters (i.e., fluorescence behavior) could be useful in reflecting alterations in lymphocytes of the cancer patients in each subgroup (at both pre- and post-surgical stages), they may also ultimately be of use as potential indicators of alteration in cellular immunity in these individuals. Follow-up studies are underway to see whether this concept can be validated. We also sought to ascertain whether shifts in ABM binding with plasma albumin could potentially be utilized as a part of an overall preliminary immunodiagnostic screening test in cancer patients. The choice to examine albumin, among the myriad of constituents in plasma, is that this protein is practically the single source of ABM binding and subsequent fluorescence in plasma (Gryzunov and Dobretsov, 1994, 1998). Our earlier studies showed that within plasma, albumin is nearly alone in binding with ABM with a very high level of selectivity (Kalnina et al., 1996, 2004, 2007). The distribution of ABM fluorescence (intensity) within fractions of human plasma was seen to be albumin >>> globulins >> non-specific binding by other components (i.e., 90%, ≈ 5%, < 1%, respectively). These widely disparate binding results were confirmed in studies wherein exogenous globulin was added to plasma samples and there was no shift in fluorescence intensity or *F*max. Clearly, only significant shifts in albumin levels or alterations/ conformational changes in albumin itself seemed to have a major impact on these ABM fluorescence endpoints. In the present study, the differences in total albumin concentrations, **pre- and post-surgery**, among the cancer patients in each group did not seem to correlate well with the relative changes in ABM fluorescence (relative to values in control subjects' plasma). This apparent "extra diminution" in fluorescence strongly suggested that there was either a novel competition for probe by other substances in the patients' plasma or that the albumin in these patients had undergone modification(s) that affected its ability to bind ABM. The fact there were substantive changes in binding constant (*K*a) values lends support to the latter viewpoint. However, this finding in and of itself does not outright preclude the possibility of the former event having occurred as well.These shifts in ABM binding constants in the plasma samples from the cancer patients, as noted earlier, could be due to a generic decreased binding by/conformational changes in their albumin molecules. Structural or functional alterations of albumin could be manifest as "shifts" away from normal "main" binding sites with high affinity for the probe to other binding sites with far lower affinities and specificities. Such shifts would be in agreement with the observations of Togashi and Ryder (2006) that albumin molecules are known to contain different binding sites (i.e., classes) for various probes. As Petitpas et al. (2001b, 2003) noted, albumin normally carries a variety of endogenous ligands like nonesterified fatty acids, bilirubin, and thyroxine; however, this protein can also bind an impressive array of drug molecules, including warfarin, ibuprofen, and indomethacin, as well as their metabolites (Petitpas et al., 2001a). It seems very likely

that patients in the groups in the present study had ingested painkillers (both prescribed and retail) during the course of their disease; thus, a presence of these drugs/ metabolites on their albumin could have contributed to the noted shifts in ABM fluorescence /*K*a values. Our future studies will endeavor to recruit non-cancer patients with a "similar" history of painkiller intake in order to ascertain whether this was a main reason underlying our observations (regarding the albumin outcomes) or if there is something more inherently unique to the patient's cancer-bearing status that influenced the measured endpoints. This second standpoint is not without foundation. In oncopathology, the blood plasma content of two important unsaturated fatty acids (i.e., oleic acid and arachidonic acid) is increased, and these natural constituents also increasingly occupy binding sites on albumin (Gryzunov and Dobretsov, 1994, 1998). Both are observed to occupy binding sites distributed across the protein that happen to also be bound by medium or long-chain saturated fatty acids. The resulting restrictions imparted on the binding configurations of the protein would then account for shifts in the binding affinities at the primary sites between polyunsaturated fatty acids and their saturated or mono-unsaturated counterparts (Petitpas et al., 2001). It remains to be determined whether these alterations in fatty acid composition/binding also result in conformational changes in the albumin that impact upon ABM binding to its major (high selectivity) binding sites. As noted earlier, changes in fluorescence parameters of the cancer patients' lymphocytes could be reflective of changes in one/more inherent characteristics of their cells. In these studies, at least two, that is, proliferative activity and phenotypical character, could readily, albeit indirectly, be evaluated by examining changes in lymphocyte populations (i.e., their numbers) themselves. While the flow cytometry studies did indicate significant changes in lymphocyte (and subpopulation) levels among the cancer patients, unfortunately, the studies failed to yield overall lymphocyte (or subtype) population patterns that paralleled the concurrent changes in ABM fluorescence (i.e., Table 1 vs. Figure 2, example of this "lack of comparativeness"). Among all the subpopulation endpoints reported, only those of "CD38+%" and the "CD4+:CD8+ ratios" approached reflecting trends seen with the patients' fluorescence measurements. Specifically, the pre-surgery levels of each of these cytometric values were "maximally" reduced relative to the control subjects' values; post-surgery, these two values were increased, but in contrast to the fluorescence levels, these values did not reattain (or surpass) counterpart control levels. In light of the cancer patients' post-surgical (1) persistent lower numbers of lymphocytes (both total and within subclasses) and (2) fluorescence values that were uniformly significantly greater than in control subjects' cells, we surmise some factor(s) about these patients' lymphocytes (i.e., some undefined phenotypical characteristics) can cause amplification of the ABM fluorescent response. The fact that this "disconnect" between these two parameters is most predominant during the post-operative period strongly suggests that these as yet-undefined modifying factors in the cancer patients might be related to their general immune response to the surgical procedure. Our future studies will need to recruit non-cancer patients with a "similar" history of surgical intervention/protocols (such as among patients suffering enterocolitis, undergoing local biopsies for non-cancer disorders, etc.) to ascertain whether the surgical procedure itself was a main reason for our observations (regarding the "disconnect") or whether, as with the albumin findings, there is something more inherently unique to a cancer-bearing status that influenced the measured endpoints. As expected, the CD4+:CD8+ ratios were seen to be increased in the cancer patients after they had undergone their respective operation. This would be expected as it is well accepted that CD4+ helper

cells stimulate and CD8+ (suppressor and cytotoxic) cells inhibit the immune response during the healing process. While that explanation for any potential changes in the phenotypic characteristics of these patients' lymphocytes is somewhat straightforward, what is less clear is the basis for the post-surgical increase in CD38+% values and why, to begin with, they are lower than in the control groups. This is because, most often, increased levels of CD38+ cells are associated with patients suffering with lymphocytic leukemias than with the solid tumors (such as those associated with gastrointestinal cancers (Kalnina et al., 2009). In general, CD38 is expressed primarily on B-lymphocytes and T-lymphocytes, as well as stem/germ cells, the CD38 ligand is an ADP-ribosyl cyclase enzyme that regulates the activation and growth of these lymphoid (as well as myeloid) cells . The data in the current study clearly show no evidence of any B-lymphocyte-based leukemia (Kalnina et al., 2009) (i.e., CD16+ cell levels were lower in patients' pre- and post-surgery blood samples than in controls) among the cancer patients. Thus, we conclude that the increase in CD38+ cell levels is more probably due to an increased presence of CD38+ T-lymphocytes. We conclude from our findings that the increase in CD38+ cell levels post-surgery was not likely due to absolute increases in T-lymphocytes, but in their activities. Such an outcome would be in keeping with the changes in the fluorescence values for these lymphocytes. For this premise to be valid, apart from showing that there are increases in relative levels of CD38-bearing T-lymphocytes due to activation during the post-surgery healing process, there still needs to be an explanation as to why these cells' levels were initially lower in the patients than in the controls. One potential explanation is in the biology of the tumors themselves, that is, they are solid tumors of the gastrointestinal system that impact on a wide variety of local cell types, including the endothelium. This particular cell type in the gut is of interest here in that there appears to be a critical relationship among endothelial cells, CD38 expression, and activation of T-lymphocytes (i.e., CD4+CD45RA+ cells. It is plausible that normal interactions between T-lymphocytes and endothelium are likely "interrupted" simply as a result of changes in accessibility (secondary to alterations in gut architecture as tumor grew). A lack of lymphocyte– endothelium interactions could help explain why there was a diminution in CD38+ cell levels before surgery; during the post-surgery recovery, angiogenic processes (i.e., during microvasculature repair/reformation at wound site) would allow for an increase in these particular cell–cell interactions—in particular, with a population of endothelial cells in very active states during the reparative processes. Future histopathology studies using biopsied samples from the gastrointestinal tracts of patients with cancers and those that underwent biopsies for non-cancer-based reasons (see earlier comments) should be useful in allowing us to verify the degree of these hypothesized cell–cell interactions. Apart from potential changes in lymphocyte–endothelium interactions as contributing factors for the reductions (vs. controls) in CD38+ cell levels—and their "recoveries" after surgical removal of the tumor—in the cancer patients, there are other possible reasons for these two observations. Among these, specifically, is the fact that patients with colorectal/gastrointestinal cancers (especially those at more advanced stages) tend to have significant levels of circulating interleukin (IL)-4 . This is critical in that it has been shown, at least with B-lymphocytes, that exposure of these cells to IL-4 reduced the amount of CD38 antigen on and in these cells; no evidence was obtained for accelerated breakdown, shedding, or internalization of CD38 molecules, or for the accumulation of CD38 molecules in the cell interior, due to IL-4 (Kalnina et al., 2009). In our ongoing studies, we will analyze patients' blood samples for IL-4 both pre- and post-

surgery to see whether its levels reflect the observed changes in the CD38+ lymphocytes and their fluorescence responses (indicative of phenotypic changes likely related to activation) in the presence of ABM. The results of the ABM studies presented here show that, as might be expected, the presence of solid tumors and surgical interventions can affect the functional activity of lymphocytes. These results are in agreement with previously- performed investigations to characterize the outer cell membrane of lymphocytes of cancer patients, patients with autoimmune disease (i.e., rheumatoid arthritis), and workers who had been contaminated during the clean up at Chernobyl (Kalnina et al., 2004, 2010a, Zvagule, 2010). Likewise, the observed changes in the ABM spectral parameters in blood plasma are probably coupled with alterations in cellular mechanisms of immune regulation in the patients here. Ongoing studies are seeking to answer this very question.

The studies here showed that spectral characteristics (fluorescence intensity) differed among the various patient sub-groups. These findings suggest likely physical (structural) and functional alterations in the patients' cells were a **function of cancer stage.** It is known that ABM fluorescence intensity can change in accordance with environment polarity and, consequently, in relation to plasma membrane microviscosity (that in turn correlates with cell lipid metabolism). There are various pathological states (i.e., cancer) in which the lipid composition and specific fatty acid content in lymphocyte membranes and blood plasma are disturbed (Kalofoutis et al., 1996). For example, colorectal cancer patients have abnormal plasma and erythrocyte fatty acid levels, as well as of their polyunsaturated metabolites (Robinson et al., 2001). Ultimately, in lymphocytes, because membrane physicochemical status and cell lipid metabolism play pivotal roles in signal transduction pathway(s) activities important in maintaining cell function (Kim et al., 1999), it would not be unexpected that disturbances in these parameters could result in altered immunocompetence in hosts with these affected cells. Fluorescence intensity of ABM in lymphocytes suspension tended to decrease with progression of cancer. Shifts in magnitude of ABM fluorescence could reflect modifications in one/more interdependent properties of cells (Kalnina et al., 2007). As seen in the studies mentioned here, at least two parameters are responsible for this phenomenon. In this studies, at least two, that is proliferative activity and phenotypical character could readily, albeit indirectly, be evaluated by examining changes in lymphocytes populations (ie., their numbers) themselves. While the flow cytometry studirs did indicate significant changes in lymphocytes (and sunpopulations) levels among the cancer patients, unfortunately, the studies failed to yield overall lymphocyte (or subtipe) population patterns that paralleled the concurrent changes in ABM fluorescence example of this "lack of comparativeness"). The studie of Milasiene (Milasiene et al., 2007) also suggest that immunosuppression covers many aspects of the complex immune system, and therefore, we have many unexpected findings.

The studies here also revealed significant changes in ABM fluorescence associated with the plasma (re: albumin) of the cancer patients. The choise to examine albumin, among the myriad of constituents in plasma, is that this protein is practically the single source of ABM binding and subsequent fluorescence in plasma. We know form earlier studies that plasma albumin binds ABM with a very high selectivity (Kalnina et al., 1996, 2004, 2007, 2009, 2010b, Zvagule et al., 2010) and that only very significant shifts in plasma albumin levels or structural changes in albumin itself seemed to impact on ABM fluorescence. In the previous study (Kalnina et al., 2009) the differences in total albumin concentrations in patients groups did not seem to correlate well with the relative changes in ABM fluorescence (relative to

values of control subjects plasma). The fluorescent method reveal the "effective" concentration of albumin (equivalent of "healthy'' albumin in blood plasma). The total concentration of albumin is conservative. In general, serious alterations in plasma albumin levels are often reflective of poor outcomes in cancer patients (Seve et al., 2007). As noted above, the changes in patient plasma albumin levels (≈14-18% below control) were far less than the recorded shifts in ABM intensities and it seemed these measures were "picking up" changes beyond those that could solely be attributed to a change in total albumin status. The additional 'binding shifts' seen with the cancer patients' plasma samples could be due, in part, to decreased binding by/conformational changes in their albumin. There are several ways in which tumor-and/or treatment-associated agents can bind to albumin and cause allosteric modifications that lead to structure and function changes: (1) tumor cells release a variety of bioactive proteins/peptide fragments - sequestration by carrier proteins (like albumin) protect these materials from clearance (and amplify their circulating levels; Kazmierczak et al., 2006); (2) plasma content of select key unsaturated fatty acids (i.e., oleic and arachidonic acids) is increased - these then increasingly occupy binding sites on albumin (Gryzunov and Dobretsov, 1994, 1998; Petitpas et al., 2001b); and, (3) an array of drugs, e.g., ibuprofen, indomethacin, etc. (and their metabolites) commonly ingested by cancer patients readily bind with albumin (Petitpas et al., 2001a, 2003). As was the case with lymphocytes, the shifts in cancer patient plasma ABM fluorescence intensity were related to disease stage. While moderate alterations in albumin-ABM signals were already noted at early (Stages II) phases of cancer, the effects were amplified as cancer evolved to Stages III-IV. It is likely that as cancer progressed, the levels of pathological/pharmacological metabolites in the patient's blood increased and their albumin could not ultimately bind them all. One consequent structural/functional alteration induced in the albumin could be a shift in ABM binding away from normal primary high affinity sites to others with lower affinities/specificities. Such shifts would be in agreement with the observations of Togashi and Ryder (2006) and Rolinski et al. (2007) who noted that albumin molecules contained different binding sites (i.e., classes) that differed in affinity, quantum yield, and degrees of polarization (i.e., higher mobility of bound probe and increased accessibility by water) for ABM and various other probes. The results of the current investigation also seemed to reflect what was predicted to occur based upon electron spin resonance (ESR) spectroscopy studies that measured structural and functional changes in serum albumin of patients with other cancers (Kazmierczak et al., 2006). Specifically, analyses of ESR spectra (using spin probes) revealed substantial differences in spectrum variables when samples from patients were compared with those from healthy hosts. For example, the increasing width of the spectral line in samples from the cancer patients indicated an alteration in albumin conformation that limited the movement of the spin probe at a binding site, as well as changes in the albumin capacity to bind spin probe, polarity of spin probe binding site, and probe mobility (Kazmierczak et al., 2006). While increased binding of tumor-/treatment-associated agents (leading to the sequelea outlined above) could be a means by which changes in albumin-ABM fluorescence evolved here, there are other means by which the albumin ability to bind the probe may have been altered. While we demonstrated here there were changes in ABM fluorescence after inter-actions with lymphocytes (and plasma albumin) obtained from gastrointestinal cancer patients, another major question that needed addressing was whether there were actual biologic/immunologic modifications associated with these alterations. As noted earlier, changes in fluorescence parameters of patient

lymphocytes could reflect changes in one/more inherent characteristics of these cells, including their phenotypical character. While the flow cytometry studies identified significant alterations in lymphocyte (and sub-populations) levels among all the cancer patients, variations in total lymphocyte levels never *clearly and consistently* paralleled the corresponding changes in ABM fluorescence for the gastric cancer subjects. In contrast, there seemed to be a good relationship between these endpoints in the colorectal patients. For now, it remains unclear why there should be a divergence in these patterns based on the cancer type itself.

The observed changes in ABM intensity in the lymphocytes might be useful to reflect current $CD4^+$ and/or $CD8^+$ status in the pacients. In this regard, the same (as above) disease-related differences in the relationships were apparent between changes in ABM fluorescence and those in $CD4^+$ levels in the patients. The noted shifts in $CD4^+$ levels were expected; cancer- related $CD4^+$ cell deficiency is a frequent finding in digestive system cancer patients (Franciosi et al, 2002). In our previous investigations, $CD4^+$:$CD8^+$ ratios tended to parallel ABM fluorescence levels (i.e., lowest among patients who manifested decreased fluorescence in their lymphocyte suspensions (Kalnina et al, 2007, 2009, 2010a,2010b). In those earlier studies, $CD4^+$:$CD8^+$ ratios gradually decreased as $CD8^+$ levels increased with progression of cancer stage (Wang et al., 2004; Kalnina et al., 2007). In the studies here, the shifts seem to depend more on decreases in $CD4^+$ levels as each disease became metastatic. These outcomes would be in keeping with the studies by Tancini et al. (1990) and (McMillan et al. (1997) that indicated that decreases in $CD4^+$:$CD8^+$ ratios in gastric cancer patients mainly depended on increases in $CD8^+$ T-cytotoxic cells in patients with early stage disease whereas it was due to decreases in $CD4^+$ T-helper cells in those with metastases (later stage disease). Thus, at least clearly for colorectal cancer patients, our results suggest that measures of ABM fluorescence intensity values for lymphocytes (and to a lesser extent, for plasma albumin) could potentially be used in clinical immunological screenings (instead of more expensive routine tests) to provide a snapshot of immune status in these cancer patients. Whether the utility of these measures could/would extend to human disease states remains to be determined.

8. Conclusion

Fluorescence behaviour of ABM could be useful to reflecting alterations in lymphocytes in each subgroup and they may ultimately be of use as potential indicators of alterations in cellular immunity in individuals. We also sought to ascertain whether shifts in ABM binding with plasma albumin could be potentially utilized as part of an overall preliminary immunodiagnostic screening test in cancer patients. Taken together it would appear that progression of cancer is associated with changes of immune function and more specifically a reduction in absolute number of CD4 + T-lymphocytes and either an increase or not change in the absolute count of CD8+ T-lynphocytes. Study suggests that higher number of absolute lymphocytes count and ratio CD4+: CD8+ have beneficial effect on overall survival of patients with advanced tumor. Overall survival depends also on quantitative parameters of cellular immunity of cancer patients. Thus, immune status of the immune system of patients with advanced tumor before treatment is important for its survival. The immunosuppression and metastatic spread are interconnected. The low plasma albumin level also were identified as bad independent marker of prognosis. Fluorescent based method is pertinent to pathway profiling, target validation, and clinical diagnosis,

prediction of therapeutic effacy, and monitoring of treatment outcomes. ABM fluorescence intensity values for plasma albumin and lymphocytes (as reflection of their functional activity) might be useful tool in the evolution of the immune status of pacients. Taken together, all the results showed that measures of ABM spectral characteristics could potentially be a useful tool to estimate the immune status of gastrointestinal patients. Compared to many commonly used diagnostic protocols, this fluorescence based method is less expensive and not very time consumming, technically simple and 100 times more sensitive than standard absorbance based methods.

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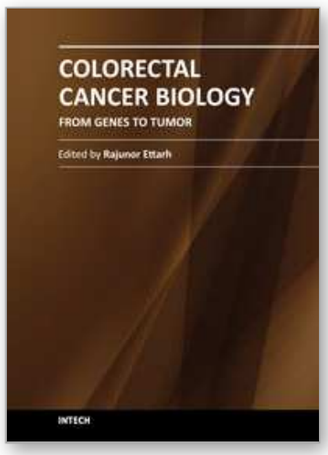

Colorectal Cancer Biology - From Genes to Tumor

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Colorectal cancer is a common disease, affecting millions worldwide and represents a global health problem. Effective therapeutic solutions and control measures for the disease will come from the collective research efforts of clinicians and scientists worldwide. This book presents the current status of the strides being made to understand the fundamental scientific basis of colorectal cancer. It provides contributions from scientists, clinicians and investigators from 20 different countries. The four sections of this volume examine the evidence and data in relation to genes and various polymorphisms, tumor microenvironment and infections associated with colorectal cancer. An increasingly better appreciation of the complex inter-connected basic biology of colorectal cancer will translate into effective measures for management and treatment of the disease. Research scientists and investigators as well as clinicians searching for a good understanding of the disease will find this book useful.

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