DOI: http://dx.doi.org/10.5915/26-2-16366

Cell-Mediated Immunity in Cancer: Prognostic Significance

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Abstract

Cell-mediated immunity (CMI) was studied in 100 patients with cancer and 20 healthy control volunteers with comparable age and sex ratios. Delayed hypersensitivity was estimated by DNCB and PPD cutaneous reactivity in vivo while lymphocyte subpopulations were estimated by peripheral blood lymphocyte (PBL), total rosette forming cells (TRFC) and active rosette forming cells (ARFC) in the peripheral blood. Purified protein derivative (PPD) and Di-nitrochlorobenzene (DNCB) cutaneous reactivity was positive in 4 percent and 64 percent respectively. TRFC values were expressed as a percentage of PBL and were found to be significantly decreased in cancer Stage I (35.5 percent; $p< 0.05$), Stage II (46.6 percent), Stage III (46.2 percent) and Stage IV (45 percent); the decrease in Stages II, III, and IV was insignificant as compared to the control group (51.5 percent). On the contrary, ARFC levels expressed as a percentage of PBL were significantly decreased in cancer Stage I (21.5%, $p< 0.05$), but significantly increased in Stage II (35.6 percent), Stage III (33.5 percent) and Stage IV (32.4 percent) as compared to control group (23.6 percent). Since DNCB cutaneous reactivity showed good immunological reserve in advancing stages of cancer in our study, a linear correlation could be established between DNCB cutaneous reactivity and ARFC levels. Since ARFC sub-population is the functionally active T-lymphocyte sub-set against malignant cells in vivo, these cells increased with proliferation of malignant cell mass in vivo, whereas PBL and TRFC levels do not exhibit significant variation. Parameters of cell-mediated immunity in vitro TRFC and ARFC were significantly decreased in lymphoreticular and mesenchymal malignancies ($p < 0.001$). However, ARFC levels were significanlty increased in cancer of gastrointestinal tract ($p < 0.05$), head and neck ($p< 0.05$) and breast ($p< 0.001$) as compared to control group. Linear correlation was found with the percentage of DNCB positive reactors in each group.

Key words: Cell-mediated immunity, cancer.

Cell mediated immune response in cancer patients has important implications in determining tumor development, progression, early, primary or recurrent malignancy and in understanding and augmenting the immune defense mechanism in order to guide conventional and immunotherapeutic managment of cancer.[1-5] Peripheral blood lymphocytes (PBL) from cancer patients are cytotoxic to inherent tumors in vivo.[6] Reduction in the PBL number and functional capactity increased the rate of progression of neoplasms.[7] Active T-cell rosette (ARFC) assay identifies a subpopulation of T-lymphocytes with high avidity for sheep red cells. They are Ia^+ but lack a F_c receptor and can be classified as either OKT_4^+ or OKT_8^+. Isolated active T-rosette subpopulation is capable of recognizing and killing allogeneic cells and can modulate B-cell immunoglobulin production. ARFC is directly correlated in vivo with specific cutaneous reactivity to an antigen. Linear correlation is found with DNCB cutaneous hypersensitivity. Active and late rosette forming T-lymphocytes are different T-cell subpopulations. ARFC count is an index of

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cells actively involved in CMI.[8-11] Lymphocyte anergy is seen in late stages of cancer.[12,13] DNCB and PPD cutaneous reactivity are important discriminatory factors in the assessment of patients with solid tumors. Negative response is associated with metastasis and early recurrence. A higher proportion of DNCB reactors were free of malignant disease in six months.[14-17] T-cell sub-populations were correlated with DNCB cutaneous reactivity because in 80 percent of the patients, T-cells mediated delayed hypersensitivity response in vivo. Linear correlation was also found between circulating TRFC and histopathological stage of tumors.[18] However, in squamous cell carcinoma of head and neck, depressed T-cell count was due to soluble immune-response suppressor factor.[19,20] We have identified ARFC sub-population as an early marker of lymphocyte stimulation which mediates cutaneous delayed hypersensitivity response to tumor antigens, and is also an index of cells actively involved in CMI. We have proved our observations by correlating ARFC with PBL, TRFC, DNCB cutaneous reactivity in vivo, as well as with tumor stage and histology.

Materials and methods

Patients were randomly selected from out-patient departments and wards of J. N. Medical College and Hospital, A. M. U., Aligarh, (U.P.), India, from January 1985 to July 1987. Blood samples were taken from these patients for routine investigations, i.e. complete hemogram with ESR, and also for estimation of PBL, TRFC and ARFC counts. Confirmation of malignancy was done by TNM classification of the American Joint Committee on Cancer, Chicago, 1962. Histopathological typing and grading of tumors was also done after microscopic examination of paraffin embedded sections. Twenty paramedical healthy volunteers were selected with comparable age and sex ratios.

Tests for CMI in vitro:

T-lymphocyte count in peripheral blood was done as total rosette forming cells (TRFC).[21] Half a ml of venous blood with 62.5 i.u. of heparin were layered onto 2 ml of Histopaque 1077 (Lymphoprep. SIGMA) and centrifuged at 1,500 rpm for 30 minutes at 20°C.

The leukocyte-rich plasma was pipetted off and cells were centrifuged three times with MEM (pH = 7.2) at 1,500 rpm for 15 minutes to make a final suspension of 5×10^6 cells/ml. One vol. of this suspension was mixed with 1 percent sheep erythrocyte suspension in MEM, incubated at 37°C for 10 minutes, later centrifuged for five minutes at 1,500 rpm and incubated at 4°C for 30 minutes. Two hundred lymphocytes were counted, and active rosette forming cells (ARFC) were identified and expressed as a percentage. Suspension was incubated for 24 hours at 4°C and TRFC were counted similarly and expressed as percentage. Absolute count of PBL as cells/cu. mm was calculated from total leukocyte and differential leukocyte counts. ARFC and TRFC were tabulated as cell/cu. mm as well as a percentage of PBL. Student-t test was applied and p value was calculated.

Test for CMI in vivo:

PPD skin test was done by injecting 0.1 ml of PPD intradermally (SPAN 10; Tu/0.1 ml), and a 10 mm reaction was considered positive after 48 hours.

DNCB skin test was done by the technique of Eilber and Morton (1970).[17] After cleaning the skin with spirit, metal rings of 2.5 cm diameter were applied to the skin, 4 cm above and below the antecubital fossa. Fifty ug/0.1 ml of DNCB solution in acetone (sensitizing dose) and 2000 ug/0.1 ml of DNCB solution (challenging dose) was applied respectively to the two sites. After the solution dried up, the rings were removed and the sites were covered with adhesive plaster and kept dry.

The "irritant reaction" was assessed after removing the plaster after 48 hours, and sensitivities were reassessed after 14 days. As the irritant dose and vesicant dose are the same for a given person, we noted the reaction to the irritant dose.

If no reaction was observed, a rechallenge dose of 2000 ug/0.1 ml and 50 ug/0.1 ml of DNCB solution was applied to the ipsilateral arm, occluded for 24 hours and assessed after 48 hours.

Negative reaction- No reaction or slight erythema or induration observed 48 hours after applying 50 ug/0.01 ml of DNCb solution.

Positive reaction- Erythema and induration observed at both sites where 2000 ug/0.1 ml and 50 ug/0.1 ml of DNCB solution was applied.

Results

Cancer patients were selected from the out-patient departments and wards of J.N. Medical College and Hospital, A.M.U. Aligarh, (U.P.), India from January 1985 to July 1987. Incidence of malignancy in Aligarh district, calculated from our hospital-based study was 0.93/1000 admissions. Malignancy was confirmed in 100 patients by histopathology. Staging of cancer was done by TNM classification. CMI was tested in vivo by PPD and DNCB cutaneous reactivity and in vitro by estimation of circulating PBL, TRFC and ARFC levels. Results of CMI from cancer patients were compared with control group.

Male and female ratio in cancer patients was 57:43 and mean age was 52.8 ± 16.70 years.

CMI results in 100 patients with cancer were sub-divided into different anatomical sites of cancer and compared with the control group (Table 1).

1) Control group (20 cases): Mean PBL count was 1997 ± 795/cumm. while mean TRFC and ARFC counts were 51.5 percent and 23.6 percent of PBL. The ratio of TRFC:ARFC was 2.12:1. All cases were positive for PPD and DNCB cutaneous reactivity.

2) Head and Neck Cancer (45 patients): Analysis of 45 patients revealed carcinoma of thelarynx (8); basal cell carcinoma (2); squamous cell carcinoma of the oral cavity

(26); ameloblastic carcinoma of jaw (2); mucoepidermoid carcinoma of parotid gland (4) and thyroid carcinoma (3). The ratio of TRFC:ARFC was 1.48:1. A decrease in TRFC (47.7 percent) and a significant increase in ARFC (32.2 percent) were observed in comparison with control values ($p< 0.05$). DNCB skin test was positive in 68.8 percent of the patients while 8.8 percent were positive by PPD skin test.

3) Gastrointestinal Cancer (31 patients): Analysis of these patients revealed esophageal carcinoma (8); carcinoma of the stomach (5); lymphoma-jejunum (1); carcinoma of the rectum and anal canal (11); carcinoma of the gallbladder (5): and carcinoma at the head of pancreas (1). The TRFC:ARFC ratio was 1.27:1, which was significantly decreased as compared to the control group ($p< 0.01$). A highly significant decrease was observed in TRFC level (38.8 percent; $p< 0.001$); while ARFC level was significantly increased (30.4 percent, $p< 0.05$) compared to the control group. DNCB cutaneous reactivity was positive in 58 percent of the patients while PPD skin test was negative in all patients.

4) Breast Cancer (12 patients): All patients were diagnosed as infiltrating duct carcinoma of the breast with productive fibrosis. The TRFC:ARFC ratio was 1.57:1, which was less than the control group. TRFC counts were significantly increased as compared to the control group ($p< 0.01$), however, a marked increase was observed in ARFC levels (32.9 percent; $p<0.001$). DNCB skin test was positive in 75 percent of the patients.

5) Genito-urinary Cancer (6 patients): Adeno-carcinoma of prostate was diagnosed in three patients, transitional cell carcinoma of urinary bladder in one patient and squamous cell carcinoma of the penis in two patients. TRFC to ARFC ratio was 1.61:1, which was significantly decreased as compared to the control group ($p< 0.05$). TRFC and ARFC counts were 52.6 percent and 32.6 percent respectively. DNCB cutaneous reactivity was positive in 66.6 percent patients.

6) Miscellaneous group (6 patients): Lymphocytic lymphoma was diagnosed in three patients and liposarcoma in the other three patients. PBL, TRFC and ARFC counts were significantly decreased as compared to the control group. TRFC level was 22.2 percent while ARFC level was 18.3 percent of PBL ($p<0.001$). The TRFC:ARFC ratio was 1.21:1 which was significantly less than the control group ($p<0.001$). DNCB skin test was positive in only 33.3 percent of the patients.

PBL and TRFC levels were decreased in all types of malignancy except breast carcinoma. ARFC sub-population was increased in all patients with malignancy except the lymphoma and sarcoma groups. Reduction of TRFC:AFRC ratio indicates increase of ARFC sub-population in circulation which is a good immunological reserve in cancer patients corroborated by DNCB skin reactivity.

Analysis of 100 cancer patients according to clinical stage of cancer revealed the following results (Table 2):

(1) a. Stage I (13 patients): mean PBL count was decreased. TRFC counts (35.5%) were decreased as compared to the control group ($p< 0.05$) while ARFC count was slightly decreased (21.5 percent). TRFC:ARFC ratio was significantly decreased compared to control ($p< 0.01$). DNCB skin test and PPD skin test were positive in 61.5 percent and 23 percent of the patients respectively.

(2) Stage II (20 patients): Mean PBL count was slightly decreased. A similar decrease was observed in TRFC count (46.6 percent). ARFC count was significantly increased (35.6 percent, $p< 0.01$). DNCB skin test was positive in 68.1 percent of patients while PPD test was positive in 4.5 percent of the patients.

(3) Stage III (20 patients): PBL count was decreased. However, the TRFC count was also decreased (46.2 percent); on the contrary, ARFC count was significantly increased in comparison with the control group (33.5 percent; $p< 0.05$). Significant reduction in TRFC:ARFC ratio was observed (1.71:1; $p< 0.05$). DNCB skin test was positive in 65 percent of the patients.

(4) Stage IV (45 patients): PBL count was increased but the difference from the control group was insignificant. TRFC level was decreased (45 percent) while ARFC level increased (32.4 percent; $p< 0.05$) as compared to the control group. TRFC:ARFC ratio was decreased (1.38:1; $p< 0.05$). DNCB skin test was positive in 62.2 percent of the patients.

Analysis of the results reveal that in the control group ARFC sub-population of lymphocytes constitutes half the total T-lymphocyte count. In cancer, there is relative lymphopenia with a reduction in T-lymphocyte sub-populations. ARFC sub-population is decreased in the initial stage of cancer. A latent period exists between the neoplastic transformation in vivo and activation of cell-mediated immune response followed by stimulation of ARFC sub-population identified as Ia^+ cells, OKT_8^+ capable of recognizing and killing allogeneic cells. ARFC begin to increase in Stages II and III, and the level declines in Stage IV with exhaustion or anergy of immunological reserve in patients with terminal cancer. The ARFC count is directly proportional to DNCB skin test in vivo. An important conclusion can be made from our study that ARFC sub-population is an important parameter of cell mediated immunity in cancer patients. These cells are not only capable of immediate antigen recognition and cellular cytotoxicity against cancer cells but are also important mediators in delayed hypersensitivity response in cancer patients.

Discussion

CMI develops against malignant tumors in vivo in response to certain "neo-antigens" on the tumor cell surface which are not recognized as "self."[5,6] The process of malignant transformation is a complex phenomenon, and this critical problem remains to be investigated despite adequate work done on T-lymphocyte sub-populations. We have tried to evaluate the relationship between ARFC and TRFC sub-

Table 1. Estimation of cell mediated immunity by PPD+ and DNCB++ cutaneous reactivity in vivo and PBL*, TRFC** and ARFC*** sub-populations in vitro in patients with different anatomical sites of cancer, and in controls.

Type of cancer	No. of patients	Age (Yrs.)	M/F[P]	PPD		DNCB		PBL Cells/c mm	TRFC Cells/c mm	TRFC / PBL %	ARFC Cells/c mm	ARFC / PBL %	TRFC / ARFC ratio
				+	%	+	%						
Control	20	34±14@	12/8	20	100	20	100	1997±795@	1029±461@	52	473±302@	24	2.2
Head and neck	45	52 ± 14	31/14	4	9	31	69	1708 ± 602ϒ	815 ± 513ϑ	48	550 ± 385ϒ	32	1.5ϒ
GIT	31	49 ± 10	15/16	0	0	18	58	1848 ± 473ϑ	718 ± 454"	39	562 ± 403ϒ	30	1.3^
Breast	12	47 ± 7	0/12	0	0	9	75	2814 ± 827"	1456 ± 432^	52	928 ± 392"	33	1.6ϒ
GUT	6	60 ± 12	6/0	0	0	4	67	1500 ± 623^	790 ± 357ϑ	53	489 ± 248ϑ	33	1.6ϒ
Misc	6	42 ± 22	5/1	0	0	2	33	1720 ± 522ϒ	382 ± 169"	22	316 ± 161"	18	1.2@
Total	100	52.8 ± 17	57/43	4	4	64	64	1865 ± 532ϑ	831 ± 368ϒ	45	598 ± 294ϒ	32	1.4ϒ

+Purified protein derivative ++Dinitrochlorobenzene
* Peripheral blood lymphocytes ** Total rosette forming cells *** Active rosette forming cells
[P]Male/female
@Mean ± SD
GIT = gastrointestinal tract; GUT = genitourinary tract; Misc = miscellaneous
ϑStatistically insignificant ϒp<0.05 ^p<0.01 "p<0.001 @p<0.0001

Table 2. Estimation of cell-mediated immunity by PPD⁺ and DNCB⁺⁺ cutaneous reactivity in vivo and PBL*, TRFC** and ARFC*** sub-populations in vitro in patients with different stages of cancer, and in controls

Clinical stage of cancer	No. of patients	PPD +	PPD %	DNCB +	DNCB %	PBL Cells/c mm	TRFC Cells/c mm	TRFC / PBL %	ARFC Cells/c mm	ARFC / PBL %	TRFC:ARFC Ratio
Control	20	20	100	20	100	1997 ± 795	1029 ± 461	52	473 ± 302	24	2.1:1
Stage 1	13	3	23	8	62	1664 ± 365ϒ	591 ± 211"	36ϒ	359 ± 71ϑ	22ϒ	1.7:1^
Stage 2	22	1	5	15	68	1740 ± 673ϒ	812 ± 311ϒ	47ϑ	621 ± 384^	36^	1.3:1^
Stage 3	20	0	0	13	65	1827 ± 302ϑ	845 ± 403ϒ	46ϑ	613 ± 271ϒ	34ϒ	1.4:1ϒ
Stage 4	45	0	0	28	62	2002 ± 788ϑ	904 ± 548ϑ	45ϑ	650 ± 450ϒ	32ϒ	1.4:1ϒ
Total	100	4	4	64	64	1865 ± 532ϑ	831 ± 368ϒ	45	598 ± 294ϒ	32	1.4:1"

⁺Purified protein derivative ⁺⁺Dinitrochlorobenzene
*Peripheral blood lymphocytes ** Total rosette forming cells *** Active rosette forming cells
ᴾMale/female
@Mean ± SD
GIT = gastrointestinal tract; GUT = genitourinary tract; Misc = miscellaneous
ϑStatistically insignificant ϒp<0.05 ^p<0.01 "p<0.001 @p<0.0001

populations and the growth and proliferation of malignant tumors.[4,12,18] Since PBL, TRFC and ARFC are intricately linked with delayed hypersensitivity reaction, hence DNCB and PPD skin tests were also performed on cancer patients to evaluate the efferent limb of CMI.

In our study of 100 cancer patients (Table 1), TRFC and ARFC levels were significantly decreased in lymphomas and sarcomas ($p<0.001$) while PBL and ARFC were significantly increased in breast carcinoma ($p< 0.001$), which is compatible with other workers.[14,18] Decrease in PBL and TRFC levels in squamous cell carcinoma of head and neck in our study can also be explained on the basis of the soluble immune suppressor factor emphasized by other authors.[19,20] However, in our study, ARFC levels remained higher than control values in gastrointestinal and genito-urinary cancers, and also adenocarcinoma of head and neck, which is consistent with the other workers.[8-11] Lymphocyte sub-populations were decreased in the early stage of cancer. (Table 2) In cancer Stage I, TRFC levels were decreased in greater proportion ($p< 0.001$) as compared to ARFC levels. Sixty-one and one-half percent of the patients were DNCB positive reactors and 23 percent were PPD positive reactors. Depression in CMI could trigger off progressive malignant transformation due to ineffective host defense.[12] However, effective CMI may combat tumor development and progression with prolongation of the "latent" phase, wherein the tumor remains occult.[4] In our study, a significant observation was the decrease of TRFC:ARFC ratio from 2.10:1 (control) to 1.65:1 (malignancy, stage I). This phenomenon indicates a greater decrease in TRFC level (35.5 percent) than ARFC level (21.5 percent) in comparison to control values. Hence, clinically detectable tumors are preceded by decreased T-lymphocyte sub-population in peripheral blood.[1,7]

In cancer Stage II, ARFC sub-population rapidly increased in peripheral blood in our study. TRFC:ARFC ratio significantly decreased to 1.30:1 ($p<0.01$). Marked increase in positive DNCB reactors was observed (68.1 percent) compatible with other workers.[1,7]

Cancer Stage III is categorized by a greater tumor burden and a rapid turnover of tumor cells. ARFC levels remain significantly higher than the control group ($p< 0.05$), but lower in comparison with Stage II. DNCB positive reactors were only 65 percent.

In cancer Stage IV, with dissemination of malignancy, DNCB positive reactors decreased to 62.2 percent, while TRFC (45 percent) and ARFC levels (32.4 percent) also declined. However, ARFC level remained higher than in the control group. In cancer Stage IV, 38 percent of the patients showed a state of anergy. However, 62 percent patients manifested a good immunological reserve. Even though ARFC are functionally active sub-population of T-lymphocytes, which are capable of recognizing and killing allogeneic cells,[8-11] these cells do not increase in proportion to the proliferating tumor cells. Thus, the overwhelming tumor mass in Stage IV may cause functional incapacitation of

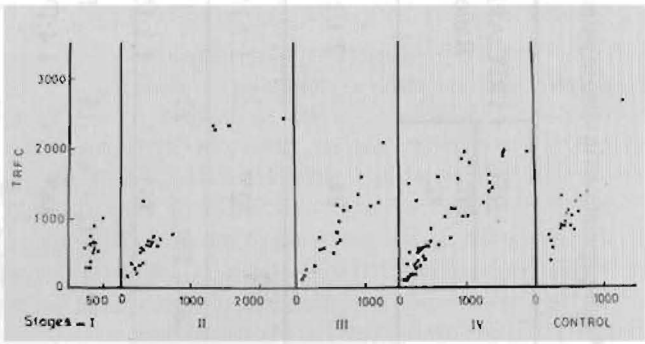

Figure 1. Total rosette forming cells:active rosette forming cells ratio in controlls and in patients with various stages of cancer.

ARFC sub-population.[13] In our study, the TRFC:ARFC ratio remains constant in Stage IV as compared to Stage III due to an insufficient increase in the ARFC sub-population in terminal cancer patients, corroborating the work done by other authors.[12,18] Linear correlation is observed between positive DNCB cutaneous reactivity and increased levels of ARFC sub-population (Table 2).

Progression of malignant proliferation from Stage I to Stage II is preceded by an increase in the ARFC sub-population which constitutes the functionally active cytotoxic T-lymphocyte subset. However, with dissemination of cancer ARFC levels either remain stationary in patients with good immunological reserve or decrease as a prognostic indicator of impending anergic state.

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