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Prognostic Significance of Circulating Immune Complexes in Cancer Patients

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Circulating immune complexes (CIC) were estimated in 100 cancer patients and 25 healthy control volunteers by means of the polyethylene glycol (PEG) precipitation test and latex agglutination inhibition (LAI) test. Pathological levels of CIC were found in 47% of the patients by PEG precipitation test and in 59% of the patients by LAI test; both tests were positive in 33% of the patients. Consequently, the use of the two assays resulted in 73% seropositivity for CIC. The PEG precipitation test detects antigen-antibody complexes formed in the ratio of 2:1 (Ag_2Ab), while the LAI test could detect immune complexes formed over an extended range of antigen-antibody ratio including complexes as small as 8S. CIC values were significantly higher by combined assays ($P<0.001$) as compared to individual assays ($P<0.01$) when compared with the control group. It was found that 75% of post-operative follow-up patients became seronegative for CIC in the combined assays, whereas the 25% of post-operative patients who remained seropositive for CIC showed recurrence within three months after surgery. Immune-complex deposition was demonstrated on malignant cells *in vitro* by direct immunofluorescence studies in 73.3% of patients, while 60% of patients revealed complement-fixing antigen-antibody complexes. It was found that 20% of patients showing positive immunofluorescence with anti-C_3-antisera had decreased levels of CIC. Complement-mediated cytotoxic injury results in reduction of tumor cell mass and subsequent decrease in CIC. Necrotizing and leucocytoclastic vasculitis in the tumor mass was initiated by raised CIC levels *in vivo* in 71% of patients. Necrosis of malignant tumors was seen in 58% of patients, and hemorrhage in 36% of patients. These changes were considered to be an aftermath of immuno-complex vasculitis initiated by CIC.

Key words: Immune complex — Cancer patient

Tumorigenesis represents an escape from normal immune surveillance. 'Blocking factors' in the sera of cancer patients are circulating immune complexes[1] and their level in serum is directly dependent upon tumor size, so that a high level implies a poor prognosis. Cancer sera contains immune complexes of intermediate size and the reported incidence is 16–52% as compared to 19% in control sera.[2,3] Studies done in patients with Hodgkin's disease, non-Hodgkin's lymphomas and leukemias have shown that CIC[4] appear at the time of tumor spread and breakdown of cell-mediated defence.[4–6] Estimation of CIC in breast cancer demonstrated significantly higher pre-operative levels which declined to normal after mastectomy.[7–11] Raised CIC levels have been noted in neuroblastoma,[12] Burkitt's lymphoma, cancer or the lung[13] and uterine cervix[14] (63% versus 13% in control) ovarian cancer[15] and renal cell carcinoma.[16] Increased CIC levels were also found in carcinoma of larynx[17,18] and oral cavity.[19] In colorectal cancer, raised post-operative CIC levels corroborate transient CEA elevations which occur with occult tumor recurrence.[20] In patients with large, rapidly disseminating cancers the mean CIC values were 75% as compared to 22% in small or regressive tumors.[21] The purpose of our study was to demonstrate immune complexes of different physico-chemical properties, sizes and molecular configurations by combining PEG precipitation and LAI tests. Combined assays cover a larger immune-complex spectrum, and should be more sensitive prognostic indices as compared to individual assays used by other workers.[1–27] Increased CIC levels trigger immune-complex vasculitis, which causes necrosis and hemorrhage in tumors, as demonstrated by our study of 100 histological sections of various tumors. Complement-fixing immune complexes are deposited on tumor cell surfaces, causing complement-mediated cytotoxic injury to tumor cells, as demonstrated by our immunofluorescence studies. Consequently spontaneous regression of tumor is an aftermath of immune complex-mediated injury to tumor cells *in vivo*.[28–31]

MATERIALS AND METHOD

Patients were selected from the out-patients department and wards of J.N. Medical College & Hospital, A.M.U., Aligarh (U.P.), India. The diagnosis of cancer was established by histopathological examination of

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[4] Abbreviations: CTC, circulating immune complexes; SD, standard deviation; PEG, polyethylene glycol; C_3, third component of complement; LAI, latex agglutination inhibition, FITC, fluorescein isothiocyanate; IF, immunofluorescence.

biopsy and surgically resected speciments. Staging of cancer was done according to the American Joint Committee for Cancer (TNM classification). Post-operative follow-up studies were done at three months after surgery in 20 cases. For the control group, 25 blood samples were drawn from healthy paramedical volunteers matched for age and sex with the study group.

Estimation of circulating immune complexes by PEG precipitation test This test was based on selective precipitation of soluble circulating antigen-antibody complexes in 4.166% high-molecular-weight polyethylene glycol. Under these conditions neither monomeric IgG nor antigen alone was precipitated. Aggregated antigen-antibody complexes are precipitated selectively by steric exclusion of immuno-complexes from the polymer domain. CIC precipitated by PEG have an antigen-to-antibody ratio of 2:1 i.e. Ag_2Ab. These immune complexes are formed in antigen excess.

Test serum was diluted 1:3 with borate buffer at pH 8.4, and 0.22 ml of diluted serum was taken in two test tubes marked A (control) and B (test). Then 2 ml of borate buffer was added to tube A, while 2 ml of 4.166% PEG (mol. weight 6,000) in borate buffer was added to tube B. Both tubes were shaken well and kept at 37°C for one hour. Observations were made at 450 nm in a spectrophotometer previously adjusted with plain borate buffer.

PEG precipitation index
$= (E_{450}$ with PEG $- E_{450}$ with buffer $\times 1000)$.

E_{450} is the light absorbance measured at 450 nm in a spectrophotometer (Shimadzu UV-150-02). The control value of PEG precipitation index was taken as the mean value of 25 control sera $\pm$ SD. The criterion for a pathological value of CIC in cancer sera was a PEG index > control value + 2 SD.

LAI test[23] Rheumatoid factor (RF)-positive serum was obtained from patients with rheumatoid arthritis (titer 128) and used as the source of RF. All test sera were screened and only RF-negative test sera were included in our study. CIC inhibit the agglutination of latex particles by RF.

RF-positive serum was diluted 1:64 in sterile saline, then 25 μl of diluted RF-positive serum, 25 μl of test serum and 25 μl of latex-RF reagent were mixed. The mixture was stirred and the plate rotated gently and observed for agglutination up to 5 min. Inhibitory activity was expressed in μg equivalents of agglutinated latex/ml. The LAI test was positive if complete inhibition of agglutination of latex particles was caused by test sera, i.e., μg equivalents of agglutinated latex/ml was nil. The LAI test was negative if 1–5 μg equivalents of agglutinated latex/ml was observed, or if any agglutinated latex was seen microscopically.

Direct detection of antigen-antibody complexes by immunofluorescent technique on impression smears from tumors[29–31] Direct immunofluorescence was studied on imprint smears from malignant tumors.[28–30] Antigen-antibody complexes were demonstrated on imprint smears by using polyvalent anti IgG + IgA + IgM antisera and anti-C_3-antisera tagged with FITC. Fixed antigen-antibody complexes were demonstrated by fluorescence microscopy.

RESULTS

CIC were studied in 100 cases of cancer by using the PEG precipitation test and LAI test. The results were compared with those for a control group of 25 healthy volunteers. Follow-up studies were done in 20 post-operative cases. Mean age was 45.58 $\pm$ 2.5 yr in cancer patients and 40 $\pm$ 2.5 yr in the control group. Male: female ratio in both cancer and control groups was 1:08. The incidence of cancer in Aligarh district (U.P.), India was 0.95/1000 population from 1st Jan. 1987 to 31st Dec. 1987. Further analysis of cancer patients was done according to the clinical stages and antomical stites of cancer (Table I). Serum samples from 25 control cases were assayed in order to compute a suitable threshold value of this test. A mean PEG index of 20.36 $\pm$ 6.40 was observed for normal serum. Hence PEG index > 33.16, representing control + 2 SD, was taken as the criterion of pathological CIC levels in sera of cancer patients. It was found that 47% of total cancer cases were seropositive by PEG test and 59% by LAI test (Fig. 1). By combining both these tests, 73% seropositivity was obtained. No positive case was observed in the control group by either test individually.

Analysis of 100 cancer cases according to clinical stages showed mean PEG index values of 23.77 $\pm$ 10.95 in stage I, 28.11 $\pm$ 17.07 in stage II, 37.36 $\pm$ 36.00 in stage III and 70.88 $\pm$ 33.39 in stage IV. Seropositivity for pathological levels of CIC was 11.11% in stage I, 35.13% in stage II, 50% in stage III and 83.33% in stage IV by PEG precipitation test. The LAI test revealed 55.5% seropositivity in stage I, 54.05% in stage II, 58.33% in stage III and 72.22% in stage IV as compared to nil in the control group Combination of the two assays increased the sensitivity of CIC detection as follows: 66.66% in stage I, 64.86% in stage II, 69.44% in stage III and 100% in stage IV. These values were highly significantly increased ($P < 0.001$) as compared to each assay individually ($P < 0.01$), when compared to the control group (Table I, Fig. 1). CIC levels estimated in different anatomical sites of cancer revealed the following results: 54.82% of head and neck cancer patients seropositive for pathological levels of CIC by each assay separately and 60.29% by the combination of PEG and

Table I (Fig. 1). Estimation of CIC in Malignancy by Combination of PEG Precipitation Test and LAI Test in Different Clinical Stages and Anatomical Sites of Cancer

S. No.	Clinical stage	No. of cases	Seropositivity for CIC[a]						Combination of LAI & PEG		P value
			LAI test		PEG test		LAI & PEG				
			No.	%	No.	%	No.	%	No.	%	
1. i)	I	9	5	55.5	1	11.1	nil		6	66.6	
ii)	II	37	20	54.0	13	35.1	9	24.3	24	64.8	
iii)	III	36	21	58.3	18	50	14	38.8	25	69.4	
iv)	IV	18	13	72.2	15	83.3	10	55.5	18	100	
	Total	100	59	59	47	47	33	33	73	73	
2. Anatomical sites											
i)	Head & Neck	31	17	54.8	17	54.8	15	48.3	19	60.2	
ii)	GIT	21	10	47.8	8	38.0	3	14.2	15	71.4	
iii)	GUT	23	15	65.2	10	43.4	9	39.1	16	69.5	
iv)	Breast	12	8	66.6	8	66.6	5	41.6	11	91.6	
v)	Lymphomas & sarcomas	13	9	69.2	4	30.7	1	7.6	12	92.3	
3. Total		100	59	59	47	47	33	33	73	73	
4. Control		25	0	0	0	0	—	—	—	—	3:4 = $P<0.001$
5. Follow-up											
i)	Pre-operative	20	15	75	16	80	11	55	20	100	5(i):(ii) = $P<0.01$
ii)	Post-operative	20	4	20	5	25	4	20	5	25	

a) Pathological value of CIC: (i) PEG precipitation test = PEG index > control + 2 SD. (ii) LAI test = complete inhibition of agglutination of latex measured in μg/ml.

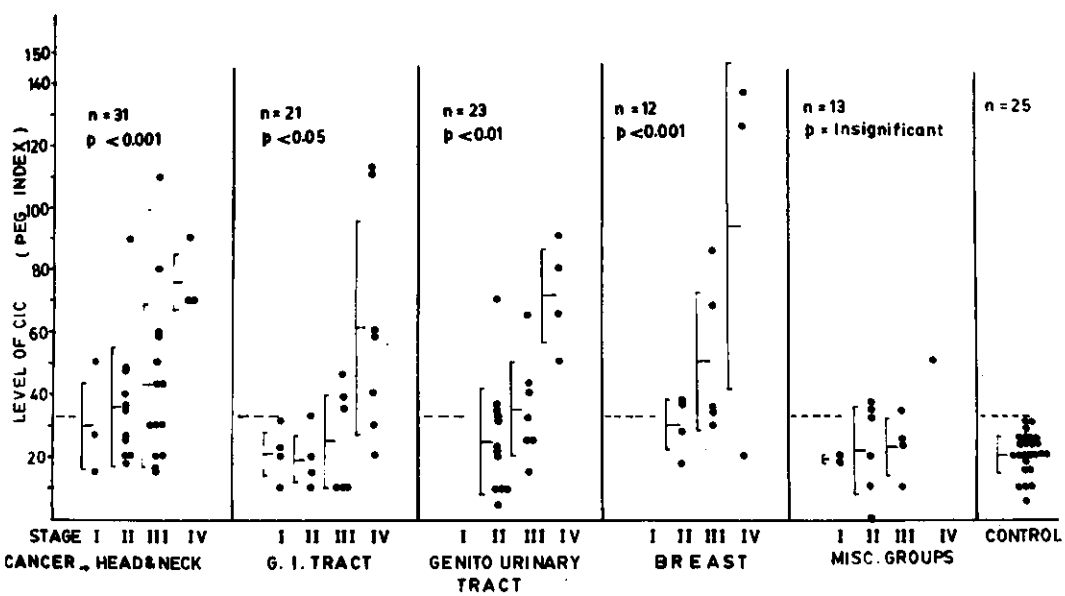

Fig. 1. Measurement of circulating immune complexes (CIC) in different cancers in relation to control. *P*-value = individual group vs. control. Positive cases > mean + 2 SD of control (indicated by dotted line).

LAI tests; 47.80% of gastrointestinal cancer patients seropositive by LAI test and 38.09% by PEG test, with 71.42% seropositivity for the combination; 65.20% of genitourinary cancer patients seropositive by LAI test and 43.48% by PEG test, with a combined assay value of 69.56%; 66.66% seropositivity in breast cancer observed

by each test, and 91.66% in combination. Lastly 69.20% lymphoma and sarcoma patients were seropositive for CIC by LAI test, while 30.76% were seropositive for CIC by the PEG precipitation test, while 92.30% seropositivity for CIC was observed by a combination of the two assays (Table I).

Post-operative follow-up studies were done in 20 patients. Seropositivity for CIC decreased from 75% to 20% by LAI test and from 80% to 25% by PEG precipitation test post-operatively. Combined assays revealed a decline in CIC positivity from 100% to 25% within 3 months after radical surgery.

Immune complex vasculitis was studied in paraffin-embedded histological sections of tumor tissue from 100 cases of cancer (Table II). It was found that 71% of patients had histological evidence of vasculitis; 13% showed mild vasculitis, 20% showed leucocytoclastic vasculitis (endothelial swelling and leucocytic infiltrate in the vessel walls) (Fig. 2), and 38% showed necrotizing vasculitis in the blood vessels supplying malignant tumors (Fig. 3). In cancer patients with necrotizing vasculitis, CIC were positive in the sera of 66% of cases by PEG precipitation test and 77% of cases by LAI test. The combination of both assays revealed 100% seropositivity for CIC in cancer patients manifesting immune-complex vasculitis. The thrombogenic phenomenon which accompanies necrotizing vasculitis leads to infarction and necrosis of malignant cells *in vivo*, leading to reduction in tumor mass: 58% of cancer patients had histological evidence of extensive necrosis around blood vessels in the tumor mass (Fig. 4), while 36% of patients showed histological evidence of extensive hemorrhage (Fig. 5) around blood vessels showing changes of necrotizing vasculitis (Fig. 6).

Table II (Figs. 2–6). Tissue Injury Suggestive of Immune Complex Phenomenon in Malignancy (Histological Interpretation of 100 Cases)

S. No.		Immune complex vasculitis	Necrosis	Hemorrhage
	Total cases (100)	Cases %	Cases %	Cases %
1.	Mild vasculitis	13		—
2.	Leucocytoclastic vasculitis	20	20	—
3.	Necrotizing vasculitis	38	38	36
	Total	71	58	36

Pathological levels of CIC cause immune-complex vasculitis in the blood vessels of the tumor which further leads to necrosis and hemorrhage of tumor cells. Direct immunofluorescence (IF) studies were done on 15 impression smears from malignant tumors using polyvalent antigammaglobulin (anti IgG + IgA + IgM) antisera and anti-C_3-antisera linked with FITC (Table III). It was found that 73.3% of cases showed positive immunofluorescence with polyvalent antigammaglobulin antisera (Fig. 7) and 60% of cases showed positive immunofluorescence with anti-C_3-antisera (Fig. 8). Antigen-antibody complexes were deposited on the surface of malignant cells with complement fixation in 60% of cases. The

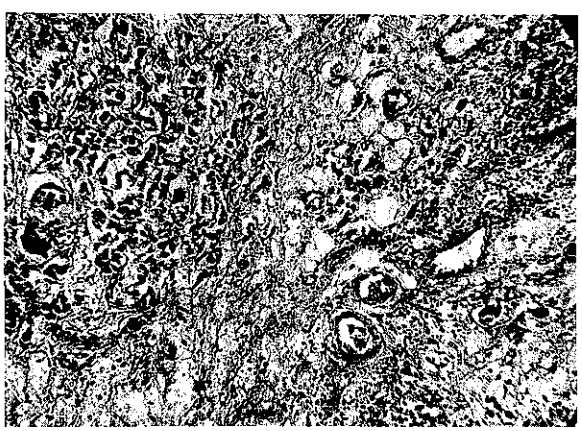

Fig. 2. Photomicrograph of hematoxylin and eosin (H&E)-stained histological section showing infiltrating duct carcinoma of breast. Leucocytoclastic vasculitis is seen in medium-sized arterioles (arrow). Magnification 1:100 (low-power Nikon).

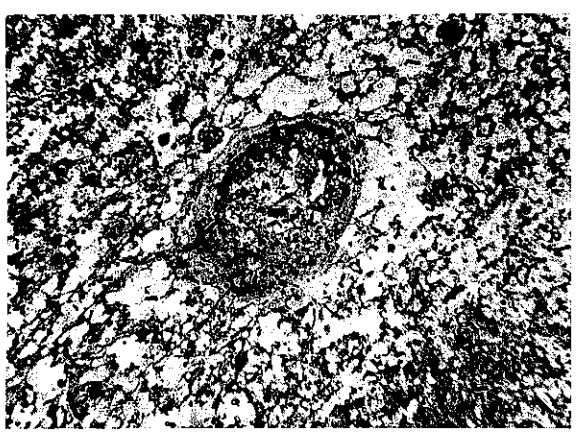

Fig. 3. Photomicrograph (H&E stain). Immune-complex vasculitis (leucocytoclastic type) in renal cell carcinoma.

Fig. 4. Photomicrograph of H&E-stained histological section showing mucus-secreting adenocarcinoma of colon. Necrosis is seen in the tumor mass (arrow) magnificantion 1:100 (low-power Nikon).

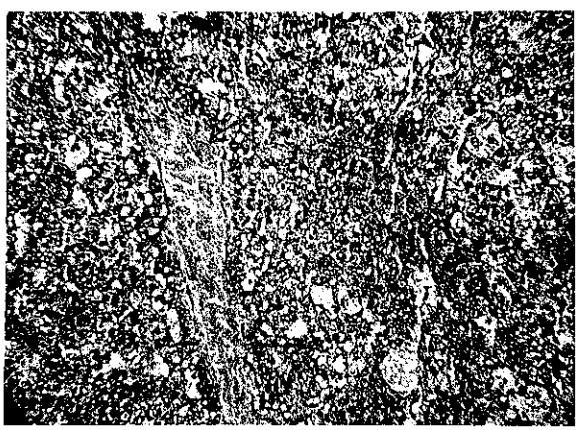

Fig. 5. Photomicrograph of H&E-stained histological section showing renal cell carcinoma (clear cell type). Hemorrhage is seen owing to rupture of dilated vein (arrow) magnification 1:100 (low-power Nikon).

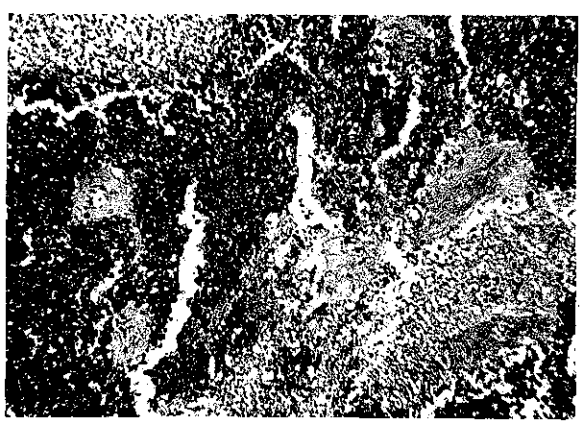

Fig. 6. Photomicrograph of H&E-stained section showing Ewing's sarcoma. Hemorrhage is seen around medium-sized arterioles with necrotizing vasculitis (arrow) and focal necrosis (arrow).

control group consisted of 15 impression smears from normal organs. Negative background fluorescence was observed in all cases. Seropositivity for CIC was 53.33% by combined PEG and LAI assays in those patients who showed deposition of antigen-antibody complexes on tumor cells *in vitro*. On the contrary, seropositivity for CIC was 33.33% in patients who showed deposition of antigen-antibody complexes which also fixed complement. Reduction of CIC by 20% occurred in those cases in which complement-fixing immune complexes were deposited on malignant cells, as demonstrated *in vitro* by IF studies. Hence it is deduced that complement-fixing immune complexes cause complement-mediated cytotoxic injury to malignant cells *in vivo* resulting in a reduction of tumor cell mass and a subsequent decrease in CIC levels.

DISCUSSION

CIC have been detected and quantitated in the sera of cancer patients by many techniques, such as Raji cell assay, C_{1q} and L_{1210} assay.[25-27] However in our study, the PEG precipitation test and LAI test were used. These are relatively simple tests which utilize physicochemical and biological properties of soluble immune complexes for their detection in serum samples from cancer patients.

We found CIC positivity by PEG assay in 47% of cases as compared to 24.6% and 65% seropositivity demonstrated by other workers.[25] As regards the different clinical stages of cancer, PEG index was significantly raised in stage III ($P<0.01$) and highly significantly raised in stage IV ($P<0.001$) as compared to the control group (Table II, Fig. 1). Significant elevation of CIC was also found by PEG assay when the results in stage I were compared with stage III ($P<0.01$) and stage IV ($P<0.001$). These results are consistent with those obtained by other workers.[1-4]

The frequency of positivity by LAI test was 59%, which was significantly higher compared with the results of other authors.[23-26] This finding also corroborated the earlier report that the LAI test could detect soluble complexes formed over an extended range of antigen-antibody ratio, including those as small as 8S.[25] The LAI test failed to detect immune complexes in 41% of cases, out of which the PEG precipitation test was positive in 14%.[27] Hence 14% of sera samples may have IgM antibody which binds complement efficiently. The combination of the PEG precipitation test and LAI test could detect CIC in 75% of cases, whereas individually the PEG technique could detect 47% and the LAI technique 59% of a total of 100 cancer cases studied. Our results revealed significantly increased values of CIC as compared to other workers,[5-21] because even highly complicated and sensitive tests such as Raji cell assay,[2] C_{1q}[23]

Table III (Figs. 7 and 8). Detection of Antigen-Antibody Complexes and C_3 Component of Complement on Malignant Cells *in vitro* by Direct Immunofluorescent Technique

S. No.	Type of malignancy	IF with FITC linked anti-(IgG+IgA+IgM) antisera	IF with FITC linked anti-C_3-antisera	PEG test Seropositivity for CIC=PEG Index 33.16 PEG	Index	LAI test
1.	Ca oral cavity	−	−	20	−	−
2.	Ca maxilla	−	−	40	+	−
3.	Ca larynx	−	−	90	+	−
4.	Ca tongue	−	−	35	+	−
*5.	Seminoma testis	+	−	34	+	+
*6.	Ca Breast	+++	+++	28	−	−
*7.	Ca Breast	+++	+++	85	+	−
*8.	Ca Breast	+++	+++	30	−	−
*9.	Ca Breast	+++	+++	68	+	−
*10.	Chondro-sarcoma (Iliac crest)	++	++	25	−	−
*11.	Ca Ovary	+	+	40	+	+
*12.	Ca Ovary	++	−	43	+	+
*13.	Ca Stomach	++	++	39	+	+
*14.	Liposarcoma	++	++	50	+	−
*15.	Lymphoma	++	++	20	−	−
	Total	11 (73.3%)	9 (60%)	10 (66.6%)		4 (26.6%)
* CIC seropositivity by combined PEG precipitation and LAI tests in patients with positive IF *in vitro*.		53.3%	33.3%			

Fig. 7. Direct immunofluorescence with FITC-linked anti-IgG+IgA+IgM antisera, demonstrating cell-bound immune complexes on imprint smear of breast carcinoma.

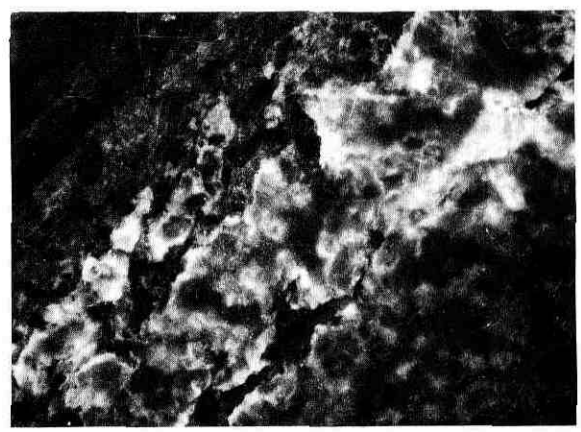

Fig. 8. Direct immunofluorescence with FITC-linked anti-C_3-antisera, demonstrating complement fixation on cell-bound immune complexes on imprint smear of breast carcinoma.

and L_{1210}[26] could only detect specific immune complexes comprising a fraction of the total CIC spectrum. The combination of two simple, sensitive and specific assays, LAI and PEG precipitation, could detect a wider range and an appreciable proportion of total CIC. Other workers[1-21] have studied CIC in cancers of various organs and sites. Different types of assays were used in each study. Hence a valid comparison of data was not possible among different stages and types of cancer studied previously. Our compiled study of 100 cancer cases

using both PEG precipitation and LAI tests for detecting CIC in the same serum increased the sensitivity to 73%. Detection of CIC over an extended range of antigen excess was also possible by using the combined assays. This finding is new, and was not taken into consideration by other workers.[5-21] Although CIC block the antigen effector sites of cytotoxic T lymphocytes, denoting an ineffective afferent limb of cell-mediated immunity, as discussed by other workers,[2,4] we have shown by our study that complement fixation by immune complexes on tumor cells causes complement-mediated cytotoxic injury to cancer cells, resulting in reduction of tumor cell mass *in vivo*, which appears as spontaneous regression of tumors. CIC levels were decreased in 20% of patients showing deposition of complement-fixing immune complexes on malignant cells by IF studies,[28-30] corroborating the significance of complement-mediated cytotoxicity to tumor cells *in vivo*. Immune complex vasculitis[31] caused initially by increased CIC levels *in vivo*, subsequently leads to necrosis in 58% and hemorrhage in 36% of malignant tumors *in vivo*, further reducing the malignant cell load.

A multiple role of CIC in cancer patients has been demonstrated by the finding that CIC levels rise in serum of cancer patients with proliferation of malignant tumors *in vivo*.[5-21] Sensitive detection of a wider immune complex spectrum has significant therapeutic and prognostic importance in monitoring of cancer patients, so the combination of PEG precipitation and LAI tests could be practically useful. CIC also appear to have a role in spontaneous regression of tumors by initiating complement-mediated cytotoxicity and immune complex vasculitis in malignant tumors.

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