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Pre-operative and Post-operative Evaluation of Circulating Immune Complexes in Patients with Gastric Adenocarcinoma.

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SUMMARY **Circulating immune complexes (CICs) in the sera of patients with histologically proven adenocarcinoma of stomach were sequentially studied. Serial CICs levels, quantitated using a sensitive method $F(ab')_2$ anti-C_3 ELISA, were measured before surgery and in a post-operative follow up. CICs could be detected in 85% of the patients pre-operatively, while ten days after surgery positivity decreased to 71%. Thirty days after surgery, the mean CIC levels decreased significantly and positivity fell to 46%. The results indicate that removal of primary tumor mass results in a sharp decline of CIC levels.**

Elevated serum levels of circulating immune complexes (CICs) have been detected in many malignancies [1-4] and have been reviewed by Baldwin and Robin. [1] CICs levels have been reported highest in patients with residual tumor after surgical resection and in those with rapidly progressing malignant disease while there is a tendency for levels to be normal in remission. [1] CICs concentrations have been found to correlate with tumor volume in viral and chemical induced tumors in experimental animals. [5,6] Hellstrom *et al.* [7] have provided indirect evidence that antigen-antibody complexes may be responsible for interference with or blocking of cell mediated tumor immunity. In addition, occasional case reports of immune complex nephritis in malignancy have also appeared. [8] The measurement of immune complexes using sensitive techniques, may therefore, be useful in diagnosis or prognosis of malignant diseases.

The present study was designed to investigate the effect of removal of tumor (antigen) load on the concentration of CICs using a highly sensitive ELISA method in a group of patients with gastric adenocarcinoma and their comparison with normal controls.

MATERIALS AND METHODS

Antisera

Anti-human C_3 was obtained from Behringewerke, Germany and $F(ab')_2$ fragment of anti-C_3 prepared by standard procedures of pepsin digestion and gel chromatography. Horseradish peroxidase conjugated anti-human γ-specific IgG was purchased from Dakopatts, Denmark.

Preparation of aggregated human gamma globulin (AHG) for standard reference curve

Human IgG, purchased from Sigma Chemicals, was dissolved in phosphate buffered saline at a concentration of 10 mg/ml and heated for 18 min at 63° C. The solution was quickly cooled down to 30° C and saturated Na_2SO_4 added at a final concentration of 40 percent to remove monomeric IgG. The solution was centrifuged at 3,000 X g for 30 min and supernatant dialysed against phosphate buffered saline. The dialysate was centrifuged at 100,000 X g for 90 min; the pellet resuspended in PBS and again centrifuged at 3,500 X g for 20 min. The supernatant was collected as aggregated human gamma globulin and concentration determined by ultraviolet spectrophotometry.

Buffers

i) Coating buffer : 0.05M carbonate-bicarbonate buffer pH 9.6

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ii) ELISA buffer: 0.15M phosphate buffered saline pH 7.2 containing 0.05 percent Tween 20, 0.05 percent gelatin and 0.1 percent human serum albumin. iii) Substrate buffer: 0.05M citrate-phosphate buffer pH 5.0 containing 0.004 percent H_2O_2 (v/v). iv) Dilution buffer: 0.05M borate buffered saline pH 8.3 containing 1 percent HSA.

Human sera

The present study included twenty eight patients of pathologically proven gastric adenocarcinoma. The study was performed without selection except those patients who had evidence of widespread disease both pre-operatively or intra-operatively, were excluded from the study. Venous blood was obtained from each patient in the morning before surgery, 10 and 30 days after surgery (POD). Samples were also obtained from twelve age-matched healthy control subjects. All sera were frozen at -70° C and thawed only once before use.

Quantitation of circulating immune complexes (CICs) by modified F(ab')$_2$ anti-C_3 ELISA

The method employed was a modification of Jordon *et al.*[9] Briefly, polystyrene plates (Dynatech, Singapore) were sensitized with rabbit F(ab')$_2$ anti-C_3 by incubating with 200 µl of 30 µg/ml of coating buffer for 2 hr at room temperature followed by overnight incubation at 4° C. The residual sites were blocked by incubation with ELISA buffer for 1 hr at room temperature followed by washing with the same buffer. Serial 2-fold dilutions of AHG and normal human serum were made in dilution buffer to establish a standard dilution curve. Test sera were twice precipitated at a final concentration of 2 percent polyethylene glycol (PEG) and volume made up to original. Dilutions of these PEG precipitated sera (1:10 and 1:20) were made with dilution buffer containing 0.01M EDTA, added to F(ab')$_2$ anti-C_3 coated wells along with AHG/NHS and incubated for 2 hr at 37° C. The wells were washed with ELISA buffer and incubated with 1:1,000 dilution of peroxidase conjugated anti-human IgG for 2 hr at 37° C followed by washing. The development of colour was achieved by incubation with 34 mg of o-phenylenediamine in 100 ml of substrate buffer. The reaction was stopped by the addition of 2M H_2SO_4 and optical density recorded on ELISA processor II using 492 nm filter. A selected positive control serum was included in each run and within-run and between-day coefficient of variation (CV) determined.

Statistics

Student's *t*-test was used for unpaired sera (control and patient) while the paired *t*-test was utilized for paired sera (pre-operative and post-operative).

RESULTS

The age range was 28-54 years (mean = 41 ± 9) for twelve healthy controls and 36-60 years (mean = 49 ± 8) for twenty-eight patients with adenocarcinoma of the stomach.

The modified F(ab')$_2$ anti-C_3 ELISA was used and found to be sensitive with the AHG/NHS system at 10 µg/ml of serum. The test was specific since no binding to monomeric IgG was observed. Reproducibility was good as the within-run and between-days CV for control serum was 3 percent and 6 percent respectively. After establishing a reference curve (Fig. 1) with AHG/NHS, the CICs were quantitatively estimated in serum samples from control subjects and gastric adenocarcinoma patients subjected to curative resection of the primary mass. Results are expressed in µg equivalent of AHG/ml.

Fig. 2 shows the results obtained for CICs using the F(ab')$_2$ anti-C_3 ELISA for controls, pre-operative patients and the 10 day and 30 day post-operative values of carcinoma patients. The CIC values for the normal healthy control group ranged from 11.0 to 21.0 µg/ml with a mean value of 16.5 ± 3.44 and a value of 23.38 µg was considered as positive, i.e. 2 standard deviations above the mean value of normal sera tested. Eight-five percent of the patients had elevated levels of CICs on the pre-operative day with a mean value

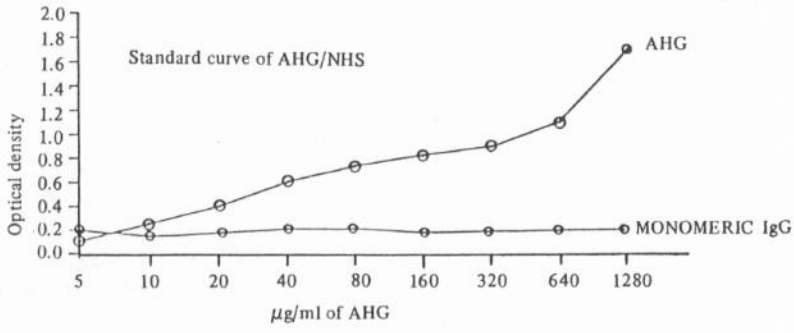

Fig. 1 Standard curve for aggregated human gammaglobulin in human serum (AHG/NHS) obtained as binding of horse raddish peroxidase conjugated anti human IgG to increasing amounts of AHG/NHS previously bound to F(ab')$_2$ anti–C_3. Binding of monomeric IgG/NHS is also shown along with. Details are described under methods.

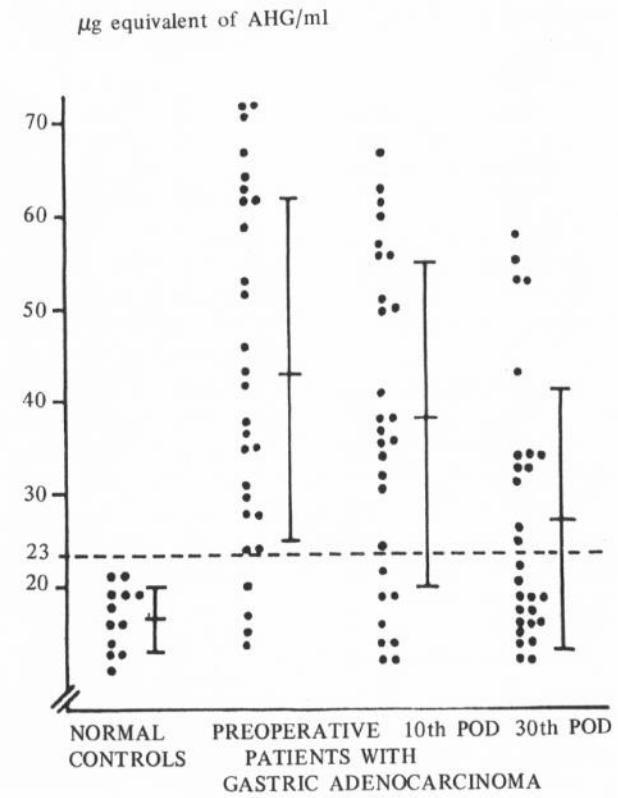

Fig. 2 Levels of circulating immune complexes measured by F(ab')$_2$ anti–C3 ELISA in control subjects and gastric adenocarcinoma patients on pre–operative, 10th and 30th post–operative days (POD). A positive value was defined as 23.38 μg/ml and is indicated by broken lines.

of 43.0 ± 18.8 μg/ml (range = 13.5-72.0). The 10 day post-operative value showed a declining trend and the percentage of positivity fell to 71 percent with a mean value of 37.71 ± 17.5 μg/ml (range = 12.0-66.5). The 30 day post-operative value fell to a significantly low mean of 27.0 ± 14.1 μg/ml (range = 12.0-57.5) and the positivity fell to 46 percent.

Mean pre-operative levels showed significant variation ($P < 0.01$) as compared to normal control values. When 10 and 30 day post-operative values were compared to their corresponding pre-operative values using the paired *t*-test, differences were found to be statistically significant ($P < 0.01$) and highly significant ($P < 0.001$) respectively.

DISCUSSION

CICs have been quantitatively estimated in different classes of carcinoma patients by various methods like PEG precipitation [10] and C1q binding assays. [11,12] However, all theses methods suffer from the disadvantage of non-specificity and false positive results, [13] thereby presenting a serious limitation to the use of CICs as a prognostic or diagnostic tool in malignant diseases. Raji cell ELISA was found to be equally sensitive, specific and as reproducible as that of Raji cell RIA. However, the F(ab')$_2$ anti-C$_3$ ELISA, which did not require culture of Raji cells, was less sensitive than the Raji cell RIA (serum CICs detected at 16 μg/ml in the ELISA compared to 9.6 μg/ml in the RIA). [9] In the present study, the introduction of an additional step of PEG precipitation of sera for CICs analysis was found to enhance the sensitivity of the assay system (10 μg/ml). Since PEG precipitates only CICs and not the uncomplexed C$_3$ (data not shown), inhibition of binding of uncomplexed C$_3$ to F(ab')$_2$ anti-C$_3$ was avoided. The modified assay system was used to analyse sera from patients of gastric adenocarcinoma subjected to surgical resection. This assay was sensitive enough to distinguish pathological sera from normal sera (none of the normal sera gave a positive value).

Our results demonstrate that CIC levels were elevated in patients with gastric adenocarcinoma as compared to age matched normal controls. Elevated CICs have been reported in colorectal carcinoma [12] and gastric carcinoma patients. [11] Surgical resection resulted in return to normal values for a few patients at 10 day post-operative while the majority of patients showed CIC values in the normal range at 30 day post-opeative. There is now considerable evidence for elevated CICs during tumor-growth [14,15] and that CICs decrease after removing the antigenic load [16] or during remission. [1] Our observations were consistent with reports of elevated CIC levels with advanced disease or relapse and the occurance of normal levels during remission. [14-16] However, observations of relative decrease in CICs during tumor metastasis [17] have been explained on the basis of decreased antibody production in the immunocompromised host with a large tumor burden.

The role of CICs in cancer remains uncertain. Immune complexes were shown to interfere with antigen recognition and inactivation of various effector cells after antigen recognition. [18] Immunosuppressive effects of CICs *in vivo* were found to be related to antibody Fc receptors. [19,20] Other studies have reported

that CICs may suppress the antigen specific antibody synthesis by B-cells.[21] CICs were also reported to inhibit T-cell and B-cell cooperation in T-dependent antibody production.[22] All the above observations point towards an implied role for immune complexes in modulating cell to cell interactions in host immune response to any immunogenic tumor.

Presently, a larger study with an extended follow up is underway to characterize tumor specific antigens from CICs and to more thoroughly evaluate the use of CICs as a prognostic predictor for tumorigenic patients.

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