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ANTICOMPLEMENTARY ACTIVITY IN HUMAN SERUM OF LUNG CANCER PATIENTS AND ITS POSSIBLE CLINICAL SIGNIFICANCE

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Objective: To identify whether the level of anticomplementary activity in serum is different between lung cancer patients and normal subjects. Method: With a sensitive immune haemolytic assay, the anticomplementary activity in the sera of 50 normal subjects and 61 lung cancer patients were demonstrated. Results: It was found that all samples contained complement-inhibition activity, although some were of low degree. Increased anticomplementary activity was found to be associated significantly with lung cancer. With the progression of cancer the anticomplementary activity in sera increased in different lung cancer patients. Conclusion: Such a higher anticomplementary activity in sera of lung cancer patients might be one of the immunosuppressive contents induced by the tumor cells.

Key words: Anticomplementary activity, Lung cancer, Immunosuppression.

The immunosuppression induced by tumor cells has been demonstrated to be an important mechanism for tumor cells escaping from immunologic rejection.[1] Many reports have demonstrated the immunosuppressive properties induced by tumor cells that affect both B and T cell function. Recently Kumar et al. using a combination of Northern blot and RT-PCR assay have found that in some cells and tumors mRNAs for complement inhibitors are overexpressed.[2,3] They compared the expression of mRNAs coding for three complement inhibitors, i.e., complement cytolysis inhibitor(CLI), decay accelerating factor(DAF), and CD59, in normal tissues with that in human tumor cells and solid tumors and found that the distribution of mRNAs for complement inhibitors in tumor cell lines is somewhat different from that in normal tissues. Another evidence indicated that the expression of these mRNAs shows a mutually exclusive relationship between the expression of CLI and that of CD59 or DAF, with the possible exception of the lung, where all these mRNAs are equally expressed. Although there were significant differences between normal and tumor cells in terms of distribution of these mRNAs coding for these complement inhibitors,[2] the following questions are still worthwhile to be noticed: 1. Whether the level of anticomplementary activity in serum is different between the sera of patients with lung cancer and that of normal subjects? 2. Do the level of anticomplementary activity in sera of lung cancer patients change with the time course of disease? and what are, if any, these changes? To clarify these questions will be of significance in evaluating the results of anticomplementary activity in sera of lung cancer patients.

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MATERIALS AND METHODS

Serum

Serum samples from patients with lung cancer (PLC, n=61; age, 50±19.5 years; male/female, 49/12)

were collected from those inpatients at the General Hospital of Taiyuan Iron and Steel Company. All the patients were consistent with the diagnostic criteria of lung cancers.[4] For comparison, sera from healthy subjects (HS, n=50; age, 41±12.5 years; male/female, 40/10) were obtained from healthy blood donors of Taiyuan Central Blood Bank. All the HS were verified to be in good health by routine examination including thoracic roentgenography liver and renal function tests, etc. The characteristics of HS and PLC are shown in Table 1. All of the serum samples were heated at 56℃ for 30 min and stored at -30℃.

Table 1. Characteristics of healthy subjects and patients with lung cancer ($\bar{x}\pm s$)

	Healthy subjects	Patients of LC
Number	50	61
Age (years)	41± 12.5	50± 19.5
Sex (F/M)	40/10	49/12
Stage I*	–	3
Stage II*	–	25
Stage IIIa*	–	17
Stage IIIb*	–	6
Stage IV*	–	7
BLT	–	3

LC: lung cancer; F: female; M: male; BLT: benign lung tumor. *Staging of lung cancer by 4th World Conference of Lung Cancer.

Preparation of Standard Inactivated Sera

The sera of PLC, containing 30 individual samples, were pooled, inactivated at 56℃for 30 min and used as standard inactivated sera (SIS) in the studies. The pooled SIS were centrifuged at 2000 g for 10 min, then the supernatants were divided into small aliquots and stored at -30℃ for use.

Haemolysin

Rabbit antisheep erythrocyte antiserum was prepared as described by Kabat and Mayer.[5] The antiserum was heated at 56℃ for 30 min and added glycerin at 1:1 dilution to the antiserum. The rabbit anti-SRBC glycerinated haemolysin was stored at 4℃ and used it at 1:2000 dilution in barbitone-buffered saline (BBS).[5]

Red Blood Cells

Sheep red blood cells (SRBC) were collected by venepuncture via jugular vein and added to Alsevers solution. The blood was stored at 4℃for two weeks for later use. The cells were washed three times and made to 3% solution (v/v) in BBS prior to assay.

Complement

A pooled fresh guinea pig sera was aliquoted for use as the complement source in this study. The sera were adsorbed three times with washed SRBC and frozen at -70℃. In the present experiment, guinea pig complement was used, diluted appropriatly in BBS to achieve an optimal 75% control heamolysis.

Micro-haemolytic Assay

This assay was used to characterize the anti-complementary activity in sera of human, and its relationship to lung cancer. Based on the micro-haemolytic assay, we added inactivated serum (IS) in the reactance. 3% SRBC were sensitized optimally with hemolysin for 30 min at 22℃. Assay was performed in U-shaped of a polyvinylchloride micro-titration plate (Flow Laboratories, Mclean, VA, USA). The required amount of serum and complement were put in each well and the volume was adjusted to 150 μl with BBS. 50 μl of the sensitized SRBC was then added to each well with the plates occasionally agitated and incubated at 37℃ for 1 h. The method of feeding is shown in Table 2. Following centrifugation at 4℃ (500 g for 10 min), 100 μl of each supernatant was transferred to flat bottomed wells of polystyrene micro ELISA plates (Dynatech, Sydney). The optical densities were measured at 410 nm using a microplate reader (Moleuclar Devices Corp. Menlo Park, CA. USA). The percentage of haemolysis was calculated using equation 1.

$$\% \text{ lysis} = \frac{\bar{x}\,(WCH_{75} \text{ or } EW) - \bar{x} \text{ blank control}}{\bar{x}\,WCH_{100}} \times 100 \quad (1)$$

Where $\bar{x}$ is the mean optical density of each triplicate; WCH_{75} is the OD value of 75% sensitized SRBC haemolytic well; EW is the OD value of the experiment well; WCH_{100} is the OD value of 100% sensitized SRBC haemolytic well. The percentage of

inhibition of haemolysis was calculated using equation 2.

$$\% \text{ Inhibition} = \left[1 - \frac{\% \text{ lysis experiment well}}{\% \text{ lysis } CH_{75}}\right] \times 100 \quad (2)$$

Where the percentage of lysis is calculated using equation. Whether SIS has a inhibitic effect and to what extent it can inhibit the complement are indicated by the variation of haemolysis %, the anticomplementary activity is expressed as inhibition %.

Table 2. The method of feeding for micro-haemolytic assay (μl)

Factors	EW	BC	ECW	WCH_{75}	WCH_{100}
BBS	50	150	100	100	100
Complement $(CH_{75})^*$	50	–	50	50	–
Complement $(CH_{100})^*$	–	–	–	–	50
Serum (1:10)	50	–	50	–	–
SSRBC	50	50	–	50	50

EW: experiment well; BC: blank control; EC: experiment control; WCH_{75}: well of 75% SSRBC haemolysis; WCH_{100}: well of 100% SSRBC haemolysis; BBS: barbitone-buffered saline; SSRBC: 3% sensitized sheep red blood cells; *Complement concentration to cause 75% SSRBC haemolysis; **Complement concentration to cause 100% SSRBC haemolysis.

Studies Using the Micro-haemolytic Assay

Using the micro-haemolytic assay, the following studies were made: 1. The mean and SD of inhibition % for SIS: Micro-haemolysis assay was performed with SIS once a day for 20 days calculating the means and standard deviations of inhibition %. 2. Optimal temperature for anticomplementary activity: Incubation of the complement with SIS was performed at 4℃, 25℃, 37℃ for 25 min prior to use in the assay. 3. Stability of anticomplementary activity: SIS was heated at 56℃for 30 min, or frozen rethawed five times prior to use in the assay. 4. Effect of SIS on the ability of SRBC to bind haemolysin: SRBC were incubated with SIS for 30 min at 37℃, washed twice with BBS prior to sensitization, and use in the assay. 5. Effect of SIS on the ability of sensitized SRBC to fix complement: Sensitized SRBC were incubated with SIS for 30 min at 37℃, and washed twice prior to the addition of complement in the assay. 6. Species specificity of anticomplementary activity: The assay was performed using human, rabbit, mouse and guinea pig sera as the complemen source, respectively.

Statistical Analysis

The percentages of inhibition of complement were normalized by square root antisine transformation, then the significance of group differences determined by analysis of variance. Significance of differences between each two groups in many groups was calculated using q-test, and that between two groups was analysed using Student's t-test.

RESULTS

Level of Anticomplementary Activity

Anticomplementary activity was found to be present in 111 samples of normal and abnormal human sera, with the percentage complement inhibition ranged in 12% to 44%. Figure 1 shows the scatter diagram of inhibitory percentages for these samples. A significant increase for anticomplementary activity was seen in sera of lung cancer patients as compared with that of the HS. There were no significant differences in the level of anticomplementary activity between HS and BLT.

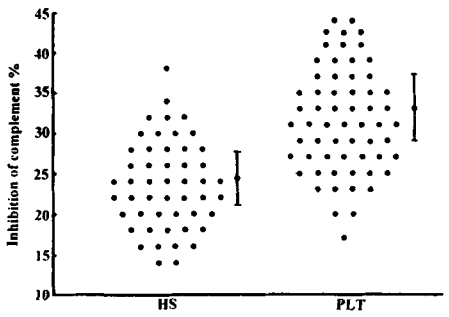

Fig. 1. Inhibition of complement in III sera
HS: health subjects; PLT: patients with lung tumors; ECP: esophageal cancer patients.

Changing Trend of the Percent Inhibition of Haemolysis in Sera from Lung Cancer Patients

with the Progressive Stage of Cancer

Sixty-one patients with lung tumor were divided into six groups according to the grading criteria of lung cancer.[4] It was shown that there was an increasing tendency of the inhibition % of haemolysis by complement in sera of lung cancer patients with progression of the cancer. The level of anticomplementary activity in each group are summarized in Table 3.

Characteristics of SIS Anticomplementary Activity

The results of the studies using the microhaemolytic assay were as follows:

The Mean and SD of Inhibition % for SIS

The mean and SD of inhibition % for SIS dirived from 20 assays were 31.5±0.05 and then the 31.2% of inhibition for SIS as 100% in the following studies.

Table 3. Anticomplementary activity present in the sera from lung cancer patients with different grades and HS

Groups	Number	Inhibition % of haemolysis	
		$\bar{x}^a$	s^b
HS	50	25.1	0.16
BLT	3	26.4	0.19
Stage I of LC	3	27.8	0.18*
Stage II of LC	25	30.5	0.26**
Stage IIIa of LC	17	33.4	0.24**
Stage IIIb of LC	6	34.9	0.19**
Stage IV of LC	7	36.0	0.21**

[a] Mean percentage of inhibition calculated using equation 2. (see Materials and Method);

[b]SD, Standard Deviation; *$P<0.05$ as compared with HS; **$P<0.01$ as compared with HS; BLT: Benign Lung Tumor; LC: Lung Cancer

Optimal Temperature for Anticomplementary Activity

For complement/SIS, at incubation temperature of 4℃, 65% inhibition % haemolysis occurred; while at 37℃, 100% inhibition occurred.

Stability of Anticomplementary Activity

No loss of anticomplementary activity was measurable after heating SIS at 56℃ for 30 min, or after five cycles of freezing and rethawing.

Effect of SIS on the Ability of SRBC to Bind Haemolysin

The degree of haemolysin was unaffected by the treatment of SRBC with SIS prior to sensitization.

Effect of SIS on the Ability of Sensitized SRBC to Fix Complement

No significant difference was observed in the ability of sensitized SRBC treated with SIS to fix complement, when compared with complement fixation by untreated sensitized cells.

Species Specificity of Anticomplementary Activity

SIS was shown to have anticomplementary activity against pooled rabbit, mouse and guinea pig complement ten times, three times and twice as effective as against pooled human complement, respectively.

DISCUSSION

One of the physiological roles of the complement system is the lysis of foreign cells, including virus-infected and tumor cells.[6] This cytolytic process is activated by the interaction of more than twenty plasma proteins in two pathways: the classical pathway that is triggered by antigen-antibody complexes and the alternative pathway that is triggered by foreign surfaces, including that of tumor cells.[6] The final steps in the binding of complement proteins to the cell membrane are common to both pathway and consist of the sequential assembly of the C5b, C6, C7, C8 and C9 components into the membrane attack complex (MAC). Then this poreforming MAC inserts into the lipid bilayer of the plasma membrane and causes cell lysis.[6] But, when tumor cells occurred, overexpression of complement inhibitor mRNAs by tumor cells might lead to overexpression of the corresponding proteins. Among them all the CLI, DAF and CD59 could prevent the assembly of MAC and contribute to the

resistance of tumor cells to complementmediated cytotoxicity.[7-9] Perhapes it is one of the main reasons that result in preventing some tumor cells from the defence action of complement in serum of the host. In the study by Kumar S. et al.[2] they speculated that the overexpression of mRNAs coding for three complement inhibitors may be lead to production of the corresponding proteins. But their studies were limited to an analysis of mRNA levels, and hence the results obtained could not be immediately extrapolated to the expression of the corresponding proteins. Consequently, it is not able to assess the level of anticomplementary activity through detection of complement inhibitors during development of cancer cells. The method we set up in this study may fill up this defect. With the use of sensitive micro-haemolysis assay, we can detect directly the level of anticomplementary activity in sera of patients with lung cancer.

In the present study, we have demonstrated that the anticomplementary activity in the sera of PLC was increased significantly, which may account for the resistance of cancer cells to complement-mediated cytolysis. It is commonly considered that the mechanisms of immuno-rejection for cancer cells are very complicated and far less clarified. In our study, we have found that the level of anticomplementary activity in serum of PLC began to increase even in the early stage of disease and increased significantly there after with deterioration of pathologic state. These results suggest to us that the complement-inhibitors found in the sera of patients with cancer may also play an important role in tumor immunity. The increase of anticomplementary activity might be one of the mechanisms responsible for the immuno-suppression produced by tumor cells and thus could provide an effective defence from host attack by ADCC for tumor cells. The role of anti-tumor antibodies has long been controversial. Some authors regarded the anti-tumor antibody as a blocking one which does not combine with complement. However, from the studies by Kumar et al. and ours, there is another possibility that the production of anticomplementary factors induced by tumor cells might also be related to restrict the action of anti-tumor antibody. According to this hypothesis, it might be beneficial to attach the use of antibodies to complement inhibitors in the treatment of tumors with anti-tumor antibodies.

Another purpose of this study is to characterize the nature of the serum anticomplementary factors and the mechanism of their action. SIS was shown to be definitely complement inhibitory and species-specific. In addition, it was demonstrated that the anticomplementary factors in serum is not madiated by masking antibody binding sites or complement binding sites.

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