

Detection of Antibody and Complement Complexed *in Vivo* on Membranes of Human Cancer Cells by Mixed Hemadsorption Techniques¹

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SUMMARY

The mixed hemadsorption (MHA) techniques demonstrated antibody and complement fixed *in vivo* to the surface of human cancer cells. Tumors from 12 cancer patients and normal tissues from 5 cancer patients and 8 patients with cerebrovascular or cardiac diseases were collected from biopsy and autopsy for *in vitro* testing. Antiserum to human whole immunoglobulins and antiserum to human C3 were used in the MHA techniques. Positive MHA patterns were demonstrated on the surface of cancer cells by both methods. Positive reactions ranged from 12 to 32% in mixed hemadsorption for antibody detection and from 10 to 34% in mixed hemadsorption for complement component 3 detection. Normal tissues obtained from cancer patients or from patients who died of causes other than cancer rarely exhibited distinct MHA reactivity.

Collectively, the data suggest that most human cancers are antigenic in the autologous host and that tumor-associated antigens of cancer cells react *in vivo* with their humoral antibody to fix complement.

INTRODUCTION

In the past several years, evidence has accumulated indicating that an *in vivo* immune reaction occurs on membranes of cancer cells. The *in vivo* coating of animal tumor cells with antibodies has been shown by various methods including bound C1 studies (22), absorption of isotope-conjugated globulin (20, 21), and antibody-elution techniques (14, 15, 17). Similar results were obtained in human tumor systems by membrane immunofluorescence (6) and antibody-elution techniques (2, 13, 19).

Recently, we demonstrated the *in vivo* reaction of anti-

body and complement to surface antigens of human cancer cells using the IA tests (3, 4). In these studies, cancer cells obtained directly from biopsy or autopsy showed positive IA reactivity without adding serum or complement. The reactivity was enhanced by the addition of purified human C2 and C3. Normal cells from patients who had nonmalignant disease did not show the reactivity. When SRBC that do not carry IA receptors were used as the indicator cells instead of human erythrocytes, the cancer cells did not show the reactivity.

To confirm these earlier findings, we again studied human cancer cells obtained from biopsy and autopsy by the MHA techniques. This highly sensitive immunoassay (16, 18) detected the antibody and complement separately and confirmed our previous findings.

MATERIALS AND METHODS

Preparation of Single Cells from Biopsy and Autopsy Materials

Twelve tumor specimens from cancer patients, 5 normal tissue samples from cancer patients, and 8 normal tissue specimens from patients with cardiovascular or cerebrovascular diseases were obtained at biopsy or autopsy. Surgical specimens were acquired from the operating room immediately after removal and were stored at 0–4°. Autopsy specimens were collected within 4 to 5 hr of death and were stored in a like manner. All specimens were further processed within 2 to 3 hr after receipt. Single cells from these specimens were prepared and stored as previously described (4, 9).

Mixed Hemadsorption Techniques

Ab-MHA. The basic technique described by Fagraeus and Espmark (1) and Metzgar *et al.* (8) for mixed hemadsorption in tissue culture was modified to detect antibody on the surface of suspended single cells. Single cells stored in a –190° liquid nitrogen freezer were thawed rapidly at 37°, washed once with Roswell Park Memorial Institute Medium 1640 (Microbiological Associates, Bethesda, Md.) containing 20 to 40% human agamma serum (prepared by

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Cohn cold ethanol technique; Biocell Laboratory, Culver City, Calif.) and 3 times with HA-VB⁵, and resuspended in HA-VB for use as target cells. HA-VB was used as the diluent for the further procedures. The indicator system was prepared as follows. SRBC adjusted to 2.0% concentration in HA-VB were mixed with an equal volume of serially diluted baboon antiserum to SRBC and incubated at 37° for 60 min. The dilution of the baboon antiserum to give the subagglutination of SRBC, *i.e.*, $\frac{1}{32}$, was determined by the observation of the reactive mixture under a microscope. SRBC of 2% concentration, sensitized by baboon anti-SRBC serum diluted 32 times, were washed 3 times and adjusted to 0.5%. The sensitized SRBC were added to an equal volume of serially diluted goat antiserum to human immunoglobulins (Cappel Laboratories, Inc., Downingtown, Pa.), incubated at 37° for 60 min, washed 3 times, and adjusted to 0.5%. The optimal dilution of the goat antiserum to human immunoglobulins was determined next by using a model system composed of human cultured cells and human antibody to these cells. The SRBC sensitized by baboon anti-SRBC and serially diluted goat anti-human immunoglobulins (SRBC-baboon γ -globulin-goat anti-human γ -globulin) were added to an equal volume of cultured human sarcoma cells (1×10^6 /ml) complexed by human anti-heterologous membrane antigen (5) antibody. After incubation at 37° for 10 min with agitation and for 20 min without agitation, the hemadsorption patterns were observed under a microscope. The optimal dilution (1/128) that gave the strongest pattern was determined. The lower dilution of antiimmunoglobulins decreased the hemadsorption pattern because of the increased agglutination of the indicator erythrocytes themselves.

The indicator erythrocytes were prepared in this manner. One-fortieth ml of the 0.5% indicator erythrocytes was added to 0.05 ml of the target cell suspension containing 2.5×10^4 cells and was incubated at 37° for 10 min with agitation and for 20 min without agitation. The MHA patterns were determined by microscopy as the percentage of target cells that positively adsorbed 2 or more indicator cells whether single or clumped in a count of 50 target cells. Any contaminating lymphocytes could be excluded from the count of each tumor cell sample because of their obvious differences in morphology and size.

C3-MHA. In the C3-MHA test, target cells were prepared just as described for the Ab-MHA test, except that the cells were washed and resuspended in glucose-gelatin-Veronal buffer (10) instead of HA-VB. Glucose-gelatin-Veronal buffer was used as the diluent for the further procedures. The indicator system was prepared as follows. SRBC were treated by a 1/750 dilution of a rabbit antiserum to SRBC and conjugated with human complement C1 and 4 according to the method described by

⁵ The abbreviations used are: IA, immune adherence; SRBC, sheep erythrocytes; MHA, mixed hemadsorption; Ab-MHA, mixed hemadsorption for antibody detection; HA-VB, 0.05 M Veronal-buffered 0.87% NaCl solution (pH 7.5) containing 0.2% human serum albumin (Merck, Sharp & Dohme, West Point, Pa.), 0.00015 M CaCl₂, 0.005 M MgCl₂, 0.0057 M KCl, and 0.1% glucose; C3-MHA, mixed hemadsorption for complement component 3 detection; Sh.EAC 1423, sheep erythrocyte coated by rabbit antiserum coated by human complement components 1, 4, 2, and 3.

Nishioka and Linscott (11) except that fresh human cord serum was used as the complement source instead of fresh guinea pig serum. The SRBC coated by rabbit antiserum and C1 and C4 were adjusted to 2.0% concentration and were mixed with an equal volume of purified human complement C2 and C3 (Cordis Laboratories, Miami, Fla.) at 200 units of each component per ml. The reactive mixture was incubated at 30° for 30 min. After incubation, the Sh.EAC1423 cells were washed 2 times and resuspended to 0.5% concentration. The optimal dilution of goat antiserum to human β 1C globulin (C3) (1/16) was determined in the same manner as described for Ab-MHA technique using complexes of cultured human sarcoma cells/anti-heterologous membrane antigen antibody/human C1, C4, C2, and C3 as the model target. The Sh.EAC1423 cells of 0.5% concentration were sensitized with an equal volume of a 1/16 dilution of a goat antiserum to human β 1C globulin by incubation at 37° for 60 min, washed 2 times, and resuspended to 0.5% concentration (Sh.EA1423-anti- β 1C cells).

The C3-MHA test was performed as described above for the Ab-MHA test.

RESULTS

Ab-MHA on Cancer Cells and Noncancer Cells. The results of the Ab-MHA test on tumors and nonmalignant tissues are shown in Table 1 and Fig. 1. The percentage of MHA-positive cancer cells ranged from 12 to 32%, while that of the nonmalignant cells was 0 to 4% at the most. In cell preparations of R. C. melanoma, D. H. osteosarcoma, R. M. liposarcoma, R. H. osteosarcoma, and R. P. normal spleen, rosette formations of sensitized SRBC were observed around a certain number of lymphoid cells contained in the cell preparations. The rosette formation occurred only with sensitized SRBC even in the absence of anti-human immunoglobulins, whereas MHA patterns on cancer cells were completely negative without the antiimmunoglobulins. Nonsensitized SRBC did not cause either MHA or rosette formation with or without anti-human immunoglobulins.

Differentiation between tumor cells and lymphoid cells was pathologically determined after the preparation was stained with Wright's solution. The MHA reactivity was not changed by the staining technique (Fig. 1).

C3-MHA on Cancer Cells and Noncancer Cells. The results of the C3-MHA test are also shown in Table 1 and Fig. 1. The percentage of MHA-positive cancer cells ranged from 10 to 34%, whereas that of the normal cells was negligible except for 1 sample (R.P. spleen, 14%). Sh.EAC1423 cells not pretreated by anti- β 1C globulin did not cause the MHA reactivity either on cancer cells or on noncancer cells, but they caused rosette formation around 6% of the R. P. spleen cells. Neither SRBC coated by rabbit antiserum cells nor SRBC cells were adsorbed by cancer cells with or without anti- β 1C globulin.

The reactivity of both Ab-MHA and C3-MHA was tested against the tumor cells from 5 cancer patients and against autologous normal tissues adjacent to or distant from their tumor sites. The MHA reactivity of the normal tissues was

low or negative in both tests, whereas distinct MHA reactivity was observed on tumor cells from these patients (Table 2). These results suggest that the fixation of antibody and C3 to the surface of cancer cells was indeed tumor associated.

DISCUSSION

Two types of the mixed hemadsorption techniques (Ab-MHA and C3-MHA) demonstrated the presence of antibodies and C3 on membranes of cancer cells obtained from biopsy and autopsy tissues. These findings were previously suggested in IA studies (3, 4). The results indicate that there are complement-fixing antibodies on the surface of cancer cells.

Before starting this study, we assumed that the reactivity of the C3-MHA on the surface of cancer cells would be the same as or less than that of the Ab-MHA because C3 fixes to antigen/antibody/C1,4,2 complexes; nevertheless, the reactivity of C3-MHA was higher than that of Ab-MHA in

most cases [exceptions, J. A. and S. N. melanomas and D. H. osteosarcoma (Table 1)]. These results might be explained as follows: (a) the sensitivity of C3-MHA might be greater than that of Ab-MHA because a single molecule of antibody could fix multiple molecules of C3 in the presence of C1, 4, and 2 (7); (b) nonspecific incorporation of β 1C globulin into membranes of cancer cells (12) might have occurred and could be responsible for the high C3-MHA reactivity; (c) despite the fact that MHA detect IgM more sensitively than IgG (16), the Ab-MHA technique used in this study might have detected IgG primarily because the antisera to SRBC were obtained from a hyperimmunized baboon and contained a high proportion of IgG (agglutination technique; K. Irie, unpublished data). Theoretically, the C3-MHA technique should be able to detect not only C3 fixed by IgG antibodies but also C3 fixed by IgM antibodies on the surface of cancer cells.

The relationship of antibodies and complement on cancer cells to the clinical course and prognosis of cancer patients remains to be determined. However, tests using antibodies specific to human IgG, IgM, and IgA might characterize

Table 1
Ab-MHA and C3-MHA reactivity of cancer cells and noncancer cells

Biopsy or autopsy	Patient	Diagnosis	Organ	Ab-MHA (%)	C3-MHA (%)
B	J. W. A.	Melanoma	Lung metastasis	22	30
B	J. F.	Melanoma	Lung metastasis	28	34
B	J. A.	Melanoma	Lymph node metastasis	18	18
A	R. C.	Melanoma	Lymph node metastasis	12	16
B	S. N.	Melanoma	Spleen metastasis	32	18
B	D. H.	Osteosarcoma	Left humerus	22	12
B	S. Z.	Synovial sarcoma	Left elbow	12	16
B	R. M.	Liposarcoma	i.p. metastasis	18	22
A	R. H.	Osteosarcoma	Retroperitoneal metastasis	12	18
B	M. V.	Carcinoma	Pancreas	24	26
A	G. V.	Hodgkin's disease	Liver metastasis	14	16
B	G. M.	Ganglio-neuroblastoma		16	22
A	V. W.	Cerebrovascular accident	Liver	2	0
A	M. C.	Tetralogy of Fallot	Liver	0	0
A	M. M.	Heart attack	Liver	0	0
A	J. H.	Coronary insufficiency	Spleen	0	0
A	M. V.	Heart attack	Spleen	0	2
A	R. P.	Tetralogy of Fallot	Spleen	4	14
A	R. W.	Cerebrovascular accident	Kidney	0	0
A	W. B.	Cerebral hemorrhage	Kidney	0	0

Table 2
MHA reactivity of cancer cells and autologous normal cells

Biopsy or autopsy	Patient	Diagnosis	Cancer cells		Normal cells			
			Organ	Ab-MHA (%)	C3-MHA (%)	Organ	Ab-MHA (%)	C3-MHA (%)
A	R. H.	Osteosarcoma	Retroperitoneal metastasis	12	18	Liver	4	0
A	T. R.	Reticulum cell sarcoma	Cervical lymphnode metastasis	18	16	Spleen	2	0
A	L. M.	Breast carcinoma	Kidney metastasis	14	12	Kidney	0	0
B	S. N.	Melanoma	Spleen metastasis	30	20	Spleen	6	4
A	C. B.	Melanoma	Chest wall metastasis	16	12	Kidney	0	0

the nature of the antibodies fixed on the surface of cancer cells. Antibodies against complement components other than C3 could detect each component fixed on cancer cells using the MHA techniques. Furthermore, a detailed study of complement components on cancer cells would be helpful in the analysis of *in vivo* complement-dependent cytotoxicity of cancer cells and to define the interaction between complement components and lymphocyte cytotoxicity. Thus would the clinical implications and the prognostic significances of antibody and complement on the surface of cancer cells be more clearly understood.

As described in "Results," a certain number of lymphoid cells contaminated the tumor cell preparations and showed rosette formation when exposed to sensitized SRBC in several cases. This phenomenon needs further study to characterize these lymphoid cells (B-cells or mononuclear cells such as macrophages?), to ascertain the interaction between these lymphoid cells and tumor cells coated by antibodies and complement, and to understand better the relationship between this interaction and the cancer patient's prognosis.

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Fig. 1. Mixed hemadsorption pattern of J. F. melanoma cell. Wright's solution, $\times 800$. A, positive pattern of Ab-MHA; B, positive pattern of C3-MHA; C, negative pattern of Ab-MHA without anti-immunoglobulins. Negative pattern of C3-MHA without anti- β 1C globulin was essentially the same.

