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10. The discrepancy between the duration of PAD and of the inhibitory effect has at least two possible explanations. First, interneurons like the one tested probably receive a mixture of pre- and postsynaptic inhibition (*5*), and the latter may involve long polysynaptic pathways. Second and more likely, although the presynaptic conductance change measured in our experiments is a possible mechanism for presynaptic inhibition, it is not necessarily the only one. If, for example, inhibition of transmitter release involved changes in the amount of C^{2+} available at the terminal, the effect could outlast measurable changes in membrane potential or conductance.
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Evidence for in vivo Reaction of Antibody and Complement to Surface Antigens of Human Cancer Cells

Abstract. *The immune adherence test was used to determine whether antibody and complement in cancer patients are fixed in vivo to tumor cells. Human erythrocytes adhered in vitro to the surface of human cancer cells obtained from autopsy and biopsy. Adherence was enhanced by further addition of the C2 and C3 components of complement, and was diminished by preliminary treatment with antibody to C3 (that is, to β1C-globulin). The results suggest that tumor associated membrane antigens form complexes in vivo with antibodies and complement.*

Recent studies have shown that malignant human tumors possess tumor specific surface antigens and that patients with tumors do produce humoral antibodies against these antigens (*1*). Various immunologic techniques indicated that humoral antibodies from the serums of cancer patients react with antigens on the membranes of cancer cells in vitro. However, it has not been established whether such reactions take place in vivo. In fact, it has been suggested that antibodies cannot cross the capillary barrier in sufficient amounts to influence tumor growth patterns.

We assumed that, if tumor associated antigens formed complexes with antibody and complement on the membranes of cancer cells in vivo, such complexes would be detectable by the immune adherence (IA) phenomenon in vitro. Ever since Nelson's discovery that normal human erythrocytes adhered to complexes of antigen, antibody, and complement on a cell membrane (*2*), this sensitive immunoassay has been used to detect and characterize membrane antigens in animal systems (*3*) and, more recently, in human systems (*4*). We have tested the in vitro reactivity of various human cancer cells obtained from biopsy and autopsy.

Biopsy specimens from 12 patients with various malignancies and normal tissues from 8 patients who died of cerebrovascular or cardiac diseases were used. Free cells were prepared as described (*1*). The cells were frozen at 1°C per minute in a fluid containing RPMI medium 1640, 10 percent dimethyl sulfoxide, 20 to 40 percent human serum from which the γ-globulin has been removed (human agamma serum; Irvine Scientific Sales), penicillin, streptomycin, and Fungizone (Grand Island Biological) and stored at − 190°C (liquid nitrogen freezer). The cells were thawed rapidly at 37°C in a shaker bath, washed once with a medium containing RPMI 1640 and human agamma serum (20 to 40 percent) and three times with barbital-buffered saline containing Ca^{2+}, Mg^{2+}, K^+, glucose, and human albumin (buffered saline 1) (*4*), and resuspended in this buffered saline for use as target cells. The contaminating erythrocytes were eliminated by freezing and thawing and subsequent low-speed centrifugation.

Human erythrocytes carrying IA receptors from a healthy O-type donor were used as indicator cells (*4*). Sheep erythrocytes were prepared by the same procedure and used as a control for the indicator cells.

A portion (50 μl) of the human erythrocyte suspension (4×10^6 cells) was added directly to 25 μl of the target cell suspension (2.5×10^4 cells) without any serum or complement. This mixture was incubated at 37°C for 10 minutes with agitation, and for 20 minutes without agitation. One drop of this mixture was placed on a glass slide, covered by a cover slip, and viewed through the microscope. The IA patterns were determined by the percentage of target cells that positively adhered to human erythrocytes in a count of 50 cells. Adherence of at least one erythrocyte to the target cell was taken as an IA-positive reaction. To distinguish a true positive from a pseudopositive pattern, the cover slip covering the reaction mixture was lightly tapped with a pencil. The erythrocytes adjacent, by chance, to target cells moved away, while the truly positive ones remained attached.

In order to prove that the attachment of erythrocytes to cancer cells was indeed the IA phenomenon, three experiments were performed. In the first, the reactivity of the cancer cells was enhanced by adding purified human complement 2 (C2) and complement 3 (C3); in the second, the reactivity was inhibited by prior treatment with antibody to human β1C-globulin (that is, antibody to human C3) (*5*); and in the third, the specific adherence activity was tested against sheep erythrocytes to the target cancer cells (*2*).

The first experiment was necessary because in the IA phenomenon a human erythrocyte adheres to a C3 site in a complex of antigen, antibody, and complement components C1, C4, C2, and C3 (fixed in that order) (*2*). The C5 and subsequent components are not essential. Since it is known that C2 decays rapidly from the complex at 37°C unless stabilized by C3 (*6*), additional complement was added to overcome the possible loss of C2 during preparation of the cell suspensions, and

subsequent freezing, thawing, and washing. An augmentation of erythrocyte adherence would indicate that the tumor cells had complexes of antigen, antibody, C1, and C4 and that the adherence reaction being observed was actually the IA phenomenon. Barbital-buffered saline 2, which contained glucose, gelatin, Ca^{2+}, and Mg^{2+} was used for the diluent. A portion (25 μl) of target cell suspension containing 2.5×10^4 cells was mixed with 25 μl of purified human C2 and C3 (Cordis Laboratories) containing 5 hemolytic complement (CH_{50}) units of each component, and incubated at 30°C for 30 minutes, with agitation. After the incubation, 25 μl of human erythrocyte suspension (4×10^6 cells) in the buffered saline 2 was added to the reaction mixture. The IA test was performed as described.

The second experiment was done to confirm that the attachment of human erythrocytes to cancer cells actually involved IA. For this experiment, target cells were mixed with 100 μl of diluted (1 : 30) goat antiserum to human β1C-globulin (Hyland Laboratories) in buffered saline 1 and incubated at 37°C for 60 minutes. The cells were washed once with buffered saline 1, mixed with human erythrocytes in suspension, and examined for adherence. Inhibition of adherence would occur if the C3 sites were blocked by antiserum to β1C-globulin.

Last, to exclude the possibility of nonspecific erythrocyte adherence, we performed the IA test on sheep erythrocytes, which do not carry IA receptors. The IA test was done as before except that sheep erythrocytes were used as the indicator cells.

Ten to 40 percent of the cell population was IA positive in all tumors tested, although the IA reactivity of the noncancer cells was negligible (Tables 1 and 2). None of the cells from the tumor specimens or from the nonmalignant control specimens showed any significant adherence with sheep erythrocytes (Table 1). The addition of C2 and C3 increased the IA patterns in the tumors, but not in the noncancer control tissues. Prior treatment of tumor cells with goat antiserum to β1C inhibited the IA reactivity in 11 tumors of the 12. These data demonstrate that the adherence of human erythrocytes to human cancer cells is complement dependent and as such is based on the principle of IA (*2*).

Normal tissues from cancer patients adjacent to or distant from tumor sites did not show any clear IA reactivity even after the addition of C2 and C3, whereas the IA reactivity of tumor cells from the same patients became distinct (Table 2). This result suggested that the antibody fixation on the cancer cells was tumor associated. However, other possible causes for this reaction were investigated. Such a reaction might occur as an autoimmune reaction elicited by necrotic tumor materials on the cell surface or as an immune response to bacterial or viral antigens incorporated on or into the cancer cells. Although neither of these can be completely eliminated, necrotic tissue was removed from the biopsy and autopsy material at the time of the cell preparation. Four of the 12 tumor specimens were tested for bacterial contamination in culture and, in each case, were free of bacteria (*7*) even though each was IA positive.

Taken together, these observations indicate, first of all, that tumor associated surface antigens are present in various human malignant tumors, that these surface antigens do react with humoral antibodies in vivo, and that the resultant complexes of antigen and antibody formed fix complement. Obviously, the vascular capillary barriers surrounding a tumor are crossed by the

Table 1. Immune adherence reactivity of cancer cells and noncancer cells.

Source	Patient	Diagnosis	Organ	IA reactivity (%)			
				No prior treatment	Prior treatment		With sheep erythrocytes
					With C2 and C3	With anti-β1C	
Biopsy	J.W.A.	Melanoma	Lung metastasis	34	56	6	0
Biopsy	J.F.	Melanoma	Lung metastasis	40	48	8	0
Biopsy	J.A.	Melanoma	Lymph node metastasis	22	38	4	0
Autopsy	R.C.	Melanoma	Lymph node metastasis	18	30	8	0
Biopsy	S.N.	Melanoma	Spleen metastasis	18	34	4	2
Biopsy	D.H.	Osteosarcoma	Left humerus	26	36	8	0
Biopsy	S.Z.	Synovial sarcoma	Left elbow	18	32	6	0
Biopsy	R.M.	Liposarcoma	Intraabdominal metastasis	14	42	10	0
Autopsy	R.H.	Osteosarcoma	Retroperitoneal metastasis	12	24	0	0
Biopsy	M.V.	Carcinoma	Pancreas	30	42	4	0
Autopsy	G.V.	Hodgkin's disease	Liver metastasis	20	28	0	2
Biopsy	G.M.	Ganglio-neuroblastoma		22	30	8	4
Autopsy	V.W.	Cerebrovascular accident	Liver	0	0		0
Autopsy	M.C.	Tetralogy of Fallot	Liver	6	2	6	4
Autopsy	M.M.	Heart attack	Liver	4	4	4	2
Autopsy	J.H.	Coronary insufficiency	Spleen	0	0		0
Autopsy	M.V.	Heart attack	Spleen	2	2	2	2
Autopsy	R.P.	Tetralogy of Fallot	Spleen	4	2	4	0
Autopsy	R.W.	Cerebrovascular accident	Kidney	0	0		0
Autopsy	W.B.	Cerebral hemorrhage	Kidney	0	0		0

Table 2. Immune adherence of normal cells from cancer patients. Parentheses indicate IA reactivity of tumor cells from the same patients, which was simultaneously tested after the addition of C2 and C3.

Source	Patient	Diagnosis	Normal cells from	IA with additional C2 and C3
Biopsy	S.N.	Melanoma	Spleen	0% (34%, spleen metastases)
Biopsy	M.M.	Melanoma	Skin	2% (40%, skin metastases)
Autopsy	R.H.	Osteosarcoma	Liver	0% (24%, retroperitoneum metastases)
Biopsy	H.G.	Osteosarcoma	Skin	0% (30%, sternum primary)
Autopsy	T.R.	Reticulum cell sarcoma	Spleen	0% (26%, cervical lymph node metastases)

antibody and complement in vivo. Whether these complexes of surface antigens, antibody, and complement act to suppress or to accelerate lymphocyte cytotoxicity and complement-dependent cytolysis of tumor cells in vivo under particular conditions is not yet known. However, B (bone marrow derived) lymphocytes and neutrophils that have receptors for cell-bound C3 (*8*) may react to C3 complexed on tumor cells and play an important role in the interaction of tumor and lymphoid cells. This interaction would be a significant factor for prognosis in the cancer patient.

The above-described IA techniques should be applicable to immunological studies of human autoimmune diseases such as certain types of thyroiditis and glomerulonephritis as well as to cancer studies.

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Interspecies Conversion of Clostridium botulinum Type C to Clostridium novyi Type A by Bacteriophage

Abstract. *When* Clostridium botulinum *type C is cured of its prophage it simultaneously ceases to produce toxin. This nontoxigenic culture can then be converted to another toxigenic bacterial species,* Clostridium novyi *type A or to toxigenic* Clostridium botulinum *types C or D, by specific bacteriophages. The toxigenicity and type of toxin produced by these cultures depends upon the continued presence of these bacteriophages.*

Clostridium botulinum and *Clostridium novyi* are pathogenic anaerobes that are characterized by their ability to produce powerful toxins. The *C. botulinum* group produce neuroparalytic toxins that are responsible for botulism in man and animals. Wound botulism has been reported in man, but botulism usually occurs in both man and animals from the ingestion of food containing toxin produced by one of the several known types of *C. botulinum*. *Clostridium novyi* also produces lethal toxins; it is often found in gas gangrene infections of man and animals and causes necrotic hepatitis, osteomyelitis, hemaglobinuria, and bighead diseases in animals.

Clostridium novyi is divided into four types designated as types A through D, based on the production of toxins and other biologically active substances; *C. botulinum* includes a very heterogeneous group of strains that are divided into types A through G, based on the antigenic specificity of the neurotoxins that are produced. The strains of these seven types can be separated into four groups according to their physiological and serological characteristics (*1–3*). The strains of *C. botulinum* types C and D form one of these groups and share some of the same characteristics possessed by *C. novyi* type A (*1, 2*). The main difference between the species is in the toxins produced.

It has been reported previously that the change from nontoxigenicity to toxigenicity in *C. botulinum* types C and D requires the active and continued participation of bacteriophages (*4–7*).

This report provides evidence that a nontoxigenic, phage-sensitive strain of *C. botulinum* type C can be converted to another toxigenic species, *C. novyi* type A, after infection by phage NA1 from *C. novyi* type A. In addition, this same nontoxigenic, phage-sensitive bacterial strain can be converted back to toxigenic *C. botulinum* type C after infection by phage 3C of *C. botulinum* type C or to toxigenic *C. botulinum* type D by infection with phage 1D of *C. botulinum* type D. Three immunologically distinct toxins can therefore be produced by a common bacterial strain following infection by specific bacteriophages. Furthermore, the persistence of the toxigenic characteristic and type of toxin produced requires the continued presence of these same bacteriophages.

Spores of toxigenic *C. botulinum* type C strain 162 were treated with heat at 70°C for 15 minutes, diluted, and plated on trypticase-yeast-glucose (TYG) agar (*8*). Isolated colonies were cultured in TYG broth and tested for toxigenicity by the mouse assay (*8*) and for sensitivity to the bacteriophages of the toxigenic parent culture by the agar-layer procedure (*8*). Of the 40 isolates tested, one strain designated as HS37 had simultaneously lost its prophage and ceased to produce toxin. Strain HS37 was subcultured about 20 times in fortified egg-meat medium (*9*) over a period of 1½ years, and it re-

Table 1. Infection of cured nontoxigenic bacterial strain HS37 with *Clostridium botulinum* type C phage 3C, type D phage 1D, and *Clostridium novyi* type A phages NAI and NA2 and their effect on toxigenicity.

Phage	Number of cultures			Toxin neutralized by antiserum of
	Toxigenic	Produced phage*	Tested	
$3C^{tox+}$	40	40	40	*C. botulinum* type C
$1D^{tox+}$	40	40	40	*C. botulinum* type D
$NA1^{tox+}$	40	40	40	*C. novyi* types A and B
$NA2^{tox-}$	0	40	40	

* Cultures were also resistant to infection by homologous phage.