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Antibody Response of Syngeneic Mice to Membrane Antigens from NDV-Infected Lymphoma[1] (38694)

MONROE D. EATON AND SUSAN J. P. ALMQUIST

Department of Medical Microbiology, Stanford University School of Medicine, Stanford, California 94305

Homogenates from transplantable virus-induced tumors that have been infected with viruses of influenza, Newcastle disease (NDV), or parainfluenza I produce tumor transplant resistance in syngeneic mice (1–3), but similar preparations from uninfected tumor cells are not immunogenic. We now report that cytotoxic and complement fixing antibodies are found in inbred C_3H/Bi mice immunized with certain cell fractions of an NDV-infected syngeneic lymphoma induced by the Gross leukemia virus. The results indicate that the immunogenic helper effect of NDV may extend to more than one leukemia antigen. The simultaneous presence of antibodies in syngeneic mice and rejection of tumor transplants suggests a possible relation of antibodies to resistance rather than to a blocking effect *in vivo*.

Materials and Methods. The C_3H/Bi strain of mice, the ascites lymphoma, NDV strain IS1, and the method of growing NDV in primary cultures of lymphoma cells have been described in a previous publication (1). The infected lymphoma cells were processed by centrifugation at 800 *g* to sediment intact cells. The supernatant was then centrifuged at 5000 *g* to obtain small fragments of membranes, and again for 90 min at 5×10^4 *g* to sediment free virus particles.

Homogenates and crude membrane fractions from NDV-infected (NDV-CM) or uninfected cells (CM) were obtained by allowing the lymphoma cells to swell for 5 min in 20 vol of 0.01 *M* Tris buffer pH 7.6 containing 0.01 *M* sodium azide and 0.015 *M* iodoacetate as preservatives (4). Cells were then disrupted by 20 strokes in a Dounce homogenizer. After adding 1/20 vol of a stabilizing solution consisting of 0.04 *M* $MgCl_2$ and 0.1 *M* NaCl, the nuclei and intact cells were removed by centrifugation at 2000 rpm for 30 sec in an International refrigerated centrifuge. Additional membranes were obtained by washing the sediment once in 0.025 *M* Tris, 0.01 *M* NaCl and 0.004 *M* $MgCl_2$. The membrane fraction was concentrated by centrifugation at 10^4 *g* for 20 min and the sediment resuspended in 0.15 *M* NaCl or EDTA saline at a volume twice that of the original cells. The resulting suspension contains membranes from about 2.5×10^8 cells/ml. The supernatant from uninfected cells was concentrated fivefold by pressure filtration on a PM10 Amicon membrane. Tests for leukemia virus in this fraction were done by complement fixation and inoculation of tissue cultures (5). Triton X-100 extracts were prepared by suspending the membranes from 1.5×10^9 cells in 12 ml of Tris saline pH 7.6 containing 0.25% detergent and homogenizing at 4° in a Potter grinder. After centrifuging at 10^4 *g* the supernatant was treated with an equal volume of saturated $(NH_4)_2SO_4$. The resulting precipitate was dissolved in 6 ml of water and dialyzed against 0.02 *M* phosphate buffer pH 7.4 to remove $(NH_4)_2SO_4$ and part of the Triton X-100 which was also precipitated.

The antigens for complement fixation (CF) were derived as described above from homogenates of washed uninfected cells. The CM homogenate 1:20 was added to serum dilutions and 1.6–1.8 units of guinea pig complement each in a volume of 0.25 ml. After incubation at 35° for 1 hr 0.25 ml of 2% sensitized sheep erythrocytes were added. Serum titers were stated as the highest final dilution of serum at which some erythrocytes remained. The uninfected CM fraction gave no CF with rabbit antiviral sera against NDV, Sendai, or influenza viruses which were being used in the laboratory. On the other hand NDV-CM gave a strong reaction with NDV rabbit or mouse

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TABLE I. IMMUNE RESPONSE OF MICE TO VARIOUS FRACTIONS OF C_3H/Bi LYMPHOMA AND OTHER IMMUNOGENS.

Immunogen (C_3H/Bi Mice)	No. of injections[a]	Transplant Rejection (%)	Antibody titers of serum	
			Cytotoxic	Comp. Fix.
Uninfected lymphoma				
membrane (CM)	5	0/14 (0)[b]	<10	<20
NDV-infected lymphoma				
NDV-CM	4	10/12 (83)[b]	<10	160
NDV-CM	4 + 3[c]	5/6 (83)[c]	160	160
homogenate minus NDV-CM	5	2/18 (11)	<10	160
triton extract of NDV-CM	4	3/12 (25)	20	80
NDV-tissue culture supernate:				
5×10^3 *g* sediment	4	8/13 (60)	40	80
5×10^4 *g* sediment	4	3/13 (23)	<10	80
Egg-pass NDV	3	1/11 (9)	—	<20
None	—	(<20)[d]	<10	<10
C_3H/Bi spleen, lymphoma in C_3H/HeJ mice	5	20/23 (87)[e]	20	40

[a] Whole cell equivalent of each dose was approximately 4×10^7.

[b] Results of challenge with 50 cells (partly reported in Ref. 1) number surviving/number challenged.

[c] Survivors of challenge after four immunizing injections received three booster doses.

[d] Control mice in numbers equal to experimental groups showed a survival rate of 20% or less (1).

[e] Challenge dose 10^7 cells: rejection due to histocompatibility differences. Serum was absorbed to remove H antibodies.

antibody but none with normal serum or mouse antibody to Sendai virus.

Cytotoxicity was determined by the dye exclusion method using toluidine blue 0.1% (6). Cells were incubated with serum dilutions and rabbit complement 1:20. Most counts of dead cells were done at 3 hr but comparable results were obtained at 24 hr. The cytotoxic titers of the unheated sera were 1:80 or 1:160. Cytotoxic antibody was absorbed by adding 10^8 cells or the equivalent amount of CM per ml of a 1:5 dilution of serum. The absorption was repeated. Inhibition of cytotoxicity was done by adding equal parts of undiluted homogenate, prepared without iodoacetate or NaN_3, to serum dilutions. Where the antigen was cytotoxic, dilutions were made and added to a constant serum dilution.

Mice were immunized by four or more injections of NDV-CM given ip at intervals of three weeks. Complete Freund's adjuvant was mixed with the material for the first injection. C_3H/HeJ mice which are allogeneic with respect to the C_3H/Bi strain were immunized without use of NDV by giving one injection of normal C_3H/Bi splenic tissue (to produce immunity to the H antigens) followed by increasing doses of ascites lymphoma up to 10^7 cells. Mouse blood was obtained by cardiac puncture under ether anaesthesia. Sera from six or more mice per group were pooled.

Results. Immune responses of mice receiving uninfected CM, NDV-CM, or various other fractions from NDV-infected lymphoma cells are summarized in Table I. CM failed to stimulate tumor transplant rejection or detectable antibodies. NDV-CM did produce protection against tumor transplant and relatively high titer antibodies. However, the appearance of cytotoxic antibodies was irregular. One group of mice as shown in the table developed tumor transplant resistance without cytotoxic antibodies but these appeared after three booster doses following challenge. In several other groups of mice not shown in the table cytotoxic and complement fixing antibodies were produced by multiple injections of NDV-CM. The immunogenic effect of the 5×10^3 *g* tissue culture sediment is attributable to membrane fragments from disrupted NDV-infected cells. No transplant resistance was

TABLE II. CYTOTOXICITY ABSORPTION OR INHIBITION.

Serum absorbed with	Estimated cell equivalent	Serum dilution[a]	% Cell death	
			Unabs.	Absorbed[b]
Whole lymph. cells	10^{8c}	10	79	10
Whole lymph. cells	10^{8c}	40	75	11
Washed CM	10^{8d}	10	37	5
Washed CM	10^{8d}	40	24	7
Whole Krebs-2 cells	10^{8c}	10	87	64
Whole Krebs-2 cells	10^{8c}	40	35	40
Whole lymph homogenate	4×10^{7d}	10	72	10
Whole lymph homogenate	4×10^{7d}	40	40	7
Homogenate minus membrane	4×10^{7d}	10	35	30
Homogenate minus membrane	4×10^{7d}	40	39	8
Triton extract CM	6×10^{7d}	40	35	9
Triton extract CM	1.5×10^{7}	40	35	25

[a] Highest dilution of serum showing cytotoxicity was 1:80–1:160.
[b] Range of cell death with normal mouse serum, <5–20%.
[c] Serum 1:5 absorbed twice with one fifth volume of packed cells.
[d] Equal volumes of antigen suspension and serum dilutions incubated 1 hr. Insoluble part removed by centrifugation.

produced by the cell homogenate from which membranes had been removed, by the 5×10^4 *g* sediment (virus) from tissue culture supernatants previously centrifuged at 5×10^3 *g*, or by the triton extract of NDV-CM. Despite the lack of resistance to challenge, complement fixing antibodies appeared in the serum of mice receiving these fractions. Egg passage NDV produced no transplant resistance or antibodies against uninfected CM. To compare the antibody response obtained by allogeneic immunization without NDV, C_3H/HeJ mice were treated as described in Materials and Methods. Due to the immune response to H antigens, these mice resisted challenge with 10^7 cells but had relatively low titers of tumor specific antibodies after the serum had been absorbed with C_3H/Bi erythrocytes and splenic tissue.

Absorption or inhibition of cytotoxic antibodies in the sera of mice immunized with NDV-CM was observed with several fractions of uninfected lymphoma cells. Whole living cells and washed CM reduced the serum cytotoxicity titer from 80–160 to less than 10 (Table II). On the other hand absorption with the unrelated Krebs-2 ascites cells did not reduce cytotoxicity. The whole lymphoma homogenate and the homogenate supernatant without membranes both showed inhibition of cytotoxicity when added in equal parts to serum dilutions except that no inhibition was obtained with the homogenate supernatant and 1:10 serum, indicating, in this case, an excess of antibody. Similar antibody excess and cytotoxicity was seen with the triton extract at a high dilution and serum 1:40 but not with extract at lower dilution. Soluble antigen–antibody complexes by themselves fix complent but this was not a significant factor in the inhibition of cytotoxicity since complement dependent lysis was observed at the higher antibody–antigen ratios.

Unabsorbed serum gave CF with antigens from several cell fractions other than CM, also with the high speed sediment from tissue culture supernatants, and cell free ascites fluid from mice carrying the lymphoma (Tables III and IV). To determine whether the membrane antigens were antigenically related to the small particle and soluble fractions, antisera were absorbed with whole cells and washed CM. Complement fixation was reduced eightfold by washed CM (Table III) but the absorbed serum gave residual reactions with the whole homogenate, the homogenate minus mem-

TABLE III. COMPLEMENT FIXATION OF ANTISERUM ABSORBED WITH WASHED CM.

CF Antigen[b]	Serum titer[a] Unabsorbed	Absorbed[c]
Washed membrane	80	10
Whole homogenate	40	20
2×10^4 *g* supernate	80	20
Ascites fluid filtrate	40	20
Krebs-2 membrane	<10	—
Washed membrane and:		
serum abs. K_2 membrane	40	20
serum abs. K_2 cells	80	40

[a] Serum from mice immunized with NDV-CM (Table I).

[b] CF antigens prepared directly from mouse tumors do not react with antibodies against proteins of calf serum used in tissue culture medium.

[c] Absorbed sera were often anticomplementary. Results presented are limited to those having titers at least four times the anticomplementary control.

TABLE IV. COMPLEMENT FIXATION WITH VIRUS AND SOLUBLE FRACTIONS FROM LYMPHOMA.

Treatment before centrifuging at $10^5 \times g$	Antigen titer[a] $10^5 \times g$ sediment	$10^5 \times g$ supernatant
Homogenate minus membranes[b]	80	40
Filtered homogenate[b]	40	10
Ascites fluid filtrate	20	10
Tissue culture 4×10^3 *g* supernatant[c]	20	—

[a] Against 1:20 dilution of serum from mice immunized with NDV-CM.

[b] Homogenate centrifuged at $10^4 \times g$ to remove membranes and 0.45 μm filtrate of this. Both concentrated fivefold on an Amicon membrane.

[c] Against serum from allogeneic mice immunized with C_3H/Bi spleen and uninfected lymphoma cells to avoid reaction with NDV.

branes (2×10^4 *g* supernatant), and filtered ascites fluid. Some nonspecific absorption, twofold reduction of titer, was observed with Krebs-2 cells or membranes although the latter did not fix complement with the antilymphoma serum. Thus a twofold reduction in titer for the other antigens must be regarded as due to nonspecific absorption.

Evidence for the presence of CF antibodies to antigens the particle size of the leukemia virus and also to "soluble" antigens not sedimented at $10^5 \times g$ is presented in Table IV. These were present in the membrane-free homogenate, in the ascites fluid, and the virus fraction of the NDV-lymphoma tissue culture supernatant. The latter was tested with allogeneic antiserum having no antibodies to NDV. Swiss mouse embryo tissue cultures inoculated with filtrates of ascitic fluid, or homogenate failed to give CF reactions. It remains to be determined whether this was due to failure of replication of the leukemia virus or to absence of antiviral antibodies from the sera.

Discussion. The observations reported in this paper should facilitate studies on the role of humoral immunity in Gross virus leukemia. Our highly malignant ascites lymphoma has an LD50 of 5–10 cells in C_3H/Bi mice and repeated minimal doses of living cells are not immunogenic (1). Others have obtained cytotoxic antibodies for syngeneic tumor but not antiviral antibodies in mice immunized with an allogeneic strain of Gross leukemia (7, 8). Published results are quite different with Moloney virus which often produces regressing lymphomas or sarcomas. Antibodies of high titer can be produced in syngeneic hosts by living cells or homogenates (9, 10). Direct cytotoxicity (9), antibody dependent cell mediated cytotoxicity (11), and passive immunity to tumor transplant (10) have been demonstrated with sera from syngeneic mice carrying progressing or regressing tumors induced by the Moloney virus. It should be noted that treatment of tumor cells with allogeneic or xenogeneic antibody may result in rejection rather than enhancement *in vivo*. Such rejection has been noted with mouse antiserum against Ehrlich ascites tumor (12) and with serum from rats bearing Gross lymphoma (13). These findings on anti-tumor antibodies need to be reconciled with the generally accepted evidence that tumor rejection is cell mediated.

In the present study we observed three types of response to leukemia antigens. The membranes of NDV-infected cells stimulated both transplant immunity and CF antibodies. The latter reacted with all fractions

of uninfected cells. Some infected cell fractions stimulated antibodies but not transplant immunity. This may have been due to the absence of transplant rejection antigen in these preparations or to the development of blocking antibodies. This remains to be investigated. The infected membrane fraction when solubilized in Triton X-100 produced CF and cytotoxic antibodies but lost the ability to produce transplant rejection possibly because of partial dissociation of the NDV-tumor-antigen complex. CF antibodies produced by the high speed sediments from the tissue culture supernatants may be directed against the leukemia virus envelope rather than cell surface antigens. In the third category are the negative results of attempts at immunization with membrane and other fractions of uninfected lymphoma cells. All of these fixed complement with antibodies that could partially be absorbed with uninfected membranes, thus establishing a relation to the cell surface and its associated virus. Purified, soluble Gross leukemia cell surface antigens have been reported to give serological reactions but no transplant immunity and only a very weak antibody response (14) in syngeneic mice whereas irradiated whole cells are immunogenic. Also it should be noted that antibody response to the Gross leukemia virus is irregular in both syngeneic and allogeneic systems (7, 8). Serological reactions without immunogenicity are characteristic of haptenes. Yet it is not possible that the many antigens, viral and cellular, of Gross leukemia (15) all behave as haptenes in syngeneic mice. Various explanations such as tolerance, multiple molecular forms and instability of the antigens, or immunosuppression by a factor in homogenates have been considered. Although it is conceivable that myxovirus proteins might form an immunogenic hybrid antigen with one tumor cell constituent (1–3) our results indicate several antigens are reactive with the antibodies produced by NDV-CM. NDV may affect immunogenicity in some way other than by acting as a carrier.

Summary. Antibodies giving complement dependent cytotoxicity and complement fixation with several cell fractions were obtained by immunizing mice with membranes from a syngeneic ascites lymphoma infected with NDV. Some NDV-infected cell fractions stimulated tumor transplant resistance as well as antibodies. Others gave only antibodies. No immune response was found with membranes or homogenates from uninfected cells even though these gave CF with immune serum.

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