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Autoimmunity Induced by Injection of Virus-Modified Cell Membrane Antigens in Syngeneic Mice

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C_3H/Bi mice developed autoantibodies after repeated inoculations of isolated membranes from primary tissue cultures of a syngeneic ascites lymphoma in which Newcastle disease virus had grown. This was in addition to the tumor transplantation resistance and cytotoxic antibodies previously demonstrated. The complement-fixing antibodies were completely removed from sera by adsorption with ascites tumor cells but only partially by normal mouse liver powder or C_3H/Bi erythrocytes. With continued immunization, antibodies to deoxynucleoprotein and heterophile reagins also appeared. After several months, mice showing these serological reactions died with a wasting disease characterized by loss of lymphoid tissue and scarred, atrophied kidneys. No significant antibody response or autoimmune disease occurred in mice receiving membranes from uninfected syngeneic ascites lymphoma.

Enhancement of immunogenicity of cell-free preparations from tumors infected with noncogenic viruses has been studied in several laboratories (2, 4, 11). Recent work on infection with an avian myxovirus of human leukemia and mammary tumor cells in tissue culture suggests possible use of such antigens in adjunct immunotherapy of human cancer (15). A clinical trial of this method has been reported by one group of investigators (A. A. Green, A. G. Webster, and K. Smith, Proc. Am. Assoc. Cancer Res. **16**: 271, 1975). Chemical modification of tumor or normal cell antigens to enhance immunogenicity has also been explored (16, 18; K. R. Madyastha and F. Rolf, Proc. Am. Assoc. Cancer Res. **16**: 137, 1975). Several workers have found membranes or homogenates from uninfected cells not to be immunogenic when tested in syngeneic animals.

The plasma membrane fraction from ascites lymphoma infected in vitro with Newcastle disease virus (NDV) was found to contain the major portion of antigen-stimulating tumor-specific transplant resistance (4) and cytotoxic antibodies (3) in syngeneic mice. The sera of these mice also contained antibodies that fixed complement with several fractions of uninfected ascites lymphoma cells, including high-speed-centrifuged sediments and supernatants from filtered homogenates or tumor ascites fluid. Injection of cytoplasmic fractions or homogenates minus membranes from NDV-infected lymphoma did not stimulate tumor transplant resistance but did produce complement-fixing (CF) antibodies reactive with the membranes.

It was also found that autoantibodies were in part involved in the CF reactions, and certain groups of mice were then held for prolonged observation. Some animals developed serological and pathological reactions characteristic of autoimmune disease, which is the subject of this report.

MATERIALS AND METHODS

Ascites lymphoma. Transplantable leukemia produced by the Gross virus in C_3H/Bi ($C_3Hf/Bi/Gs/L$, Snell) mice was converted to the ascites derivative by repeated intraperitoneal (i.p.) passage. The 50% lethal dose of this tumor is approximately 10 cells.

Virus. NDV strain IS_1 was propagated in the allantoic sac of chicken embryos. The method of growing NDV in primary cultures of lymphoma cells has been described in a previous publication (4). Infected lymphoma cells were centrifuged from the culture medium at $800 \times g$ and washed.

Immunogens. Crude plasma membrane fractions (NDV-CM) were prepared from infected cells by disruption in a hypotonic solution containing 0.01 M tris(hydroxymethyl)aminomethane, pH 7.6, 0.01 M NaN_3, and 0.015 M iodoacetate and separated from intact cells and nuclei by differential centrifugation as previously described (4). The membrane fraction was then concentrated at $10^4 \times g$ in a Sorvall centrifuge, resuspended in phosphate-buffered saline to give a concentration of membranes equivalent to about 2.5×10^8 cells/ml, and stored at −20°C. Control preparations were made from uninfected cells (CM).

Immunization. C_3H/Bi mice at least 2 months old received four or more i.p. injections of 0.2 ml of suspended NDV-CM or uninfected CM at intervals of 3 weeks. Complete Freund adjuvant in equal volume was added only for the first injection. Blood was obtained by cardiac puncture, and sera from six or

more mice per group were pooled. Sera from rabbits immunized with allantoic-passage NDV were used for titration of viral antigen in NDV-CM.

CF. Antigens were well-dispersed membranes of uninfected ascites lymphoma cells (CM), Krebs 2 ascites tumor cells, normal C_3H/Bi spleen cells, or homogenates of liver, lung, or kidney cells. Cells were disrupted in 20 volumes of hypotonic solution as described above, and the resulting suspension of membranes was stored at 4°C. Fresh antigens were prepared every 3 to 4 weeks. CF with anti-mouse rabbit serum was used to check the antigen content of membrane suspensions. The test was performed with 1.6 to 1.8 units of guinea pig complement as described (3) and a 1:20 dilution of the membrane suspension or homogenate. Antibody adsorption was done with washed mouse or sheep erythrocytes, saline-insoluble mouse liver powder, or intact ascites lymphoma cells. One-tenth volume of packed cells or powder and diluted serum were incubated at 25°C for 1 h and centrifuged, and the absorption was repeated.

Agglutination and hemolysis of sheep erythrocytes. Dilutions of mouse sera were incubated with 0.5% sheep erythrocytes for 1 h at 37°C and then at 4°C overnight. In a similar procedure, mouse serum agglutination of erythrocytes sensitized with rabbit hemolysin as for the CF test was observed. In other tests for hemolysis and agglutination, guinea pig complement (1:10) was added after preliminary incubation at 37°C of unsensitized sheep erythrocytes with mouse serum, and hemolysis or agglutination was read after the cells had settled.

Nuclear preparations. The nuclei from disrupted ascites lymphoma cells were separated from membranes and cytoplasmic components by sucrose density centrifugation (4). Washed intact nuclei and deoxyribose nucleoprotein (DNP) fraction insoluble in 0.15 M NaCl (9) were used as antigens for CF.

RESULTS

Viral antigens on the membranes of infected cells. Washed NDV-CM were tested for the presence of viral antigen by hemagglutination inhibition and CF with anti-NDV rabbit serum; finely dispersed membranes gave an agglutination pattern with 0.25% chicken erythrocytes to a titer of 80. The saline wash did not agglutinate beyond a dilution of 1:10. Agglutination was inhibited by anti-NDV serum in proportion to the dilution of membranes (Table 1). Treatment of the membranes with 0.5% Triton X-100 did not dissolve them but removed the agglutinating activity, presumably by disrupting the virus.

The results of CF tests are shown in Table 2. The membrane antigen gave a titer of 160 with NDV antiserum but no significant fixation with other antiviral sera. None of these sera reacted significantly with membranes of uninfected ascites cells. Unlike its effect on hemagglutination, Triton X-100 did not completely remove viral antigen detected by CF. It has been shown previously that much of NDV IS_1 is cell associated, probably on the membrane, but can be released into the supernatant by freezing and thawing or removed by antibody (7).

TABLE 1. *Hemagglutination with membranes from NDV-infected ascites lymphoma and inhibition by NDV antiserum*

Determination	Dilution of membranes				Wash (1:10)	Triton residue (1:10)
	1:10[a]	1:20	1:40	1:80		
Hemagglutination	+++	+++	++	+	+	0
Highest dilution of NDV antiserum inhibiting[b]	640	1,280	2,560	2,560	5,120	

[a] This dilution represents membranes from about 10^7 cells/ml.

[b] At a dilution of 1:640, this serum inhibited hemagglutination by 10 hemagglutination units of free virus or less.

TABLE 2. *Viral CF antigen on isolated membranes of NDV-infected and uninfected lymphoma cells*

Rabbit antiserum[a]	Highest dilution of antigen giving CF	
	NDV-CM	CM
NDV	160	< 10
Parainfluenza (Sendai)	10	< 10
Influenza PR_8	< 10	10
	NDV-CM Triton[b]	CM-Triton[b]
NDV	40	< 10

[a] Rabbits immunized with allantoic passage virus; serum dilution, 1:20.

[b] Membranes treated with 0.5% Triton X-100 and washed.

CF antibody response to NDV-infected membranes. C_3H/Bi mice receiving syngeneic NDV-CM i.p. began to show, after two injections, CF antibodies to both uninfected tumor membranes and cell membranes from normal spleen. Antibody titers to tumor and normal tissue increased in parallel (Fig. 1). Maximum titers with CM were reached after four or five injections, at which time anti-spleen antibodies appeared to be declining gradually. Repeated injections up to a total of 13 over a period of nearly 1 year did not increase the titer. Sera collected 2 weeks after a booster injection of antigen showed no increase in antibodies. Cytotoxic antibodies and resistance to tumor transplant were present after three to five injections as previously reported (3, 4) and could not be increased by additional injections.

As shown in Table 3 (left columns), serum pools from six groups of mice were tested with uninfected lymphoma membranes and normal C_3H/Bi spleen cell membranes. Four gave high CF titers with both antigens, and two showed low titers with spleen. Serum also tested with lung and kidney homogenates gave strong posi-

tive reactions. Four of these serum pools were tested with the Krebs 2 ascites tumor membranes, but only one gave a strong reaction and one was <10. However, these sera gave high titers with allogeneic (ICR) spleen and kidney cells and slightly lower titers with liver and lung cells. This result suggests that the reactive organ antigen(s) may be reduced in the membranes of the Krebs 2 ascites cells. At the right side of Table 3 are results with four control serum pools from mice immunized with CM from ascites lymphoma not infected with NDV. Results were negative or of borderline significance. The membrane preparations may contain "internal" antigens not present on the intact cell surface, and this might account for some slight degree of immunogenicity.

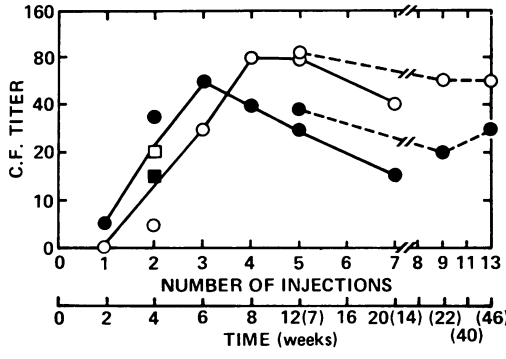

FIG. 1. *Antibody response measured by complement fixation with ascites lymphoma membranes (○, □) or normal spleen cell membranes (●, ■) of mice given multiple injections of membranes (10^7 cell equivalents per dose) from ascites lymphoma infected with NDV. Pooled sera from 8 to 10 mice. Experiment 1, solid lines; experment 2, broken lines; time indicated in parentheses. Results with control mice receiving membranes from uninfected cells on a similar immunization schedule were essentially negative (not shown). A third group (squares) was tested after two injections only.*

Heterophile antibodies. In some of the CF tests it was noted that agglutination of sheep erythrocytes had occurred after standing overnight at 4°C. To analyze this phenomenon, agglutination experiments were done with 0.5% sheep cells alone or with sheep cells sensitized with rabbit hemolysin. Two sera gave agglutination with unsensitized cells both at 37 and 4°C overnight, the reaction being somewhat stronger at 4°C (Table 4, experiments 1 and 3). Two other sera (experiments 2 and 4) gave agglutination only with sensitized erythrocytes, suggesting that rabbit immunoglobulin G may have participated in the reaction. In experiments 1 and 3, sera taken after five injections gave no agglutination but became positive after additional immunization.

When sheep erythrocytes were sensitized with sera from mice given multiple injections, and guinea pig complement was added, agglutination and partial hemolysis occurred. The former was evident at higher dilutions of mouse serum than in the absence of complement. One serum (experiment 3) showed agglutination but no hemolysis.

Absorption of CF antibody. Attempts were made to remove CF antibodies reactive with normal syngeneic spleen and retain the reaction with tumor. The results of several experiments using mouse or sheep erythrocytes and allogeneic mouse liver powder are presented in Table 5. By absorption, CF titers with CM were reduced twofold or less whereas those with normal spleen decreased three- to sixfold. Whole ascites lymphoma cells almost completely removed antibodies to CM and normal spleen, indicating that the procedure was adequate. Apparently the erythrocytes and mouse liver cells both lacked some of the antigens present

TABLE 3. *Complement fixation of mouse sera with syngeneic and allogeneic antigens*

Tissue ag[a] for CF	Immunized with NDV-CM										Immunized with CM (syngeneic ag)			
	Syngeneic ag[b]						Allogeneic ag[c]							
Lymphoma (M)	20	80	80	80	40	80		–			<10	<10	<10	<10
Krebs 2 (M)			–				<10	40	10	10	<10	(allogeneic)		
Spleen (M)	40	40	40	80	10	10	80	80	40		10±	–	–	–
Liver (H)	–	–	–	–	–	–	40	40						
Lung (H)	40	–	–	–	–	–	20	40			10±	–	–	–
Kidney (H)	80	–	–	–	–	–	80	80			10	–	–	–

[a] Antigen (ag) derived from 2.5 to 5.0 mg of tissue (wet weight) per ml. M, Crude membrane; H, homogenate.

[b] Titers with pooled sera from individual groups of five to seven mice receiving five injections of NDV-CM on a schedule similar to that shown in Fig. 1. The results of testing a single serum pool with several antigens are shown by titers in columns. Repeated tests with a given antigen and serum indicated that titers were reproducible to within one twofold dilution. –, Not tested.

[c] Tissues from ICR Swiss mice except for Krebs 2 ascites tumor.

TABLE 4. *Agglutination and complement-dependent hemolysis of sheep erythrocytes by sera from C_3H/Bi mice receiving multiple injections of NDV-CM*

Expt no.	NDV-CM injections	Mouse serum agglutination titers with:[a]			Mouse serum hemolysis titer (E + C')
		E	EA	E + C'	
1	5	<10	<10	<10	<10
	13	20[b]	40[b]	80	40
2	10	<10	20[b]	<10	80
3	5[c]	<10	<10	<10	<10
	5 + 2[d]	10[b]	40[b]	80	<10
4	8	<10	10[b]	80	40

[a] E, 0.5% sheep erythrocytes; A, rabbit anti-sheep hemolysin at dilution for CF; C', guinea pig complement, 1:30 final dilution.

[b] Agglutination at 37 and 4°C.

[c] Injections given between 1 and 3 months of age, bled at 8 months.

[d] Booster doses at age 10 and 11.5 months.

TABLE 5. *Complement fixation of mouse anti-lymphoma serum before and after absorption*[a]

Serum absorbed with:	Expts averaged	CF titer unabsorbed		CF titer absorbed[b]	
		Ascites CM	Normal spleen	Ascites CM	Normal spleen
C_3H RBC	3	46	53	33	13
Mouse liver	2	60	30	30	10
Sheep RBC	2	50	60	30	10
Ascites lymphoma	2	80	40	<10	<10

[a] Absorption procedure: 10^8 washed erythrocytes (RBC) or 0.2 g of saline-insoluble mouse liver powder was added to 1 ml of serum diluted 1:5. After standing for 1 h at 25°C with mixing, suspensions were centrifuged and the absorption was repeated.

[b] When sera became anticomplementary as a result of absorption, corrections were made; e.g., anticomplementary titer of 1:10 and titer with antigen of 1:80 were corrected by one dilution to 1:40.

in normal spleen cells. Use of multiple absorbents or spleen cells was unsatisfactory because the sera became anticomplementary.

Antinuclear antibodies. The development of antibodies to deoxyribonucleic acid (DNA) and especially to DNP is seen in autoimmune disease of certain strains of mice (17). By CF, elevated titers against DNP made from ascites lymphoma were found in sera of C_3H/Bi mice that had received three or more injections of NDV-CM over periods of 3 to 14 months (Table 6). Whole nuclei gave relatively weaker reactions, sometimes with sera of mice receiving uninfected CM. Only two of the sera had detectable antibodies against purified calf thymus DNA. Weanling mice that had received five injections before 3 months of age showed no antinuclear antibodies when bled 5 months later, but did develop positive CF reactions after two booster doses between 8 and 13 months (lines 5 and 6, Table 6). The DNP antibodies were not absorbed out by intact lymphoma cells. Control sera from mice that had received uninfected CM gave no reaction with DNP or DNA. Allogeneic mice (not shown) that had rejected the ascites lymphoma and then received multiple injections of 10^7 live untreated cells also failed to develop antinuclear antibodies.

Fatal autoimmune disease. A considerable portion of the mice showing serological reactions characteristic of autoimmune disease died after 7 to 13 months (Table 7). Not included were a number of deaths due to other causes and some in which a satisfactory autopsy could not be performed. Control animals receiving only Freund adjuvant, untreated lymphoma cell membranes, or normal spleen cells did not develop signs of autoimmunity. The disease was not found in normal stock mice kept for 8 to 12 months.

Since death from autoimmunity occurred long after mice began to develop humoral antibodies against normal syngeneic tissue, the chronic nature of the disease is obvious. Physical signs included weakness, wasting, and a humped posture such as seen in graft-versus-host reactions. In gross pathology the outstanding lesion was scarring and extreme atrophy of the kidneys, sometimes accompanied by atro-

TABLE 6. *Development of antinuclear antibodies in relation to number of injections and period of immunization*

Injections	Time bled (months)	CF titer with:			
		Nuclei[a]	DNP[a]	DNA[b]	N. spleen[c]
NDV-CM					
2	1.5(6)[d]	–	<10	<10	10
3	3 (6)	20	20	<10	40
5	3.5 (6)	20	40	20	20
7	4 (10)	<10	40	<10	80
5[e]	8 (8)	–	<10	<10	10
5 + 2[e]	13 (2)	–	40	20	10
13	14 (3)	20	80	–	80
Uninfected CM					
7	8 (9)	10	<10	<10	<10

[a] Isolated nuclei from ascites lymphoma, 10^7/ml. DNP (500 μg/ml) was prepared from these nuclei (see Materials and Methods). –, Insufficient serum for test.

[b] Calf thymus DNA (20 μg/ml) purchased from Wentworth Chemical Co.

[c] Isolated spleen cell membranes.

[d] Numbers in parentheses are numbers of mice.

[e] Same as experiment 3, Table 4.

TABLE 7. *Incidence and time of autoimmune deaths*

Expt no.	Initial no. of mice	Period observed (months)	Deaths[a] (month)	Antibodies[b]		
				Heterophile[c] E + C′	DNP	DNA
1	10	3	None[d]	<10	40	<10
	10	12	8, 9, 10[e]	80 (40)	80	<10
2	8	10	7, 7, 7, 10[f]	<10 (80)	–	<10
3	8	8	None[d]	<10	<10	<10
	7	13	9, 10, 13[g]	80 (<10)	80	20
CM	10	8	None[h]	<10	<10	<10

[a] All mice had kidney lesions, and some showed wasting several weeks before death.

[b] Tests on pooled sera. See Tables 4 and 6 for time of bleeding.

[c] See Table 4; titers by agglutination; hemolysis in parentheses additional deaths.

[d] Same group of mice as on line just below, but sera were collected before the appearance of autoimmune disease when anti-spleen antibodies were present as shown in Fig. 1.

[e] Additional deaths: one mouse died at 9 months with questionable kidney lesions, four were used in an other experiment, and two were bled out at 12 months.

[f] Additional deaths: two mice died after bleeding (7 months) one died of injury with kidneys normal, and one died with questionable kidney lesions.

[g] Additional deaths: deaths at 9, 9, 10, 13 months, autopsies unsatisfactory because of postmortem changes or cannibalism.

[h] Additional deaths: one mouse died at 8 months; no significant pathology.

phy of lymphoid tissue. The lungs, liver, and other organs usually appeared normal.

Microscopic examination of kidney sections stained with hematoxylin and eosin showed that the kidney damage involved the entire parenchyma, with participation of both mononuclear cells and polymorphonuclear neutrophils (Fig. 2).

DISCUSSION

Several aspects of the interaction of budding virus with host cell antigens have been reviewed previously (4), and it was suggested that antigen modification occurred in the membrane itself, not in the envelopes of free virus particles concentrated from tissue culture supernatant. The free virus was much less immunogenic against tumor transplant than isolated membranes of infected lymphoma cells. Furthermore, it is now apparent that antigen modification by virus is not limited to tumor antigens but may extend to some as yet unknown normal tissue antigens not related to the tumor antigen. Attempts to produce tumor transplant immunity with membrane preparations of 3T3 cells infected with influenza virus were unsuccessful (1). Conceivably, autoimmune effects might occur after injection of membranes of normal cells in which NDV had grown; this is being investigated.

Neuraminidase is known to cause modification or unmasking of antigens and has been used successfully with some tumors (16) to enhance immunogenicity. Boone (1) has reported negative results with *V. cholerae* neuraminidase and simian virus 40 sarcoma, and we have not been able to show enhancement of immunogenicity of the ascites lymphoma.

A sensitive radioimmunoassay has been used to demonstrate widespread occurrence of antibodies to leukemia virus in normal mice (12), and with some cell types carrying leukemia antigen, cytotoxic antibodies are found in "normal" serum from older mice. The CF method used by us is apparently less sensitive than radioimmunoassay because normal C_3H/Bi sera and those from mice receiving repeated injections of CM gave no CF with lymphoma cell fractions known to contain Gross virus antigens.

Autoimmunity such as that associated with lymphocytic choriomeningitis (13) or graft-versus-host reactions (14) can activate endogenous leukemia virus. Tests for lymphocytic choriomeningitis in our mice were negative, but the autoimmune disease observed by us in some respects resembles a graft-versus-host reaction. If activation of endogenous leukemia virus does occur, it must be only at the level of leukemia antigen expression on cell membranes because none of the mice immunized with NDV-CM have developed signs of leukemia.

Several viruses cause chronic infections in mice with circulating virus-antibody immune complexes and nephritis, but are not known to be associated with autoantibodies except for one report on lymphocytic choriomeningitis (8). The ascites lymphoma was found to contain lactic dehydrogenase virus, but "contaminated" membrane preparations, unless treated with a myxovirus, did not produce autoimmunity. Similar objections can be raised to the possible role of murine sarcoma and polyoma viruses. In early experiments (5), large amounts of NDV were injected with 10^2 to 10^3 intact tumor cells repeatedly for a year or more, but no autoimmune disease was observed. The high titers of anti-NDV antibodies, always present in the treated mice, would tend to eliminate circulating NDV-antibody complexes.

The agglutination of sheep erythrocytes and hemolysis by the mouse antisera in the presence of complement may be attributed to a Forssman-like antibody. The ascites lymphoma cells are lysed by rabbit antiserum against

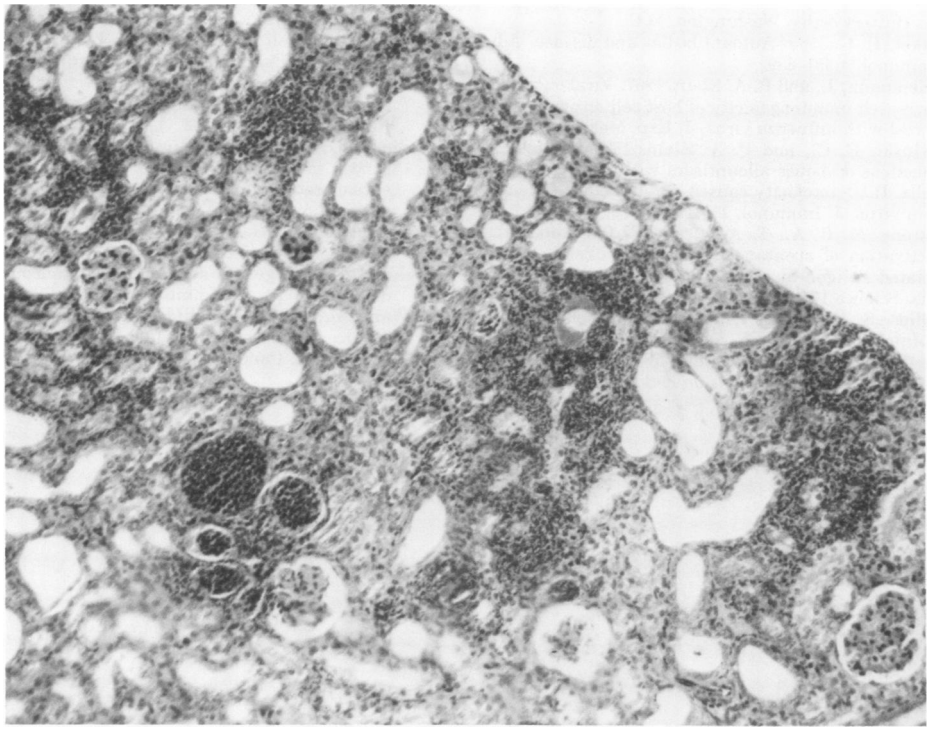

FIG. 2. *Kidney section stained with hematoxylin and eosin. Mononuclear and neutrophil infiltration and glomerulus obliterated with polymorphonuclear leukocytes. Several glomeruli appear normal or show only minor infiltration. Heavy interstitial infiltration around the tubules, which appear to be distended in several areas, some containing polymorphonuclear leukocytes. Destruction of normal kidney architecture.* ×120.

heat-treated sheep erythrocytes (6), and it is possible that immunogenicity of the heterophile antigen, in syngeneic mice, was enhanced by the action of NDV. The additional reactions with erythrocytes sensitized with rabbit hemolysin and conglutination in the presence of guinea pig complement are probably due to antiglobulins which are known to be associated with autoimmunity (10).

Antibodies to DNP were found in all groups of mice developing fatal autoimmune disease and appeared relatively early during the course of immunization with NDV-CM. Spontaneous appearance of these antibodies in aging mice has been noted (17). Since the mice studied by us were at least 8 months old by the time they died with kidney damage, it is possible that age was a factor. However, DNP antibodies appeared in immunized mice not more than 4 months old and were absent from those receiving uninfected ascites tumor cell membranes up to the age of 10 months.

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