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Autoimmunity Induced by Syngeneic Splenocyte Membranes Carrying Irreversibly Adsorbed Paramyxovirus

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Newcastle disease virus was adsorbed to a membrane fraction prepared from splenocytes, and the resulting preparation was injected into syngeneic C3H mice. Complement fixing and cytotoxic antibodies reactive with syngeneic tissue and intact cells developed, and some mice died with autoimmune disease characterized by wasting, severe kidney damage, and loss of lymphoid tissue as described previously for animals receiving the membrane fraction of a syngeneic lymphoma in which Newcastle disease virus had grown. Similar experiments were done with L929 mouse fibroblasts and allogeneic spleen membrane fractions. With syngeneic spleen tissue and L929 fibroblasts, serological evidence of autoimmunity appeared after several injections, but deaths from autoimmunity were considerably delayed unless Freund's complete adjuvant was given with the antigen. The results suggest that antigen modification occurs after adsorption of the paramyxovirus to normal tissue as well as lymphoma cell membranes.

In several autoimmune diseases, the involvement of a virus has been suspected. The prime animal model for lupus is the NZB mouse, and evidence for a type C virus as a cause has been presented (17). This disease of NZB mice is exacerbated by lymphocytic choriomeningitis or polyoma virus accompanied by an increase in antinuclear antibodies, but chronic infection with these agents does not produce the disease in other strains of mice such as C3H or BALB/c (18). A disease like lupus has been produced in C3H mice (5) by inoculating plasma membranes from tumor cells infected with Newcastle disease virus (NDV) or parainfluenza I (strain Sendai), but the involvement of other myxoviruses in autoimmunity is controversial (9). Diseases associated with these viruses that may have an autoimmune component are encephalitis, orchitis (mumps), and thyroiditis. A runting syndrome similar to graft versus host disease is sometimes caused in mice inoculated when newborn or in utero by chronic infections with lymphocytic choriomeningitis, polyoma (19), or reoviruses (8). In lymphocytic choriomeningitis, killer lymphocytes with broad specificity have been found and these may play a part in the occurrence of autoimmunity in this and other virus infections (20).

Type C leukemia viruses have been associated with a number of autoimmune reactions in rodents. Friend leukemia virus produces autohemolysins and runting after inoculation into newborn rats (12) and also xenogenization of lymphocytes (11). Autohemolysins have been associated with spontaneous hemolytic anemia (presumably viral) in NZB mice, and the nature of the responsible antibodies has been analyzed (3). A neoantigen apparently not related to the virus appears in the preleukemic spleen cells and kidneys of mice infected with a lymphomagenic virus (14). In attempts to treat or prevent spontaneous leukemia (Gross virus) in AKR mice with *Mycobacterium bovis* BCG, a runting syndrome and autoantibodies appeared in some of the mice (13). This strain carries the type C virus from birth, and it is not clear whether the runting was due to autoimmune damage by the virus or by BCG.

In a recent study in our laboratory it was shown that antigenic modification of separated lymphoma cell membranes could be produced by irreversible adsorption of paramyxoviruses (3a). This eliminated the necessity of growing virus in the cells, and a similar adsorption procedure was found to be effective with splenocyte membranes. The purpose of the present study was to demonstrate the production of autoantibodies and autoimmune disease as previously described (5) with normal splenocyte membranes that had adsorbed NDV and to compare the results with those obtained with lymphoma cell membranes.

MATERIALS AND METHODS

Mice. Male or female C3H/Bi mice inbred by continuous brother × sister mating were used in most experiments. In one series, an F_1 cross of DBA/2 × C3H/Bi was used.

Virus. NDV strain Cal was obtained and used as in previous studies (4, 5). Virus grown in the allantoic sac of chicken embryos was partially purified and concentrated by differential centrifugation. Virions suspended in phosphate-buffered saline (PBS) had titers

of 5,000 to 50,000 hemagglutination units (HAU) per ml. NDV was not lethal for mice when 1,000 HAU or less were injected by the intraperitoneal route.

Ascites lymphoma. Our C3H/Bi mice were infected with the Gross leukemia virus, and an ascites lymphoma was obtained first by intraperitoneal transplant of leukemic spleens, then by passage of tumor cells in peritoneal fluid. The 50% lethal dose for both C3H/Bi and C3H × DBA (F_1) strains is 5 to 10 cells.

Preparation of crude membranes from spleen and tumor cells. Tumor cells were allowed to swell in 20 volumes of 0.05 M Tris buffer (pH 7.4) containing sodium azide (0.01 M) and iodoacetate, then disrupted in a Dounce homogenizer with 20 strokes; 1/20 volume of a stabilizing solution, 0.04 M MgCl and 0.1 M NaCl, was added immediately and the nuclei and intact cells were removed by centrifugation at 2,000 rpm for 30 s. The membrane fraction was then sedimented at 10^4 rpm for 20 min and suspended in phosphate-buffered saline at pH 7.2 by using a volume twice that of the original cells. Each milliliter of the resulting suspension contains membranes from about 10^8 cells. These preparations to be used in complement fixation were stored at −20°C. In addition to the crude membrane antigens prepared from uninfected lymphoma cells, similar preparations were made from normal spleen as follows. The tissue was minced to small fragments in 0.01 M tris(hydroxymethyl)aminomethane (Tris) buffer, pH 7.4, containing 0.01 NaN_3 and diluted in Tris-azide to a volume 20 times the original weight of the spleens. After a brief period to allow swelling of the cells, the spleen fragments were disrupted in a Dounce homogenizer (Bellco), first with a loose pestle, then with a tight pestle using about 25 strokes in each. One-tenth volume of 1.5 M NaCl was added, and the suspension was put in the refrigerator for 1 h to allow partial precipitation of nuclear material. Separation of a membrane-enriched fraction was done by centrifuging first at 1,000 rpm for 5 min and discarding the sediment. Then the membrane fraction was obtained by centrifuging the supernatant at 10^4 rpm for 20 min. L929 mouse fibroblast (C3H) cultures were also used for similar membrane preparations.

Adsorption of concentrated virus. Membrane suspensions mixed with an equal volume of virus suspension, containing about 5,000 HAU/ml, were thoroughly dispersed in a Dounce homogenizer and allowed to remain at 25°C for 1 h. Membranes with adsorbed virus were then sedimented at 10^4 rpm for 20 min and washed in phosphate-buffered saline by suspending and centrifuging at least three times or until less than 10 HAU of virus per ml remained in the supernatant. Viral antigen irreversibly attached to the membranes was measured by complement fixation with sera of rabbits immunized with egg passage virus.

Immunization of mice. A 0.2-ml amount of the membrane suspension was injected i.p. into 6 to 10 mice at intervals of 3 to 4 weeks. In some experiments Freund's complete adjuvant (CFA; Difco Laboratories) was emulsified with the antigen and given with all injections. Control mice received crude membrane that had not been treated with virus. In some experiments, mice were challenged with 100 to 500 ascites tumor cells. Other mice receiving the immunizing injections were kept under observation for signs of autoimmune disease and for serological tests. Mice dying were autopsied, and the cause of death was recorded as due to autoimmunity as previously described (5) or to nonspecific disease such as pneumonia or enteritis. The presence of autoimmunity was confirmed by serology.

CF. The procedures for the measurements of antigen or antibody by complement fixation (CF) were the same as those used previously (4). Serum dilutions and sensitized sheep erythrocyte suspensions were made in phosphate-buffered saline containing 50 mM $MgCl_2$ and 12 mM $CaCl_2$.

The tumor cell and spleen antigens were standardized by complement fixation with serum from a rabbit immunized with mouse tissue. For antigen in the CF tests, membrane suspensions equivalent to about 0.05 g of cells per ml were used.

Cytotoxicity tests. Washed ascites tumor cells or splenocytes were prepared as 1% suspensions in Eagle minimal essential medium containing 5% calf serum, 17 mM HEPES buffer (*N*-2-hydroxyethylpiperazine-*N*′2-ethanesulfonic acid), pH 7.4, and 50 μg of gentamycin (Schering Corp.) per ml. No bicarbonate was added to the medium, because the buffering action of the HEPES was sufficient to maintain the pH above 7.0 for 18 to 24 h. Splenocytes were obtained by mincing spleen fragments, screening, and lysis of erythrocytes in isotonic NH_4Cl. All tests were done with 0.1 ml of a 1:4 dilution of mouse serum, to which was added 0.1 ml of rabbit complement 1:16 and 0.2 ml of 1% cell suspension. After 18 h of incubation at 37°C in a water bath, the proportion of dead cells was determined by the dye exclusion method, using 0.1% toluidine blue for a staining period of 3 min (7). The cytotoxic index was stated as the percentage of dead cells in the tubes with mouse serum minus the percentage of dead cells in controls with complement only. The percent dead in controls was usually 20 to 30%. Immune serum alone was not cytotoxic.

Autohemolysis. Blood from male C3H/Bi mice was collected in phosphate-buffered saline containing 100 U of heparin per ml. The erythrocytes, washed three times, were suspended at a 1% concentration in phosphate-buffered saline containing 50 mM $MgCl_2$ and 12 mM $CaCl_2$. These suspensions were always used within 24 h. Serial twofold dilutions of syngeneic mouse serum 1:4 to 1:64 were prepared in 0.1-ml amounts; 0.2 ml of erythrocyte suspension and 0.1 ml of guinea pig complement 1:4 were added. Controls consisted of mouse serum without complement or of erythrocytes alone. No hemolysis was seen at any time in the guinea pig complement at 1:4 by itself. After 3 h at 37°C the tubes were examined for hemolysis and then shaken and placed at 4°C overnight. Often a second incubation at 37°C was necessary before hemolysis became evident. Some sera showed a prozone-possibly because of an anticomplementary effect at lower dilutions.

RESULTS

Adsorption of virus. Viral antigens adsorbed irreversibly to plasma membrane fractions of splenocytes, ascites lymphoma, and L929 mouse fibroblasts were titrated with anti-NDV rabbit serum. The results in Table 1 show high titers of viral antigen as compared with slightly

TABLE 1. *Titration of viral antigens adsorbed to membrane fraction*

Antigen with or without adsorbed NDV	Highest dilution of antigen giving CF[a]	
	Rabbit anti-NDV	Rabbit anti-influenza
Spleen membrane + NDV	80–160	10[b]
Spleen membrane, no NDV	<10	ND[c]
Lymphoma membrane + NDV	80	10–20[b]
Lymphoma membrane, no NDV	<10	<10
L929 fibroblasts + NDV	160	ND
Lymphoma membrane + NDV grown	40	<10

[a] Serum dilutions 1:20 or 1:40. Undiluted antigen equivalent to membranes from about 10^8 cells.
[b] Titer with anti-influenza rabbit serum against allantoic grown virus.
[c] ND, No data.

lower titers in membranes from lymphoma cells in which NDV had grown. Untreated membranes gave no reaction with NDV antiserum. Antisera to unrelated viruses such as influenza grown in the chorioallantois gave weak reactions with membranes that had adsorbed NDV. These are attributed to the presence of chicken antibodies which could be removed by adsorption of the sera with minced chorioallantoic tissue, but such absorption did not reduce the complement fixation of NDV-treated membranes with anti-NDV serum. With an antiserum to sheep erythrocytes, weak positive reactions were obtained, indicating the presence of Forsmann antigen as previously reported (7) (not shown).

During adsorption of virus by membranes, the HA titer of the supernatant was reduced by about 50%, and a similar proportion of viral hemolysin was removed as determined by titration with guinea pig erythrocytes. Membranes after washing gave a relatively low titer by HA/HA inhibition (5) (10 HAU in the membranes of 10^7 cells), and it appears that much of the input viral antigen elutes. Despite the low HA titer, complement fixation titers of 40 to 100 were observed. It has been shown that the CF titer is proportional to the amount of virus added for adsorption (manuscript in preparation). Burnet and his colleagues (2) first observed irreversible adsorption of a portion of the input NDV to erythrocytes. Similar reactions have been studied recently in nucleated cells. When carrying adsorbed virus on the plasma membrane, such cells will adsorb antibody from antiviral serum, and they are sensitized to antiviral immune cytolysis by antibody and complement or by immune lymphocytes.

Serological responses (measured by CF and cytotoxicity), indicating development of autoimmunity after injection of spleen membrane fractions with adsorbed NDV, are presented in Tables 2 and 3. In earlier experiments CF antibody response was poor when the antigens were given without adjuvant, but better results were obtained when CFA was given with each injection. This caused the development of ascites in some mice, and it is possible that a few controls died with enteritis or pneumonia due to the effects of the adjuvant.

The results of an experiment in which mice receiving spleen membranes plus NDV and CFA were bled at intervals is presented in Table 2. After two injections, little response in CF antibodies was found, but a high cytotoxic index with splenocytes developed. After four or more injections, there were moderate to high CF titers against both ascites lymphoma and spleen antigens. CF titers of sera from mice receiving CFA and membranes without NDV were low, but after several injections titers of 10 were found consistently, and this suggested that CFA as noted by others may produce some autoimmunity when injected with syngeneic antigen homogenates. The cytotoxic reactions in these control mice were generally weaker.

Representative results of experiments done without CFA are shown in Table 3. With C3H spleen and adsorbed NDV, titers of CF antibodies were low, even after 11 injections over a period of 10 months. Cytotoxic antibodies were essentially undetectable. Some of these mice did develop autoimmune disease (Table 4), suggesting participation of a cell-mediated rather than a humoral immune response. In an experiment with NDV adsorbed to membranes of C3H fibroblasts (L929), antibodies appeared after five injections but decreased after additional immunization. It is of interest that cytotoxic antibodies for splenocytes but not lymphoma cells were found. Immunization with allogeneic Swiss spleen membranes and NDV produced a weak CF antibody response to C3H lymphoma and spleen.

Autohemolysins appeared in the sera of mice inoculated with C3H or Swiss splenocyte membrane with adsorbed NDV but not in those receiving membrane preparations without NDV. The possible relationship of these hemolysins to Paul-Bunnell antibodies will be considered in another paper. Some of these sera also contained antibodies to deoxynucleoprotein (not shown), as previously reported for immunization with NDV-lymphoma membranes (5). Mouse fibroblast membranes with NDV failed to stimulate detectable hemolysins.

Autoimmune disease. The main features of the autoimmune syndrome are wasting or runting and severe kidney damage, as previously described (5). In some groups of mice, the appearance of wasting and abnormal skin and fur were used as indicators of approaching death

TABLE 2. *CF and cytotoxic antibody response of mice receiving splenocyte membranes treated with NDV or untreated membranes*[a]

Virus adsorbed	No. of injections	No. of mice bled	CF titer[b]		Cytotoxic index		Autohemolysin titer
			ALM[c]	Spleen	ALM[c]	Spleen	
NDV	2	6	10	20	8	67	10
0	2	6	<10	<10	2	19	0
NDV	4	4	40	40	29	34	ND
0	4	5	10	10	18	0	ND
NDV[d]	6	6	40	20	32	20	ND
0[d]	6	8	20	10	11	18	ND
NDV	7	6	80	40	40	60	20
0	7	7	10	10	24	4	0

[a] All injections were with CFA (1 volume + 2 volumes of membrane suspension).
[b] Each value represents the titer of pooled serum from the number of mice indicated.
[c] ALM, Uninfected ascites lymphoma membranes.
[d] Challenged with tumor cells (see text).

TABLE 3. *Injections without CFA of NDV-treated membrane from spleen and C3H fibroblasts (L929)*

Material injected	No. of injections	Immunization period (mo)	CF titer		Cytotoxic index		Autohemolysin titer
			ALM[a]	Spleen	ALM[a]	Spleen	
C3H spleen + NDV	6	4	20	10	0	11	10
	11	10	10	10	0	0	
Swiss spleen + NDV	6	5	20	20	0	ND[b]	40
Swiss spleen, no NDV	6	5	10	10	ND	ND	0
L929 culture + NDV	5	5	20	40	0	65	0
	8	10	10	10	0	39	0
	11	16	0	0	0	12	0

[a] ALM, Ascites lymphoma membranes.
[b] ND, No data.

from autoimmunity, and all mice were sacrificed by bleeding and autopsied. In other experiments where development of symptoms was not as rapid, tests for serological evidence of autoimmunity were done periodically, and if no mice died they were bled out after a long observation period.

In the first experiment in Table 4, twelve mice received NDV membranes with CFA. Of these, five died, with an average survival time of 2.5 months, and an additional five mice having autoimmunity were sacrificed at 3.7 months. One mouse died without signs of autoimmunity (Table 4, last column), and the remaining mouse survived and was judged to be normal at the end of the experiment. (Mice surviving without disease are not shown in the table.) Of the control mice injected with CFA and untreated membrane, only two were found to have damaged kidneys when sacrificed. Five mice died with enteritis, pneumonia, or trauma late in the observation period but did not have kidney damage, and the rest were normal. In another similar experiment (Table 4, lines 3 and 4), although a few mice died from causes other than autoimmunity, the major portion survived for 5.5 months and were sacrificed. Six of nine receiving NDV membranes had scarred kidneys, and only one control showed signs of kidney damage.

In two additional experiments (Table 4, lines 5 and 6), mice given C3H spleen and NDV without CFA survived much longer, but most of them died or were sacrificed with autoimmune disease after 9 to 11 months. In the experiment with allogeneic (Swiss) spleen antigens, the average survival time was shorter (5.9 months), and none of the controls showed autoimmune disease.

Ten mice were injected with NDV membranes from cultured L929 mouse fibroblasts that were derived from C3H mice many years ago and could be considered quasi-syngeneic except for minor antigen changes. These mice survived for over 2 years without signs of autoimmune disease. However, autoantibodies were found after five monthly injections (Table 3), diminishing in titer at 16 months. At the time of sacrifice, moderate kidney damage was found in four mice. Among nonspecific deaths of mice between 24 and 28 months, one was caused by cardiac puncture, and one apparently by polyoma virus. These showed questionable kidney damage, and

TABLE 4. *Autoimmune disease in C3H/Bi mice inoculated with adsorbed NDV on membrane from syngeneic splenocytes, allogeneic spleen, L929 mouse fibroblasts, and C3H × DBA/2 spleen*

Material injected	CFA	Initial no. of mice	Injection no.	Period observed (mo)	AST[a]	Deaths/total	
						Autoimmune	Other cause
C3H/Bi spleen + NDV	+	12	5	3.7	2.5	5/12	1/12
					S	5/12	
C3H/Bi spleen no NDV	+	12	5	3.7	S	2/12	5/12
C3H/Bi spleen + NDV	+	9	7	5.5	S	6/9	3/9
C3H/Bi spleen no NDV	+	10	7	5.5	S	1/10	2/10
C3H/Bi + NDV	0	6	16	15	11.1	4/6	1/6
					S	1/6	
C3H/Bi + NDV	0	6	10	10	9	3/6	1/6
Swiss spleen + NDV	0	10	10	8.5	5.9	7/10	3/10
Swiss spleen no NDV	0	6	8	8.5	S	0/6	1/6
L929 + NDV	0	10	11	28	S	4/10	4/10[b]
C3H × DBA/2 + NDV[c]	0	10	5	5	3.0	4/10	5/10
C3H × DBA/2 no NDV[c]	0	12	5	5	3.1	4/12	4/12
					S	4/12	

[a] AST, Average survival time in months; S, sacrificed by bleeding at end of observation period.
[b] Survival times were 24, 27, 27, and 28 months.
[c] Results of two experiments combined.

in two others at autopsy the kidneys were normal on gross examination. In this experiment the effects of aging must be considered.

In an additional investigation of the effect of allogeneic antigen, C3H/Bi mice were injected with splenocyte membranes from C3H × DBA/2 F_1 hybrids with and without absorbed NDV. The results were quite different from those of other experiments. Many of the mice died with wasting and scarred atrophic kidneys after a short period and only 3 or 4 injections. There was essentially no difference between the pathology and death rate of mice receiving adsorbed NDV preparations and those without NDV. Several mice had enlarged spleens instead of the atrophy seen in those receiving syngeneic tissue, and an additional feature was the presence of hemorrhagic adrenals in practically all cases of autoimmune disease. These results indicate that the autoimmunity was attributable to the presence of allogeneic antigen in the same membrane with syngeneic antigen and not to the adsorbed NDV.

Tests for tumor transplant resistance. Mice in the groups used for the study of antibody response (top lines of Table 2) after six injections were challenged with 500 ascites lymphoma cells. All died: nine mice with NDV membranes had an average survival time of 24 days, eight receiving untreated spleen membranes had an average survival time of 21.6 days, and seven having no injections survived an average of 17 days. Since no mice survived, resistance to tumor challenge was considered doubtful. In other experiments, using a smaller challenge dose of 50 cells, a few mice survived but there was no difference in survival rate or average survival time between those injected with NDV membranes and those with no injection.

DISCUSSION

The purpose of this study was to determine whether normal splenocyte membranes with adsorbed NDV would produce autoimmunity like that caused by membranes from lymphoma cells in which virus had grown (5) or by isolated lymphoma membranes with adsorbed virus. Provided that CFA was given with all injections, autoimmune response was similar to that with tumor cell membranes but development of autoimmune disease with splenocyte or L929 fibroblast membranes in the absence of CFA was slower than with lymphoma membranes. It is perhaps not surprising that CFA given with membranes without virus caused autoimmune disease in a few control mice. CFA mixed with organ homogenates has long been used in studies of experimental allergic encephalitis or nephritis.

It is doubtful that NDV released in mice can produce autoimmunity by itself. In previous experiments, large amounts of NDV (1:20 infected allantoic fluid) were injected with 10^2 to 10^3 intact tumor cells repeatedly for 1 year or more (6), but no autoimmune disease was observed. The high titers of anti-NDV antibodies always present after the first injection would tend to eliminate circulating NDV and virus antibody complexes. No occurrence known to us of autoimmunity has been described by other investigators who have injected free NDV into animals. The amount of irreversibly adsorbed virus used in the present experiment is considerably less

than that of free virus described earlier (6). Injection of cells in small numbers after treatment with NDV into NDV immune mice resulted in destruction of the tumor cells, but in these experiments the amount of cellular antigen was apparently too small to produce autoimmunity.

Although antibodies against ascites tumor cells were produced by injection of splenocyte membrane with adsorbed NDV, these were considered to be due to the antigens also present in normal spleen, and no convincing evidence of resistance to tumor transplant could be obtained. However, such resistance was found in mice immunized with lymphoma membrane and adsorbed NDV (manuscript in preparation). The constant presence of Gross virus antigens in the lymphoma membranes might have an enhancing effect and thus account for more rapid development of autoimmunity in addition to tumor immunity.

Autoimmunity was also produced with allogeneic tissue from Swiss mice and C3H × DBA/2 F_1 hybrids. The disease resulting from injection of hybrid splenocyte membranes with or without virus was similar but not identical to that from syngeneic membranes. The presence of alloantigen as well as syngeneic antigen in the membranes may have produced a helper effect which was independent of the adsorbed virus. It is not known whether NDV acts as an antigenic helper or modifies antigens on the cell membrane so that they appear foreign to the immunologic systems of the host. Murine lymphoma cells treated with iodoacetamide (1) were shown to produce tumor immunity in syngeneic mice and also autoantibodies reactive with thymocytes from BALB/c (the syngeneic host) AKR and DBA mice but not with other tissues except brain. A crude membrane fraction isolated from mouse tumor cells when treated with dimethyl sulfate produced tumor protection in syngeneic mice (15). There is evidence that analogous modifying biochemical reactions occur with viruses (see literature referred to in reference 5). Recent studies in this laboratory have indicated that the F (fusion) envelope protein of paramyxovirus is a major factor in antigen modification (3a). If the protein is inactivated or used in the uncleaved nonhemolytic form, even though the hemagglutinin and neuraminidase are intact, the autoimmune response in syngeneic mice is reduced. These observations favor an antigen modification hypothesis rather than a helper effect.

There is a similarity of the autoimmune disease described in this and other papers to graft versus host reactions. The runting disease in mice with chronic virus infections may have an autoimmune component produced by in vivo xenogenization. Destruction of lymphoid tissue and autohemolysins has been described in rats inoculated when newborn with Friend leukemia virus (12), and it was suggested that interaction of virus with lymphoid cells in vivo was responsible for autoimmunity. The autohemolysin found by us has characteristics similar to Paul-Bunnell antibodies, and the corresponding antigen has been found in some lymphomas (7, 10).

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