Target cells of the Friend virus complex : studies in nude and anti-[mu] suppressed mice
by Kenneth Wayne Lee

A thesis submitted in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in Microbiology
Montana State University

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ABSTRACT

The nature of the target cell for the virus in the Friend virus complex remains unclear. The Friend virus complex was assayed for its capacity to induce splenic foci or splenomegaly in thymus deficient (nude) mice and neonatally suppressed B-type Balb/c mice. Following challenge with either B or NB-tropic virus, nude mice developed typical symptoms of Friend leukemia. No overt symptoms were evident in nude mice challenged with N-tropic virus 30 days or more after birth, unless they were injected with relatively high doses of virus. This refractory state could be overcome by supplementing the Friend virus with Moloney leukemia virus. Anti-μ suppressed mice remained totally refractory to the disease process when challenged with N-tropic virus, while mice challenged with NB-tropic virus were partially refractory to the disease. On the other hand, anti-μ suppressed mice challenged with B-tropic virus exhibited typical symptoms of Friend virus leukemia. Mice neonatally suppressed with anti-immunoglobulin (devoid of anti-μ) were partially refractory to the disease when challenged with N-tropic virus, but not NB-tropic virus. In addition to distinguishing virus tropism, these results support the hypothesis that the target cell for one of the virus in the Friend virus complex is an immunoglobulin-bearing B-lymphocyte or its precursor. The B-lymphocyte appears to be the target cell for the helper component of the Friend virus complex.

INTRODUCTION

In 1957, Charlotte Friend described the development of a leukemia in Swiss mice following an inoculation with cell free extracts of Erlich acites carcinoma (34). DeHarven and Friend (38) later described the presence of virus-like particles in the spleen, bone marrow, and liver of leukemic mice. This report was followed by a detailed description of the morphology of the virus and its identification as the etiologic agent of the leukemia (39). The neoplastic process initiated by the Friend virus is characterized by splenomegaly, resulting from a proliferation of reticulum cells, and erythroblastosis (60). In the terminal phases of the disease there is massive enlargement of the liver and spleen and infiltration of the bone marrow with resultant failure of normal hematopoeisis (5).

It has been demonstrated by several laboratories (5, 21, 22, 24, 29, 56, 68, 71) that Friend virus is a complex that contains at least two distinct virus which have been named on the basis of their pathogenic properties, spleen focus forming virus (SFFV) and lymphatic leukemia virus (LLV). An investigation of the relationship between LLV and SFFV indicated that SFFV is helper dependent for spleen focus formation (5). SFFV appears incapable of carrying out its entire infectious cycle and of inducing erythroleukemia in the absence of coinfection with LLV. Thus LLV serves a helper function, supplying factors missing from the SFFV genome which permits the production of infectious virions (25,54).

Specific host genes have a marked influence on the virus-induced oncogenesis in mice (53). The host range of Friend virus has been investigated extensively and many studies have been performed to determine the genetic factors that control susceptibility and resistance to Friend virus. These studies have been well summarized by Lilly (54) and reviewed by Lilly and Pincus (55).

The disease induced in mice by Friend virus is a model of a neoplastic condition, susceptibility to which is a multiple-gene trait (54). There are at least eight genes that are known which govern the susceptibility to Friend virus (53). Of these, two are major loci, Fv-1 and Fv-2, which govern the response to the virus. Susceptibility to SFFV is regulated by Fv-2 while Fv-1 influences susceptibility to the helper component of the Friend virus complex. An additional gene, Rgv-1, associated with the H-2 locus, plays a modifying role during the late stages of the disease. Rgv-1 influences the disease by affecting quantitatively the splenomegaly response to antigens associated with virus infection. The remaining five genes that are known include two additional histocompatibility genes, H-4 and H-7, and three genes which affect erythropoiesis, W, Sl, and f (53).

Naturally occuring Murine leukemia viruses may be classified into three groups according to their ability to replicate in NIH Swiss and Balb/c mouse embryo cultures (82). The difference in response in the two cell lines is not absolute, but relative, being of the order of

100 to 1000-fold (79). Strains of virus replicating best in each cell line are termed N and B-tropic, respectively. Strains of virus which replicate equally well in both cell lines are termed NB-tropic. NB-tropic Friend virus complexes arise after forced passage of either B or N-tropic virus through mice strains which are restrictive for that strain of virus (37). The ability of the cell lines to support replication of the three types of virus is genetically determined. The gene responsible for susceptibility of in vitro cultures appears to be identical to the Fv-1 locus (67). This gene determines susceptibility to LLV, whereas SSFV susceptibility is governed by the Fv-2 loci (54, 64).

In man, the clinical hematological and physiopathological data that are presently available in chronic granulocytic leukemia, polycythemia vera, and the erythroblastic component of erythroleukemia are compatible with the Friend physiopathological model (43). The general interest in this disease as a model for human leukemia prompted the present research into the nature of the target cell for Friend virus induced leukemia.

Morphological transformation of as yet unidentified target cells occurs in vivo after infection of susceptible mice by Friend virus. Several lines of evidence suggest that a B lymphocyte may be the target cell for Friend virus infection. Infection of mice with Friend Leukemia virus results in a marked immunologic impairment (4, 8-15, 19, 33, 44, 69, 70, 78, 80). Previous studies showed that the immune

response to sheep erythrocytes as well as to other antigens such as E. coli LPS, was severely depressed in Friend virus infected mice (49, 13). Ceglowski and co-workers (13) have shown that a marked suppression of humoral immune responses to unrelated antigens is a predictable consequence of virus induced leukemogenesis. It has become clear that the immunosuppressive event occurs quite early in the disease process and is observed shortly after infection of a susceptible host (13). The precise mechanism by which leukemia viruses mediate their immunosuppressive effect has not, as yet, been elucidated. However, a number of studies concerning the mechanism of Friend virus induced immunosuppression in mice pointed toward the antibody precursor cells derived from the bone marrow as a major target for the immunodepressive properties of the virus (9, 16-18, 35, 40, 41, 49, 62, 73). In addition, other studies showed that virus-like particles are present in lymphoid cells presumed to contain or secrete antibodies (48). This agrees with later studies (45) which used electron microscopy to show virus particles budding from the lymphocyte surface and those studies which showed the presence of virus-like particles in Plaque-forming cells (PFC's) (47). It has been postulated that only cells that are infected by virus at an early stage are arrested in their function and maturation, whereas mature antibody-forming cells may be infected but not affected, and thus maintain their normal functions (33, 62). It has not been shown at exactly what stage of development or maturation the

potential antibody-forming cell may be infected with the virus. However, Manning et al, (57) report that neonatally initiated anti-μ suppression will render mice refractory to infection with Friend virus.

The effects of neonatally initiated injection of anti-μ has been well reviewed by Manning (58). Briefly, this treatment suppresses the formation of IgM, IgG_1, and IgG_2 in both conventionally reared and germfree mice. Production of IgA suppression requires somewhat higher dosages of suppressive antibody and is a rather unstable condition tending toward recovery. Similar panspecific immunosuppressive effects of anti-μ antibodies have been observed in mouse cell cultures as well as in cell culture and in vivo systems for several other species. More recently, Manning et al, (59) have shown that neonatally initiated injection of anti-μ antiserum in mice also suppresses the formation of reagenic antibodies. These observations support the hypothesis that immunoglobulin producing cells of all classes arise from IgM bearing precursors. The mechanism whereby anti-μ exerts its suppressive effect remains unknown.

The results reported by Manning et al, (57) would suggest that the target cell for Friend virus infection is a lymphoid cell which has differentiated to at least the IgM bearing state. It is presumed that viral replication and cellular transformation depend upon lymphocyte differentiation to the stage of immunoglobulin production.

T lymphocytes are not infected with Friend virus (16, 18, 36);

however, T lymphocyte function may be depressed (32). Prolonged skin graft survival times were observed in Friend virus infected mice, even when donor mice differed at the major histocompatibility locus (H-2). Quantitative in vitro assays for cell-mediated immunity revealed a marked impairment of cellular immunity for Friend virus infected mice (32). Thus both B and T lymphocyte functions appear to be affected by Friend virus infection, though perhaps not by the same mechanism or to the same degree.

In studies in which T lymphocyte deficient mice were infected with Friend virus, conflicting results have been reported. Stutman and Dupuy (74) using ALS and Axelrad et al, (3) using ATS have shown that T cells may function to inhibit or even destroy lymphoid cells having undergone malignant transformation. In agreement with these observations, Steeves and Grundke-Iqbal (73), reported that the nude mouse, described by Flanagan (30) and shown to be congentially thymus deficient (66), is at least as susceptible to Friend virus infection as its phenotypically normal littermates. However, Kouttab (50) reported that the nude mouse remains refractory to the typical symptoms of leukemia when challenged with Friend virus.

Although a pronounced immune depression follows Friend virus infection and evidence is available to suggest the lymphoid nature of the target cell, substantial evidence indicates that the primary target for Friend virus infection is not a lymphoid precursor but a

hematopoeitic stem cell of the erythrocytic lineage (31, 75, 77). Thus the immune defect may be a secondary event following infection of hematopoeitic elements.

Because of the incompleteness and the frequent discrepancies present in previous work on the nature of the target for Friend virus infection, studies reported here were initiated in an attempt to more conclusively define the nature of the target cell for Friend virus. Of primary interest was to quantitatively assess the lymphoid nature of the target cell using selectively suppressed B cell deficient mice. In addition, a reassessment of the infection characteristics of Friend virus in thymus deficient (nude) mice was undertaken. These studies were done for all three known strains of Friend virus.

The results of this study confirm that the target cell for at least one of the virus in the Friend virus complex is a lymphoid cell of the B cell line or its precursor. Evidence is also presented which suggest that the lymphocyte is the target cell for the helper component of the virus complex.

MATERIALS AND METHODS

Animals

Inbred conventionally reared Balb/c male and female mice were used. These mice were originally obtained in 1966 from Baylor Medical School (Houston, Texas) in the specific pathogen free state and were conventionalized in 1967. They have since been maintained by random brother-sister mating in our laboratory. The homozygous nude (nu/nu) mice and their phenotypically normal littermates (+/nu and +/+) were the offspring of heterozygous (nu/+) animals obtained by crossing nude males with females from our Balb/c colony. The nude gene is now in its tenth and eleventh cross-intercross generation onto the Balb/c background.

The nude mice and littermates were housed in a clean environment with individual filter cap cage isolation. All mice received sterilized Wayne laboratory animal chow and acidified-chlorinated water (63), ad libitum. All mice were weaned 21-24 days after birth. Mice were 4-8 weeks old at the start of experiments and groups of mice were age and sex matched within a particular experiment.

Viruses

Friend virus complexes of the three known tropisms were used. The NB-tropic (FV-NB) and B-tropic (FV-B) virus were a gift from Dr. Bruce Chesebro and were originally obtained from the stock of Dr. Frank Lilly. The passage history was such to maintain the stated tropism.

The N-tropic virus (FV-N) was obtained from Dr. A. Howard Fieldsteel in 1968 without prior passage history. It has since been maintained in our laboratory by frequent mouse passage. For experimental work, a virus stock was prepared for each virus by passage in Balb/c mice. Spleen homogenates (20%) were prepared in phosphate buffered saline (PBS) 14-30 days following virus infection. The crude homogenate was clarified by centrifugation at 1500 RPM at 4^{o} C for 10 minutes and stored at -70^{o} C in 1-2 ml aliquotes. Each virus preparation was titrated in Balb/c mice using the focus-forming assay described by Axelrad and Steeves (2). In addition, the mean infective dose (ID_{50}) as determined by spleen weight increase 24 days postinfection was calculated using the method of Reed and Muench (52).

The N-tropic characteristics of the virus preparation designated N-tropic were maintained even after passage in the B-type Balb/c mice. This was confirmed by Dr. Chesebro by simultaneous titration of the virus preparation in both N-type (CFW) and B-type (Balb/c) mice. The titer (FFU/ml) of the virus preparation was 100-500 fold greater in the N-type mouse than in the B-type mouse.

Another gift from Dr. Chesebro were the helper virus preparations, Tennant virus (B/T-L) and Moloney leukemia virus (MLV). MLV was prepared as tissue culture supernatants from clone 1 cells. These cells produced MLV with a titer of 1-2 X 10^{6} PFU/ml. Tennant virus was prepared as 10% organ supernatants from leukemic mice which had received

B/T-L at birth. The titer of this preparation was 0.5-1.7 X 10^3PFU/ml.

Virus Inoculation

Viruses from 20% spleen homogenates were thawed at 37° C and reclarified by low speed centrifugation as above. The supernatant was diluted in PBS and 0.25 or 0.50 ml was injected into the lateral tail vein of each mouse. Assay for spleen focus-forming activity was done in mice after intravenous inoculation of the various dilutions of the virus. Spleens were removed nine days later, fixed in Bouin's solution, and the macroscopic white foci visible under the spleen capsule were counted directly.

Preparation of Antigen

The antigen used to induce anti-μ production in rabbits was prepared using a modification of the procedure described by Krøll and Andersen (51). Briefly, mouse sera were reacted in gel diffusion against monospecific antisera directed toward the heavy chain, μ, of mouse IgM. Precipitin bands were allowed to form for 72 hours at room temperature, then the bands were cut out and the excess unreacted protein was removed by washing with several changes of PBS. The remaining precipitin bands and gel were then homogenized and lyophilized. Each injection consisted of the amount of protein that was contained in seven complete bands.

The anti-μ initially used to form the precipitin bands had been prepared in goats and was purchased from Meloy Laboratories (Spring-

field, Va.). This antiserum was used at a 1:2.5 dilution. All subsequent antigen preparations were made using adsorbed antisera harvested from rabbits which had responded to challenge with the primary antigen. This was done to avoid stimulation of a humoral response to goat antigen or to as yet unseen mouse antigens. A similar procedure was followed for preparation of antigens for the remaining classes of immunoglobulins, however, the respective class-specific antisera were substituted for anti-μ.

Preparation of Antisera

Antisera were prepared by subcutaeous injection of rabbits with the above described antigens. Each animal received two weekly injections in complete Freund's adjuvant (GIBCO, Grand Island, N.Y.), then weekly injections in incomplete Freund's adjuvant until serum precipitin titers ceased to rise. Thereafter injections were at ten-day intervals in incomplete Freund's adjuvant. Rabbits were bled by cardiac puncture also at ten-day intervals, but staggered by five days from the injection schedule. All antisera were routinely adsorbed with mouse erythrocytes thrice at a 2-3% concentration of packed and washed cells. Light chain specificity and antibodies directed toward mouse serum factors other than IgM were removed by passing the rabbit sera over affinity-binding columns. These columns had conjugated to them serum harvested from mice which had been suppressed with anti-μ from birth to age 42 days. This adsorbant contained all mouse protein except IgM.

The ligand was conjugated to Sepharose 4-B (Pharmacia, Piscataway, N.J.) using a modification of the procedure described by Bing (6). Briefly, activation was achieved by adding CNBr at a concentration of 100mg/ml of packed beads to well-washed Sepharose 4-B which had been suspended in an equal volume of distilled water. The entire solution was maintained at pH 11 using 4 N NaOH until the reaction was complete (ca. 10 minutes). The temperature was maintained at 20^{o} C by the slow addition of ice as required. When the reaction was complete, the solution was cooled rapidly by the addition of ice. The beads were then washed with 7-10 volumes of cold borate buffer (pH 9). Ligand which was suspended in the same borate buffer was mixed with an equal volume of activated Sepharose beads immediately after washing. Coupling was allowed to proceed for 16-24 hours in the cold or 2-4 hours at room temperature with constant gently agitation. Unreacted ligand was removed by washing the coupled beads with PBS until the 280 adsorbancy of the wash was 0.02 or less.

Specificity of all adsorbed antisera was checked by immunoelectrophoresis and Ouchterlony gel diffusion using commercial (Meloy) class-specific antiserum standards. Multiple passages through the column were made until no other specificity could be detected. Specific activities of the antisera were evaluated in Ouchterlony gel diffusion plates (65). The antisera used for suppression were capable of producing precipitin bands at dilutions of 1:32 or greater when

reacted against normal mouse sera diluted 1:4 or purified IgM standard (0.3 $A_{280\ nm}$ units/ml). Similar procedures were followed for each of the other immunoglobulins.

Suppressive Treatment

Each litter of mice was divided into three groups of two mice each. One group received anti-μ injections intraperitoneally, another was similarly treated with normal rabbit sera (NRS) while the third group received no treatment. Neonatally treated animals were injected with 0.05 ml of antisera within 24 hours of birth (day 0). The size of subsequent injections, given at 2-4 day intervals, was increased slowly until the total desired dose was achieved. At no time did a single injection exceed 0.50 ml. A similar schedule was followed for suppression with anti-Ig antibodies. These antisera contained antibodies directed toward the major immunoglobulin classes except IgM. No specificity other than immunoglobulin could be detected in these preparations.

Humoral Immunological Assays

Serum immunoglobulin levels were determined and antibody-forming cells elicited by a specific antigen, sheep erythrocytes (SRBC), were enumerated in groups of mice which had received the various treatments. Antibody forming cells were enumerated using a slide modification of the Jerne plaque assay (61). For animals suppressed with anti-μ, only direct PFC's were determined whereas; both direct and facilitated PFC's

were determined for those animals suppressed for immunoglobulins other than IgM. The mean number of PFC's was expressed as either the number of PFC's / 10^6 nucleated cells or number of PFC's per spleen.

Serum immunoglobulin levels were determined using the semiquantitative serial dilution Ouchterlony technique of Arnason, et al, (1). These levels were reported as the reciprocal of the highest two-fold serum dilution producing a distinct band against a constant dilution of commercial (Meloy) class-specific antiserum. Mean serum levels of groups were calculated as simple numerical averages of the individual values.

Assay for Direct Effect of Anti-μ on Friend Virus

To test the direct effect of anti-μ on Friend virus, both in vivo and in vitro, tests were performed. To test the direct effects of anti-μ in vitro, one ml of Friend virus suspension (FV-NB) was incubated with one ml of anti-μ (titer 1:64) at 37° C for 4 hours. Serial ten-fold dilutions were then prepared in PBS and the virus titer was determined in Balb/c mice. This procedure was repeated substituting either NRS or PBS for anti-μ. In vivo effects of anti-μ directly upon Friend virus were determined by challenging mice with virus after the mice had been treated from birth with a sub-suppressive level of anti-μ.

Effect of Helper Virus on Friend Virus Infection in Nude Mice

Thymus deficient (nude) mice were challenged with N-tropic Friend virus then injected with either Tennant or Moloney leukemia virus.

Each of these viruses have the capacity to act as helper for the SFFV component of the Friend virus complex (24,27-29,76). Friend virus inoculation was as previously described. A 1:10 dilution of the original Tennant virus preparation was made in PBS and 0.25 ml of this was injected intrapertoneally into each mouse immediately following challenge with FV-N. Mice were similarly treated with FV-N then challenged intravenously with a 1:12 dilution of MLV twelve hours later. Nine days later the mice were killed, the spleens fixed in Bouin's solution, and the number of macroscopically visible foci on the spleen surfaces was determined.

Spleen Index Determination

Induction of splenomegaly by Friend virus was determined using a spleen weight assay. Since different groups may have exhibited splenomegaly due to treatment, a spleen index (51) was calculated using the following mathematical relationship:

$$SI = \frac{\text{Experimental Spleen Weight / Experimental Body Weight}}{\text{Control Spleen Weight / Control Body Weight}}$$

Infection was considered evident when a spleen index exceeded that index calculated for a control group which exhibited maximum variation in spleen weight.

The spleen index assay has its value in that it accounts for individual variation within a group. Also, the index is a direct reflection of the degree of splenomegaly. For example, a spleen index of 2 would reflect a doubling in spleen weight.

Presentation of Data and Statistical Methods

The results are presented as the actual individual number of foci present on the spleen surface or as the arithmetic of the spleen weights within a treatment group. The non-parametric distribution-free test (Kruskal-Wallis) (42) was used to determine if significant differences among treatment groups were evident. Differences were considered to be significant when probability (p) values less than 0.10 were obtained.

RESULTS

Relationship Between Focus-Forming Assay and Spleen Weight Assay

Friend virus infectivity titers have been reported to be measurable by several methods including spleen weight increase and spleen focus formation. Axelrad and Steeves (2) reported that Friend virus, when inoculated intravenously into mice, induces discrete macroscopically visible foci in the intact spleen within nine days of infection. Their results showed that the mean number of foci induced per spleen is directly proportional to the dose of virus administered. In addition, they showed that holding animals longer than nine days resulted in increased formation of foci which were then too numerous to count or they became confluent. Chirigos, et al, (20) have shown that three measurable parameters, foci, splenomegaly, and death are related to virus dose. They also have shown that spleen weight and the number of foci progressively increase with time.

The results of the present study presented in Table 1 are not unlike those reported for previous investigations. The FFU titer is directly proportional to the ID_{50} titer for all virus strains studied. The nine day FFU titer represents only a fraction of the end point mean infective dose.

The susceptibility of Balb/c mice to infection was a function of the virus strain, regardless of the assay procedure. The ability of the assay methods to resolve differences in virus titer is reproducible within a factor of two or better (2,20). Therefore, the relative suscep-

Table 1. Relationship between spleen focus-forming units (FFU) and mean infective dose (ID_{50}) of three strains of Friend virus.

VIRUS	FFU/ml[a]	ID_{50}/ml[b]	ID_{50}/FFU
FV-N	4.8×10^3	7.5×10^4	15.6
FV-NB	2.4×10^5	3.6×10^6	15.0
FV-B	2.1×10^5	2.8×10^6	13.5

[a]Known dilutions of virus preparations from 20% spleen extracts were injected i.v. into groups of 5-10 Balb/c mice. Nine days later the mice were killed, the spleens fixed in Bouin's fluid, and the macroscopically visible foci on the spleen surface of each spleen were counted. One FFU is that amount of virus required to induce the formation of one focus per spleen, on the average, in a specified host.

[b]Known dilutions of virus preparations were injected i.v. into groups of 5-6 Balb/c mice. Twenty-four days later the mice were killed and the spleen weights were determined. A spleen weighing more than 0.25 gm. was considered infected. The ID_{50} was calculated using the method of Reed and Muench.

tibility of Balb/c mice reported here indirectly verifies that viruses of different tropism were used. Balb/c mice exhibited an approximate 50-fold greater susceptibility to either B or NB-tropic virus than to N-tropic virus.

Effect of Anti-μ Suppression on Friend Virus as Determined by Peripheral Nucleated Cell Count

Manning, et al, (57) have presented evidence that neonatally initiated immunosuppression of Balb/c mice with rabbit anti-μ antibodies renders them refractory to Friend virus leukemia. They showed that mice so treated failed to develop typical leukemia leukocytosis, splenomegaly, or splenic foci upon injection of 2.2 ID_{50} doses of Friend leukemia virus. It is now known that the virus used in their studies was a complex which exhibits N-tropic characteristics. It is this virus preparation that has been designated N-tropic for the present study. Our initial experiments were designed to repeat the observation that anti-μ suppressed mice remain refractory to challenge with low doses of Friend virus, and to determine if the refractory state was also evident for virus of B or NB-tropism.

The results obtained from the present study (Table 2) substantiate those reported earlier by Manning and coworkers. Neonatally initiated injection of anti-μ antibodies to Balb/c mice rendered the mice refractory to infection with low doses of N/tropic Friend virus. In addition, the results indicate that the refractory state is induced for NB-tropic virus as well as N-tropic virus. This refractory state is present even

Table 2. Mean absolute peripheral nucleated cell count and spleen weight of neonatally anti-μ suppressed and control mice following challenge with low doses of Friend virus.[a]

		TREATMENT								
		Anti-μ			NRS			None		
Virus	Challenge Dose (ID_{50})	No. Mice	Nucleated Cell Count	Spleen Weight	No. Mice	Nucleated Cell Count	Spleen Weight	No. Mice	Nucleated Cell Count	Spleen Weight
FV-N	1.9	0/4	11,464	0.12	2/3	25,861	0.96	4/7	10,859	0.52
FV-NB	9.0	0/6	14,741	0.15	3/4	54,132	0.20+	7/7	81,285	1.50
FV-B	7.0	2/4	42,823	0.71	2/4	50,507	0.11+	1/8	8,162* (25,671)	0.12 (0.53)
None	0.0	0/5	11,550	0.20		NT		0/11	8,195	0.10

[a] Balb/c mice, untreated or treated with anti-μ or NRS were injected i.p. with known concentrations of N, NB, or B-tropic Friend virus. Sixty days later survivors were killed and the spleen weights determined. Nucleated cell counts were performed at weekly intervals and are reported as the mean of 4-6 mice in thousands per cubic millimeter.

\+ Mean of survivors only.

* Mean calculated omitting the one infected mouse. Numbers in parentheses are the means calculated with the one mouse included.

60 days post infection. Equivical results were obtained when anti-μ suppressed mice were challenged with B-tropic virus. Despite the size of the challenge dose, few of the mice developed symptoms of leukemia. However, at no time was there any indication that anti-μ suppression altered the disease process induced by B-tropic virus.

Direct Effect of Anti-μ Antisera on Friend Virus

It is possible that some contaminating antibody or even anti-μ itself exerts a direct anti-viral effect on Friend virus, or that anti-μ may stimulate in vivo production of an anti-viral substance. To test the first of these possibilities, Friend virus was incubated in vitro in the presence of anti-μ, NRS, or PBS. These suspensions were then titrated in Balb/c mice after a four hour incubation period at 37° C. The results presented in Tables 3 and 4 clearly indicate that the anti-μ antibodies or rabbit sera have no direct anti-viral activity for Friend virus. Although an exact end-point for FFU was not reached (Table 3) the number of mice showing evidence of splenic foci was equal for all three treatment groups. In addition, so significant difference is evident among the spleen indices of the three groups (Table 4).

To test the possibility that anti-μ antibodies stimulate anti-viral responses in vivo, mice were treated with anti-μ at a level which was not totally suppressive. The mice were then challenged with concentrations of virus, either N or NB-tropic. In neither case was evidence obtained that indicated an anti-viral substance produced in response to

Table 3. Effect of *in vitro* incubation on Friend virus in the presence of anti-μ, NRS, or PBS as determined by spleen focus formation in Balb/c mice.[a]

	TREATMENT					
	Anti-μ		NRS		PBS	
Virus Dilution	No. Positive / No. Challenged	FFU Spleen	No. Positive / No. Challenged	FFU Spleen	No. Positive / No. Challenged	FFU Spleen
10^{-1}	6/6	TNTC[b]	5/5	TNTC	5/5	TNTC
10^{-2}	5/5	TNTC	5/5	TNTC	5/5	TNTC
10^{-3}	3/4	0,T,T,T[c]	3/5	0,0, T,T,T	3/5	0,0, T,T,T
None	0/5	0,0,0, 0,0	0/5	0,0,0, 0,0	0/5	0,0,0, 0,0

[a] One ml of an NB-tropic virus preparation was incubated with one ml of anti-μ (titered 1:64), NRS or PBS. After four hours incubation at 37° C., known dilutions of the suspensions were injected i.v. into Balb/c mice. Nine days later the mice were killed, the spleens fixed in Bouin's fluid, and the number of macroscopically visible foci on the spleen surfaces were determined.

[b] TNTC indicates all spleens within the group had too numerous foci to accurately count.

[c] T indicates the number of foci on one surface of an individual spleen exceed 50, which was considered too numerous to accurately count.

Table 4. Effect of *in vitro* incubation of Friend virus in the presence of anti-μ, NRS, and PBS as determined by the splenomegaly response in Balb/c mice.[a]

	TREATMENT								
	Anti-μ			NRS			PBS		
Virus Dilution	Average Body Weight	Average Spleen Weight	Spleen Index[b]	Average Body Weight	Average Spleen Weight	Spleen Index	Average Body Weight	Average Spleen Weight	Spleen Index
10^{-1}	19.89	1.16	8.94	22.08	1.16	8.05	21.95	1.03	7.19
10^{-2}	21.12	0.34	2.47	23.33	0.37	2.43	20.74	0.29	2.14
10^{-3}	20.80	0.17	1.25	20.39	0.20	1.50	19.75	0.19	1.47
None							20.78	0.13 ± 0.04	1.00[c] ± 0.34

[a] One ml of an NB-tropic virus preparation was incubated with one ml of anti-μ, NRS, or PBS. After four hours incubation at 37° C, known dilutions of suspensions were injected i.v into groups of 4-6 Balb/c mice. Nine days later the mice were killed and the spleen weights determined.

[b] $\text{Spleen Index} = \frac{\text{Experimental Spleen Weight / Experimental Body Weight}}{\text{Control Spleen Weight / Control Body Weight}}$

[c] Infection was considered evident when a spleen index exceeded that index calculated for a control group which exhibited maximum variation in the spleen weight.

anti-μ injections. Regardless of treatment, mice remained equally susceptible to infection when challenged with either NB-tropic (Table 5) or N-tropic virus (Tables 6 and 7). In both cases there is an apparent slight decrease in susceptibility to virus infection in the anti-μ treated animals. This may be an indication that an anti-viral substance is induce in vivo following injections of anti-μ. Since the effect is only minor, it is suggested that the anti-μ treatment did cause a slight decrease in the number of available target cells for virus infection and multiplication.

Humoral Immunological Competence of Mice Treated with Subsuppressive Levels of Anti-μ

Neonatally initiated anti-μ treatment has been shown to lower the levels of all serum immunoglobulins as well as render mice unresponsive to a specific antigenic challenge. To insure that the mice were not totally suppressed for humoral immunity, the serum immunoglobulin levels as well as specific antibody forming cells to a specific antigen (SRBC) were enumerated. Immunoglobulin levels of all classes were equal to those levels of control groups (Table 8) and the mice were equally capable of responding to a specific antigenic challenge (Table 9). It was concluded that the anti-μ treatment schedule was insufficient to totally suppress the humoral immunity in mice.

The level of anti-rabbit precipitins is substantially lower in anti-μ treated and untreated mice as compared to NRS treated mice.

Table 5. The development of foci in the spleens of mice treated with subsuppressive levels of anti-μ then injected with an NB-tropic Friend virus complex.[a]

Challenge Dose (FFU)	TREATMENT: Anti-μ No. Positive / No. Challenged	Anti-μ FFU Spleen	NRS No. Positive / No. Challenged	NRS FFU Spleen	None No. Positive / No. Challenged	None FFU Spleen
500	NT[b]	NT	5/5	TNTC[c]	5/5	TNTC
50	3/3	TNTC	4/5	TNTC	4/5	TNTC
5	1/2	0,35	5/5	4,16,16, 21,T	2/5	0,0,0, 4,T
None	0/6	0,0,0, 0,0,0	0/4	0,0,0,0	0/2	0,0

[a] Balb/c mice, untreated or treated with subsuppressive levels of anti-μ or NRS were injected with known concentrations of NB-tropic Friend virus. Nine days later the mice were killed, the spleens fixed in Bouin's fluid and the number of macroscopically visible foci on the spleen surfaces was determined.

[b] NT indicates test group was not included.

[c] TNTC indicates all spleens within a group had too numerous foci to accurately count.

Table 6. The development of foci in the spleens of mice treated with subsuppressive levels of anti-μ then injected with an N-tropic Friend virus complex.[a]

	TREATMENT					
	Anti-μ		NRS		None	
Challenge Dose (FFU)	No. Positive / No. Challenged	FFU / Spleen	No. Positive / No. Challenged	FFU / Spleen	No. Positive / No. Challenged	FFU / Spleen
100	4/4	TNTC[b]	3/3	TNTC	2/2	TNTC
10	3/4	0,2, 19,25	3/3	6,22,T[c]	5/5	3,5,20, 27,T
None	0/2	0,0	0/4	0,0,0,0	0/6	0,0,0, 0,0,0

[a] Balb/c mice, untreated or treated with subsuppressive levels of anti-μ or NRS were injected with known concentrations of N-tropic Friend virus. Nine days later the mice were killed, the spleens fixed in Bouin's fluid, and the number of macroscopically visible foci on the spleen surface was determined.

[b] TNTC indicates all spleens within the group had too numberous foci to accurately count.

[c] T indicates the number of foci on an individual spleen exceeded 50, which was considered too numerous to accurately count.

Table 7. The development of splenomegaly in mice treated with subsuppressive levels of anti-μ then injected with an N-tropic Friend virus complex.[a]

	TREATMENT								
	Anti-μ			NRS			None		
Challenge Dose (FFU)	Average Body Weight	Average Spleen Weight	Spleen Index[b]	Average Body Weight	Average Spleen Weight	Spleen Index	Average Body Weight	Average Spleen Weight	Spleen Index
100	17.82	0.26	1.83	19.15	0.31	2.14	23.49	0.23	1.57
10	19.26	0.15	0.98	19.09	0.19	1.31	22.51	0.19	1.35
None	21.33	0.17 ± 0.10	1.00 ± 0.59	19.80	0.15 ± 0.07	1.00 ± 0.47	20.78	0.13 ± 0.04	1.00 ± 0.34

[a] Balb/c mice, untreated or treated with subsuppressive levels of anti-μ or NRS were injected with known concentrations of N-tropic Friend Virus. Nine days later the mice were killed and the spleen weight was determined for groups of 4-6 mice.

[b] $$\text{Spleen Index} = \frac{\text{Experimental Spleen Weight / Experimental Body Weight}}{\text{Control Spleen Weight / Control Body Weight}}$$

Infection was considered evident when a spleen index exceeded that index calculated for a control group which exhibited maximum variation in spleen weight.

Table 8. Serum immunoglobulin levels of mice injected with subsuppressive levels of anti-μ.

Treatment	No. Mice	IMMUNOGLOBULIN CLASS[a] IgM	IgA	IgG_1	IgG_2	Free Anti-μ	Anti Rabbit
Anti-μ	14	11 ± 8	11 ± 8	969 ± 533	302 ± 177	0	26 ± 21
NRS	3	9 ± 6	6 ± 6	853 ± 296	192 ± 111	0	85 ± 37
None	3	8 ± 0	19 ± 12	256 ± 0	256 ± 0	0	0

[a] Numerical average of highest individual reciprocal serum dilution, ± variance, producing pricipitin band in Ouchterlony gel diffusion test to detect serum immunoglobulin level, free anti-μ, and antibodies specific for normal rabbit serum.

Table 9. Direct plaque forming cells of mice injected with subsuppressive levels of anti-μ.[a]

Treatment	No. Mice	DIRECT PLAQUES PFC's/10^6	DIRECT PLAQUES PFC's/Spleen
Anti-μ	3	195 ± 74	39,793 ± 5,859
NRS	4	339 ± 126	86,420 ± 30,982
None	3	233 ± 193	36,666 ± 31,832
None	2	0.65 ± 0.18	88 ± 18

[a]Balb/c mice untreated or treated with subsuppressive levels of anti-μ or NRS were immunized with 0.25 mls. of a 10% Sheep eryhtrocyte (SRBC) suspension. Five days later the mice were assayed for SRBC-specific plaque-forming cells. Results are expressed as the number of direct IgM producing PFC's/10^6 and PFC's/spleen.

Therefore, it is suggested that the suppressive schedule was such that a slight degree of unresponsiveness was achieved. These results support the hypothesis put forth earlier to account for the slight decrease in FFU in anti-μ injected mice when challenged with Friend virus (Tables 5 and 6).

These data support the hypothesis that the effect of anti-μ on Friend virus infection must be mediated through its effect upon the host and not upon the virus complex itself.

Effect of Anti-μ Suppression on Friend Virus Infection in Mice

Thus far, our data suggest that anti-μ treated and suppressed mice are rendered refractory to infection with low doses of N and NB-tropic Friend virus but not to B-tropic virus. Also, this effect is mediated through an effect on the host rather than a direct effect of the antisera on the virus complex. The next series of experiments was designed to determine at what level of virus challenge anti-μ suppressed mice remain refractory to typical Friend leukemia symptoms, and to determine more conclusively the effect of anti-μ suppression upon B-tropic Friend virus. Suppressed and control animals were injected with variable known doses of virus of each known tropism, and the FFU and splenomegaly responses were determined nine days following virus challenge. The results of these studies are presented in the following six tables (Tables 10-15). These data support the infectivity characteristics indicated in our initial experiment and the studies reported by

Manning, et al. Anti-μ suppressed mice remain refractory to typical Friend leukemia symptoms when challenged with N-tropic Friend virus. This refractory state is evident using two different criteria for infection, spleen focus formation(Table 10) and spleen weight increase (Table 11).

From the results shown in Table 10, experiment 2, it appears the refractory state can be overcome when larger doses of N-tropic virus complex are injected. The mice that exhibited symptoms of infection, however, had residual levels of serum IgM indicating suppression was not achieved. This was true for all mice showing symptoms of infection with the exception of one mouse, which had only three foci on the spleen surface. Chirigos, et al, have reported that a few spleens from normal mice have been observed to contain 1 to 3 foci (20). They propose that the foci observed in the spleens of normal mice represent occassional groups of reticulum cells present in the subcapsular area of the spleen. Also, the foci observed in the one suppressed spleen may be the result of a subclinical abortive infection. Therefore, we conclude that if mice are rendered devoid of serum IgM and lymphocytes capable of producing antibody to a specific antigenic challenge, they are also rendered refractory to the development of typical Friend leukemia symptoms by N-tropic virus. This refractory state is evident even at relatively large challenge doses of virus.

The data presented in Tables 12 and 13 clearly show that anti-μ

Table 10. The development of foci in the spleens of anti-μ suppressed mice following intravenous injection of an N-tropic Friend virus complex.[a]

EXPERIMENT 1 FFU/SPLEEN				EXPERIMENT 2 FFU/SPLEEN			
Challenge Dose (FFU)	Treatment			Challenge Dose (FFU)	Treatment		
	Anti-μ	NRS	None		Anti-μ[b]	NRS	None
100	0,0,0	TNTC[c]	TNTC	750	0,0,T,T[d], T,T	TNTC	TNTC
10.	0,0,0	2,8,16, 18	0,2,4, 18	75	0,0,0,0, 3,7,T	TNTC	TNTC
1	NT[e]	0,0,0	0,0,0,2	7.5	0,0,0,0, 0,0,0	2,3,5	5,8,10

[a] Balb/c mice, untreated or treated with anti-μ or NRS were injected i.v. with known concentrations of Friend virus. Nine days later the mice were killed, the spleens fixed in Bouin's fluid and the number of macroscopically visible foci on the spleen surfaces was determined.

[b] With the exception of one mouse, all mice showing evidence of infection also exhibited residual levels of serum IgM indicating suppression was not achieved.

[c] TNTC indicates all spleens within the group had too numerous foci to accurately count.

[d] T indicates the number of foci on an individual spleen exceeded 50, which was considered too numerous to count.

[e] NT indicates test group not included.

Table 11. The development of splenomegaly in anti-μ suppressed mice following intravenous injection of N-tropic Friend virus complex.[a]

	TREATMENT								
	Anti-μ			NRS			None		
Challenge Dose (FFU)	Average Body Weight	Average Spleen Weight	Spleen Index[b]	Average Body Weight	Average Spleen Weight	Spleen Index	Average Body Weight	Average Spleen Weight	Spleen Index
750	20.03	0.28	1.74	17.63	0.42	3.14	NT[d]	NT	NT
75	18.30	0.15	1.02	16.68	0.19	1.50	18.82	0.19	1.61
7.5	15.81	0.13	1.02	16.07	0.11	0.91	18.87	0.14	1.19
None	16.14	0.13 ± 0.04	1.00[c] ± 0.31	19.80	0.15 ± 0.07	1.00 ± 0.47	20.78	0.13 ± 0.04	1.00 ± 0.34

[a] Groups of 3-8 Balb/c mice, untreated or treated with anti-μ or NRS, were injected intravenously with known concentrations of a Friend virus complex. Nine days later the mice were killed and the spleen weights were determined.

[b, c] see Table 4.

[d] NT indicates test group was not included.

Table 12. The development of foci in the spleens of anti-μ suppressed mice following intravenous injection of an NB-tropic Friend virus complex.[a]

	TREATMENT					
	Anti-μ		NRS		None	
Challenge Dose (FFU)	No. Positive / No. Challenged	FFU / Spleen	No. Positive / No. Challenged	FFU / Spleen	No. Positive / No. Challenged	FFU / Spleen
500	4/4	TNTC[b]	3/3	TNTC	3/3	TNTC
50	2/3	0,3,7	4/4	23,T,T,T[c]	3/3	TNTC
5	0/3	0,0,0	2/2	5,12	2/2	4,5
None	0/2	0,0	0/2	0,0	0/2	0,0

a, b and c see Table 10.

Table 13. The development of splenomegaly in anti-μ suppressed mice following intravenous injection of NB-tropic Friend virus complex.[a]

	TREATMENT								
	Anti-μ			NRS			None		
Challenge Dose (FFU)	Average Body Weight	Average Spleen Weight	Spleen Index[b]	Average Body Weight	Average Spleen Weight	Spleen Index	Average Body Weight	Average Spleen Weight	Spleen Index
500	21.57	0.60	3.49	19.58	0.69	4.65	22.71	1.10	7.74
50	16.50	0.11	0.84	21.70	0.23	1.40	18.75	0.21	1.79
5	20.23	0.14	0.87	25.35	0.16	0.84	20.76	0.13	1.00
None	21.33	0.17 ± 0.10	1.00[c] ± 0.59	19.80	0.15 ± 0.07	1.00 ± 0.47	20.78	0.13 ± 0.04	1.00 ± 0.34

a, b, and c see Table 11.

suppressed mice remain refractory to infection when challenged with low doses of NB-tropic Friend virus. A significant reduction or total elimination of foci on the spleen surfaces was evident in suppressed mice as compared to both NRS and untreated controls. Also indicated is that this refractory state may be overcome when the challenge dose of virus is increased by a factor of ten or more.

As our earlier studies had indicated, at no time were anti-μ suppressed mice refractory to infection with B-tropic Friend virus (Tables 14 and 15). No significant difference was evident in the number of splenic foci among the various treatment groups, even at low virus challenge. The splenomegaly induced by B-tropic virus, as reflected in the spleen index, was consistantly smaller for the suppressed mice than for control groups when large doses were injected (Table 15). No consideration was given these values since spleen indices for the next lower challenge dose were consistant. In addition, the FFU titers were not significantly different.

Collectively, the data presented in Tables 10-15 indicate relative degrees of susceptibility of anti-μ suppressed mice to Friend virus infection. Anti-μ suppressed mice are rendered totally refractory to infection with N-tropic viruses, whereas B-tropic viruses induce typical leukemia symptoms. Of interest, is the observation that NB-tropic virus infection characteristics in anti-μ suppressed mice are intermediate between those of N-tropic and B-tropic Friend virus. It is

Table 14. The development of foci in the spleens of anti-μ suppressed mice following intravenous injection of B-tropic Friend virus complex.[a]

	TREATMENT					
	Anti-μ		NRS		None	
Challenge Dose (FFU)	No. Positive / No. Challenged	FFU / Spleen	No. Positive / No. Challenged	FFU / Spleen	No. Positive / No. Challenged	FFU / Spleen
500	5/6	TNTC[b]	7/9	TNTC	8/8	TNTC
50	5/8	0,0,0,3, 6,T,T,T[c]	6/7	0,6,6,8, 20,26,T	5/6	0,14,14, T,T,T
5	1/7	0,0,0,0, 0,0,6	1/4	0,0,0,10	6/8	0,0,2,4,6, 6,12,16

a, b and c see Table 10.