Antigenic Behavior of the S-63 Mouse Leukemia Virus

By Eric R. Brown, Irving Greenspan and Steven O. Schwartz

STUDIES OF ANTIBODIES in mice injected with S-63 leukemia virus have been reported.1 This study represents an extension of that work (table 1) and a study of the correlation between in vitro and in vivo protection tests (table 2).

The S-63 virus was originally isolated by DNA extraction of ascites cells obtained from mice in which this form of leukemia had been maintained by cell passage in our laboratories for several years. Serum obtained from survivors of S-63 virus-injected mice protects newborn mice that have been injected with this agent.

Comparative studies were performed between the protection afforded by convalescent sera and antisera produced in rabbits. In some experiments, in order to avoid mouse tissue antibodies, newborn rabbits were first made immunologically tolerant to normal mouse tissues and were later challenged with extracts of leukemic tissues.

Because this virus had apparently been carried in the ascites cells in a latent form, immunologic studies were also undertaken to determine the relation of the virus to the carrier cell.

METHODS

Preparation of Antigens

S-63 Virus Antigen: Young adult ICR mice, approximately 20 Gm. in weight, previously injected with the virus, were killed at a time when gross symptoms of leukemia were apparent (2 to 4 weeks after inoculation). At that time the animals had splenomegaly, hepatomegaly, thymic enlargement and a leukocytosis averaging 50,000 WBC/cu. mm.

Twenty per cent homogenates of their leukemic tissues were prepared by mincing the tissues with scissors in Hank’s balanced salt solution (HBSS) containing 1.5 mg. per cent hyaluronidase at a pH of 7.2. The homogenate was digested for 1 hour at room temperature, ground in a Ten Broek glass homogenizer, and centrifuged for 20 minutes at 900 g. The supernatant fluid was clarified through a Selas CFF porcelain filter, then diluted 1 to 5 with HBSS. The clarified solution was passed through an 0.45 μ Millipore filter and was then centrifuged at 100,000 g for 1 hour in a Spinco Model L centrifuge.

From the Hematology Department of The Hektoen Institute for Medical Research of the Cook County Hospital and Northwestern University Medical School, Chicago, Illinois.

Supported by a grant from the McCormick Fund and USPHS Grants No. CA 03321 and No. CRTY 5092.

First submitted Jan. 14, 1965; accepted for publication May 18, 1965.

Eric R. Brown, Ph.D.: Senior Investigator, Department of Hematology, The Hektoen Institute for Medical Research, Chicago, Illinois. Irving Greenspan, M.D.: Senior Investigator, Department of Hematology, The Hektoen Institute for Medical Research, Chicago, Illinois. Steven O. Schwartz, M.D.: Professor of Medicine, Northwestern University Medical School, and Director, Department of Hematology of The Hektoen Institute for Medical Research, Chicago, Illinois.

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Blood, Vol. 27, No. 1 (January), 1966
The resulting pellet was suspended again in HBSS on the basis of 1 ml of diluent/Gm. of original tissue weight. This resuspended pellet was used as the viral antigen. For animal inoculations, 0.1 ml (0.5 mg N/ml) was given intraperitoneally to newborn mice and 0.2 ml intravenously to young 20 Gm. adult ICR mice.

Normal Tissue Antigen: Normal tissue antigens were prepared from untreated, uninoculated ICR mice using spleens, livers and lymph nodes. Twenty per cent suspensions of these tissues in HBSS were prepared, and after mincing, the tissues were gently ground in a Ten Broek Tissue grinder. The cells were then washed 3 times in HBSS, resuspended, and counted.

Ascites Cells: The ascites cells were obtained from an established cell-transmitted line of leukemia carried in our laboratory. They were washed 3 times in HBSS and resuspended for use.

Induction of Immune Tolerance in Newborn Rabbits

Immune tolerance was induced in newborn rabbits* by a modification of the method reported by Garb and his colleagues. Newborn albino rabbits were injected intraperitoneally with fresh normal tissue antigen adjusted to contain approximately 250,000 cells/cu. mm. Daily injections were given starting with 0.1 ml and increasing the dose by 0.1 cc. daily until 1.0 ml was reached. This dose was continued for 4 consecutive days and thereafter once weekly for 10 weeks. At the end of 14 weeks the rabbits were tested for antibodies against normal mouse tissue antigens by microprecipitin, immunodiffusion, immunofluorescence, and a modified passive cutaneous anaphylaxis test. Immune-tolerant animals—that is, those whose sera failed to react against normal mouse tissue antigens—were used for purposes of challenge with either the S-63 virus antigen or ascites cells.

Production of Antibodies in Immune-Tolerant Rabbits

To produce specific immunity to leukemic tissues, the immune-tolerant rabbits were given suspensions of ascites cells (1,500,000 cells/cu. mm. in HBSS). The first injections consisted of 1.0 ml intramuscularly and 0.5 ml intravenously. One week later the cell suspension was mixed with an equal volume of Freund’s incomplete adjuvant and 2.0 ml. of the mixture were given intramuscularly. This was repeated a week later. Fourteen to 21 days after the last challenge, the animals were bled and the serum was tested for activity against both normal and ascites cells.

To produce immunity to the viral antigen, immune-tolerant rabbits were treated in a manner similar to those given ascites cells. Here the first dose of antigen consisted of 2.0 ml (0.5 mg N/ml) of resuspended virus pellet given intramuscularly and 0.5 ml given intravenously. One and 2 weeks later 2.0 ml of the material, mixed with an equal volume of Freund’s incomplete adjuvant, were given intramuscularly.

Production of Antibodies in Normal Rabbits

Normal female rabbits comparable in age to the immune-tolerant rabbits at the time of challenge were used for producing antibodies. Those rabbits received the same dose of leukemic antigens as the animals in the immune-tolerant experiments.

Testing for Antibodies

Microprecipitin Test: The microprecipitin test was used as a screening test. The technic, as well as the advantages and disadvantages of this test, have been previously discussed. Immunodiffusion: The immunodiffusion test has been slightly modified since it was previously described. Noble Agar (Difco), 7/10 per cent, suspended in physiologic

*Thirty-eight newborn rabbits were originally injected. Seven survived to maturity. Four were used for antiascites cell and three for S-63 virus injection. The antisera produced in these rabbits were individually tested for immune tolerance and then pooled.
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saline solution, containing 0.005 per cent cadmium acetate, was prepared. After dis-
solving the agar, Merthiolate was added to a final dilution of 1:10,000. Petrie dishes
were coated with a mixture of glycerin and alcohol (1:2.5) and 25 ml of agar was
poured into each dish. A 5-hole or 7-hole Feinberg agar-gel cutter (Colab) was used
for cutting the wells. All plates were held a minimum of 10 days, and the wells were
recharged at least 3 times (every third day) in order to obtain maximum saturation of
the agar.

Passive Cutaneous Anaphylactic Test (PCA): The classic PCA test described by Ovary was
used.

Immunofluorescence: Impression slides were prepared by gently pressing a clean glass
slide against the cut surface of leukemic and normal tissues. Test and control sera were
conjugated with fluorescein isothiocyanate dye. Lissamine-Rhodamine RB-200 counter-
stain was used to eliminate nonspecific fluorescence.

Preparation of Gamma Globulin

Gamma globulin was prepared by Cohn's cold-alcohol technic. It was lyophilized in
an Amico freeze-drying apparatus and was stored at +5 C. in sealed vials until used.

Protection

Neutralization Tests: Newborn ICR mice were used as the test animals in this study
of neutralization. One ml. of test serum was mixed with 0.2 ml. of viral pellet (2.0 mg.
N/ml.) and incubated for 1 hour at 37 C. in a water bath. At the end of the incubation
period, each animal received 0.2 ml. of the serum-virus mixture intraperitoneally. As
controls, the virus pellets were similarly incubated with normal rabbit serum and physio-
logic saline solution.

Protection Studies of Newborn ICR Mice: Newborn animals were given 0.1 ml. doses
of antibody-containing serum intraperitoneally once a day for 3 days. On the third day
they also received 0.1 ml. of virus pellet (0.5 mg. N/ml.). Other groups of newborn
animals were given 0.1 ml. (0.5 mg. N/ml.) of the virus pellet intraperitoneally, fol-
lowed by 10 mg. of gamma globulin derived from various types of sera. This dose of
gamma globulin was continued for 10 days (table 2). Control animals were treated with
normal rabbit gamma globulin or physiologic saline solution.

Protection Studies in Young Adult ICR Mice: Gamma globulin, 100 mg., obtained from
immune-tolerant rabbit serum was given young ICR mice in doses of 10 mg./day. The
accompanying graph shows the treatment schedule, the time of challenge, and the
survival rate at 5 months. The protective effect of the gamma globulin was challenged
with 1,500,000 ascites cells in this experiment.

RESULTS

Newborn and young adult ICR mice reacted differently to the S-63 virus. Splenomegaly,
lymphadenopathy, hepatomegaly and leukocytosis from 50,000 to over 100,000 cu.mm. developed in both in 2 to 3 weeks. The newborn
animals died in 2 to 3 weeks. However, only 20 per cent of the young adult
animals died of leukemia within this period, whereas the rest recovered in 6
to 8 weeks. The convalescent serum for antibody testing was obtained from
this recovered group. The results are shown in table 1. Antibodies reacting
with the S-63 virus antigen were demonstrated only in the serum from con-
valescent animals. These antibodies could not be demonstrated during the
period of maximal leukocytosis. None of the sera showed demonstrable anti-
bodies against normal mouse tissue antigens. Neutralization and protection
tests using this convalescent serum confirmed the results of the in vitro tests.
The convalescent serum appeared to protect the newborn mouse (table 2)
Table 1.—Specificity of Antibody Reaction

<table>
<thead>
<tr>
<th>Mouse Serum</th>
<th>No. of Mice</th>
<th>Normal Mouse Tissue Antigens</th>
<th>S-63 Leukemic Mouse Tissue Antigens</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MP</td>
<td>PCA</td>
</tr>
<tr>
<td>Normal</td>
<td>37</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S-63 infected*</td>
<td>28</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Convalescent</td>
<td>52</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

MP = microprecipitin.
PCA = passive cutaneous anaphylaxis.
IF = immunofluorescent.
ID = immunodiffusion.
*Serum obtained at time of maximum leukocytosis.

Table 2.—Protection Studies on Newborn Mice Against S-63 Virus

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>Dead of Leukemia</th>
<th>% Protection</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>12/12</td>
<td>0</td>
</tr>
<tr>
<td>Saline solution</td>
<td>20/21</td>
<td>5</td>
</tr>
<tr>
<td>Normal mouse serum</td>
<td>15/15</td>
<td>0</td>
</tr>
<tr>
<td>Convalescent mouse serum</td>
<td>4/39</td>
<td>90</td>
</tr>
</tbody>
</table>

*Newborns injected for 3 days before, and 7 days after virus challenge.

Table 3.—Protection Studies on Newborn Mice Against S-63 Virus; Virus Pellet and Test Substance Incubated before Injection

<table>
<thead>
<tr>
<th>Test Material</th>
<th>Dead of Leukemia</th>
<th>% Protection</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>11/11</td>
<td>0</td>
</tr>
<tr>
<td>Saline solution</td>
<td>9/9</td>
<td>0</td>
</tr>
<tr>
<td>Normal mouse serum</td>
<td>12/13</td>
<td>9.2</td>
</tr>
<tr>
<td>Convalescent mouse serum</td>
<td>5/21</td>
<td>76.2</td>
</tr>
</tbody>
</table>

when the serum was given before and during viral challenge. The virus could also be made inactive by in vitro incubation with the convalescent serum (table 3).

The results of antibody studies of serum obtained from rabbits made immune-tolerant to normal ICR mouse tissues and subsequently challenged with leukemic antigens are shown in table 4. Antisera from immune-tolerant rabbits failed to react with normal mouse tissues but did react with the specific leukemic antigen. Antiserum produced in normal rabbits against ascites cells or the S-63 virus reacted with both normal tissue and leukemic mouse antigens even after adsorption of the serum against normal mouse tissues. However, cross-protection studies using immune-tolerant antisera showed that antiascites cell sera would not protect mice against S-63 virus infection, and vice versa (table 5).

Studies of the protective capability of gamma globulin obtained from anti-ascites cell sera prepared in the immune-tolerant rabbit against ascites cells
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Table 4.—Specificity of Antiserum Produced in Immune-Tolerant Rabbits

<table>
<thead>
<tr>
<th>Tests</th>
<th>Normal Mouse Tissue Antigens</th>
<th>Mouse Ascites Cell Antigen</th>
<th>S-63 Mouse Leukemia Virus Antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PCA</td>
<td>MP</td>
<td>ID</td>
</tr>
<tr>
<td>I. Immune-tolerant rabbit</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>antiascites cells</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>anti-S-63 virus</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>II. Nonimmune-tolerant</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rabbit antiascites cells</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>anti-S-63 virus</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>III. Nonimmune-tolerant</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rabbit adsorbed against nor-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mal tissue antigen antiascites cells</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>anti-S-63 virus</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 5.—Failure of Cross-Protection with Serum Prepared in Immune-Tolerant Rabbits

<table>
<thead>
<tr>
<th>In Vivo Tests</th>
<th>Died</th>
<th>% Protection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antiascites serum + S-63 virus</td>
<td>12/12</td>
<td>0</td>
</tr>
<tr>
<td>Anti-S-63 serum + 1,500,000 ascites cells</td>
<td>18/18</td>
<td>0</td>
</tr>
<tr>
<td>Saline solution + S-63 virus</td>
<td>8/9</td>
<td>11</td>
</tr>
<tr>
<td>Saline solution + 1,500,000 ascites cells</td>
<td>10/10</td>
<td>0</td>
</tr>
</tbody>
</table>

are shown in the accompanying graph. One and a half million ascites cells were used as the challenging dose. This number of cells produces leukemia in 90 to 100 per cent of injected animals in 3 weeks. All animals received a total of 100 mg. of gamma globulin in 10 equally divided doses. When the mice were pretreated or treated at the time of the cell injection, the degree of protection was high. Even more significant is the fact that it was possible to reverse the progression of the leukemia even after the appearance of objective indications of the disease. As might be expected, control of the disease became more difficult with each passing day. The 46 per cent protection in the group of animals treated on the fourth day after evidences of leukemia became manifest, compared with 30 per cent protection of the group started on the third day, is somewhat skewed, because in one of the three experimental groups the survival was 80 per cent. In the two other groups, the survival was under 20 per cent.

Protection studies with S-63 virus and immune-tolerant anti-S-63 gamma globulin in newborn mice showed (table 6) that this gamma globulin protected newborn animals. The degree of protection was similar to that achieved by convalescent mouse sera.

DISCUSSION

The S-63 virus kills newborn ICR mice in 15 to 21 days; however, there develops in young adult mice generalized lymphadenopathy, splenomegaly,
Table 6.—Protection of Newborn Mice Against S-63 Virus by Means of Antisera Produced in Immune-Tolerant Rabbits

<table>
<thead>
<tr>
<th>Treatment</th>
<th>In Vivo Tests</th>
<th>% Protection</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>22/24</td>
<td>8.4</td>
</tr>
<tr>
<td>Nonimmune-tolerant rabbit gamma globulin</td>
<td>18/20</td>
<td>10.0</td>
</tr>
<tr>
<td>Immune-tolerant anti-S-36 rabbit gamma globulin</td>
<td>2/21</td>
<td>90.5</td>
</tr>
</tbody>
</table>

and leukocytosis of 50,000 to 90,000 white blood cells/cu.mm. made up predominantly of lymphoid cells. Leukemia develops in about 20 per cent of these animals, and they die in 4 to 5 weeks. The surviving animals recover, with a gradual stabilization and eventual subsidence of abnormalities. As previously reported,1 demonstrable antibody to the virus develops in the animals that recover in 6 to 8 weeks. A correlation seems to exist between the appearance of antibody and the drop in white blood cell count.

Antibodies obtained from convalescent mice protect newborn animals from the lethal effects of the virus (table 2). The convalescent antibodies do not cross-react with normal tissues from noninfected ICR mice (table 1), and adult mice that recover from the S-63 infection are not susceptible to reinfection with the virus.

Difficulties were encountered in the induction of immune tolerance to normal mouse tissues in the rabbit. The toxicity of the mouse tissue for the newborn rabbit is such that less than one in five injected rabbits survived. A satisfactory method has not been found for improving the survival rate which does not compromise the degree of immune tolerance. Furthermore, not all rabbits surviving the course of normal tissue injections become immune-tolerant. It is necessary to test sera for antibodies to normal tissues from each animal before challenge with the leukemic antigens.

Antibodies produced in nonimmune-tolerant rabbits against the S-63 virus reacted against normal and leukemic tissues (table 4). Similarly, antibodies produced against the ascites cells also reacted against normal tissues, and in protection studies were more toxic to the mice than antibodies produced in immune-tolerant animals.

Immunologic studies were carried out to determine the relation of the virus to the parent ascites cell. The virus had apparently been carried in an inactive form in the ascites cell before isolation and the antigenic relation of the virus to the cell was obscure. Antibodies produced in immune-tolerant animals against either ascites cells or S-63 virus failed to cross react (table 5). Antibodies produced in convalescent mice also failed to react with the ascites cells. This suggests that the parent cell is antigenically different from the virus isolated from it. These studies define 3 antigens: normal tissue antigens, ascites cell antigens, and a specific and distinct antigen to the virus.

Levi8 prepared antibodies in immune-tolerant rabbits using spontaneous AKR lymphatic leukemia cells. She showed that in this system antibodies to leukemia could be differentiated from normal mouse (AKR) tissue antigens.
The antibodies produced in immune-tolerant rabbits were not toxic to the recipient mice, a finding we also observed in the S-63 system.

Experiments were performed in order to determine the protective capability of leukemic antibodies produced in immune-tolerant rabbits (fig. 1). The antibodies were administered at various stages of infection to evaluate their therapeutic potential. One and a half million cells produced leukemia in 90 to 100 per cent of young ICR mice. The mice were given 100 mg. of gamma globulin derived from various sources. Those receiving gamma globulin from immunized immune-tolerant rabbits showed strong capability to resist the development of leukemia. Mice receiving similar doses of normal rabbit gamma globulin showed minimal protection. The time of administration of the antibody has a decisive relation to its protective effect. Pretreatment or treatment within one to two days after the appearance of symptoms gave the best chance of survival.

Protection studies with convalescent mouse sera, or with antibodies produced in immune-tolerant rabbits showed that such antibodies can modify the development of leukemia. The degree of modification depends on the amount of antibody, time of treatment, and toxicity of the antibody to the recipient. Several explanations are possible regarding the difficulties in reversing the progress of the disease after the third day of symptoms in protection studies against the ascites cells: (1) the ascites cells neutralize the limited amount of gamma globulin available, allowing other ascites cells to propagate freely; (2) leukemic changes progress because of sufficient “autonomy” of surviving ascites cells; (3) virus is released in sufficiently large quantities from the cells to perpetuate the progress of the infection.

**SUMMARY**

The present studies are an extension of earlier reports on the antibody response of mice to a leukemogenic virus (S-63). These investigations were designed to show whether a correlation exists between in vivo and in vitro determinations, and whether protective antibodies against the virus could be produced in immune-tolerant animals. Viral neutralization and protection studies in mice were compared with passive cutaneous anaphylaxis (PCA), immunodiffusion, microprecipitin, and immunofluorescent tests using similar antigens and antibodies. As shown in tables 1, 2 and 3, antibody could be demonstrated by both in vivo and in vitro methods, and these studies showed (table 2) that the antibody was protective.

The S-63 leukemia virus was isolated in 1963 from a mouse ascites cell leukemia line. That cell line had been carried in the laboratory for over 3 years. The virus has been carried in ICR mice since its isolation. Immunologic studies were carried out to determine the relation between the virus and the originating ascites cell. Studies of antibodies produced in normal rabbits against the S-63 virus were unsatisfactory for purposes of these experiments because of cross reaction with normal mouse tissue. Immune-tolerant animals have yielded valuable information:

Antibodies produced in immune-tolerant rabbits injected with the S-63 virus do not cross react against intact ascites cells from which the virus was
Injection of 1,500,000 ascites cells GROUP

Control

-3 0 5 +10 Days in relation to cell injection

Rabbit

10 mg/day

Gamma Globulin

Immune-tolerant

Gamma Globulin

Fig. 1.—Protection with immune-tolerant rabbit gamma globulin.

originally isolated. Conversely, antibodies similarly produced against the ascites cells do not react against the virus antigen.

Antibodies produced in immune-tolerant rabbits against ascites cells protect mice against ascites cell-induced leukemia, but not against the disease induced by S-63 virus. Mice challenged with ascites cells and treated with antiascites cell gamma globulin are protected against the disease. The best therapeutic advantage is obtained when treatment is started either at the time of cell inoculation or in the early stages of the disease.

Antibodies which protect newborn animals from infection develop in adult ICR mice recovering from S-63 virus leukemia. These antibodies produced by convalescent mice are similar, from the standpoint of protection, to those produced in immune-tolerant rabbits.

SUMMARIO IN INTERLINGUA

Le hic-reportate studios representa un extension de previe reportos relative al responsa anticorporee de muses contra virus leucemogenic (S-63). Le investigationes esseva concipite pro determinar si il existe un correlation inter le observationes in vivo e in vitro e si anticorpore anti le virus pote esser producite in animales con tolerantia immunologic. Studios del neutralisation de virus e del protection contra illo, effectuate in muses, esseva comparate con tests de passive anaphylaxia cutanea, de immunodiffusion, de microprecipitina, e de immunofluorescentia utilisante simile antigenos e anticorpores.

Le datos summarisate in tabulas 1, 2, e 3 indica que anticorpore poteva esser demonstrate tanto in vivo como etiam in vitro e que—vide specificamente tabula 2—le anticorpore esseva un anticorpore protective.

Le virus de leucemia S-63 esseva isolate in 1963 ab un linea de cellulas leucemia de ascites murin. Ille linea cellular habeva essite mantenite in le laboratorio durante plus que 3 annos. Le virus habeva essite portate per
muses ICR deposit su isolation. Esseva effectuate studios immunologic pro
determinar le relation inter le virus e su originatoris cellulas de ascites. Studios
del anticorpore producite in conilios normal contra le virus S-63 esseva inade-
quate pro le objectivos de iste experimentos in consequentia del phenomeno
de reaction cruciate con normal tissu murin. Animales a tolerantia immunolo-
logic ha producite information de grande valor:

Le anticorpore producite in conilios a tolerantia immunologic post inje-
tiones con le virus S-63 monstrava nulle reaction cruciate contra le intacte
cellulars de ascites ab le quales le virus habeva essite isolate originalmente.
Conversemente, anticorpore producite similemente contra le cellulars de ascites
monstrava nulle reaction contra le antigeno viral.

Anticorpore produce in conilios a tolerantia immunologic contra cellulars
de ascites protege muses contra leucemia a induction per cellulars de ascites
sed non contra le morbo inducite per virus S-63. Muses provocate per cellulars
de ascites e tractate con globulina gamma anti cellulars de ascites es protegete
contra le morbo. Le optime effecto therapeutic es obtenite quando le tracta-
mento es initiate al tempore del inoculation de cellulars o al minus durante
le precoce stadios del morbo.

Anticorpore capace a proteger animales neonate contra le infection se dis-
velloppa in adulte muses ICR durante lor restablimento ab leucemia per virus
S-63. Iste anticorpore producite per muses in convalescentia es simile, ab le
punto de vista de lor effecto protectori, a illo producite in conilios a tolerantia
immunologic.

ACKNOWLEDGMENT

We acknowledge our indebtedness to Dr. Peter Buinauskas, Department of Surgery,
University of Illinois School of Medicine, for his invaluable assistance.

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