Demonstration of Neutralizing Antibody to the Suckling Mouse Cataract Agent (SMCA)* (33736)

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The suckling mouse cataract agent (SMCA), an egg-lethal virus isolated from rabbit ticks in Georgia in 1961 (1), is of particular interest because it causes a high incidence of cataracts, as well as lifelong brain infection, in intracerebrally inoculated suckling mice (2). Early attempts to study the antigenic characteristics of SMCA, using an *in ovo* neutralization test, were unsatisfactory because of the low antibody titers obtained even with homologous antisera (1). It was demonstrated, however, that SMCA-infected mice conferred strong protection against SMCA-induced cataracts on their progeny (3).

A number of viruses were compared antigenically with SMCA by titration in parallel in suckling mice born to normal and SMCA-infected dams (4). Antigenic relationship was detected only with GT-48, a virus isolated from rabbit ticks, similar to SMCA in many characteristics, but lethal for suckling mice (1). Both SMCA- and GT-48-infected dams conferred a high degree of protection on their progeny against maximum doses of either virus inoculated intracerebrally.

The most likely explanation for maternally transferred protection against SMCA is the transfer of antibody. In studies reported herein, attempts were made to demonstrate antibody activity in the sera of infected mice by serum protection tests. A neutralization test employing assay of serum–virus mixtures in intracerebrally inoculated suckling mice was developed and characterized.

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Materials and Methods. Virus. The SMCA and GT-48 stocks consisted of allantoic fluid from eggs infected by methods previously described (2). Virus stock had been passaged only in embryonated eggs except for certain GT-48 virus stocks prepared in suckling mouse brain.

Mice. CFW(A) mice [average SMCA-induced cataract rate, 46% (2)] were bred in this laboratory. Swiss Ha/ICR mice, which are hardier than CFW(A) mice, but less susceptible to cataract (19% cataract rate), were obtained from the Roswell Park Memorial Institute. All newborn mice were inoculated when less than 24 hr old.

Antisera. Rabbits were inoculated with viable allantoic fluid stocks of SMCA or GT-48 with titers of $10^{6.3}$ to $10^{8.6}$ ELD_{50}/ml. For 6 weeks, the rabbits were given weekly inoculations alternately consisting of 0.5 ml of virus plus 0.5 ml of complete Freund's adjuvant (CFA) or 0.5 ml of virus alone administered intramuscularly. The rabbits were then given 6 additional weekly inoculations of virus alone or virus and CFA administered intradermally. Final bleedings were taken 14 days after the final (thirteenth) inoculation. The better of two rabbit antisera to each virus were used in all experiments.

Mouse origin antisera were pools prepared from several litters each. For SMCA antiserum, mice were inoculated intracerebrally (i.c.) as newborns with $10^{5.0}$ ELD_{50} of virus and exsanguinated between the ages of 30 and 45 days. For GT-48 antiserum, 30-day-old mice were inoculated i.c. with $10^{6.6}$ ELD_{50} of virus and exsanguinated 30 days later.

Serum neutralization tests. All sera were heated at 56° for 30 min before use. Serum and virus were diluted in 0.75% bovine albumin in phosphate buffered saline (BAPS) in an ice bath (4°). Serum–virus mixtures were incubated at 37° for 30 min and then

TABLE I. Protection of Suckling Mice Against SMCA or GT-48 Challenge by Administration of Immune Serum to Their Dams.[a]

Serum inoculated into dam	SMCA challenge dose to progeny (ELD_{50})	No. of suckling mice	Mice with cataracts	
			No.	(%)
SMCA-mouse	$10^{5.8}$	38	0	0
Normal mouse	$10^{5.8}$	19	10	53
None—subsequent litters of SMCA serum recipients	$10^{5.8}$	17	10	59
SMCA-rabbit (no. 7 prebleed)	$10^{4.6}$	15	8	53
SMCA-rabbit (no. 7 final)	$10^{4.6}$	43	0	0
GT-48-rabbit (no. 9 prebleed)	$10^{4.6}$	15	4	27
GT-48-rabbit (no. 9 final)	$10^{4.6}$	38	3	7.9
	GT-48 challenge dose to progeny ($SMLD_{50}$)		Deaths: day 4–15	
			No.	(%)
SMCA-mouse	$10^{5.5}$	29	16	55
Normal mouse	$10^{5.5}$	27	27	100
None—subsequent litters of SMCA serum recipients	$10^{5.5}$	17	17	100
SMCA-rabbit (no. 7 prebleed)	$10^{1.8}$	18	18	100
SMCA-rabbit (no. 7 final)	$10^{1.8}$	40	0	0
GT-48-rabbit (no. 9 prebleed)	$10^{1.8}$	13	13	100
GT-48-rabbit (no. 9 final)	$10^{1.8}$	39	2	5.1

[a] Dams received 0.25 ml of undiluted serum inoculated s.c. in 2 sites. Progeny (less than 24 hr old) were simultaneously inoculated i.c. with 0.01 ml of virus.

returned to 4° until assayed for residual virus.

Results. Passive protection of newborn mice with serum from SMCA-infected mice. Newborn CFW(A) mice were inoculated intraperitoneally (i.p.) or subcutaneously (s.c.) with 0.05 ml of SMCA-mouse serum each. Control animals were given normal mouse serum (NMS) i.p. or s.c. At the time of serum inoculation, each mouse was challenged by i.c. inoculation of 450 suckling mouse LD_{50} ($SMLD_{50}$) of GT-48 virus. (GT-48 was used as challenge virus because it gives a 100% lethal end point within 15 days, rather than the variable cataract end point detected at 30 days following SMCA inoculation.) Three of 16 suckling mice given SMCA serum i.p. and none of 15 mice given SMCA serum s.c. died during 15 days of observation. Thirteen of 14 suckling mice receiving normal mouse serum died (av 8.1 days). Hence, the presence of definite protective factors in the serum of SMCA-infected mice was demonstrated.

Protection of progeny following passive administration of mouse or rabbit-origin SMCA serum to the dam. CFW(A) dams with newborn litters were inoculated s.c. with 0.25 ml of mouse or rabbit-origin SMCA or GT-48 antiserum. At the same time, their litters were challenged by i.c. inoculation of SMCA or GT-48 virus and subsequently observed for SMCA-induced cataract or GT-48 virus-induced death (Table I).

Mother mice given SMCA mouse serum completely protected their young against SMCA-induced cataract and conferred partial protection against the lethal effects of a severe challenge dose of GT-48 virus. Subsequent litters of these dams were completely susceptible to SMCA or GT-48, indicating that the previous protection resulted from the passive transfer of immune factors subsequently cleared from the circulation, and not from active antigenic stimulation.

Mothers receiving rabbit antisera to SMCA or GT-48 also conferred a high degree of protection against the effects of SMCA or

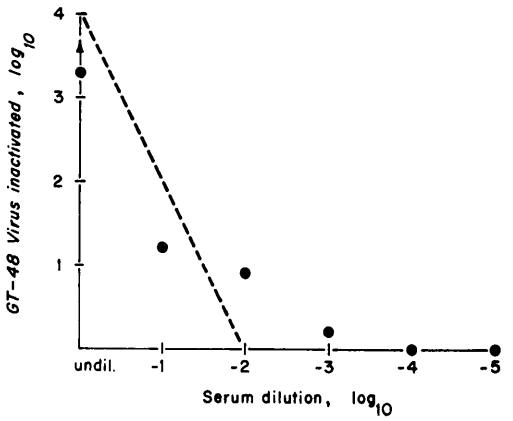

FIG. 1. Neutralization of GT-48 virus by serial dilutions of SMCA mouse antiserum.

GT-48 on their progeny. No evidence of antigenic difference was observed.

Neutralization index test for serum antibody assayed in suckling mice. The SMCA mouse antiserum, effective in direct and indirect passive protection tests against SMCA and GT-48 virus, was tested for its ability to neutralize these viruses *in vitro*. Serial 10-fold dilutions of SMCA mouse antiserum were incubated (37° for 30 min) with 10-fold dilutions of GT-48 virus. Residual infectivity was assayed in i.c. inoculated suckling mice (Fig. 1). While undiluted antiserum neutralized $\geqq 10^{3.4}$ $SMLD_{50}$ of virus, serum diluted 1:10 neutralized only $10^{1.1}$ $SMLD_{50}$.

Attempts to develop a serum neutralization test of the constant virus-varying serum dilution type, employing even very low doses of virus ($\geqq 10^{1.0}$) were unsuccessful. When minimal virus concentrations were reacted with serial 2-fold antiserum dilutions, end points extended over so many serum dilutions as to be virtually meaningless. For example, in a single neutralization test, both virus specific deaths and "protected" survivors were observed in mice inoculated with virus and serum dilutions varying from 1:2 to 1:128. Addition of guinea pig complement or fresh normal mouse serum to the reaction mixtures at a concentration of 50% did not improve the results. Hence, in all further studies, a constant serum dilution-varying virus dilution (neutralization index = NI) test was employed.

The antiviral titers in mouse and rabbit antisera were compared by NI tests assayed in parallel in IC-inoculated suckling mice and in 7-day-old chick embryos inoculated via the yolk sac (Table II). *In ovo* NI titers at end point (12 days) were 1.0 for the mouse antisera and 1.8 to $\geqq 2.8$ for the rabbit antisera. Delay of death was more prominent than absolute protection of chick embryos at day 12. NI titers determined in suckling mice were uniformly $\geqq 5.4$, as no end points were reached. The mouse assay was at least 1000 times more sensitive than the embryonated egg assay.

Use of NI test to measure antibody response in normal mice and mice under the influence of maternal protection. It was demonstrated previously that mice inoculated with SMCA protect their progeny against the cataractogenic effect of SMCA (3). These progeny could not subsequently protect their own young against SMCA. It was hypothesized that the active antibody response of the "protected" second generation was inhibited because maternal antibody limited viral replication. This hypothesis was tested by determining the antibody response of normal Swiss suckling mice and protected second generation suckling mice to i.c. chal-

TABLE II. Comparative Assay of Neutralization Index Titers in Embryonated Eggs and Suckling Mice.

		Neutralization index titer[a]		
		Embryonated eggs		
Serum	Virus	9 days postinoculation	12 days postinoculation	Suckling mice
Rabbit no.				
7 (SMCA)	GT-48	$\leqq 3.6$	2.4	$\geqq 5.4$
	SMCA	$\geqq 2.8$	$\geqq 2.8$	
9 (GT-48)	GT-48	$\leqq 3.6$	1.8	$\geqq 5.4$
	SMCA	2.5	2.0	
Mouse				
SMCA	GT-48	2.0	1.0	$\geqq 5.4$
GT-48	GT-48	0.3	1.0	$\geqq 5.4$

[a] Difference in titer ($\log_{10}$) between virus dilutions incubated for 30 min at 37° in the presence of 50% immune serum and virus incubated with an equal concentration of normal serum.

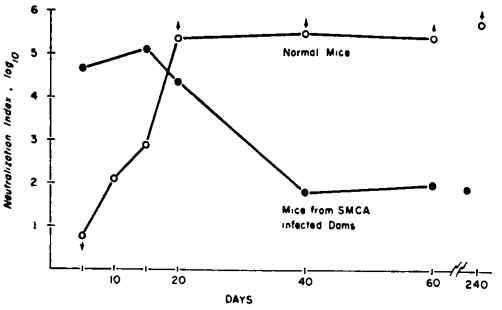

FIG. 2. Immune response of suckling mice to intracerebral inoculation of SMCA; normal control mice and mice under the influence of maternal antibody protection.

lenge with SMCA, using the suckling mouse NI test to measure antibody to GT-48 (Fig. 2). Antibody in normal mice appeared by 10 days of age, reached a maximum titer of $\geq$5.4 at 20 days, and persisted at this level for at least 240 days. Mice inoculated with SMCA under the influence of maternal protection had a high titer of antibody at age 5 days (the earliest tested) which remained elevated until 20 days. Antibody titer then declined to approximately 2.0, a titer which then persisted. The data indicate that early high levels of maternally acquired circulating antibody in protected suckling mice was followed by a diminished active antibody response to SMCA infection.

Dilution "reactivation" of GT-48 virus neutralized by SMCA antiserum. A neutralization line for the reaction of GT-48 virus with mouse SMCA antiserum could not be constructed with certainty because of failure to obtain an end point with undiluted serum, but it was obvious that whatever line is drawn will give a slope greater than 1.0 (Fig. 1, the broken line has a slope of 2.0). Such a slope suggests that dilution may reactivate serum–virus mixtures (5). In order to test this possibility, GT-48 virus (titer $10^{5.3}$ $SMLD_{50}$/0.01 ml) was incubated with an equal volume of SMCA mouse antiserum (NI = $\geq$5.5) for 30 min at 37°. The reaction mixture was then immediately inoculated undiluted and in serial 10-fold dilutions into suckling mice. The dilutions were again tested after 3- and 20-hr further incubation at 37°. All dilutions were performed in 50% mouse serum in BAPS to eliminate any possible effect of nonspecific mouse serum factors. Dilutions of serum–virus mixtures consistently revealed virus activity not detected in the undiluted serum–virus mixtures (Table III). About $10^{3.0}$ $SMLD_{50}$ of virus not irreversibly neutralized by antiserum was detected immediately after dilution or after 3-hr incubation of dilutions at 37°. The lower titers of virus recovered after 20-hr incubation of the dilutions at 37° presumably reflect thermal inactivation.

Discussion. Mouse antiserum to SMCA was found to be effective in passively protecting suckling mice against virus administered by the i.c. route. While passive protection of suckling mice against a variety of viruses inoculated peripherally has been demonstrated (6–8), studies employing eastern encephalitis (8) and herpes simplex (9) viruses revealed little or no passive protection to be demonstrable with i.c. virus challenge. Newborn mice were also effectively protected against the effects of i.c. challenge with SMCA or GT-48 when antisera to either virus were administered to the mother at the time of challenge. Protection of suckling mice by administration of antiserum to their dams has been reported in studies with the protozoan *Trypanasoma duttoni* (10), but failure was observed in similar studies employing mouse encephalomyelitis virus (7). Rabbit and mouse origin antisera administered a mouse dams were both effective in protecting progeny against SMCA or GT-48 challenge. We know of no previous demonstration of protection of suckling mice by administration of antiserum of heterologous species origin to their dams.

The SMCA mouse antiserum had a very high antibody titer when tested undiluted by the neutralization index method with assay in i.c. inoculated newborn mice. The SMCA mouse antiserum with an NI titer of only 1.0 in tests assayed in embryonated eggs had an NI titer of $\geq$5.4 in tests assayed in newborn mice. Greater sensitivity of the newborn mouse compared to the embryonated egg for assay of neutralizing antibody has been previously reported in studies with influenza

TABLE III. "Reactivation" of a GT-48 Virus–Antiserum Mixture Diluted after Incubation.[a]

Inoculum	No. of mice inoculated	No. of mice dead days 4–15	Mean day of death
Serum–virus mixture incubated 30 min at 37° and inoculated without dilution	10	2	14.5
Serum–virus mixture serially diluted in ice bath after incubation and inoculated immediately			
1:10	9	9	10.0
1:100	8	8	9.9
1:1000	9	5	10.7
Serum–virus mixture diluted after primary incubation, dilutions incubated 3 hr at 37° prior to inoculation			
1:10	8	7	11.6
1:100	12	10	10.3
1:1000	9	4	10.8
Serum–virus mixture diluted after primary incubation, dilutions incubated 20 hr at 37° prior to inoculation			
1:10	4	4	5.5
1:100	11	2	6.0
1:1000	10	0	—

[a] Undiluted mouse SMCA mixed with an equal amount of GT-48 suckling mouse brain virus, concentration $10^{6.3}$ $SMLD_{50}$/0.01 ml.

(11, 12) and mumps (13) viruses. Suggested explanations for the phenomenon include: (i) residual antibody in the reaction mixture is less diluted in the mouse and protects it more effectively; (ii) the mouse is less sensitive in detecting small amounts of nonneutralized virus than the more susceptible embryonated egg; or (iii) virus–antibody dissociation is less likely to occur in the suckling mouse, as the reaction mixture is less diluted.

Virus neutralizing capacity was lost disproportionately following even minimal dilution of SMCA antisera, suggesting that dilution reactivation might occur. The neutralizing capacity of antisera dilutions was not enhanced by addition of fresh mouse serum or guinea pig complement, which contain substances reported to enhance the neutralization of other viruses (14, 18). Reactivation of virus activity was readily demonstrated immediately following dilution of mixtures of undiluted SMCA antiserum and GT-48 virus. Approximately 1% of the virus activity neutralized in the presence of undiluted serum was recovered following dilution. The 99% of virus irreversibly neutralized may explain the maximum NI titers of approximately 2.0 $\log_{10}$ often obtained by assay of serum–virus mixtures in embryonated eggs. Dilution reactivation of neutralized virus has been described previously with influenza (12, 19, 20), fowl plague (21), vaccinia (22), and Coxsackie (23) viruses.

The NI test, assayed in suckling mice, provided a useful means for studying the immune response of SMCA-inoculated suckling mice. Normal newborn mice inoculated with SMCA i.c. developed low levels of antibody by 10 days and maximal titers by 20 days after inoculation. The NI titer persisted at this level for at least 240 days, explaining the continued ability of such mice to confer protection on their progeny (3). The SMCA-inoculated progeny of SMCA-infected mothers had a high titer of antibody at 5 days of age, at a time when no antibody was detected in virus-inoculated normal newborns. This antibody, presumably maternally acquired, persisted for about 20 days, after which it diminished. From 40 days until at least 240 days of age, a low NI titer (approx 2.0) persisted in these mice, possibly reflecting a diminished active antibody response to the reduced viral replication (3). Antibody in

this low titer is apparently ineffective in protecting progeny against virus challenge or, indeed, in preventing an active immune response to a subsequent direct virus challenge (3).

Summary. Previous studies have revealed that SMCA-infected mice confer protection against the effects of SMCA or GT-48 virus infection on their progeny, despite the fact that tests using an *in ovo* assay system consistently revealed very low antibody titers in the sera of infected mice. In the present studies, protective activity in the serum of infected mice was demonstrated by its ability to confer passive protection on GT-48 virus-inoculated suckling mice. Mouse or rabbit origin antisera were also demonstrated to confer passive protection on SMCA or GT-48 virus-inoculated suckling mice when inoculated into the dam at the time of challenge of the progeny. The neutralizing antibody in antisera prepared in mice and rabbits was assayed by means of a neutralization index test using GT-48 virus and assay of serum–virus mixtures in i.c. inoculated suckling mice. This test proved to be reliable and sensitive. When serum was diluted, neutralizing capacity decreased disproportionately, even when such accessory factors as fresh mouse serum or guinea pig complement were added. Virus–antibody dissociation was demonstrated following dilution of a reaction mixture of SMCA mouse antiserum and GT-48 virus. Approximately 1% of apparently previously neutralized virus activity was recovered immediately upon dilution of the serum–virus mixture at 4°. The antibody response in SMCA-inoculated newborn mice was studied. Normal mice inoculated with SMCA as newborns have been shown to acquire a lifelong ability to protect their progeny against SMCA. Such mice had no detectable circulating antibody at 5 days of age, but developed high titers (NI >5.0) of antibody by 20 days, which then persisted until at least 240 days of age. Newborn mice inoculated with SMCA under the influence of maternal immune protection cannot subsequently confer protection on their own progeny. These mice had high-titered circulating antibody at 5 days of age that diminished to a persisting low level (NI $=$ 2.0) by 40 of age.

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