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Resistance of Mice to Infection with Friend Disease Virus After Subcutaneous Injection of Friend Virus and Friend Spleen Cells

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Swiss mice injected subcutaneously with suspensions of spleen cells or an extract of spleens from mice infected with Friend virus develop resistance to subsequent intravenous inoculation of Friend virus. A single injection of either Friend virus or Friend cells induces resistance. Immunized mice display resistance when challenged 6 months after immunization and survive for at least 20 weeks after infection. Neutralization tests indicate that serum, but not lymphoid cells of resistant animals, can neutralize Friend virus. In vitro neutralization tests indicate that residence of virus within the peritoneal cavity of immune mice for 1 h sharply reduces the infective titer of the virus.

Resistance to infection with Rauscher virus or Friend virus has been induced in mice by immunization with Formalin-killed virus (6, 7) but not by administration of a strain of Friend virus which gives rise to regressive disease (15). Resistance to Friend disease may, however, be produced by infection with Rawson-Parr virus (4, 17), the lymphatic leukemia virus (5) isolated by Steeves et al. (19) and by murine sarcoma virus of Moloney (16). Nonspecific resistance to infection with Friend virus may be produced by immunization with an attenuated strain of *Mycobacterium bovis* (9).

Intravenous or intraperitoneal injection of Friend virus produces florid infection in mice (11), but lesser degrees of infection follow subcutaneous introduction of virus. Old et al. (12) found that the mortality rates were considerably reduced among mice injected subcutaneously with either large numbers of spleen cells from mice with Friend disease (Friend cells) or a high concentration of virus in comparison to those after intravenous injection of similar amounts of infectious material. The surviving animals developed antibodies cytotoxic for Friend cells.

Studies of resistance produced in mice by subcutaneous injection of either Friend virus or Friend cells are presented in this investigation. Mice so immunized are resistant to challenge with virus administered intravenously and develop antibodies capable of neutralizing Friend virus.

MATERIALS AND METHODS

Mice. Outbred Swiss mice were obtained from the colony maintained at the Rocky Mountain Laboratory (RML), Hamilton, Mont.

Virus. Friend virus (FV) was obtained from R. W. Sidwell and has been passed continuously in RML mice for a period of years. Pools of virus were prepared from the spleens of mice infected 4 weeks previously by intravenous (i.v.) injection of FV. A suspension of 10 or 20% spleen tissues was prepared and centrifuged (7). The clarified supernatant fluid was distributed in 1.0-ml amounts in ampoules which were sealed and frozen at −70 C. The mean lethal dose (LD_{50}) of the virus pools used in these experiments varied from $10^{-3.7}$ to $10^{-4.1}$.

Immunization. Mice were immunized by subcutaneous (s.c.) injection of either Friend spleen cells (FC) or FV. Suspensions of spleen cells prepared by teasing the spleen or sieving the organ through a stainless steel screen obviously yield both infected cells and virus. For purposes of discussion the term FV is used to describe the supernatant fluid obtained after centrifugation at 4,300 × *g* for 30 min of a suspension of spleen cells from infected mice, and FC to describe the suspension itself. Initially, mice were immunized by the method described by Old et al. (12) utilizing 3 s.c. injections of FC suspended in balanced salt solution and administered at intervals of 3 weeks. About 10^7, 5×10^7, and 10×10^7 cells were used for the first, second, and third injections, respectively. Subsequently, a single s.c. injection of either FC or FV was found to induce resistance.

Challenge tests. Groups of mice immunized by these methods, as well as normal mice of the same age, were challenged i.v. with FV. In some experiments only a single dilution of virus contained in 0.2

ml of inoculum was employed, but in others various dilutions of virus were administered to groups of immune and normal animals. Initially, tests were terminated 11 days after infection of the animals. At this time splenomegaly was not marked, but the presence and number of foci in the spleen (2, 13) of each animal was used as a criterion of the presence of Friend disease (FD). However, in most of the studies the animals were autopsied 28 days after infection, and in these experiments the degree of splenomegaly as well as the occurrence of splenic foci were determined. Splenomegaly was considered to be present if the weight of the test spleen was at least two times greater than the average weight of the spleens of the control animals. In some experiments survival for 20 weeks after challenge was used as a determinant of resistance.

In vitro neutralization. Tests were done with sera from individual normal and immune mice. The sera were diluted 1:4, mixed with equal quantities of a $10^{-2.3}$ dilution of FV, incubated for 30 min at 37 C, and 0.4 ml of the mixture was injected i.v. into groups of five mice each. The recipient animals were held for 21 days before they were autopsied and spleen weights and foci were recorded. Spleen and lymph node cells from the animals from which sera were obtained were tested for their ability to neutralize virus. The spleen, the inguinal, axillary, and mesenteric lymph nodes from individual mice were pooled and sieved through a 60-mesh stainless steel screen. The cells were suspended in Eagle minimal essential medium (MEM) and washed two times by centrifugation. The number of cells was determined and equal amounts of cell suspension and a $10^{-2.3}$ dilution of FV were mixed. After incubation for 30 min at 37 C, 0.4 ml of the cell-virus suspension was injected i.v. into each of five mice. The recipients were examined 21 days later.

In vivo neutralization. Five normal and five immune mice were each injected i.p. with 1.5 ml of a 10^{-1} dilution of FV. One hour later the animals were killed and the peritoneal fluid was harvested. Peritoneal fluids from immune and normal mice were pooled separately. The cells were separated from the fluid by centrifugation at 200 × *g* for 15 min and washed twice. The cells and fluid from each group of mice were injected i.v. in 0.2-ml amounts into groups of 10 animals each. The fluids were used whole and in dilutions of 10^{-1} and 10^{-2} and the cell suspensions were adjusted to contain either 1.6×10^6 to 1.9×10^6 (from immune mice) or 1.6×10^5 to 1.9×10^5 (from normal mice) cells per ml. The animals were autopsied 20 days later and the spleen weights and number of foci per spleen were determined. To control the experiment, a sample of FV was kept at 37 C for 1 h. Dilutions of 10^{-1}, 10^{-2}, and 10^{-3} were prepared, and 0.2-ml amounts were injected i.v. into groups of 10 mice each.

RESULTS

Immunization with multiple injections of suspensions of FD spleen cells. Mice immunized by 3 s.c. injections of FC were divided into 3 groups and challenged by i.v. injection of various dilutions of FV 49 days after the last dose of FC (89 days after the primary immunizing dose). Included as controls in the experiment (Table 1) were groups of normal mice of the same age infected with the same dilutions of FV and groups of uninfected normal and immunized mice. All animals were sacrificed 28 days after infection, the spleen and body weights were determined, and the number of splenic foci was counted. The spleen weights of uninfected normal and immune mice were almost identical and these spleens appeared to be normal in other aspects. There was marked splenomegaly among all groups of unimmunized mice infected with FV, whereas only minimal splenomegaly was observed in 1 of the 36 immunized mice

TABLE 1. *Resistance of mice immunized by 3 subcutaneous injections of Friend spleen cell suspensions to infection with FV administered i.v. 49 days after last immunizing injection*

Group	No. of mice	Immune status	Dilution of FV used for challenge	Spleen wt		Degree of splenomegaly[a]	No. of splenic foci			
				Avg (g)	Range (g)		0	1-10	10-50	Confluent
1	10	Normal	10^{-1}	2.65	0.29-4.55	13.25×[b]	1			9
2	10	Normal	10^{-2}	1.78	1.13-2.80	8.9×[c]				10
3	8	Normal	10^{-3}	1.25	0.43-2.66	6.2×[d]				8
4	8	Immune[e]	10^{-1}	0.22	0.16-0.28	0.96×	6	2		
5	9	Immune	10^{-2}	0.19	0.13-0.30	0.83×	2	7		
6	10	Immune	10^{-3}	0.26	0.14-0.57	1.13×	4	5		
7	9	Normal	0	0.20	0.14-0.30[f]		9			
8	9	Immune	0	0.23	0.15-0.34		9			

[a] Average spleen weight of infected mice per average spleen weight of uninfected mice.
[b] 1 versus 4: $p < 0.001$.
[c] 2 versus 5: $p < 0.001$.
[d] 3 versus 6: $p < 0.005$.
[e] Three s.c. injections of FC at 3-week intervals; 10^7, 5×10^7, and 10×10^7 FC at the first, second, and third injections, respectively.
[f] 7 versus 8: $p < 0.30$.

challenged with FV. A total of 27 of 28 infected, unimmunized mice had confluent foci in the spleen.

Immunization with a single injection of FC. Mice were injected s.c. with 1.2×10^6 FC. After an interval of 67 days, groups of immunized and normal mice of the same age were infected by i.v. injection of varying doses of FV. A group of normal and a group of immunized mice were retained as unchallenged controls. All mice were sacrificed 4 weeks after infection, the spleen and body weights were determined, and the number of splenic foci was recorded. The results are shown in Table 2. Splenomegaly was present in the normal mice challenged with FV (groups 1, 2, 3). Among the 28 mice in these groups, spleens of 24 animals weighed two times more than the average spleen of uninfected, unimmunized animals (group 7). Foci were confluent on the spleens of 27 of 28 mice in these three groups. Only one mouse in the immunized, challenged group had splenomegaly; 18 of 24 had no splenic foci, and only one had confluent foci. One of the uninfected, immunized mice in group 8 had an enlarged spleen with confluent foci, indicating that the immunizing material had produced FD which was manifest 95 days after the s.c. injection of FC.

Immunization with a single injection of FV. Mice were immunized by s.c. injection with 0.1 ml of a 1:10 dilution of FV. After an interval of 114 days, three groups of eight immune animals and three groups of eight normal animals of the same age were segregated. One group of immune mice and one group of normal mice were not infected with FV, but separate groups of normal and immune mice were infected i.v. with 0.2 ml of either a 10^{-2} or 10^{-3} dilution of FV. All mice were sacrificed 28 days later. The results are shown in Table 3. It was

TABLE 2. *Immunization of mice with a single s.c. injection of FC followed 67 days later by challenge with virus i.v. and autopsy 28 days after challenge*

Group	No. of mice	Immune status	Immunizing agent	Dilution of FV used for challenge	Spleen wt		Degree of splenomegaly[a]	No. of splenic foci			
					Avg (g)	Range (g)		0	1–10	10–100	Confluent
1	10	Normal		10^{-1}	2.65	0.29–4.55	12.6×[b]		1		9
2	10	Normal		10^{-2}	1.78	1.13–2.80	8.5×[c]				10
3	8	Normal		10^{-3}	1.25	0.43–2.66	6.0×[d]				8
4	8	Immune[e]	1.2×10^6 FC	10^{-1}	0.41	0.16–1.93	1.1×	8			
5	8	Immune	1.2×10^6 FC	10^{-2}	0.21	0.14–0.52	0.6×	6	2		
6	8	Immune	1.2×10^6 FC	10^{-3}	0.29	0.10–0.77	0.8×	5	2	1	
7	10	Normal		0	0.21	0.10–0.31		10			
8	10	Immune	1.2×10^6 FC	0	0.38	0.14–2.09[f]		9			1

[a] Average spleen weight of infected mice per average spleen weight of uninfected mice.
[b] 1 versus 4: $p < 0.001$.
[c] 2 versus 5: $p < 0.001$.
[d] 3 versus 6: $p < 0.005$.
[e] Immunized by s.c. injection of 1.2×10^6 FC.
[f] 8 versus 7: $p < 0.25$.

TABLE 3. *Immunization with a single s.c. injection of FV followed 114 days later by challenge with virus i.v. and autopsy 28 days after challenge*

Group	No. of mice	Immune Status	Dilution of FV used for challenge	Spleen wt		Degree of splenomegaly[a]	No. of splenic foci			
				Avg (g)	Range (g)		0	1–10	10–100	Confluent
1	8	Immune[b]	10^{-2}	0.32	0.21–0.58	1.1×[c]	5	3		
2	8	Immune	10^{-3}	0.33	0.13–0.67	1.1×[d]	6	2		
3	8	Normal	10^{-2}	1.95	0.58–3.46	7.8×				8
4	8	Normal	10^{-3}	1.04	0.23–1.97	4.2×		1	1	6
5	8	Immune	0	0.29	0.10–0.58		8			
6	7	Normal	0	0.25	0.17–0.40[e]		7			

[a] Average spleen weight of infected mice per average spleen weight of uninfected mice.
[b] Immunized by s.c. injection of 0.1 ml of a 10^{-1} dilution of the FV pool.
[c] 1 versus 3: $p < 0.001$.
[d] 2 versus 4: $p < 0.005$.
[e] 5 versus 6: $p < 0.7$.

found that FV given i.v. produced typical findings of FD in normal animals, whereas animals immunized by a single s.c. injection of FV were resistant to i.v. infection with the virus.

Survival of immunized mice. In the following experiment three different FV or FC preparations were used to immunize groups of 50 mice each. The animals were injected s.c. on days 0 and 21 with an inoculum of 0.1 ml which consisted of either a 10% suspension in MEM of spleen tissue from mice infected with FC, the supernatant fluid obtained after centrifugation of the 10% suspension at 4,300 × *g* for 30 min, or with cells from the suspension washed eight times with MEM. Mice of group 1 received supernatant fluid, those in group 2 the 10% suspension of spleen cells, and those of group 3 the washed cells. The number of cells contained in the inoculum of 10% spleen suspension given on day 0 was 1.3×10^7 and on day 21 was 2.5×10^7. The number of washed cells injected was 7.4×10^6 on day 0 and 2.0×10^7 on day 21. A group of unimmunized mice was retained as controls (group 4). Twenty-one days after the last s.c. injection 27 mice from each group, including the controls, were injected i.v. with 0.2 ml of a $10^{-2.3}$ dilution of FV. Twenty animals of each group were observed for 20 weeks in order to obtain death rates. The results are shown in Fig. 1. The other seven mice in each group were autopsied 11 days after infection and the number of foci in the spleens were observed. All of the unimmunized mice had splenic foci but none was observed in the spleens of the immunized animals.

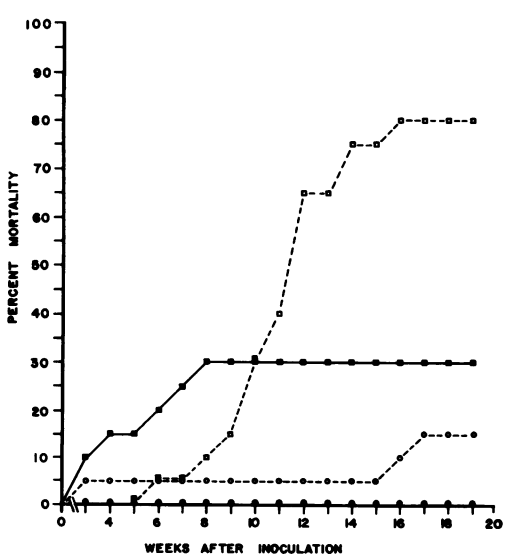

FIG. 1. *Percent mortality among mice challenged with FV injected i.v. after immunization by s.c. injection of: group 1 (●), supernatant fluid from a 10% suspension of spleens of mice with FD; group 2 (○), 10% suspensions of FD spleen; group 3 (■), 8× washed cells of above suspension; group 4 (□), unimmunized controls.*

During the observation period of 20 weeks, 80% of the infected, unimmunized animals succumbed, whereas only negligible numbers of mice immunized with the spleen suspension or supernatant fluid developed FD. Only 30% of those immunized with the washed cell suspension died.

Persistence of resistance. Six months after the initial immunization, the remaining mice were challenged by i.v. injection of 0.2 ml of a $10^{-2.3}$ dilution of FV. Unfortunately, the number of mice in groups 1 and 2 had been depleted after exposure to an unrelated organism and only four animals remained in each group. Ten normal mice of the same age were also injected with the same dose of virus. The animals were held for 28 days at which time they were killed and the spleen and body weights were determined. The data given in Table 4 show that mice immunized by s.c. injection of spleen cell suspension or spleen cell extracts from mice with FV are resistant to infection with FV administered 6 months later.

In vitro neutralization. A total of 36 mice immunized by 3 s.c. injections of FC and 5 normal mice were used to determine the capacity of either serum or lymphoid cells to neutralize FV. The sera and cells were collected 100 days after the mice had received the last immunizing injection. The results are shown in Table 5. Of the 36 immunized donors, only 1 had marked splenomegaly at the time cells and sera were obtained. Among the 9 mice yielding serum which did not neutralize virus the mean spleen weight was 0.47 g and among the 17 mice yielding neutralizing serum the mean spleen weight was 0.24 g. The sera of at least 75% of the immune mice afforded protection to recipient mice as determined by the numbers of mice developing splenic foci, and 86% were protective as judged by the number of animals developing

TABLE 4. *Results obtained following i.v. injection of FV into mice immunized 6 months previously by 2 s.c. injections of a 10% suspension of FC, the supernatant fluid from this suspension (S), or washed spleen cells (WFC)*

Group	No. of mice	Immunizing agent	Avg spleen wt (g)	Spleen/body wt ratio × 100 (%)
1	4	S	0.19[a]	0.46
2	4	FC	0.23[b]	0.55
3	15	WFC	0.20[c]	0.50
4	10		1.18[d]	3.22

[a, b] a & b versus d = $p < 0.005$.
[c, d] c versus d = $p < 0.001$.

splenomegaly, whereas 1.2×10^7 to 6.9×10^7 cells were not capable of neutralizing FV. However, since the strain of mice used in this investigation is outbred the results of tests with lymphoid cells are not definitive.

In vivo neutralization. In this experiment an attempt was made to determine the effect of residence of FV in the peritoneal cavity of normal mice and of mice immunized by s.c. injection of FC. The test was done 130 days after the first of 2 s.c. injections of 10^6 FC given at 3-week intervals, and was performed as previously described. The animals were examined 21 days after injection and the results are shown in Table 6.

TABLE 5. *In vitro neutralization of FV by sera and lymphoid cells taken from mice 100 days after completion of an immunization program consisting of three subcutaneous injections of FC given at 3-week intervals*

Group	Immune status of donors	No. of donors	Neutralization of FV with donor:	No. of recipient groups with:	
				Splenomegaly	Splenic foci
1	+	36	Serum	5/36	9/36
			Cells	32/36	33/36
2	−	5	Serum	5/5	5/5
			Cells	5/5	5/5

The results indicate that FV resident in the peritoneal cavity of normal mice for 1 h was not decreased in amount in comparison to FV maintained in vitro at 37 C for the same period of time. On the other hand, residence of virus in the peritoneal cavity of specifically immune mice resulted in a marked decrease in the amount of virus present. Neither the normal nor the peritoneal exudate cells contained virus in amounts comparable to those found in the supernatant fluids. The number of recipient animals displaying splenic foci was significantly greater among those mice injected with 1.6×10^6 peritoneal cells from normal donors than among mice injected with 1.9×10^6 peritoneal cells from immune donors.

DISCUSSION

Immunization of mice against infection with FV administered i.v. can be satisfactorily accomplished by s.c. injection of either virus or spleen cells obtained from mice infected with FV. A single s.c. injection of either cells or virus suffices to immunize animals against subsequent infection. This immunity protects mice against infection with at least 100 infectious doses of virus (Table 1). Mice immunized by a single s.c. injection of 1.2×10^6 spleen cells from mice with FD are resistant to 2 logs of virus when challenged 67 days after immunization. Likewise, mice immunized by a single s.c.

TABLE 6. *In vivo neutralization of FV after i.p. injection of FV into normal and FD-immune mice. Results were obtained by i.v. injection of groups of mice with the peritoneal exudates harvested 1 h after the test mice had been injected with FV*

Recipient group	No. of mice	i.v. Injection of:		Dilution of PF or no. of PC	Spleen wt		Degree of splenomegaly	No. of splenic foci			
		Preparation	Donor		Avg (g)	Range (g)		0	1–10	10–100	Confluent
1	10	PF[a]	Immune mice	Whole	0.37	0.17–0.92	2.1×[d, e]	2	5	2	1
2	10	PF	Immune mice	1:10	0.31	0.14–0.50	1.7×	5	5		
3	10	PF	Immune mice	1:100	0.22	0.17–0.28	1.2×	7	3		
4	9	PF	Normal mice	Whole	1.87	0.33–3.34	10.5×			1	8
5	10	PF	Normal mice	1:10	1.02	0.18–2.58	5.7×			1	9
6	10	PF	Normal mice	1:100	0.31	0.20–0.59	1.7×	2	2	5	1
7	10	Virus[b]		1:10	2.38	0.47–3.12	13.2×				10
8	10	Virus		1:100	0.95	0.24–2.52	5.3×		3	1	6
9	10	Virus		1:1000	0.36	0.19–0.69	2.0×	3	2	3	2
10	10	PC[c]	Immune mice	1.9×10^6	0.36	0.20–0.96	2.0×	4	2	2	2
11	10	PC	Immune mice	1.9×10^5	0.23	0.14–0.32	1.3×	7	3		
12	10	PC	Normal mice	1.6×10^6	0.53	0.24–1.96	2.9×	2		1	6
13	10	PC	Normal mice	1.6×10^3	0.27	0.17–0.67	1.5×	5	1	2	2
14	9				0.18	0.11–0.24		9			

[a] Supernatant fluid of peritoneal exudate.
[b] FV maintained at 37 C for 1 h.
[c] Cells of peritoneal exudate.
[d] Average weight of spleen of infected mice per average weight of spleen of uninfected mice.
[e] Mice autopsied 21 days after injection.

injection of 0.1 ml of a 1:10 dilution of FV are immune when challenged 114 days after immunization.

Not only are mice resistant to the development of splenic foci and splenomegaly, but survival is extended for at least 20 weeks after i.v. injection of FV. Animals immunized with a suspension of spleen cells from mice with Friend disease, with the supernatant fluid from this suspension, or FC washed eight times survive for 20 weeks after i.v. injection of FV. Animals immunized with the above preparations and challenged 6 months after immunization are resistant to challenge. It is of interest to note that after challenge with FV most of the deaths among animals immunized with FC occurred before deaths occurred in the control mice.

Studies of the mortality rate due to s.c. injection of FV or FC have not been specifically carried out but some idea of the effect of live material administered by this route can be obtained from other observations. Thus, among the 92 immunized mice included in the tables, only 3 had enlarged spleens at the time of autopsy. At least 84% of mice immunized with washed spleen cells survived for 42 days after immunization and 14 of 23 animals survived for at least 6 months after receiving an s.c. injection of cells. In one experiment the mortality rate was about 35% and in others the mortality rates were approximately 10% 6 months after immunization. These figures are to be compared with those reported by Old et al. (12) and Rich et al. (15).

Lilly (10) has reported upon the resistance of certain strains of mice to the F-S and F-B strains of FV. F-S is a strain of FV adapted to Swiss mice and F-B is a strain adapted to BALB/c mice. Infection of BALB/c mice with F-S virus does not result in FD among them except when high concentrations of F-S are administered, but induces resistance to a subsequent inoculation of F-B virus. Rawson and Parr (17) and Bendinelli and Nordini (4) have shown that Rawson-Parr virus (RPV) produces resistance to subsequent infections with FV. The former authors did not find neutralizing FV antibodies in the plasma of RPV-infected mice, but the latter workers attribute resistance of RPV-infected mice to infections with FV to neutralizing antibodies against the latter agent. Other studies have shown that strains of Rauscher and Friend virus maintained on tissue cultures will also produce resistance in mice to infection with standard FV (3, 8, 18, 20). The studies of Old et al. (12) yielded results similar to the present ones in terms of the infectivity of FC or FV injected s.c. into mice. Large numbers of animals infected by this route did not develop leukemia, but developed antibodies cytotoxic for FC.

Resistance which develops after s.c. injection of FC or FV appears to be related to humoral immunity. It is generally agreed that FD is initiated by infection of the reticulum cells in the spleen and that involvement of the lymph nodes is absent or minimal (14). Administration of FV by the s.c. route may result in a minimal spread of virus through the circulation and in a prompt response in the regional lymph nodes. The vast majority of mice inoculated by this method are capable of preventing FV from spreading to the spleen from the site of s.c. injection. The number of cells or amount of virus utilized to induce resistance by s.c. injection in these experiments contains at least 3 logs of infectious material as shown by the development of FD in mice after i.v. injection. Neutralization tests show that serum from immunized mice is capable of neutralizing virus in vitro and studies of the effect of residence of FV in the peritoneal cavity of immune mice indicate that FV is neutralized in vivo. No evidence of delayed hypersensitivity has been found in immune mice using either plasma virus, infected spleen cells, or peritoneal exudate cells as antigen in footpad tests (C. L. Larson, unpublished data). Transfer of spleen cells from immune mice to normal mice does not result in transfer of resistance. These results do not differ significantly from those obtained by Fink et al. (6) and Friend (7) in their studies of resistance caused by immunization of mice with Formalin-killed Rauscher virus or FV. A recent review by Allison (1) emphasizes that infections due to different viruses may be controlled by either humoral immunity or cell-mediated immunity. Infections due to FV appear to be controlled by the former mechanism.

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