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Cellular Immunity Against Semliki Forest Virus in Mice

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Intracutaneous immunization of BALB/c mice with purified inactivated Semliki Forest virus resulted in cellular immunity without detectable antibodies. The animals were protected against subcutaneous challenge, from which the challenge virus spreads slowly. After intraperitoneal challenge, which permits a rapid virus spread, the protection was marginal. Stimulation of the intraperitoneal cell population with thioglycolate before challenge resulted in complete protection. The protection could be transferred to normal mice with peripheral lymph node cells, but not with spleen cells. The course of the infection in immunized and normal mice was also studied. Semliki Forest virus does not multiply in peritoneal cells in vivo. In immunized mice part of the challenge virus in the peritoneal cavity was rapidly eliminated and viremia was reduced. After challenge, immunized mice produced less antibody than normal mice.

After intracutaneous immunization of mice with a purified inactivated Semliki Forest virus (SFV) antigen, a delayed-type hypersensitivity (DTH) without detectable antibodies could be demonstrated by footpad swelling. This DTH was transferable to normal mice with lymph node cells (8). It is of interest to know whether such a DTH provides protection. In virus infections this could, in principle, be achieved by lymphokines like interferon II and macrophage-activating factor. Protection by cytotoxic T cells is less probable because inactivated virus seems quite ineffective in recruiting these cells (3).

In this paper it is shown that after intracutaneous immunization mice without antibodies were protected against virulent SFV. The course of the virus infection in immunized mice is described.

MATERIALS AND METHODS

Virus strains, the preparation of purified virus antigens, and general virological and immunological methods have been described earlier (8).

Virulent virus. The strain SF/LS10/CI/A (1) was passed once through BALB/c mice. A brain suspension was kept as a stock in ampoules above liquid nitrogen. Batches of virulent virus were prepared on monolayers of L cells. After 24 h the supernatant contained 1.7×10^6 plaque-forming units (PFU) per ml. Ampoules of each batch were kept above fluid nitrogen. The intraperitoneal (i.p.) and subcutaneous (s.c.) 50% lethal doses (LD_{50}) for male BALB/c mice, as determined by the method of Reed and Muench (11), were 1 to 2 and 15 PFU, respectively.

Neutralizing antibodies. Serum obtained from mice at 7 days after infection with 3,000 PFU of avirulent virus (MRS MP 192/7) (6) was pooled and kept in small portions at −20°C. With this serum a plaque reduction of 50% was observed at a dilution of 1:100, which can be described as an antibody concentration of 100 plaque-neutralizing doses (PND_{50}). Guinea pig complement did not increase the titer. Treatment with mercaptoethanol reduced the titer to 1:2, which indicated that immunoglobulin M was the main antibody.

Animal experiments. Inbred male BALB/c mice (10 to 14 weeks old) from our own animal house were used throughout these experiments. Blood was obtained by retro-orbital puncture. For virus titrations clean organs (spleen, brain) were homogenized in Eagle medium in a Potter Elvehjem homogenizer, freeze-thawed twice, and centrifuged at $100 \times g$. Virus was titrated in the supernatant and expressed as virus in the whole organ. Peritoneal contents were obtained after injection of 2 ml of Eagle medium into the peritoneal cavity. After slight massage, the animal was killed, the abdomen was opened, and the fluid was collected with a pipette (1.2 to 1.8 ml). After centrifugation the supernatant was used for virus titration, if necessary by inoculating the total harvest. The sedimented cells were taken up in Eagle medium, freeze-thawed twice, and centrifuged, and the supernatant was used for titration.

For the infectious center assays the sedimented cells were passed through nylon gauze to disperse clumps and washed twice with Eagle medium. Various dilutions containing 10, 100, or 1,000 cells were transferred to 35-mm monolayers.

Cell suspensions. Preparation of cell suspensions is described in another paper (8).

RESULTS

Passive protection by antibody. To determine how much antibody was required for protection, dilutions of a serum pool from recently immunized mice, containing immunoglobulin M antibodies, were given intravenously to normal

mice in amounts of 0.25 ml. As the blood volume of mice is about 1.2 ml, the final dilution was five times greater. After 2 h the recipient mice were s.c. challenged with 100 PFU (7 LD_{50}) of virulent virus (Table 1). At 2 h after the serum injection, antibodies could only be detected in animals receiving the 1:5 dilution. However, the 1:320 dilution still protected five of six mice, showing that a serum concentration of 1:15 PND_{50} does protect mice.

The protection may be due to complete neutralization of the virus inoculum or to a limited virus multiplication giving rise to new antibodies. No viremia was observed at 48 h in animals receiving serum dilutions of 1:5 or 1:20. In an increasing fraction of animals receiving higher dilutions viremia was observed. In two of four animals with a serum titer of 2.5 PND_{50} before challenge (Table 1), the antibodies were decreased 8 days after challenge (less than 20% plaque reduction by undiluted serum). In all other groups titers of 3 or more PND_{50} had developed during infection, confirming that virus multiplication had occurred to the level of a significant antigen stimulus.

Protection of mice with DTH without antibodies against i.p. challenge. After intracutaneous immunization with 300 to 10,000 hemagglutinating units (HAU) of purified SFV antigen, a DTH without antibodies detectable by plaque reduction was observed (8). The absence of antibodies was confirmed by mouse protection tests with 0.25 ml of undiluted serum as described above. The DTH (footpad swelling) was biphasic, with peaks at 6 and 12 days. To test whether such a DTH would correlate with protection, groups of mice were immunized intracutaneously with 300 HAU and i.p. challenged on days 7 and 12 with 10 LD_{50} of virulent SFV. A marginal protection was observed, with about 20 and 40% survivors, respectively.

As it was known that virus spreads quickly from the peritoneal cavity (see below; Table 2), the chance of an effective collision between virus and T cells might be small. To attract T cells to the peritoneal cavity and stimulate macrophages, 0.5 or 1 ml of thioglycolate was i.p. injected at 2 days before challenge. It was shown with over 100 nonimmunized mice that the procedure itself does not induce nonspecific protection. Figure 1 shows that thioglycolate enhanced protection in mice 7 days after immunization with 300 HAU of SFV antigen. From Fig. 2 and 3 it can be seen that immunization with 3,000 and 10,000 HAU was more effective. Neutralizing antibodies (>1 PND_{50}) could not be demonstrated at the time of challenge, nor did 0.25 ml of undiluted serum of these mice taken at day 6 or 9 protect acceptor mice (<0.01 PND_{50}). To exclude the possibility of antibody as the cause of protection, still another group of mice was immunized with 10,000 HAU after pretreatment with 2 mg of cyclophosphamide to suppress antibody formation and challenged 10 days later. All animals survived (Fig. 3).

Protection of immunized mice with DTH without antibodies against s.c. challenge. Virulent virus spread more slowly after s.c. than

TABLE 2. *PFU in serum at 45 and 180 min after s.c. or i.p. infection of normal mice with 175 PFU of virulent SFV*

Mouse no.	PFU/ml of serum after:			
	i.p. infection		s.c. infection	
	45 min	180 min	45 min	180 min
1	30	124	ND[a]	ND
2	43	90	ND	ND
3	30	120	ND	5

[a] ND, Not detected (<10 PFU/ml).

TABLE 1. *Protection of mice by 0.25 ml of dilutions of an immune serum (immunoglobulin M) with a titer of 1:100 after s.c. challenge with 100 PFU (7 LD_{50}) of virulent SFV*

Serum dilution	PND_{50} in serum after 2 h	No. of mice		PND_{50} in serum of four mice at day 8
		Surviving at day 20	With viremia at 48 h	
1:5	2.5	12/12	0/3	UD, UD, 10, 10
1:20	UD[a]	11/12	0/3	3, 30, >100, >100
1:80	UD	11/12	1/3	3, >100, >100, >100
1:320	UD	5/6	2/3	ND
1:1280	UD	1/6		ND
Phosphate-buffered saline	ND[b]	2/12	3/3	>100, >100, >100, >100
2-Mercaptoethanol (1:5)	ND	3/8		ND

[a] UD, Undetectable by plaque reduction.
[b] ND, Not done.

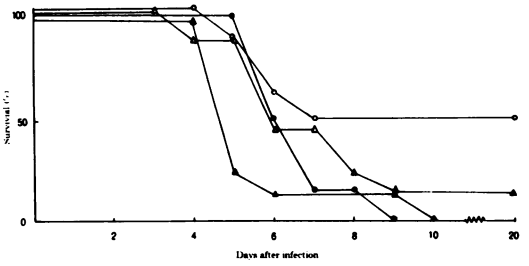

FIG. 1. *Enhancement of survival in immunized mice by thioglycolate. A total of 16 mice were immunized intracutaneously with 300 HAU of inactivated SFV. Controls received phosphate-buffered saline. At day 5 eight immunized and eight control mice were injected i.p. with 1.0 ml of thioglycolate broth. At day 7 all mice were infected i.p. with 10 LD_{50} of SFV. Symbols: ○, 300 HAU with thioglycolate i.p.; ●, control; △, 300 HAU with no thioglycolate; ▲, control.*

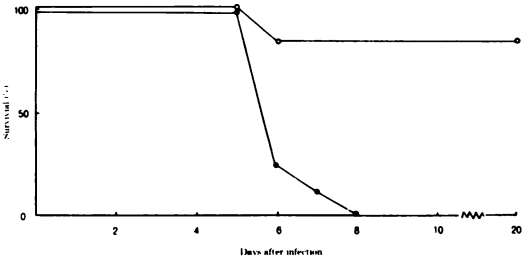

FIG. 2. *Protection of mice against 10 LD_{50} of SFV after intracutaneous immunization and i.p. injection of thioglycolate broth. Mice were immunized intracutaneously with 3,000 HAU of inactivated SFV. Controls received phosphate-buffered saline. At day 5 all animals were injected i.p. with 1.0 ml of thioglycolate broth. At day 7 10 LD_{50} of SFV was given i.p. Symbols: ○, immunized animals (8 mice); ●, control animals (8 mice).*

after i.p. injection (Table 2). A local reaction at the s.c. injection site may enhance early contact between specific T lymphocytes and viral antigens. Therefore attempts were made to demonstrate protection against s.c. challenge (Fig. 4). Seven of eight immunized mice survived, whereas all control animals died.

Transfer of primary immunity with peripheral lymph node cells. Mice were immunized with 10,000 HAU of SFV antigen. At day 6 suspensions of spleen cells and peripheral lymph node cells were prepared and intravenously transferred to normal mice. At 3 h after transfer, the recipient mice were s.c. challenged with 100 PFU (7 LD_{50}) of virulent SFV. Figure 5 shows that the lymph node cells protected four of six mice, whereas spleen cells gave hardly any protection. Lymph node cells taken at 11 days after immunization protected six of eight mice. Lymph node cells from normal animals gave no protection, as only one of eight animals survived.

Course of SFV infection in immune and control mice. Animals were immunized with 3,000 HAU and i.p. challenged with 20 PFU (10 LD_{50}) of SFV. The virus titers in blood, spleen, and brain were determined at various times (Table 3). Virus was present in blood and spleen after 1 day and began to disappear on day 2. At this stage there was apparently no difference between immunized mice and controls. However, in immunized mice the brain was reached later, and on day 5 the virus titers were much lower than those in controls.

Virus titers in the peritoneal cavity and in serum were also determined in the first hours after infection with 32 PFU of virulent SFV (Table 4). In control mice at 3 h, that is, before multiplication could have occurred, the virus inoculum was in large part recovered from the

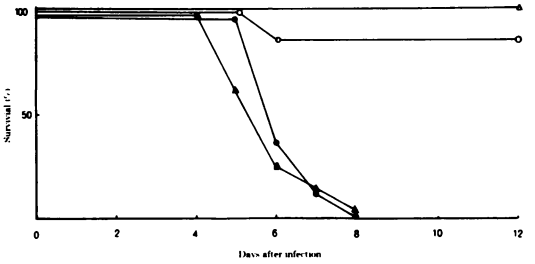

FIG. 3. *Protection against 10 LD_{50} of SFV after intracutaneous immunization with and without cyclophosphamide. A total of 16 mice were immunized intracutaneously with 10,000 HAU of inactivated SFV. Controls received phosphate-buffered saline. Eight immunized and eight control mice were pretreated with 2 mg of cyclophosphamide. Cyclophosphamide-treated animals were i.p. infected with 10 LD_{50} of SFV at day 10. The other mice were infected at day 7 with the same dose. All animals received 0.5 ml of thioglycolate at 2 days before infection. Symbols: ○, 10,000 HAU given intracutaneously (8 mice); ●, control (8 mice); △, 10,000 HAU given intracutaneously plus 2 mg of cyclophosphamide (8 mice); ▲, control (8 mice).*

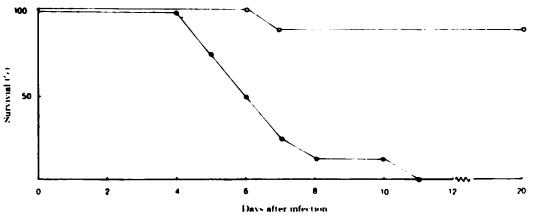

FIG. 4. *Protection of mice against s.c. infection with 7 LD_{50} of SFV. Eight mice were immunized intracutaneously with 10,000 HAU of inactivated SFV; controls received phosphate-buffered saline. All mice were treated with 2 mg of cyclophosphamide. At day 11 the mice were infected s.c. with 100 PFU (7 LD_{50}) of SFV. Symbols: ○, immunized animals (8 mice); ●, control animals (8 mice).*

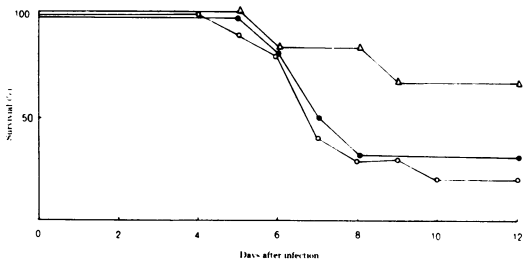

FIG. 5. *Transfer of protection against virulent infection with peripheral lymph node cells. Donor mice were immunized with 10,000 HAU of inactivated SFV. At day 6 the animals were killed. Pooled cell suspensions of peripheral lymph nodes and spleens were intravenously transferred to naive mice. After 3 h recipient mice were infected s.c. with 100 PFU (7 LD_{50}) of SFV. Symbols: ○, 10^8 immune spleen cells (10 mice); ●, 10^8 control spleen cells (10 mice); △, 5 × 10^7 immune peripheral lymph node cells (6 mice).*

peritoneal cavity. This shows that the virus did not easily find a sensitive host cell in the peritoneal cavity. In immune animals part of the virus was inactivated early. At 24 h the amount of virus in the peritoneal cavity was much lower than in controls and viremia was reduced.

Interaction of SFV with peritoneal cells in vivo. The persistence of the virus in the peritoneal cavity for 3 h suggested that the virus did not mulitply in, or was inactivated by, peritoneal macrophages. Groen et al. (5) showed that SFV multiplies in stimulated macrophages in vitro, but does so less in stimulated cells from immune animals. Pusztai et al. (9) showed that, in vivo, SFV outside the brain multiplies mainly in vascular endothelium and in muscle cells. We studied in vivo multiplication in peritoneal cells, by determining the virus content of peritoneal cells harvested 24 h after infection. The cells

TABLE 3. *Virus titers in whole blood, brain, and spleen on consecutive days after i.p. infection with 10 LD_{50} of SFV in immunized and control animals*[a]

Day	PFU/ml in blood		PFU in brain		PFU in spleen	
	Immunized animals	Control animals	Immunized animals	Control animals	Immunized animals	Control animals
1	<16[b]	30	<82	<82	100	250
	16	82	<82	<82	1,600	1,100
	33	82	<82	<82	2,100	1,000
2	<16	16	<82	<82	<82	<82
	16	82	<82	<82	41	<82
	<16	<16	<82	<82	<82	330
3	<16	<16	<82	4.8×10^4	<82	82
	<16	<16	<82	1.1×10^3	<82	<82
	<16	<16	<82	7.4×10^3	<82	<82
5	<16	<16	1.1×10^4	3.2×10^6	16	<82
	<16	<16	2.1×10^3	6.0×10^7	<82	<82
	<16	<16	8.2×10^1	7.5×10^7		

[a] All immunized and control mice received thioglycolate at 48 h before challenge.
[b] A value of <16 means that a titer of <16 PFU/ml of citrated blood was detected.

TABLE 4. *Virus titers in peritoneal cavity and serum in seven immunized and control mice at 3 and 24 h after i.p. infection*[a]

Mouse no.	Virus titer[b]							
	Peritoneal cavity				Serum			
	3 h		24 h		3 h		24 h	
	Control animals	Immunized animals	Control animals	Immunized animals	Control animals	Immunized animals	Control animals	Immunized animals
1	25	<5	<10	10	<2	2.5	20	<5
2	35	<5	3,950	<10	<2	<2	9,000	<5
3	15	15	650	10	3	<2	65	<5
4	15	<5	1,580	120	43	<2	25	<5
5	15	<5	40,200	1,960	3	<2	4,500	30
6	ND[c]	<5	490	30	<2.5	<2	340	<5
7	ND	5	ND	<10	<2	<2	ND	<5

[a] All immunized and control mice received thioglycolate at 48 h before challenge.
[b] Titer for peritoneal cavity is in PFU/2 ml; titer for serum is in PFU/ml.
[c] ND, Not done.

TABLE 5. *Antibody titers (PND_{50}) in immunized and control mice at various times after challenge with 25 to 32 PFU of SFV*[a]

Day after i.p. challenge	PND_{50}[b]				
	Control animals		Immunized animals		*P* values[c]
	Values for individual animals	Mean ± standard deviation	Values for individual animals	Mean ± standard deviation	
4	0.1, 2, 2, 1.4, 2, 5, 5, 6, 6, 10, 0.1, 0.1, 2, 0.1, 1, 1.5, 2.5, 4, 4, 8	3.14 ± 0.62	0.1, 0.1, 2, 0.1, 0.1, 1, 3, 3, 4, 0.1, 0.1, 0.1, 0.3, 0.3, 1, 1.5, 2.5, 4, 5	1.48 ± 0.37	0.05
6	1,000, 1,000, 1,000, 1,000, 1,000, 1,000, 1,000, 450, 7	828 ± 119	25, 30, 50, 70, 70, 120, 280, 1,000, 1,000, 1,000, 1,000, 1,000, 1,000, 1,000, 1,000	576 ± 121	
8	Survivors rare (always >1,000)		0.1, 0.1, 2, 2, 3, 10, 25, 30, 100, 100		

[a] All immunized and control mice received thioglycolate at 48 h before challenge.

[b] The data were compiled from various experiments. When no antibody was detected, the titer is given as 0.1, which is the lowest level of detection.

[c] The *P* value was calculated from a sign test rather than from means.

were washed, freeze-thawed, and tested for virus. In other cases washed cells were used for a center of infection assay on monolayers. In both tests less than 1 plaque per 1,000 cells was found, corresponding to the expected quantities of adhering virus not eliminated by washing. No difference was found for immune and control animals.

Normal and thioglycolate-stimulated peritoneal cells from control and immunized mice incubated in vitro with SFV and seeded on monolayers did not give indications of virus multiplication.

Antibody formation after challenge. After challenge of normal mice with virulent SFV, antibodies begin to appear after 4 days, but they were unable to save the animals (2, 12). Our immunized mice with DTH might be protected by cellular immunity (T-effector cells) or by an enhanced antibody production during infection under the influence of T-helper cells. The presence of such T-helper cells in immunized mice was demonstrated by the rapid antibody production by acceptor mice which had received spleen cells and 10,000 HAU of inactivated antigen (data not shown). Lymph node cells had much less effect. Whether enhanced antibody production would also occur after challenge with a low virus dose is another matter.

Antibodies were determined on days 4, 6, and 8 after i.p. challenge for immunized mice and on days 4 and 6 for surviving control mice (Table 5). On day 4 less antibody was present in immunized mice than in control mice. At day 6 the difference was small. After 8 days all control mice were dead, but in 2 of 10 immunized mice no antibody was detected.

DISCUSSION

Mice immunized intracutaneously with inactivated SFV virus develop DTH (as shown by footpad swelling) without detectable antibodies.

Such animals are protected against s.c. challenge with virulent SFV (Fig. 4), and this protection can be transferred to normal animals with lymph node cells but not with spleen cells (Fig. 5). This is parallel to the finding that lymph node cells transferred DTH to normal mice, but spleen cells did not (8). Possibly the immune response to the intracutaneous virus antigen is mainly localized to the draining lymph nodes. These data point to the mechanisms of cellular immunity (T-effector or T-helper cells) as the cause of protection. After s.c. challenge the spread of virus is slow (Table 2), presumably due to some local virus adsorption and proliferation. This local reaction seems to give the circulating T cells an opportunity to react with the virus. After i.p. challenge the protection was marginal unless thioglycolate was injected at 2 days before challenge (Fig. 1). Thioglycolate stimulates macrophages (10, 14) and causes a local inflammation which attracts recently committed T lymphocytes (7) and also macrophages. In in vitro experiments it was shown that human lymphocytes in combination with macrophages produce the highest amount of interferon (4). From the foregoing information, the enhancing activity of thioglycolate is interpreted as an argument for the cellular cause of protection. Without thioglycolate the number of T cells in the peritoneal cavity is too low to prevent the rapid spread of virus to the blood and to the central nervous system. With thioglycolate the virus recovery from the peritoneal cavity and in

serum is materially reduced at 3 and 24 h after challenge (Table 4). Some virus still reaches the brain, but here also multiplication is much less than in controls (Table 3).

It is of interest that in normal mice the virus inoculum is in large part recovered from the peritoneal cavity (Table 4), suggesting that the virus does not easily find a host cell and is not taken up by peritoneal macrophages even after thioglycolate stimulation. It was also shown that it does not multiply in peritoneal cells in vivo.

Very low quantities of antibody can protect mice (Table 1) if present at the moment of challenge. Such antibodies could not be demonstrated after intracutaneous immunization.

The protection of immunized animals does not seem to be due to enhanced general antibody production by T-helper cells, as the antibody titer in serum at 4 days is less in immunized than in control mice (Table 5). Control mice with high antibody titers on day 6 die, whereas immunized animals with lower titers survive. An occasional immunized mouse survives without antibodies to day 8 (Table 5), showing that the virus inoculum can be eliminated before it reaches the level of antigenic stimulation.

The early elimination of a large part of the challenge virus in immunized mice (Table 4) seems to be due to the mechanisms of cellular immunity. Abortive infection under the influence of interferon II or enhanced phagocytosis due to macrophage-activating factor are possible explanations. Antibodies might still be involved in the final recovery of many immunized animals if, under the influence of T-helper cells, antibody was formed locally at the site(s) of virus multiplication. This might explain the lower serum titers in immunized animals. As interferon II is a potent inhibitor of antibody synthesis (13) in vivo and in vitro, local antibody synthesis is not very probable. Virus multiplication in the brain of normal mice continues in the presence of high serum titers. The elimination of virus from the brain of immunized animals might again be due to T-effector cells, unless the hypothesis of local antibody production is extended to the brain.

Intracutaneous immunization with purified SFV antigen can produce cellular immunity. Whether such cellular immunity plays an active role in the natural infection remains open.

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