INFECTION AND IMMUNITY, Feb. 1979, p. 219-223
0019-9567/79/02-0219/05$02.00/0

Vol. 23, No. 2

Delayed-Type Hypersensitivity Against Semliki Forest Virus in Mice

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Received for publication 30 October 1978

After intracutaneous immunization with purified inactivated Semliki Forest virus, a delayed-type hypersensitivity without detectable antibodies in serum was obtained in BALB/c mice. Low doses of antigen given intraperitoneally induced antibodies. Intracutaneous immunization with much higher doses induced no specific antibodies, but a footpad swelling was observed after challenge with homologous antigen. Pretreatment with cyclophosphamide before immunization enhanced footpad swelling. Microscopic examination of footpads from sensitized mice at 24 h after challenge showed a mononuclear infiltrate. The delayed-type reaction could be transferred to syngenic mice with lymph node cells, but not with spleen cells or serum. The biphasic character of the delayed-type hypersensitivity is discussed.

Cellular immunity seems to be important in infections with viruses maturing at the membrane of the host cell (2). Semliki Forest virus (SFV) belongs to this group, and glycoproteins of the envelope appear at the cell membrane (1, 9, 18). Neutralizing antibodies appear early and are directed against the carbohydrate moiety of the glycoproteins (11). SFV, with its repeating surface units, might well be a partially thymus-independent antigen, as shown in nude mice for the related Sindbis virus (4).

In mice, specific T cells cytotoxic for SFV-infected cells in vitro appear at 3 to 6 days after infection (16). Spleen cells and serum taken 6 or 7 days after virulent (16) or avirulent (14) infection can protect acceptor mice. In vivo no cellular immunity or delayed-type hypersensitivity (DTH) has yet been demonstrated, however, and their importance in recovery and immunity is not known.

The induction of DTH without antibodies depends on antigen structure, on the dose, and on the route of presentation of the antigen (12, 17). In the experiments described in this paper we tried to induce DTH against purified inactivated SFV. In this way presentation on host cell membranes is avoided, and any effects can be ascribed to the antigen proper.

The inactivated virus antigen was given in different doses and by various routes. In some experiments cyclophosphamide was used. Cyclophosphamide augments DTH against other antigens (13).

MATERIALS AND METHODS

Virus strains. The virulent strain SF/LS10CI/A was received from C. J. Bradish (3). A large plaque variant of this strain was used for plaque reduction tests. The avirulent strain was MRS MP 192/7 (10) which had been passed seven times over HEL and Vero cells.

Cells and media. L cells cultured in the medium of Yamane et al. (20) with 0.01 M HEPES (*N*-2-hydroxyethyl piperazine-*N'*-ethanesulfonic acid) buffer and 10% calf serum were used throughout. Overlay medium contained the medium of Yamane et al. (20) with 0.7% agarose (Indubiose; l'Industrie Biologique Française) and antibiotics. Phosphate-buffered saline (PBS) (6) was used as diluent.

Hemagglutination. Hemagglutination was done with goose erythrocytes as described by Clarke and Casals (5). Hemagglutinating units (HAU) were calculated from the hemagglutinating titer, and 1 HAU was defined as the quantity of virus agglutinating 0.3 ml of 0.5% (vol/vol) goose erythrocytes. One HAU represents about 10^5 plaque-forming units.

Plaque titration. Plaque titration and plaque reduction tests were done in plastic palettes (24-well tissue culture cluster dish [catalog no. 3524; Costar, Cambridge, Mass.]). Dilutions were made in a 96-well palette (catalog no. 3596; Costar). Infectious center assays were done in 6-well palettes (catalog no. 3506; Costar). Virus-containing fluids were titrated in a six-fold series by adding 0.05-ml quantities to six monolayers. After 40 min of adsorption at room temperature, 0.3 ml of overlay medium was added. After 24 h plaques were developed with 0.2 ml of 0.01% neutral red in 0.7% agarose. For plaque reduction, 0.05-ml serum dilutions were mixed with 0.05 ml of virus and incubated at 37°C for 1 h. A 1-ml portion of Eagle medium was added, and 6 × 0.05 ml of each dilution was transferred to monolayers. Controls contained about 50 plaques per 16-mm cup.

Animal experiment. 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.

Cell suspensions. Clean spleens or peripheral lymph nodes were collected in Eagle medium at 4°C, passed through nylon gauze, and washed once. In a sample of the suspension the fraction of viable cells was counted in the presence of trypan blue. The suspension was brought to the required concentration and injected within 2 h after organ collection.

Assay for DTH. DTH reactions were measured as the increase in footpad thickness (footpad swelling test) at 24 h after injecting an eliciting dose of diluted virus antigen in a volume of 0.05 ml of PBS. The thickness of the footpad was measured with a semi-electronic footpad meter (19) with a sensitivity of 0.01 mm. Reactions were recorded compared with the day when the test dose of antigen was injected, rather than the day upon which the reaction was measured.

Microscopic analysis of the cell types in sections of footpads and draining lymph nodes in the groins of immunized and control animals was kindly performed by A. A. van den Broek, Department of Histology, University of Groningen, The Netherlands, by the methods of Keuning et al. (13).

Preparation of virus antigen. The avirulent strain MRS MP 192/7 was inoculated at a multiplicity of about 1 on monolayers of L cells in Roux flasks. After 20 h at 37°C, the fluid was collected, and fresh medium was added. This was repeated after 6 and 12 h. The yield was 4,000 ml with 1,000 HAU/ml. The fluid was centrifuged at low speed, and the virus in the supernatant was concentrated at 80,000 × *g* at 6°C in a Measuring & Scientific Equipment model 65 centrifuge. Sediments were kept at −70°C until all portions could be collected in 15 ml of PBS, pH 8.0, and centrifuged at low speed. The supernatant with dissolved CsCl was centrifuged for 20 h at 45,000 × *g* in an SW35 rotor. The highest virus concentration was found at a density of 1.243. The virus-containing fractions were passed over a column (30 by 1 cm) of Sepharose B and collected in 4-ml fractions. Two slightly turbid fractions with 10^5 HAU/ml were diluted with PBS (pH 8) to 15 ml. This fluid was irradiated (1,600 erg/per cm^2) in a moving petri dish (15-cm diameter) for 15 or 30 min without loss in HAU. No residual infectivity could be detected in 0.5 ml of undiluted virus suspension. Various batches with titers of 10^5 to 10^6 HAU were prepared and kept in 0.3-ml portions over fluid nitrogen. Standardized thawing in ice water, dilution in ice-cold PBS, and keeping at 0°C until injection were necessary precautions for reproducibility.

Cyclophosphamide (Koch Light, Colnbrook, England) was dissolved in PBS, pH 7.2, immediately before use. A volume of 0.5 ml was given intraperitoneally 8 h before immunization.

RESULTS

DTH against SFV antigen. (i) Intraperitoneal immunization. After intraperitoneal immunization even very small doses induced antibody (Fig. 1). A positive footpad swelling was only obtained after immunization with doses above 10 HAU. The footpad swelling was maximal at 3 h but still present at 24 h. This may have been due partly to an Arthus reaction, but a DTH reaction cannot be excluded. Figure 1 also shows that pretreatment with cyclophosphamide suppressed antibody formation with lower immunizing doses. In those animals immunized with 300 HAU a footpad swelling (maximal at 24 h) was obtained. Intraperitoneal immunization seemed to be unsuitable for obtaining DTH without antibodies unless cyclophosphamide was used.

(ii) Intracutaneous immunization. Intracutaneous immunization with 0.3, 3, 30, 300, and 3,000 HAU with or without Freund complete adjuvant (FCA) did not induce antibodies in 90 mice. Only one of five animals immunized with 18,000 HAU produced antibodies. A positive footpad swelling was present at 7 days after intracutaneous immunization without FCA with doses above 30 HAU (Fig. 2) but not after immunization with FCA. This footpad swelling developed with time as shown in Fig. 3. There was a toxic effect of the antigen in controls and immunized mice at 3 and 6 h and a clear-cut difference at 24 h. At 48 h after the challenge, no antibodies could be demonstrated in animals immunized with 300 or 3,000 HAU, showing that the footpad challenge did not induce antibodies which could have participated in the reaction. The footpad swelling test is specific; other antigens gave no reaction.

About 5 days after immunization the footpad swelling was maximal. It decreased on days 6 to 9 but rose again on days 10 to 14. This biphasic character was observed after immunization with (Fig. 4) or without cyclophosphamide pretreatment. The late footpad swelling after 10 days developed somewhat slower than in the first week.

Pretreatment with cyclophosphamide resulted in enhanced footpad swelling as shown in Fig. 4 (compare Fig. 2). The maximum was somewhat later (day 7 instead of day 5), but the

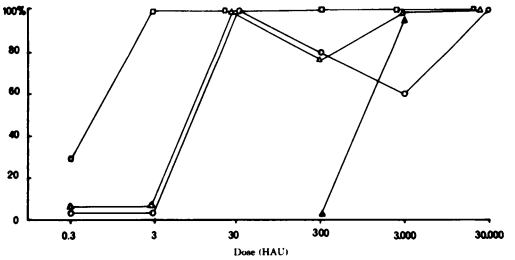

FIG. 1. *Neutralizing antibodies after intraperitoneal immunization with different doses of purified inactivated SFV. Percentage of mice with neutralizing antibodies in undiluted serum after 5 days* (○), *after 7 days* (△), *after 10 days* (□), *and after 7 days in mice receiving, at 8 h before immunization, 2 mg (100 mg/kg) of cyclophosphamide* (▲).

biphasic character is also obvious.

The histological results are given in Table 1. They are in agreement with an interpretation of the footpad swelling as a DTH reaction. The histology of the lymph nodes in the groin of immunized animals before and after challenge in the footpad was also studied. At 7 days after immunization the paracortical fields were active and rich in lymphoid cells. At 24 h after challenge the number of lymphoid cells was much smaller, which could have been due to binding of reactive T lymphocytes by the antigen in the footpad.

Transfer of DTH with lymph node cells. Donor mice were immunized with 300 HAU of SFV antigen. After 7 days the footpad was challenged with 150 HAU. The mean footpad swelling was 6.2 by 0.1 mm. The serum did not

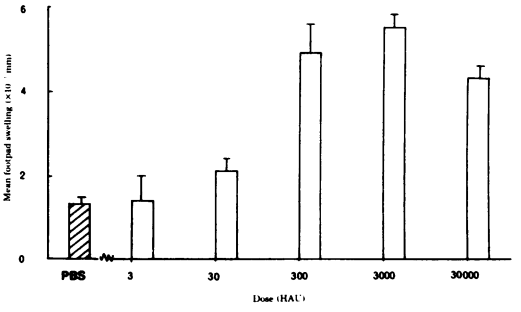

FIG. 2. *Footpad swelling after intracutaneous immunization with different doses of inactivated SFV. Groups of six to eight mice were immunized with 3, 30, 300, 3,000, and 30,000 HAU of inactivated SFV (white columns). Control mice received PBS (hatched column). After 7 days the mice were challenged with 450 HAU. And after an additional 24 h the footpad swelling was measured. Vertical bars represent one standard error of the mean.*

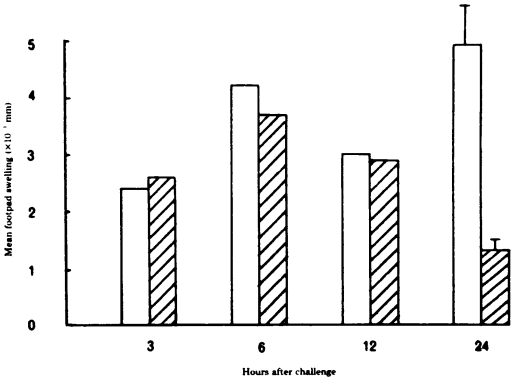

FIG. 3. *Development of footpad swelling after challenge. Six mice were immunized intracutaneously with 300 HAU of inactivated SFV (white columns). Control mice received PBS (hatched columns). After 7 days the mice were challenged with 150 HAU of SFV. Footpad swelling was measured at 3, 6, 12, and 24 h after challenge. Vertical bars represent one standard error of the mean.*

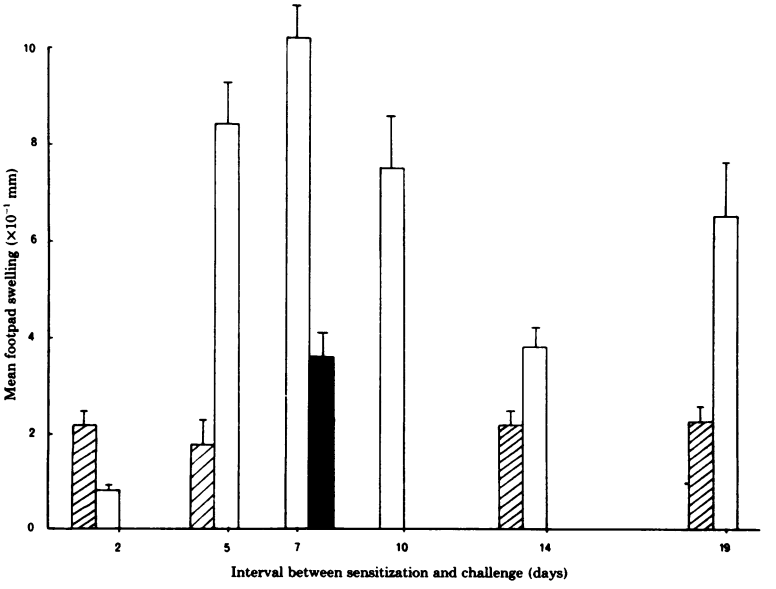

FIG. 4. *Influence of cyclophosphamide on DTH. Mice were immunized with 10,000 HAU of inactivated SFV after pretreatment with 2 mg of cyclophosphamide or without pretreatment. After various times groups of 5 mice were challenged with 450 HAU. Footpad swelling was measured at 24 h (white columns) and in some groups also after 3 h (hatched columns). Footpad swelling at 24 h after challenge in mice receiving no cyclophosphamide is shown by the black column. Vertical bars represent one standard error of the mean.*

TABLE 1. *Histology of footpads*

Intracutaneous immunization	Footpad swelling ($\times 10^{-1}$ mm)		Presence of a mononuclear infiltrate at 24 h after challenge with 50 HAU[a]
	3 h	24 h	
600 HAU	2.0, 1.6[b]	3.3, 3.8	+ +
Control	1.1, 2.4	0.5, 1.7	− −
600 HAU + 2 mg of cyclophosphamide	0.5, 1.9	4.1, 6.8	++ ++
Control	2.3, 2.0	1.5, 1.3	± −

[a] Symbols: ++, dense infiltration with mononuclear cells; +, many mononuclear cells visible; ±, some mononuclear cells visible; −, absence of mononuclear cells.

[b] Values for two individual animals are given in each case.

contain antibodies and was not protective when 0.25 ml was injected intravenously into control mice. Spleen cells and lymph node cells were transferred into acceptor mice which were challenged 3 h after transfer with 150 HAU in the left footpad (Fig. 5). It is obvious that peripheral lymph node cells but not spleen cells confer a DTH to acceptor mice. This experiment was repeated after immunization with cyclophosphamide pretreatment with the same result.

DISCUSSION

The data presented suggest that after intracutaneous immunization with purified inactivated SFV a state of DTH without detectable circulating antibodies can be obtained (Fig. 2). This DTH can be transferred with lymph node cells.

A purified antigen was desirable to exclude concomitant immune reactions against L-cell antigens as much as possible.

With intracutaneous immunization the antigen was injected in the neighborhood of the draining lymph nodes in groins and axillae. The virus antigen is probably trapped in these lymph nodes with their high T-cell content. That the T-cell response is probably localized in these lymph nodes is supported by the results of the transfer experiments in which lymph node cells were active, in contrast to spleen cells. The addition of FCA to the antigen at immunization produced neither neutralizing antibodies nor DTH. Presumably FCA destroys the envelope of the labile virus.

Intraperitoneal immunization induces an appreciable antibody production. A considerable part of the antigen presumably enters the circulation (7) and may be largely trapped in the spleen where B cells predominate. Direct contact with B cells of this partially thymus-independent antigen may favor the formation of neutralizing antibody, which indeed proved to consist mainly of immunoglobulin M. Pretreatment with cyclophosphamide before intraperitoneal immunization abolished this antibody production, but only with lower immunizing doses. In the experiments with intracutaneous immunization cyclophosphamide was used to exclude antibody formation completely. Furthermore, it enhanced the DTH reaction.

The second increase in footpad swelling in week 3 after immunization is of interest. Gardner and Blanden (8) described an analogous biphasic character for the presence of cytotoxic T cells in spleens after ectromelia infection. They suggest a differentiation of T-effector cells to T-memory cells. Such an explanation might fit our results. Pretreatment with the low dose of cyclophosphamide decreases B cells and certain T-suppressor cells. This might explain the increase in

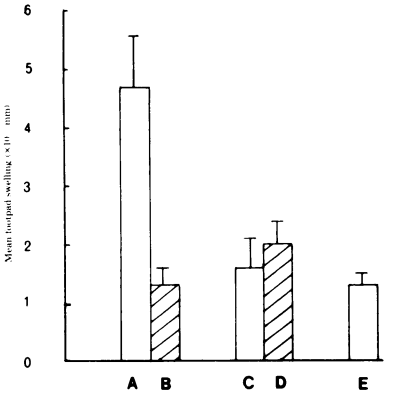

FIG. 5. *Passive transfer of DTH. Donor mice were immunized intracutaneously with 300 HAU of inactivated SFV. After 7 days they were challenged with 150 HAU of SFV. Mean footpad swelling after 24 h was 6.2 by 0.1 mm. At day 8 cell suspensions of peripheral lymph nodes and spleens were prepared from killed animals after bleeding. Naive recipient mice of cells or decomplemented donor serum were challenged with 150 HAU at 3 h after transfer. The footpad swelling was measured after an additional 18 h. (Bar A) Recipients of* 5×10^7 *immune peripheral lymph node cells (4 mice); (bar B) recipients of* 5×10^7 *control peripheral lymph node cells (6 mice); (bar C) recipients of* 5×10^7 *immune spleen cells (8 mice); (bar D) recipients of* 5×10^7 *control spleen cells (6 mice); (bar E) recipients of 0.25 ml of undiluted serum (6 mice). Vertical bars represent one standard error of the mean.*

DTH (15). The DTH in this case is also biphasic (Fig. 4). The somewhat slower development of the DTH at 19 days might be due to a somewhat slower reaction of memory cells compared with effector cells.

ACKNOWLEDGMENTS

We are very grateful to A. A. van den Broek for the histological work and to K. C. Winkler and J. M. N. Willers for methodological support and helpful discussions.

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