Delayed-Type Hypersensitivity Responses in Mice Infected with St. Louis Encephalitis Virus: Kinetics of the Response and Effects of Immunoregulatory Agents

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Labeled monocyte infiltration techniques have been used to study delayed-type hypersensitivity responses in mice immunized with St. Louis encephalitis virus. A delayed 24- to 48-h inflammatory response occurred 6 to 7 days after immunization. This response can be potentiated by cyclophosphamide treatment, by BCG administration, or by splenectomy. Treatments known to selectivity inhibit T-cell function suppressed the response.

The development of central nervous system lesions after St. Louis encephalitis (SLE) virus infection has long been associated with cellular infiltrates and inflammatory responses (18). Recent investigations have demonstrated that thymus-dependent cell (T-cell)-mediated responses play both protective and immunopathological roles in mice infected with a number of flaviviruses related to SLE (1, 9, 12, 15). In perhaps the best-studied model of virus disease in mice, lymphocytic choriomeningitis, the development of T-cell-mediated central nervous system disease is clearly associated with the development of peripheral footpad inflammatory responses (5). Peripheral delayed inflammatory responses in mice immunized against other viruses, however, are typically slight and difficult to evaluate quantitatively (4). In studies of cell-mediated response to viruses other than lymphocytic choriomeningitis and ectromelia (16), migration inhibition tests (12) or cytotoxic cell assays have usually been used (7). Direct T-cell-mediated cytotoxicity is relatively inefficient in controlling flavivirus replication in vitro (2, 7). It seems likely, therefore, that in vivo studies of the delayed inflammatory response might be useful in evaluating the immune response to flavivirus infection. We have investigated the use of labeled monocyte infiltration techniques (6, 10) for measurement of the delayed-type hypersensitivity (DTH) response in mice infected with SLE virus.

MATERIALS AND METHODS

Virus stock and control inoculum. SLE virus strain BeH 203235 was obtained from Robert Shope, Yale Arbovirus Research Unit, Yale University, New Haven, Conn. Stock virus was prepared as a 10% (wt/vol) suspension of infected suckling mouse brain in phosphate-buffered saline containing 5% bovine serum albumin (BAPS). Virus infectivity titers were determined by plaque formation on CER cells (13). The working stock virus preparation had a titer of 3.2 × 10⁶ plaque-forming units (PFU) per ml. A suspension of normal suckling mouse brain (NSMB) was prepared by the same methods as those used for preparation of virus-infected suckling mouse brain.

Mice. All mice used in this work were reared at our facility and were produced from stocks of National Institutes of Health general purpose albino mice. Mice were free of intercurrent infections with mouse pathogens with the exception of periodic problems caused by infection with Tyzzer’s bacillus (Bacillus piliformis). All mice were weaned at 3 weeks of age, and the females were pooled, caged, and held for 4 to 6 weeks before being used in experiments.

Mouse infection. Stock virus was diluted in M199 containing 5% fetal calf serum. Mice were infected by intraperitoneal or intravenous inoculation with SLE virus in 0.2 ml of diluent. Control mice received equivalent inocula of NSMB.

DTH. DTH was assessed by measuring the inflammatory response after footpad challenge administered at intervals after infection. The 24- to 48-h dorsalventral increase in footpad thickness as measured by calibrated calipers was minimal (not exceeding 0.3 mm). Statistically valid measurements were obtained with experimental groups of 8 to 16 mice. These DTH measurements were supplemented by measurement of [3H]thymidine-labeled cellular infiltration adapted from techniques developed by Lefford (6) and Sabolovitch et al. (10). At 24 h before footpad challenge, infected mice and control mice were inoculated intraperitoneally with 20 μCi of [3H]thymidine. Each mouse was then challenged by inoculation of 0.05 ml of undiluted SLE-infected mouse brain suspension (1.6 × 10⁵ PFU) into the left footpad. Each mouse was also inoculated in the right footpad with an equivalent...
dosage of NSMB. At selected intervals after footpad challenge (usually 24 h), the footpad response of each group of mice was assessed by measuring the differential swelling of the footpads. Immediately after physical measurement, the feet were removed at the first joint and solubilized in 1 ml of NCS tissue solubilizer (Amersham/Searle Corp.), and the suspension was added to 10 ml of scintillation cocktail. After scintillation counting, data uncorrected for quenching were used for calculating thymidine incorporation. Differential counts (counts per minute [left foot] – counts per minute [right foot]) of 2,000 to 20,000 cpm of $^3$H were generally measurable in infected mice responding to footpad challenge. Such differential counts show a large statistical variance because of individual differences in thymidine incorporation. The ratios of $^3$H counted in the challenged and the control feet of individual mice were not subject to such variation. Footpad cellular infiltration ratios (CIR) were therefore computed for each individual mouse, the CIR being equal to the ratio of counts per minute measured in the left and right footpads. Tests for statistical significance were based on the Student's $t$ test adapted for use with paired observations, $\alpha = 0.05$ (14).

Histological examinations of the inflammatory response in the feet of additional groups of infected and control mice were made 24 h after footpad challenge. Both feet were fixed and demineralized in Bouin solution, embedded in paraffin, sectioned at 6 $\mu$m, and stained with hematoxylin and eosin.

Humoral antibody determinations. Solid-phase radioimmunoassay determinations of SLE virus-specific immunoglobulin M (IgM) and IgG were performed by techniques adapted from those of Trent et al. (17). The antigen used was a sucrose-acetone-extracted preparation of SLE-Bell 203235-infected suckling mouse brain which was obtained from Nick Karabatos, World Health Organization Arbovirus Reference Laboratory, Center for Disease Control, Fort Collins, Colo. Each well of a 96-well plastic plate was coated with 32 hemagglutinating units of the SLE antigen. Triplicate wells were then incubated with a 1:100 dilution of each normal and test mouse serum. After the plates were washed, the amount of mouse IgM and IgG bound to SLE antigen was assessed by using $^{125}$I-labeled anti-mouse IgM and IgG (goat anti-mouse IgM and goat anti-mouse IgG, Bionetics Laboratory Products, Kensington, Md.). Results are expressed as the difference in $^{125}$I uptake by wells treated with test sera and control sera.

RESULTS

Preliminary investigations were made to determine the level of delayed 24-h footpad swelling and cellular infiltration during the 12 days after infection. The footpad response was maximal at 6 days postinfection. At this time, the course of inflammation and cellular infiltration was studied. Additionally, the character of the cellular infiltrate present at 24 h after challenge was examined histologically. Finally, the effects of various immunoregulatory treatments on the humoral antibody and peripheral delayed inflammatory responses were studied.

Delayed 24-h footpad swelling and cellular infiltration during the 12 days after infection. Mice were infected by intraperitoneal inoculation with 1,000 PFU of SLE virus. Control mice received equivalent inoculations of NSMB suspension. The level of the delayed 24-h inflammatory response was measured at intervals after infection (p.i.). At each interval the footpads of groups of eight mice were challenged. Differential footpad swelling and cellular infiltration were assessed 24 h later. Figure 1 presents the results obtained. Differential footpad swelling reached the highest levels on day 6 p.i. and decreased rapidly to negligible levels by day 10. Labeled CIR reached statistically significant levels on days 6 to 8, after which they declined rapidly, reaching normal control levels by day 10.

Time courses of inflammation and labeled cell infiltration. The time courses of inflammation and cellular infiltration were measured in mice infected as in the previous section and challenged 6 days p.i. The responses of groups of five immunized and five control mice were measured at intervals up to 72 h after footpad challenge. Figure 2 shows the development of the responses. All mice challenged with mouse brain suspensions exhibited a prominent nonspecific inflammatory response within minutes of footpad inoculation. Residual footpad swelling from this response was still measurable 2 to 4 h after inoculation. The swelling continued to decrease in control mice and was essentially undetectable 24 and 48 h after footpad challenge. A moderate inflammatory response was detect-
able in the SLE virus-infected mice. This response was highest 24 h after footpad challenge and, although significantly different from control values, did not exceed 0.25 mm. Labeled cellular infiltration in infected mice was not evident at 24 h after footpad challenge, was noticeable at 9 h after challenge, and was significantly different from that in control mice at 24 h ($\alpha < 0.01$).

**Character of the cellular infiltrate.** Histological examination of the footpads 24 h after challenge revealed a certain amount of inoculation trauma and accompanying polymorphonuclear cell infiltration in both feet of control animals. In the SLE virus-infected mice, however, cellular infiltration in the virus-challenged feet was three to four times that observed in the feet from control animals. The infiltrate contained a greater proportion of monocytes and small lymphocytes. In general, the cellular infiltrate in the virus-challenged feet of infected mice was composed of approximately 70% neutrophils, 20% macrophages, 10% small lymphocytes, and an occasional lymphoblast.

**Effects of immunoregulation on the peripheral 24-h delayed inflammatory response and associated humoral antibody response.** The character of the cellular infiltrates found could be taken as evidence of antibody-mediated or DTH response in that neutrophils, lymphocytes, and monocytes were present (3). To evaluate the potential contributions of the cell-mediated and humoral immune responses to the footpad inflammatory response, we studied the effects of various treatments known to potentiate or suppress DTH.

Peripheral DTH responses in mice can be enhanced by live BCG administration, by splenectomy, and by selective suppression of antibody production (8). Selective suppression of T-cell responses can be induced by administering phytohemagglutinin (PHA) followed by low doses of cyclophosphamide (11). An experiment was designed to evaluate the effects of various SLE infecting doses and immunoregulatory treatments on the development of 24-h cellular infiltration responses. At 18 days before SLE infection, a group of mice was inoculated intravenously with $10^7$ live BCG (live *M. bovis*, Pasteur strain, TMC 1011, obtained from the Trudeau Institute, Inc., Saranac Lake, N. Y.). A second group of mice was inoculated 10 days before SLE infection with intravenous PHA (Miles-Yeda Ltd., Miles Laboratories Inc., Elkhart, Ind.) (15 mg/kg) and 5 days before SLE with intravenous cyclophosphamide (Mead Johnson and Co., Evansville, Ind.) (40 mg/kg). A third group was inoculated intravenously with cyclophosphamide (200 mg/kg) 2 days before SLE infection. Two final groups were splenectomized and sham splenectomized 4 weeks before infection.

At the time of SLE infection, all groups of mice were separated into five equal portions and inoculated intravenously with SLE ($6.5 \times 10^5$, $6.5 \times 10^6$, $6.5 \times 10^7$, $6.5 \times 10^7$ PFU) and with a normal suckling mouse brain dilution equivalent to the highest SLE virus dose. Eight of the normal, PHA-cyclophosphamide, BCG-, and cyclophosphamide-treated mice and five of the splenectomized and sham-splenectomized mice of each treatment group were challenged in the footpads at intervals for 10 days p.i. The CIR were measured 24 h later. No appreciable response was noted in any of the control groups inoculated with normal suckling mouse brain suspensions. All responding groups of SLE-infected mice, regardless of the infecting dose of SLE virus administered, yielded maximum or near maximum 24-h CIR at 5 to 6 days after infection. Normal mice, sham-splenectomized

![Diagram](http://iai.asm.org/)
mice, mice treated with BCG, and mice given cyclophosphamide 2 days before SLE infection all showed a consistent SLE dose-related increase in cellular infiltration but were not significantly different from each other on day 6 p.i. Figure 3 shows the effects of SLE virus-infecting dose on the 24-h response to footpad challenge given on day 6 p.i. For simplicity of presentation, data for the normal SLE-infected mice have been excluded from the figure. Mice treated with PHA plus cyclophosphamide showed no evidence of significant labeled cell infiltration. A marked dose-dependent enhancement of the response was evident in splenectomized animals, with optimally enhanced responses being obtained with the dose of $6.5 \times 10^5$ PFU of SLE virus.

Although neither BCG nor cyclophosphamide given before infection amplified the response at 6 days p.i., both treatments prolonged the response. This is shown in Fig. 4 which presents the development of the footpad cellular infiltration response from days 3 to 10 p.i. with $6.5 \times 10^5$ PFU of virus. It is apparent from these data that splenectomy, BCG treatment, and cyclophosphamide treatment either enhance or prolong the 24-h footpad response. Treatment with PHA plus cyclophosphamide caused almost complete suppression of the 24-h response.

The data presented in Fig. 3 and 4 suggest that the 24-h labeled cellular infiltration may be T-cell mediated. Thus, the treatments known to potentiate DTH also potentiate or prolong the 24-h cellular infiltration responses, and the treatment known to suppress T-cell responses causes virtual elimination of 24-h cellular infiltration. A remaining reservation was caused by the lack of available knowledge pertaining to the effects of the immunoregulatory treatments on antibody responses to SLE virus. These effects were therefore measured. Six groups of mice were prepared. These consisted of normal mice; mice pretreated with BCG, PHA plus cyclophosphamide, or cyclophosphamide alone; and splenectomized and sham-splenectomized mice. Each group was infected by intravenous inoculation of $6.5 \times 10^5$ PFU of SLE virus. A portion of each group was used as a control and was inoculated with equivalent dilutions of NSMB suspensions. Virus-infected mice were bled in groups of four on days 4, 6, 8, and 12. Diluent-inoculated mice were bled on days 0, 6, and 12. Sera were collected and SLE-specific IgM and IgG antibody content was measured with the solid-phase radioimmunoassay technique. Figure 5 presents the radioimmunoassay data obtained. Control
sera from each group were used as base line measures for computation of \(^{125}\text{I} \) uptake. Mean solid-phase radioimmunoassay data for normal and sham-splenectomized mice did not differ significantly (Student’s t test) and have been pooled.

These data indicate that the cellular infiltration measured by footpad challenge on day 6 was probably not antibody mediated. The PHA-cyclophosphamide treatment, which suppressed the footpad response, left the antibody response essentially normal. Splenectomy, which enhanced the footpad response, resulted in suppression of humoral antibody 6 days after immunization.

In general we might postulate three possible relationships between antibody responses and CIR. The first, assuming that antibody contributes to the CIR, would lead one to expect a direct association between CIR and antibody levels on day 6 of the response. The second, assuming no relationship, would lead one to expect no association. The third, assuming that antibody responses may suppress the CIR, would lead to expectation of an inverse relationship. By using rank correlation techniques for 6-day CIR and IgG antibody levels in the five treatment groups, the latter hypothesis is barely tenable at the 5% significance level.

DISCUSSION

At 5 to 8 days p.i. with live SLE virus, mice exhibited a moderate 24-h delayed inflammatory response to footpad challenge. The cells infiltrating the challenged footpad were neutrophils, monocytes, and lymphocytes. Measurement of the kinetics of the cellular infiltrate by tritium-labeled cell techniques, originally developed to quantitate monocyte infiltration (6, 10), revealed a time course characteristic of delayed responses. Immunomodifications such as splenectomy, cyclophosphamide treatment, and BCG administration, which are known to potentiate T-cell-mediated peripheral DTH responses (16), either potentiated or prolonged the delayed 24-h response in mice. Treatment with PHA plus cyclophosphamide, a procedure developed to suppress splenic T-helper-cell responses (11), suppressed the footpad inflammatory response.

The degree of inflammation appeared to be independent of the antibody response. PHA-cyclophosphamide treatment, which suppressed the footpad response, did not alter the antibody response. Either splenectomy or cyclophosphamide treatment given 2 days before SLE infection enhanced the footpad responses but reduced the level of antibody produced at 6 to 8 days.

The footpad response after SLE infection, therefore, meets most of the criteria of the typical transitory peripheral DTH responses shown to occur after stimulation of mice with nonreplicating antigens (3, 8). The T-cell-mediated response, assessed by cytotoxicity assays or by splenocyte migration indexes, has been shown to play both protective and immunopathological roles in mice infected with a number of related flaviviruses (1, 4, 5, 12). Differentiation of the relative contributions of cell-mediated and humoral factors to immunity has been difficult, however, since the assessment of protection has usually depended on intracerebral inoculation of the virus into immunized animals which exhibit both cellular and humoral immunity. Since both peripheral delayed inflammatory responses and humoral antibody levels can be easily manipulated by established immunoregulatory techniques, it should be possible to assess the differential role of humoral and cell-mediated immunity in the development of resistance to SLE virus infection.

LITERATURE CITED


