SPECIFICITY OF THE INFLAMMATORY RESPONSE IN VIRAL ENCEPHALITIS

I. Adoptive Immunization of Immunosuppressed Mice Infected with Sindbis Virus*

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A mononuclear cell inflammatory response is characteristic of most viral infections. The recruitment of these cells has traditionally been considered a nonspecific reaction to viral cell destruction (1, 2). Despite the clinical observation that children with thymic dysplasias may fail to mount an inflammatory reaction to viral infection (3) and the in vitro observations that lymphoid cells may be specifically sensitized by viral infection (4–7), the role of a cell-mediated immune response in the induction of the inflammatory reaction has remained largely unstudied.

Our studies of the inflammatory response in acute viral encephalitis have employed Sindbis virus, a group A arbovirus. This virus causes an acute, nonfatal encephalitis in adult mice with a marked perivascular inflammatory reaction composed solely of mononuclear cells (8). Initial radioautographic and ink labeling studies showed these cells to be derived from a population of short-lived blood mononuclear cells composed largely of macrophages (9), but did not determine whether the stimulus for their recruitment was immunologically mediated or induced nonspecifically by tissue destruction.

The present studies examine the specificity of the inflammatory response employing the passive transfer of cells and sera to mice infected with Sindbis virus and treated with cyclophosphamide to ablate the inflammatory reaction.

Materials and Methods

Viruses and Antigens.—Strain AR339 of Sindbis virus (10) was prepared in chick embryo fibroblasts (CEF)[1] (11) grown in Hanks' lactalbumin hydrolysate media with 20% fetal calf

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[1] *Abbreviations used in this paper*: CEF, chick embryo fibroblasts; CFA, complete Freund's adjuvant; LCM, lymphocytic choriomeningitis; pfu, plaque-forming units; $SMLD_{50}$, suckling mouse 50% lethal doses.

serum. Monolayers of CEF developed in 1 day and were then inoculated with virus; 48 hr after inoculation the cultures (cells and media) were frozen and thawed twice, suspension was clarified by centrifugation at 1000 rpm for 10 min, and supernatant (stock virus) was stored at −70°C. Stock virus contained 10^6 plaque-forming units (pfu) per milliliter.

Control antigens included (*a*) tissue culture antigen consisting of identically processed CEF and media without virus, (*b*) complete Freund's adjuvant (CFA) (Difco Laboratories, Inc., Detroit, Mich.), and (*c*) the 17D strain of yellow fever virus (National Laboratories, Philadelphia, Pa.). Yellow fever virus was passed once intracerebrally in suckling mice, a homogenate of brains of sick mice was inoculated into amniotic cavities of 11-day old chick embryos, and eggs were harvested in 4 days. Stock yellow fever virus was a 20% homogenate of chick embryos containing 10^5 suckling mouse 50% lethal doses ($SMLD_{50}$) per milliliter by intracerebral assay (12).

Animals.—Inbred BALB/c mice (Flow Research Animals, Dublin, Va.) were used.

Adoptive Immunization.—8-wk old donor mice were inoculated subcutaneously in each footpad with 10^4 pfu of Sindbis virus, a comparable dilution of tissue culture control antigen, 10 $SMLD_{50}$ of yellow fever virus, or a 1:1 dilution of CFA. All dilutions were made in Hanks' solution, and each inoculum consisted of 0.03 ml.

Sequential examination of the draining lymph nodes showed maximum enlargement at 7 days. At this time anesthetized donors were sacrificed by exsanguination. Serum was separated for transfers and consistently contained neutralizing antibody at greater than 1:400 dilutions. Brachial, axillary, and popliteal lymph nodes were removed, and cells were dispersed by teasing in cold medium 199. The cell suspension was centrifuged at 600 rpm for 10 min and resuspended in medium 199 to a concentration of 5×10^7 cells/0.1 ml. Bone marrow was flushed from the tibias into medium 199, and cells were similarly processed. Siliconized glassware was used. For adequate cells and serum, five donors were needed per recipient.

Recipient mice, 4 wk of age, were inoculated intracerebrally with 1000 pfu of Sindbis virus or 1000 $SMLD_{50}$ of yellow fever virus on day 0 and given intraperitoneal cyclophosphamide 150 mg/kg body weight on day 1. On day 3, 10^8 cells (0.2 ml) or undiluted serum (0.25 ml) were given intravenously. Animals were sacrificed by exsanguination on day 7 or 8. Brains were removed and fixed in formalin. In studies correlating virus content and inflammation brains were split in the midsagittal plane; the right side containing the stab wound resulting from inoculation was frozen for subsequent virus titration, and the opposite half was fixed in formalin.

Viral and Antibody Assays.—Virus content of brains was determined by plaque assay in CEF cultures, and neutralizing antibody was assayed by 50% plaque reduction as described previously (8).

Histology.—Brains were embedded in paraffin, sectioned horizontally at four levels, and stained with hematoxylin and eosin. The degree of inflammatory reaction in all sections was graded under code by two persons. In whole brains the area of the stab wound was graded separately from the remaining brain. Fluorescent antibody staining was performed on acetone-fixed frozen sections using a fluorescein-conjugated mouse anti-Sindbis globulin (8).

RESULTS

Intracerebral inoculation of 1000 pfu of Sindbis virus into 4-wk old BALB/c mice caused a nonlethal infection with virus growth limited to the brain. The maximum virus content was found on day 2, and no virus was detectable after day 7 (Fig. 1). Fluorescent antibody staining showed multiple foci of viral antigen in brain parenchyma. These were present 24 hr after inoculation and persisted without significant extension until day 7. Inflammatory cells were first seen on day 3 or 4, and the reaction reached a maximum on day 7 or 8. The inflammatory reaction was characterized by the perivascular accumulation of

mononuclear cells around many small blood vessels throughout the brain (Fig. 2 *a*). At no time were polymorphonuclear cells seen in this reaction. The stab wound of inoculation was seen as a well-defined tract surrounded by an area of tissue necrosis and a localized mononuclear cell reaction which began on day 1 or 2, before the generalized inflammatory reaction. Polymorphonuclear cells were frequently seen within the stab wound reaction. Intracerebrally inoculated yellow fever virus produced a similar but more extensive perivascular reaction. Sindbis virus-neutralizing antibody was detectable at day 3 at a dilution of 1:5 and on day 6 at a dilution of greater than 1:400.

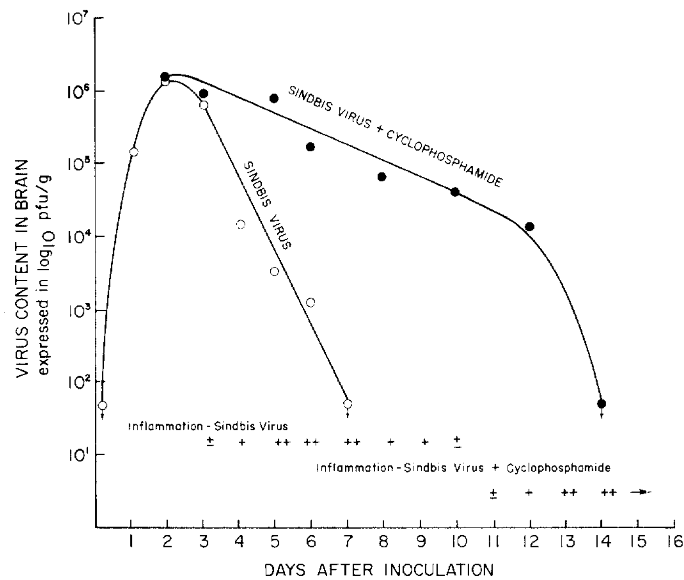
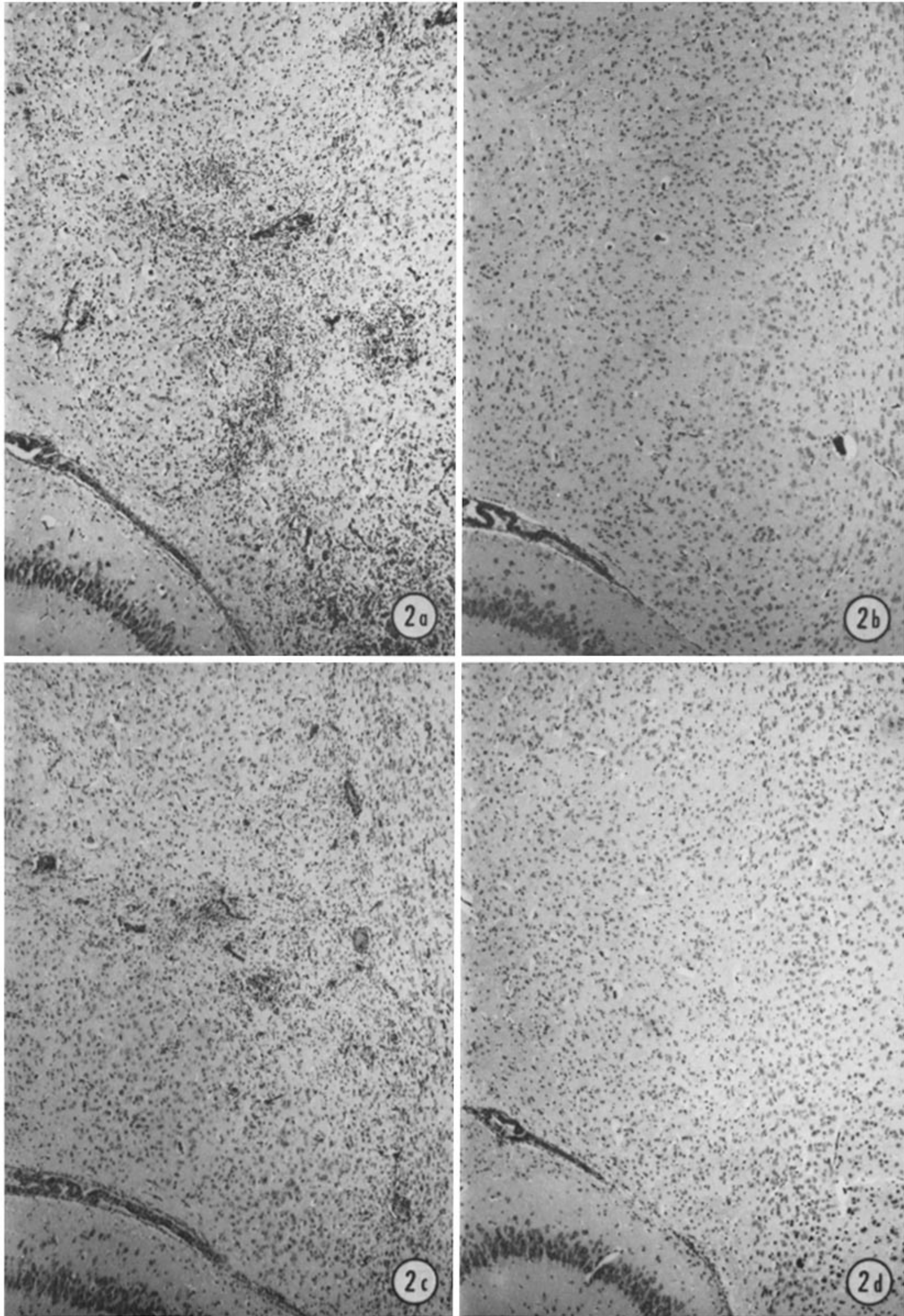


FIG. 1. Virus multiplication and development of inflammation in brains of 4-wk old BALB/c mice after intracerebral inoculation with Sindbis virus. Persistence of virus and delay in the inflammatory response are shown in mice receiving cyclophosphamide 24 hr after virus.

When a single dose of cyclophosphamide was given 24 hr after virus inoculation, virus replication was not augmented. However, virus persisted in brains through day 12 (Fig. 1), and occasional deaths occurred. Fluorescent antibody staining showed foci of viral antigen which were indistinguishable in distribution, size, and evolution from those in untreated mice except for persistence of

FIG. 2. Horizontal sections of mouse brains 8 days after intracerebral inoculation of Sindbis virus. Sections show normal inflammatory response to virus (*a*), ablation of the response by a single dose of cyclophosphamide was given 24 hr after virus (*b*), reconstitution of the response with Sindbis virus-sensitized lymph node cells and bone marrow cells given 48 hr after cyclophosphamide (*c*), and lack of reconstitution of the response with control tissue culture antigen-sensitized lymph node and bone marrow cells (*d*). Hematoxylin and eosin. × 34.


2a
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antigen beyond 7 days. No inflammatory cells were found in the areas of virus replication (Fig. 2 *b*) or around the stab wound until day 12. Neutralizing antibody was not detected until day 11 or 12.

Effect of Passive Transfer of Cells and Serum on the Inflammatory Response.—Mice inoculated intracerebrally with Sindbis virus and treated with cyclophosphamide were reconstituted on day 3 with specifically and nonspecifically sensitized cells and/or serum derived from donor mice sensitized in the footpads 7 days previously. In various experiments cells were obtained from draining lymph nodes and bone marrow. Specifically sensitized cells were obtained from donors inoculated in all four footpads with Sindbis virus, since this virus had previously been shown to undergo limited replication restricted to muscle at the inoculation site (8). Nonspecifically sensitized cells were obtained from donors inoculated with control tissue culture antigen, yellow fever virus, or CFA.

TABLE I

Effect of Passive Transfer of Specifically and Nonspecifically Sensitized Cells and Sera on the Reconstitution of the Inflammatory Response in Sindbis Virus Encephalitis

Adoptive immunization procedure*	No. of mice	Inflammatory reaction to virus					Inflammatory reaction to stab wound				
		Grade of inflammation of individual Mice‡				% with + or ++ reaction	Grade of inflammation of individual mice‡				% with + or ++ reaction
		0	±	+	++		0	±	+	++	
						%					%
Control											
Sindbis virus	20	0	0	5	15	100	0	0	2	18	100
Sindbis virus + cyclophosphamide	15	14	1	0	0	0	15	0	0	0	0
Cell transfers§											
Sindbis LN + Sindbis BM	20	0	2	5	13	90	0	1	2	7	90‖
Sindbis LN	5	1	1	3	0	60	1	3	1	0	10
Sindbis BM	5	5	0	0	0	0	1	0	2	2	80
CTCA LN + CTCA BM	10	4	5	1	0	10	0	3	4	3	70
Yellow fever LN + yellow fever BM	10	7	3	0	0	0	0	1	2	2	80‖
CFA LN + CFA BM	5	3	1	1	0	20	0	0	2	3	100
CTCA LN	4	4	0	0	0	0	4	0	0	0	0
Sindbis LN + CTCA BM	5	0	1	1	3	80	0	0	2	3	100
CTCA LN + Sindbis BM	5	3	2	0	0	0	0	1	3	1	80
Sindbis LN + Sindbis BM + Sindbis serum	6	0	1	1	4	83	0	0	2	4	100
CTCA LN + CTCA BM + Sindbis serum	5	4	0	1	0	20	0	1	2	2	80
Serum transfer											
Sindbis serum	8	6	2	0	0	0	3	0	0	0	0‖
CTCA serum	5	4	1	0	0	0	5	0	0	0	0
Yellow fever serum	5	5	0	0	0	0	2	0	0	0	0‖

* Recipients were inoculated intracerebrally with Sindbis virus on day 0, given cyclophosphamide on day 1, given transfers of cells or serum on day 3, and killed on day 8.

‡ Inflammatory response graded 0 = none, ± = equivocal, + = definite mild, ++ = moderate to marked.

§ LN = lymph node cells; BM = bone marrow cells; CTCA = control tissue culture antigen; CFA = complete Freund's adjuvant.

‖ Numbers do not total to left column since hemisphere containing inoculation site not examined histologically.

The effects of adoptive immunization with cells and/or serum on the inflammatory responses are shown in Table I. Significant reconstitution of the generalized perivascular reaction to viral infection occurred only with the transfer of Sindbis virus-sensitized lymph node cells. This effect was accentuated when Sindbis virus-sensitized lymph node cells were given in combination with bone marrow cells, even though the total number of cells transferred remained constant (i.e. 10^8 cells) (Fig. 2 *c*). This accentuation was independent of the source of bone marrow cells, and since the transfer of bone marrow cells alone caused no reconstitution of the reaction, bone marrow as not processed to remove T cells. Lymph node cells from mice sensitized with control antigens failed to significantly reconstitute the reaction (Fig. 2 *d*). The minor degrees of inflammation sometimes seen after transfer of nonspecifically sensitized lymph node cells

TABLE II

Effect of Passive Transfer of Sensitized Cells on the Reconstitution of the Viral Inflammatory Response to Yellow Fever Virus

Recipient virus inoculation*	Donor virus inoculation (LN + BM cells)	No. of mice	% Recipients with inflammatory reaction (+ or ++)
			%
Yellow fever virus	Yellow fever virus	5	80
Yellow fever virus	Sindbis virus	5	0

* Recipients were inoculated with virus on day 0, given cyclophosphamide on day 1, given transfers of sensitized cells on day 3, and killed on day 8.

may have resulted from in vivo sensitization or may have represented the beginning of a nonspecific response to tissue necrosis analogous to the stab wound reaction.

In contrast, the localized inflammatory reaction around the stab wound of inoculation was reconstituted with bone marrow cells from all sources and not with lymph node cells alone. Serum from animals sensitized with Sindbis virus, tissue culture antigen, yellow fever virus, or CFA produced no reconstitution of inflammation.

Combination of Sindbis virus immune serum with tissue culture antigen-sensitized lymph node and marrow cells also failed to reconstitute the perivascular inflammatory reaction, and no accentuation of the reaction was obtained when immune serum was combined with Sindbis virus-sensitized cells.

To test the competency of control antigen-sensitized cells, mice were inoculated intracerebrally with yellow fever virus, treated with cyclophosphamide, and reconstituted on day 3 with cells from yellow fever virus or Sindbis virus-sensitized donors (Table II). The inflammatory reaction to yellow fever virus was reconstituted only with cells from yellow fever virus-sensitized donors establishing the competency of these cells to reconstitute an inflammatory response and further confirming the specificity of the response.

Effect of Transfer of Cells and Serum on Virus Content—Sindbis virus-inoculated immunosuppressed mice showed a mean viral titer of $10^{5.7}$ pfu/g of brain 8 days after inoculation (Table III). Mice similarly treated and reconstituted with Sindbis virus-sensitized lymph node and bone marrow cells in combination showed a 1000-fold decrease in virus in brain. Animals receiving Sindbis virus-sensitized lymph node cells alone showed a similar reduction in virus, while

TABLE III

Effects of Adoptive Immunization on Virus Content, Neutralizing Antibody, and Inflammatory Response 8 Days after Intracerebral Inoculation of Sindbis Virus

Adoptive immunization procedure*	No. of mice	Virus content of brain‡	Serum-neutralizing antibody (dilution causing 50% plaque reduction)‡	% with viral inflammatory reaction (+ or ++)
		pfu/g		%
Controls				
Sindbis virus	15	$<10^{1.7}$	>1:400	100
Sindbis virus and cyclophosphamide	15	$10^{5.7}$	<1:5	0
Cell transfers				
Sindbis LN and Sindbis BM	12	$10^{2.7}$	1:10	90
Sindbis LN	5	$10^{3.0}$	1:5	60
Sindbis BM	6	$10^{5.0}$	<1:5	0
CTCA LN and CTCA BM	6	$10^{5.0}$	±1:5	10
Yellow fever LN and yellow fever BM	6	$10^{5.7}$	±1:5	0
CFA LN and CFA BM	5	$10^{5.0}$	<1:5	20
Serum transfers				
Sindbis serum	9	$10^{3.8}$	1:10	0
CTCA serum	6	$10^{5.0}$	<1:5	0
Yellow fever serum	3	$10^{5.3}$	<1:5	0

* Recipients were inoculated intracerebrally with Sindbis virus on day 0, given cyclophosphamide on day 1, given transfers of cells or serum on day 3.

‡ Brains and sera were assayed individually and mean virus content or antibody titers are shown. Variations in virus titer in brain did not exceed $10^{1.0}$fu/g within any group.

animals receiving Sindbis virus-sensitized bone marrow alone failed to show a significant decrease. Similarly, animals receiving tissue culture antigen, yellow fever virus, or CFA-sensitized lymph node and marrow cells in combination showed no reduction in virus content. Mice receiving Sindbis virus immune serum alone showed a 100-fold reduction in virus content.

Production of Antibody by Transferred Cells.—Recipients of Sindbis virus-sensitized lymph node and bone marrow cells had a mean neutralizing antibody titer of 1:10 at the time of sacrifice 5 days after cell transfer (Table III). This was equivalent to residual antibody 5 days after transfer of high titered Sindbis virus immune serum. Animals receiving only Sindbis virus-sensitized lymph

node cells showed neutralizing antibody titers of 1:5, while recipients of Sindbis virus-sensitized bone marrow had no detectable neutralizing antibody titer.

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In some instances, recipients of nonspecifically sensitized lymph node and bone marrow cells showed detectable neutralizing antibody at a 1:5 dilution. The transfer of antibody-producing capacity was further studied by transferring specifically and nonspecifically sensitized lymph node and bone marrow cells to normal uninfected mice. Mice receiving Sindbis virus-sensitized cells showed detectable levels of neutralizing antibody at a 1:5 dilution 6 days after cell transfer, while those receiving nonspecifically sensitized cells developed no demonstrable antibody. Virus was not detectable in the Sindbis virus-sensitized cells used for transfer.

DISCUSSION

These studies indicate that the induction of the inflammatory response in this viral encephalitis is immunologically specific. Furthermore, the reconstitution of the viral-induced inflammation seems dependent on a population of sensitized lymph node cells, and a synergistic effect is achieved when bone marrow cells are used in combination with the lymph node cells. Bone marrow cells alone are incapable of reconstituting this reaction but do reconstitute the reaction surrounding the stab wound at the inoculation site. Thus, optimal reconstitution of the specific mononuclear cell inflammatory response appears to require an interaction between immune lymphoid cells and bone marrow cells, while the nonspecific reaction to the stab wound is reconstituted with any adequate source of monocytes.

The majority of previous studies of the inflammatory reaction in viral encephalitis have employed lymphocytic choriomeningitis (LCM) virus (13, 14). However, the immune-mediated disease in LCM virus infection stands in contrast to the arbovirus encephalitides, where disease appears to result from the viral lysis of neural cells (15). In two experimental arbovirus infections, acceleration or accentuation of the inflammatory response in the brain has been reported by the administration of immune serum during a viremia (16, 17). On the basis of these studies, a virus-antibody reaction has been postulated in the recruitment of the inflammatory response in encephalitis (18).

The present studies do not support this hypothesis. Although the passive transfer of Sindbis virus-sensitized lymph node cells did reconstitute the neutralizing antibody-forming capacity of the immunosuppressed recipient, there

is no evidence that antibody played an essential role in recruitment of the perivascular inflammatory response. The passive transfer of Sindbis virus immune serum did not accentuate or accelerate the inflammatory response reconstituted with Sindbis virus-sensitized lymph node cells; further, immune serum failed to reconstitute inflammation when combined with control antigen-sensitized cells.

The cells needed for the specific reconstitution of the viral inflammatory response are similar to those needed for the reconstitution of delayed hypersensitivity-type reactions. Studies of the tuberculin reaction using thymectomized-irradiated animals indicate that the expression of the cellular infiltrate requires an interaction between thymus-derived, antigen-sensitized lymphocytes and bone marrow cells (19). Similar cell cooperation has been demonstrated in the mononuclear cell inflammatory reaction in contact sensitivity (20). In other studies not specifically directed at the mononuclear cell inflammatory response, cell cooperation has been demonstrated in the passive transfer of resistance to *Listeria monocytogenes* (21) and in the recovery mechanism of mousepox virus infections (22, 23).

The inflammatory response appeared to play some role in the clearance of virus from the brain. With reconstitution of inflammation by the transfer of specifically sensitized lymph node cells, a significant reduction in virus was found, and this reduction was of greater magnitude than was achieved by the transfer of immune serum. Although specifically sensitized lymph node and bone marrow cells were found to produce antibody 5 days after passive transfer, the amount was no greater than residual circulating antibody 5 days after the passive transfer of high titered immune serum. Thus, the reduction in virus content of brains appeared to be more closely related to the inflammatory reaction than to antibody production. Although these data do not permit definitive separation of the effect of the cellular and humoral mechanisms on virus clearance and the recovery process, they do demonstrate that the induction of the inflammatory reaction is immunologically specific and suggest that the cellular infiltrate plays more than a passive role in the pathogenesis of disease.

SUMMARY

The viral-induced perivascular inflammatory response in Sindbis virus encephalitis of mice was shown to be immunologically specific. Mice were inoculated intracerebrally with Sindbis virus, and 24 hr later a single dose of cyclophosphamide was given which ablated the inflammatory response. 3 days after virus inoculation, cells and/or sera from specifically and nonspecifically sensitized donor mice were given, and the inflammatory reactions, virus content, and antibody response of recipients were examined 5 days later.

Reconstitution of the viral inflammatory response required virus-specific sensitized lymph node cells and was enhanced when these lymph node cells were combined with bone marrow cells. Reconstitution was not achieved with Sindbis

virus immune serum even when combined with nonspecifically sensitized cells. Combination of immune serum with Sindbis virus-sensitized cells did not produce an accentuation of the reaction.

In distinction, reconstitution of the inflammatory reaction surrounding the stab wound was reconstituted with bone marrow cells from mice inoculated with Sindbis virus or control antigens.

Reconstitution of the perivascular reaction was associated with a reduction in brain virus content. Although the transfer of Sindbis virus-sensitized lymph node cells and bone marrow cells resulted in the limited production of neutralizing antibody in the immunosuppressed recipient, the reduction in virus was significantly greater with the transfers of Sindbis virus-sensitized lymph node cells than with the passive transfer of immune serum alone.

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