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TOGAVIRUS-SPECIFIC CELLULAR IMMUNE EFFECTOR MECHANISMS

ANNUAL PROGRESS REPORT

by

Gerald A. Cole, Ph.D.

January, 1979

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

In order to assess the role of virus-specific cellular immune responses in the outcome of togavirus infections, these initial studies have focused on: 1) characterizing Sindbis virus (SV) infections in inbred strains of mice and cultured murine cells sharing the same major histocompatibility (H-2) antigens, and 2) determining the requirements and optimal conditions for inducing and measuring cell-mediated immune reactivity to SV. Following intracerebral inoculation of appropriate doses of SV (neuro-adapted AR 339), BALB/c ($H\text{-}2^d$) mice develop non-fatal paralytic disease whereas C3H/He ($H\text{-}2^k$) mice display no

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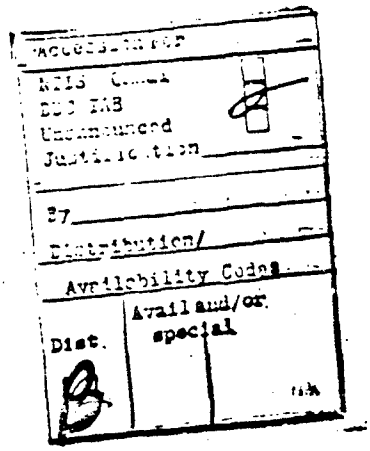
paralysis but die. In vitro, B10D2 ($H\text{-}2^d$) cells are highly susceptible and all are destroyed after exposure to a wide range of virus multiplicities (MOI), while total destruction of MC57G ($H\text{-}2^b$) cells occurs only at low MOI. L ($H\text{-}2^k$) cells develop abortive noncytopathic infections at any MOI. Splenic lymphocytes from SV-immune C57BL/6 mice gave virus-specific proliferative responses when cultured with SV-infected, H-2 identical cells but not when cultured with SV alone. Cytotoxic lymphocytes were induced in spleens of C57BL/6 mice by immunization with SV-infected, H-2 identical cells but not with virus alone. It is tentatively concluded that non-H-2 genetic background is an important determinant of host susceptibility to SV and that H-2 antigens are involved in SV-specific effector lymphocyte induction and recognition.



SUMMARY

This project was initiated to assess the role of virus-specific cellular immune responses in recovery from, and protection against togavirus infections. Initial studies have focused first on characterizing the salient features of Sindbis virus (SV) infections both in several inbred strains of mice, 6-8 weeks of age, and in cultured murine cell lines bearing the same major histocompatibility (H-2) antigens and, second, on determining the requirements and optimal conditions for inducing and measuring cell-mediated immune reactivity. The results indicate the following:

The pathogenicity of the neuro-adapted AR339 strain of SV used in these studies varies between different strains of mice infected intracerebrally. Given the same appropriate doses, BALB/c ($H\text{-}2^d$) mice all develop non-fatal paralytic disease whereas C3H/He ($H\text{-}2^k$) mice fail to display paralysis but die. Non-lethal infections of C57BL/6 ($H\text{-}2^b$) and BALB/c mice result in a prominent long-lasting lymphoid hyperplasia which, in the latter, may be related to their depressed lymphoproliferative responses to in vitro stimulation with the mitogens PHA and LPS.

On the basis of sequential immunofluorescent staining and morphological observations, the capacity of SV to infect and/or cause cytopathology in different murine fibroblastic cell lines varies markedly. BIOO2 ($H\text{-}2^d$) cells are highly susceptible and all are destroyed after exposure to a wide range of virus multiplicities (MOI). MC57G ($H\text{-}2^b$) cells appear to be equally susceptible to infection although total cell destruction (CPE) is delayed and occurs only after exposure to low MOI. L ($H\text{-}2^k$) cells develop abortive noncytopathic infections at any MOI.

Cytolytic lymphocytes were induced in spleens of C57BL/6 mice immunized 7 days previously with SV-infected H-2 identical (MC57G) cells, but not with virus alone. Although lytic activity appeared to be directed mainly against cellular rather than viral antigens, it was much less, or absent, in spleens of animals immunized with uninfected cells.

Splenic lymphocytes from C57BL/6 mice infected with SV 60 days previously, gave virus-specific proliferative responses when cultured with SV-infected, UV-irradiated, H-2 identical cells, but not when cultured with infectious SV alone.

Tentatively, it is concluded that: (1) the genetic background of the host is an important factor in determining its susceptibility to SV and possibly other alphaviruses and, (2) host cell H-2 antigens probably are involved in the induction of (and virus-specific recognition by) SV-specific effector lymphocytes.

FOREWARD

In conducting the research described in this report, the participating investigators have adhered to the "Guide for Laboratory Animal Facilities and Care" as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences - National Research Council.

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INTRODUCTION

During the course of operations by U.S. military personnel in tropical areas, infections by certain groups of togaviruses have been a persistent cause of morbidity. Consequently, the development of safe and effective vaccines against these agents is a continuing major objective of the U.S. Army Research and Development Command. In meeting this objective, it is important to have, for each relevant togavirus group, a clear understanding of the extent to which the various components of the anti-viral immune response contribute to recovery from infection and how the induction and magnitude of each component is influenced by factors intrinsic to either the host or the infecting agents. Such an understanding will provide not only the conceptual framework necessary for producing vaccines that induce the relevant immune response components, but also the basis for developing laboratory methods capable of assessing their efficacy in vaccinated personnel.

Although the presence of measurable circulating anti-viral antibodies after natural infection or vaccination is usually accepted as evidence of protective immunity, a question of long-standing is whether virus-specific cellular immune reactivity is equally important in protection, particularly when antibody levels wane or become undetectable. In view of the recent advances made in the fields of cellular immunology and immunogenetics, it is now feasible to answer this question experimentally. Such is the goal of the various studies comprising this project, which consists of two phases. The first phase, now in progress, is focused primarily on the demonstration and measurement of virus-specific T lymphocyte responses in different strains of genetically defined mice during and after, experimental infections produced by selected alpha- and flaviviruses. The results of these efforts should provide a conceptually sound basis for proceeding to the second phase which, in an anticipated collaboration with the U.S. Army Medical Research Institute of Infectious Diseases, would be directed toward evaluating togavirus-specific cellular immune reactivity in human recipients of existing pilot vaccines.

MATERIALS AND METHODS

Animals

Adult inbred 6-8 week old C_3H/HeJ ($H\text{-}2^k$), BALB/c ($H\text{-}2^d$) and C57BL/6 ($H\text{-}2^b$) female mice were purchased from the Jackson Laboratories, Bar Harbor, Maine. In addition, 1-2 day old as well as adult outbred ICR mice were obtained from Blue Spruce Farms, Blue Spruce, Maryland.

Sindbis virus (SV)

The Egypt AR339 strain of Sindbis virus, which had been neuroadapted by five serial brain-passages in suckling BALB/c mice, was received as a clarified 10% infected brain homogenate in two percent fetal calf serum (FCS) from Dr. D. Griffin, The Johns Hopkins University. This material (NSV-mp6) contained 2.75×10^7 plaque forming units (PFU) per gram of brain when assayed in cultures of chick embryo fibroblasts.

To prepare virus for these studies, 3-4 day old outbred ICR mice were inoculated intracerebrally (i.c.) with 0.02 ml of phosphate buffered saline (PBS) diluent containing $10^{2.7}$ PFU of NSV-mp5. After 30 hours 90-95% were moribund. Brains from these animals were homogenized in Hanks' balanced salt solution, pH 7.2, (HBSS) containing penicillin (200 units/ml) and streptomycin (100 µg/ml). The brain suspension (20%) was clarified by centrifugation and aliquots were stored at -70°C until used. The titer(s) of this material (NSV-mp6) in PFU per gram of brain was $10^{9.5}$ in Vero cells, and $10^{10.4}$ in BHK-21 cells. The LD_{50} was $10^{10.2}$ per gram in i.c. inoculated 3-4 day old ICR mice. Plaque morphology was heterogeneous and, in BHK-21 cells, approximately 80% were large while the remainder were small. As yet, this preparation has not been plaque-purified.

Cell lines

All cell lines used were grown and maintained as stationary cultures in plastic tissue culture flasks at 37°C and in an atmosphere of 5% CO_2 in air. All culture media (GIBCO, Grand Island, N.Y.) contained penicillin (100 units/ml), streptomycin (100 µg/ml), and gentamicin (40 µg/ml) and were supplemented with 10% heat-inactivated FCS. Of the murine lines, SV40-BALB ($H\text{-}2^d$) and MC57G ($H\text{-}2^b$) cells were grown in RPMI 1640; B10D2 ($H\text{-}2^d$) were grown in Dulbecco's modified medium; L ($H\text{-}2^k$) cells were grown in Eagle's minimal essential medium (MEM). BHK-21 and Vero cells were also grown in MEM.

SV infections in vitro

BHK-21 and murine cells were grown on 22 mm square glass coverslips contained in 35 mm diameter wells of 6-well plastic culture plates (Costar,

Cambridge, Mass.). Cell monolayers were inoculated with 0.25 ml of diluted virus at an MOI ranging from 0.02 to 600. After adsorption for 1 hr at 37°C with occasional rocking of the plates, the unabsorbed inoculum was removed, cells were refed with fresh medium, and the cultures were returned to a 37°C incubator containing 5% CO_2 in air. At various times post-inoculation, coverslips were removed from the culture plates, washed in PBS, fixed for 30 seconds in cold absolute methanol, washed in tap water, and air dried. Infected cells were identified by indirect immunofluorescence using a mouse anti-SV ascitic fluid provided by Dr. R. E. Shope, Yale University, together with a fluorescein-conjugated rabbit anti-mouse IgG (Cappel Laboratories, inc., Cochranville, Pa.).

Delayed type hypersensitivity (DTH) responses

Mice were inoculated in the left hind footpad with doses of SV virus ranging from 10^2 to 10^8 PFU. Negative control mice were similarly inoculated with an appropriate dilution of normal mouse brain. For comparison with a positive control, other mice were sensitized with 2,4-dinitro-1-fluorobenzene (DNFB; Eastman Kodak, Rochester, N.Y.) by two daily applications of 18 µg of DNFB in 0.01 ml of acetone and olive oil (4:1) to the shaved skin of their abdomens.

Seven days later, a baseline control was established by measuring and recording the thickness of the four footpads and both ears of each animal using a micrometer ("Des Schnelltaster," Pastfach, Germany). Mice in both the experimental and negative control groups were inoculated in the left front footpad with 10^3 PFU of SV and in the right front footpad with diluted normal mouse brain. The DNFB-sensitized mice were challenged on the dorsal side of left ear with 10 µg of DNFB in 0.010 ml of acetone and olive oil while the right ear received the acetone and olive oil mixture alone. Thereafter, the footpad- and ear-thicknesses of all mice were again measured at 6, 24, and 48 hr.

Lymphocyte cultures

Lymphocyte suspensions were prepared from groups of 3-5 virus-infected (primed) or normal (unprimed) mice. Lymph nodes (axillary, inguinal, brachial, and popliteal) and spleens were teased through 60-mesh stainless steel screens into chilled HBSS. The suspensions were first centrifuged for 20 seconds at 50 x g to remove clumps and then for 10 minutes at 200 x g. Erythrocytes were lysed by suspending the cell pellets in 0.83% ammonium chloride for 3 minutes at room temperature. After 3 washes in HBSS, the remaining mononuclear cells were suspended at a concentration of 2×10^6 viable cells/ml (as determined by trypan blue dye exclusion) in a standard medium of RPMI 1640 containing 10% heat-inactivated FCS, antibiotics, 5×10^{-5} M 2-mercaptoethanol, and 10 mM Hepes buffer.

For proliferation assays, 0.1 ml aliquots of primed and unprimed lymphocytes (2×10^5) were added to wells of flat-bottomed 96-well plastic microtiter plates (Costar). To these wells of putative "responders" were added, in triplicate, an equal volume of medium containing either 10^7 PFU of SV, diluted normal mouse brain, various concentrations of SV-infected "stimulator" cells (see below),

0.4 ug of phytohemagglutinin (PHA), or 0.125 µg of E. coli lipopolysaccharide (LPS). The latter two mitogens were purchased from Difco Laboratories, Detroit, Mich. After 44, 68, and 92 hours incubation at 37°C in air containing 5% CO_2, 1 uCi of tritiated thymidine (^{3}H-Tdr; New England Nuclear, Boston, Mass.; specific activity 6.7 mCi/mM) was added to each well of appropriate plates. Following an additional 4 hr incubation, lymphocytes from each well were collected on glass fiber filters (Reeve Angel, Clifton, N.J.) and washed with distilled water using a semi-automatic cell harvester (Biomedical Research Inst., Rockeville, Md.). The filters were dried and counted for radioactivity in a Packard liquid scintillation spectrometer. Incorporation of ^{3}H-Tdr by stimulated responders was always compared with that of responders cultured in medium alone.

Preparation of stimulator and target cells

Cell monolayers of the appropriate species or strain grown in plastic tissue culture flasks were infected with SV at an MOI of 8. After 1 hr at 37°C, the unadsorbed inocula were removed, fresh medium was added, and the cells were further incubated for 5 hr. The infected monolayers (and corresponding uninfected control cells) were then removed from the plastic with 0.125% trypsin, washed and suspended in HBSS at a concentration of 1×10^7 viable cells/ml.

When used as targets in cytotoxicity assays, infected and uninfected cells were labeled for 1 hr at 37°C with ^{51}Cr (New England Nuclear; 500 uCi/10^7/ml) followed by 3 washes in HBSS and resuspension in RPMI 1640 at a concentration of 2×10^5/ml.

When used as stimulators of lymphoproliferation, 1-2 ml aliquots SV-infected and uninfected cells contained in 60 mm plastic culture dish were exposed to a 30 watt ultraviolet (UV) light source (G15T8, General Electric Co., Waterford, N.Y.) for 3 minutes at a distance of 5 cm with constant agitation. This treatment rendered the cells incapable of incorporating ^{3}H-Tdr. After UV-irradiation the cells were washed once in HBSS and suspended to the desired concentration in RPMI 1640.

In some experiments, infected (and control uninfected) spleen cells, syngeneic with the responders, were used as stimulators. Spleen cell suspensions, depleted of erythrocytes, were mixed with SV (MOI of 100) for 1hr at 37°C, washed twice in HBSS, suspended in RPMI 1640 and then exposed to 2500 rads of γ-radiation from a Gammacell 40 Caesium 137 irradiation unit (Atomic Energy of Canada, Ltd., Ottawa, Canada) at a dose rate of 130 rads/min. Irradiated cells were adjusted to a final concentration of 2×10^6/ml.

Lymphocytotoxicity assays

These were performed with both H-2 identical and H-2 nonidentical target cells. For each H-2 haplotype, 0.1 ml aliquots of infected and uninfected ^{51}Cr-labeled target cells (2×10^4) were added to wells of flat-bottomed 96-well microtiter plates. Equal volumes of SV-primed and unprimed lymphocyte suspensions were then added to triplicate wells in varying lymphocyte:target ratios, usually 80, 40, 20, and 10:1. The mixtures were incubated for 15 hr at 37°C in

air containing 5% CO_2, after which 0.1 ml of culture medium from each well was transferred to corresponding 12 x 75 mm plastic tubes which were counted for radioactivity in a Packard auto-gamma spectrometer. Lysis of target cells attributable to either virus-specific or non-specific effector lymphocyte activity (release of label) was calculated using the following formula:

$$\% \text{ lysis} = 100 \times \frac{\text{CPM released in the presence of lymphocytes} - \text{CPM spontaneously released in the absence of lymphocytes}}{\text{CPM released by } H_2O \text{ lysis}}$$

Virus-specific lysis, if obtained, could be calculated by subtracting the % lysis of uninfected targets from that of infected targets.

RESULTS AND DISCUSSION

SV infection of mice

One of the first objectives of this project is to evaluate the role of cellular immune effector mechanisms in the outcome of experimental murine togavirus infections. It is desirable, therefore, to employ representative strains of alpha - and flaviviruses which, under certain conditions, are capable of producing clinical disease and/or mortality in animals having a fully developed T cell system. Since murine infections with the well characterized AR 339 strain of SV are usually self-limiting and inapparent, except in newborn or relatively young animals, (1) a neuro-adapted derivative (NSV-mp6) was chosen for the present studies because of its demonstrated ability to cause fatal paralytic disease when inoculated intracerebrally (i.c.) into young adult BALB/c mice (2).

Recent studies from this (3) and other laboratories (4,5) have shown that in inbred (syngeneic) strains of mice, both the specificity and magnitude of cytotoxic T lymphocyte (CTL) responses to members of the arenavirus, myxovirus, and poxvirus groups are dictated, in part, by genes mapping to the K and D regions of the H-2 gene complex. However, other studies (6,7) have shown no relationship between the susceptibility to togavirus infections of certain mouse strains and their H-2 genotype(s) indicating that other genetically determined host factors are involved.

With these studies in mind, we compared the clinical responses of two different inbred strains of mice to a range of i.c. doses of SV. As shown in Table 1, the type and severity of disease are markedly strain- and dose-dependent. In general, BALB/c ($H\text{-}2^d$) mice exhibit non-fatal paralytic disease as the main feature of infection whereas in C3H/HeJ ($H\text{-}2^k$) mice, SV causes much greater mortality, often without preceding paralysis. These contrasting disease patterns are most obvious after i.c. inoculation of 10^5 - 10^6 PFU of virus.

This apparent genetic influence on susceptibility of mice to an alphavirus, to our knowledge, has not been described previously and it will be of interest to determine whether it is related to their H-2 genotype and/or their background, both of which differ. Even more relevant is whether these differences in susceptibility can be correlated with differences in virus-specific cellular immune reactivity. In this context, it has been shown only recently (4,5) that mice possessing the k allele at H-2K (K^k) give poorer virus-specific CTL responses than those typing as K^d or K^b.

Another question raised by the observations summarized in Table 1 is why large i.c. doses ($10^{7.6}$PFU) of SV are less effective in producing either paralytic disease or mortality in both strains of mice. In the past, this "autointerference" phenomenon has often been associated with flavivirus infections of mice and now is usually attributed to defective interfering

(DI) virus particles which are present in the inoculum and/or accumulate in situ at foci of initial virus replication. From in vitro studies (see below), it seems likely that the host cell genotype significantly influences the extent of DI particle production which, in turn, may be another determinant of susceptibility in the intact animal.

SV infections of cultured cells

The kinetics of SV infections of several different murine cell lines exposed to a range of MOI was assessed by sequential immunofluorescence and light microscopy in order to determine: 1) whether cells originating from mice of either differing backgrounds or H-2 genotypes exhibited differences in their susceptibility to SV, and 2) what doses of virus were optimal for preparing both stimulator and target cells required, respectively, for lymphoproliferation and cytotoxicity assays. Infections of BHK-21 cells served as a point of reference because of their known permissiveness and sensitivity to SV.

The results are presented in Tables 2-5 and compare, at different times after exposure to SV, the approximate percentages of cells containing immunofluorescent antigen with the percentages of those showing evidence of cytopathology (CPE). In BHK-21 cells (Table 2) the interval between exposure to virus and the appearance of antigen positive cells, in all cases, is inversely related to MOI. There is also a significant lag between the appearance of viral antigen in the majority of cells and the first evidence of CPE. A similar picture is seen in B10D2 ($H\text{-}2^d$) cells (Table 3) except that the production of viral antigen and CPE occur almost simultaneously. The fate of MC57G ($H\text{-}2^b$) cells, once infected, depends on the MOI. Large doses of virus (600 and 60 PFU/cell), while capable of initiating infection in practically all cells (as determined by immunofluorescence at 25 hr post-infection) causes CPE in only 20-30%. Presumably, the majority of antigen-positive cells undergo abortive infection due to the presence of production of DI particles (7). At an MOI of 6, the pattern of infection resembles that seen in BHK-21 cells; CPE is detected well after the appearance of viral antigens but, eventually, all cells are destroyed. In L ($H\text{-}2^k$) cells (Table 5) infection at any MOI is abortive. No CPE is evident and, at best, only 30% of cells can be shown to contain viral antigens. Although not shown, this same pattern of infection is seen in SV 40-transformed BALB/c ($H\text{-}2^d$) fibroblasts.

Collectively, the results presented in Tables 3-5 show that genotypically different murine cells differ markedly in their capacity to support SV replication and in their susceptibility to the destructive effects of viral infection. These differences do not seem related to H-2 haplotype since the most susceptible cell line, B10D2, and one that is highly resistant, SV40-BALB, are both $H\text{-}2^d$; nor could such a relationship, if it existed, explain why C3H/HeJ ($H\text{-}2^k$) mice, from which SV-resistant L cells originate, are more prone to develop fatal disease as compared with BALB/c mice. On the other hand, it is noteworthy that although B10D2 and MC57G differ at H-2, both were derived originally from mice of the C57BL background and both are almost

equally susceptible to SV at low MOI. A tentative conclusion that would accomodate the combined in vivo and in vitro observations made thus far (Tables 1, 3-5) is that for any given strain of mouse, non-H-2 genetic background determines the level of host cell permissiveness and possibly DI particle production (7), whereas the H-2 gene complex mainly determines the class (component) and magnitude of the immune response to infection.

Interferon induction might be another factor contributing to the differences observed in vitro; however, since they do not appear to reflect what occurs in the intact infected animal, the importance of interferon in genetically determined susceptibility to SV is questionable.

These observations (Tables 3-5) are being extended in order to establish for each murine cell line, how much infectious virus is produced, especially in those situations where the MOI determines infection outcome, and also to what extent infected (antigen-positive) cells express viral antigens on their surfaces (as measured by specific antibody-binding). These are important parameters to consider when using infected and potentially virogenic cells as stimulators or targets of SV-immune effector lymphocytes.

SV-induced lymphoid hyperplasia

During the course of these studies it was observed that when sacrificed, acutely infected mice, as well as those infected up to 8 weeks previously, had markedly hyperplastic lymphoid tissues. This was most prominent in lymph nodes, which in some i.c. inoculated animals appeared hemorrhagic. For example, Table 6 shows the evolution of splenic and lymph node hyperplasia in C57BL/6 and BALB/c inoculated with SV in their footpads. Although the phenomenon was observed with the same regularity in mice given virus i.c. or intraperitoneally (i.p.), in degree, it was directly related to the infecting dose but apparently unrelated to mouse strain.

The significance of this finding is still open to speculation. From a mechanistic standpoint, chronic lymphoid hyperplasia may reflect a persisting immunogenic stimulus. That such a stimulus would be provided by persisting SV seems unlikely although this possibility will be explored. On the other hand, infection(s) with other unrelated agents that are indigenous to mice (such as Sendai or LDH viruses) may be activated by SV. This possibility will also be explored because the presence of such agents may alter SV-specific immune responses. The observed lymphoid hyperplasia may interfere with attempts to demonstrate SV-specific cellular immune reactivity in vitro since activated T lymphocytes can act non-specifically as "suppressor" cells (see below).

Depressed in vitro responses to mitogens

Measurements of proliferative responses by lymphoid cells cultured in the presence of mitogenic concentrations of PHA and LPS are useful in assessing

immunologic status. Although these responses are polyclonal and therefore non-specific, a depression in their magnitude is indicative of some dysfunction in one or more of the cell types required for "normal" responses (8).

Groups of BALB/c mice were inoculated i.c. with either; 1) 10^8 PFU of SV, a dose which caused neither paralysis nor death, 2) 10^6 PFU of SV, which caused non fatal paralytic disease in all animals by the 15th day post-infection, or 3) equivalent dilutions of normal mouse brain. Sixty days later, cultures of pooled lymph node and spleen cell suspensions from each group were tested for their ability to incorporate ^{3}H-Tdr after 48 hr in the presence of PHA or LPS. As seen in Table 7, no significant differences in incorporation (CPM) were obtained between unstimulated virus-primed and unprimed cells. However, approximately a 50% reduction in CPM occurred in mitogen stimulated lymph node and spleen cells from mice inoculated with 10^{7-8} PFU of SV, as compared with corresponding stimulated unprimed cells. A similar reduction was seen in lymph node (but not spleen) cells from mice inoculated with 100-fold less virus.

From these preliminary findings, it appears that SV may produce a dose-dependent impairment of immune function that persists beyond the period of active infection. Whether this impairment is related to the co-existing lymphoid hyperplasia described above remains to be determined. It should be noted that the SV preparation used in these studies does not appear to replicate in or inhibit the growth of a continuous line of BALB/c T cells ("Gloria"), suggesting that the observed depression in mitogen responsiveness may be due to an effect of virus on a non-lymphoid cell type such as the macrophage.

SV-specific lymphoproliferation in vitro

A series of experiments were done in order to establish the experimental conditions that are required to induce virus-specific proliferative responses by lymphoid cells from animals infected with SV weeks to months previously. Such secondary (2°) responses, if obtained, usually reflect the presence of long-lived T cell memory (3).

Initially, spleen and lymph node cells from BALB/c mice inoculated i.c. with SV 60 days previously (as described in the preceding section) were used as putative responders, and were cultured for 72 hr with either 10^7 PFU of SV, an equivalent dilution of normal mouse brain, or an equal number of gamma-irradiated normal syngeneic spleen cell "stimulators" that had been exposed for 1 hr to SV at an MOI of 100. In each case, no differences in CPM were observed between virus-primed and unprimed cells.

Next, C57BL/6 mice inoculated in their foot-pads with SV 60 days previously were used as a source of primed responders which, along with control unprimed lymphocytes, were cultured for 72 and 96 hr with virus-infected (and control uninfected) H-2 identical stimulator cells. SV-specific responses by spleen cells were obtained at both time points although

they were greater at 72 hr. The magnitude of these responses (Table 8) was dependent on the ratio of responders to stimulators; 3H-Tdr incorporation was greatest at ratios of 33 and 10:1 whereas at 3:1 it was depressed. Responses by primed lymph node cells were similar (data not shown) but less impressive because of the unusually high background CPM in virus-primed cells cultured alone, possibly the result of co-existing lymphoid hyperplasia.

In considering the impressive responses shown in Table 8 together with our failure to induce similar responses in cultures of virus-primed lymphocytes from BALB/c mice, it is not clear whether the use of a different mouse strain or route of SV inoculation can account for these contrasting outcomes. Currently, our bias is that the use of an optimal concentration of infected stimulator cells, which present both virus (or viral antigens) and the appropriate H-2 determinants, is a critical requirement for the induction of 2^{o} lymphoproliferative responses and, therefore, virus alone or in combination with relatively non-permissive spleen cell stimulators is less likely to be effective.

Our results (Table 8) differ somewhat from those reported by Griffin and her associates (9) who have been able to demonstrate significant SV-specific lymphoproliferative responses in vitro only during the 10-day period following primary (1^{o}) or 2^{o} infections. Although these workers added only infectious virus to cultures of primed lymphocytes, it is important to note that the virus-specific inductive event had to take place in vivo; in other words, proliferation in vitro occurred only when lymphocytes were obtained from acutely infected mice or from immune mice 4-5 days after reinfecting them. Thus, rather than virus-specific induction, these findings most likely represent an in vitro amplification of on-going 1^{o} or 2^{o} responses.

Delayed-type hypersensitivity

Groups of outbred ICR mice were primed with either 10^2, 10^4, 10^6, or 10^8 PFU of SV in their left hind foot-pads. Seven days later, their left front foot-pads were challenged with 10^3 PFU of SV while the right foot-pads were given an equivalent dilution of normal mouse brain. No significant SV-specific foot-pad swelling was observed in any group at 24, 48, or 72 hours after challenge. This procedure has not yet been tried in inbred mice.

Cell-mediated cytotoxicity

Several approaches have been taken to demonstrate whether primary SV infection will cause the induction of virus-specific effector lymphocytes that lyse infected target cells in vitro.

BALB/c and C57BL/6 mice were infected with 4×10^4 PFU of SV given in divided doses in all four foot-pads. At 5, 7, and 9 days post-infection, lymph node and spleen cell pools from 3 mice, as well as those from control

mice given normal mouse brain, were cultured at 4 different concentrations for 15 hours with a constant number of ^{51}Cr-labeled normal (control) and SV-infected target cells of the same or a different H-2 haplotype. No virus-specific lysis was observed.

C57BL/6 mice were infected with either 4×10^6, 4×10^4, or 4×10^2 PFU of virus. All 3 doses were administered by either the i.p. intravenous, or foot-pad route making a total of nine infected groups with 5 mice in each. Seven days later, lymph node and spleen cells from each group were assayed separately for SV-specific lytic activity. None was obtained.

C57BL/6 mice were immunized i.p. with 10^7 H-2 identical MC57G cells that were either uninfected, infected with SV, or infected with SV followed by UV-irradiation. Seven days later, lymph node and spleen cells from these and control non-immune mice were assayed for lytic activity. As shown in Table 9 both uninfected and SV-infected target cells were lysed, with only a suggestion of viral specificity exhibited by lymph node cells obtained from animals primed with non-irradiated infected cells. What seems to stand out is the considerably greater lytic activity, against both targets, in lymphoid tissues of mice immunized with SV-infected cells, particularly those that were irradiated. From these limited data, it cannot be determined whether this represents an enhanced immunogenicity of cell membrane (H-2) antigens which were physically modified by SV infection or merely an adjuvant effect of virus on a response directed primarily against non-H-2 "tumor" antigens. Experiments are now in progress that should discriminate between these two possibilities.

Comments

Thus far we have met with some successes and some failures. We attribute the latter to methodological rather than conceptual problems which we expect to resolve. Future work will continue to follow the general research plan set forth in the accompanying renewal application.

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Table I

Comparative Susceptibility of C3H/HeJ and BALB/c Mice to Graded Intracerebral Doses of Sindbis Virus[1]

Mouse strain	Log_{10} virus dose (PFU)	Lethality (%)	Mean survival time (days)	Paralysis (%)
BALB/c ($H\text{-}2^d$)	1.8	20	11	50
	3.8	20	13	100
	5.8	0	-	100
	7.8	0	-	0
C3H/HeJ ($H\text{-}2^k$)	1.8	20	11	50
	3.8	20	13	50
	5.8	80	18	0
	7.8	20	11	0

[1]Mice, 6-8 weeks old, inoculated with 0.03 ml of varying dilutions of NSV-mp6 (5 mice/dose) and observed daily for hind-leg paralysis and/or deaths for 21 days.

Table 2

Sindbis Virus Infection of BHK-21 Cells as Measured by Immunofluorescence (IF) and Cytopathology (CPE)[1]

MOI		Percent infected cells after:				
		5 hr	10 hr	15 hr	20 hr	25 hr
200	IF	5-10	80-90	80-90	75-85	90-100
	CPE	0	0-5	10-20	50-60	90-100
20	IF	10-20	80-90	80-90	75-85	90-100
	CPE	0	0	0-5	10-20	80-90
2	IF	0-5	60-70	80-90	90-100	90-100
	CPE	0	0	0-5	5-15	50-60
.02	IF	0	0-5	10-20	50-60	60-70
	CPE	0	0	0	0	0-5

[1]Sequential observations after infecting cells growing on glass coverslips with varying multiplicities (MOI) of NSV-mp6. At each time point, infected cells were compared with identically prepared uninfected cells.

Table 3

Sindbis Virus Infection of BIOD2 Cells as Measured by Immunofluorescence (IF) and Cytopathology (CPE)[1]

MOI		Percent Infected cells after:			
		7 hr	15 hr	20 hr	25 hr
100	IF	5-10	70-80	80-90	70-80
	CPE	0-5	60-70	80-90	90-100
10	IF	0-5	70-80	85-95	75-85
	CPE	0-5	60-70	85-95	90-100
1	IF	0-1	60-70	85-95	85-95
	CPE	0	20-30	85-95	90-100
.1	IF	0	40-50	85-95	85-95
	CPE	0	20-30	85-95	85-95

[1]Procedures were the same as those described for Table 2.

Table 4

Sindbis Virus Infection of MC57G Cells as Measured by Immunofluorescence (IF) and Cytopathology (CPE)[1]

MOI		Percent infected cells after:					
		8 hr	10 hr	15 hr	20 hr	25 hr	30 hr
600	IF	80-90	85-95	90-100	90-100	90-100	90-100
	CPE	0	0	10-20	20-30	20-30	20-30
60	IF	0-5	10-20	80-90	80-90	90-100	90-100
	CPE	0	0	0	10-20	20-30	40-50
6	IF	0	0-5	45-55	80-90	80-90	95-100
	CPE	0	0	10-20	20-30	70-80	90-100

[1]Procedures were the same as those described for Table 2.

Table 5

Sindbis Virus Infection of L Cells as Measured by Immunofluorescence (IF) and Cytopathology (CPE)[1]

MOI		Percent infected cells after:			
		10 hr	15 hr	20 hr	25 hr
400	IF	0-5	10-15	25-30	25-30
	CPE	0	0	0	0
200	IF	0-5	10-15	15-20	25-30
	CPE	0	0	0	0
20	IF	0-1	0-5	0-5	0-5
	CPE	0	0	0	0
.02	IF	0	0	0	0
	CPE	0	0	0	0

[1]Procedures were the same as those described for Table 2.

Table 6

Lymphoid Hyperplasia in C57BL/6 and BALB/c Mice Acutely Infected with Sindbis Virus[1]

Mouse strain	Lymphocyte source	Increases in splenic and lymph node lymphocytes[2]		
		day 5	day 7	day 9
C57BL/6 ($H\text{-}2^b$)	spleen	1.1	2.0	4.2
	lymph nodes	3.0	4.5	5.2
BALB/c ($H\text{-}2^d$)	spleen	1.0	1.9	4.1
	lymph nodes	2.5	4.0	5.5

[1]Mice, 6-8 weeks old, were inoculated with 10^4 PFU of NSV-mp6 in each footpad (4×10^4 PFU/mouse).

[2]Suspensions of pooled lymph node (popliteal, inguinal, axillary, brachial) cells and spleen cells from 6-8 normal control mice and equal numbers of mice inoculated with virus 5, 7, and 9 days previously were depleted of erythrocytes, counted, and compared. Values given are multiples of the numbers of splenic and lymph node lymphocytes in normal animals.

Table 7

Depressed Responses to Mitogens by Lymphocytes from BALB/c Mice Previously Infected with Sindbis Virus

Log_{10} virus (PFU)	^{3}H-thymidine incorporation in CPM ± S.D.					
	Lymph node			Spleen		
	unstimulated	PHA (4 µg/ml)	LPS (12.5 µg/ml)	unstimulated	PHA (4 µg/ml)	LPS (12.5 µg/ml)
None	1,766 ± 455	24,897 ± 3,178	5,142 ± 729	3,121 ± 567	12,056 ± 1,845	10,242 ± 1,578
5.8	1,240 ± 69	12,082 ± 619	2,463 ± 496	2,793 ± 204	13,639 ± 766	7,746 ± 156
7.8	1,118 ± 56	10,334 ± 1,898	2,651 ± 780	1,427 ± 140	5,133 ± 1,167	4,939 ± 140

[1]Spleen and lymph node pools were obtained from normal mice and mice intracerebrally inoculated 60 days previously with NSV-mp6. Aliquots of lymphocyte suspensions (2×10^5) were cultured in triplicate with or without mitogenic doses of phytohemagglutin (PHA) and E. coli lipopolysaccharide (LPS) for 44 hr then pulsed for 4 hr with ^{3}H-thymidine followed by harvesting and counting for incorporated label. Results are presented as counts per minute (CPM) ± the standard deviation (S.D.).

Table 8

Virus-specific Proliferative Responses by Splenic Lymphocytes from C57BL/6 Mice Previously Infected with Sindbis Virus (SV)[1]

Stimulator	responders / stimulators :	^{3}H-thymidine incorporation in CPM ± SD			
		100	33	10	3
SV.MC57G(UV)		14,197 ± 1,400	22,332 ± 270	32,561 ± 217	1,848 ± 493
MC57G(UV)		4,754 ± 301	6,785 ± 621	8,205 ± 504	3,757 ± 243

[1]Cultures of lymphocytes (responders), each containing 2 x 10^5 cells were prepared from pooled spleens of normal mice and mice infected 60 days previously with 10^4 PFU of NSV-mp6 in all 4 footpads. Added to triplicate cultures of normal and immune responders were varying numbers of syngeneic (H-2^d) MC57G cells (stimulators) which had been UV-irradiated 10 hr after a) infection with NSV-mp6 (MOI=8) or, b) exposure to an equivalent dilution of normal mouse brain. After 68 hr, all cultures were pulsed with 1 µCi of ^{3}H-thymidine for 4 hr and then the cells in each were harvested and counted for incorporated label. No difference in uptake of label (CPM) was seen between normal responders cultured with normal (uninfected) stimulators and thosecultured with infected stimulators (data not shown). Neither type of stimulator, where cultured alone, incorporated significant amounts of label (< 200 CPM). CPM of immune responders cultured in the absence of stimulators was 5,394 ± 692.

Table 9

Induction of Cytotoxic Lymphocytes in C57BL/G Mice Acutely Infected with Sindbis Virus (SV)[1]

Immunogen	Lymphocyte source	E/T ratio[2]	Percent specific lysis[3] of:	
			MC57G	SV.MC57G
SV.MC57G.UV	spleen	40	31.8	35.4
		20	29.3	17.7
	lymph nodes	40	7.9	10.1
		20	5.8	2.7
SV.MC57G	spleen	40	20.9	23.4
		20	13.2	12.8
	lymph nodes	40	9.7	20.6
		20	4.9	15.9
MC57G.UV	spleen	40	0	9.5
		20	0	0
	lymph nodes	40	0	0
		20	0	0
MC57G	spleen	40	6.4	14.2
		20	5.0	10.5
	lymph nodes	40	0	5.1
		20	0	5.4

[1]Mice, 6-8 weeks old, were inoculated intraperitoneally with 10^7 of each immunizing cell preparation. Seven days later, pooled lymph node and spleen cells from 4 mice were measured separately.

[2]Ratio of putative effector cells to a constant number (2×10^4) of target cells.

[3]Values are adjusted for non-specific (spontaneous) isotope release ($\leq$ 35%) occurring in the absence of lymphocytes as described in the text.

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