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Isolation from Mouse Spleen of Cell Populations with High Specific Infectivity for Scrapie Virus

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Spleen cells from mice infected with scrapie virus were separated into subpopulations on the basis of buoyant density in discontinuous gradients of isotonic albumin or differential adherence of cells to plastic. At three different intervals after infection, a population of "less dense" cells was found in albumin gradients that had 40- to 60-fold higher specific infectivity (cells per median lethal dose) than the total cell suspension before gradient sedimentation. The class of cells associated with high relative specific infectivity has a density characteristic of lymphoblasts, myeloblasts, and macrophages. Separation of "macrophage rich" cells on the basis of adherence to plastic did not result in significant enrichment of scrapie virus-infected cells.

Study of the pathogenesis of scrapie in the mouse by Eklund et al. (6) revealed that lymphoreticular tissues are important early sites for multiplication of scrapie virus. The virus is present in spleen and peripheral lymph nodes as early as 4 weeks after subcutaneous inoculation. Maximum titer in these tissues may be reached before virus is detected in the central nervous system. Virus appears similarly early in the spleen after intracerebral inoculation (3, 11). However, replication of scrapie virus in lymphoreticular tissue is not accompanied by any significant histopathologic change. Characterization of virus-infected cells remains difficult because the virus has not been seen in the electron microscope, cytopathic effects have not been observed in tissue culture systems, and immunological responses related to the virus have not yet been found.

The study reported here was undertaken to provide a basis for identification and separation of the lymphoreticular cells that support multiplication of scrapie virus. Its distribution in spleens of infected mice was analyzed by procedures that separate lymphocytic cells into subpopulations on the basis of buoyant density in albumin gradients or differential adherence of cells to plastic.

MATERIALS AND METHODS

Mice. Female mice of the inbred BALB/cAn strain were used throughout. A stock scrapie virus pool was prepared in mice maintained at the National Institutes of Health. All experiments and viral assays were performed with mice obtained from Simonsen Laboratories, Inc., Gilroy, Calif.

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Virus. Scrapie virus (Compton strain) used was obtained from C. J. Gibbs. A homogenate prepared from infected brain from the eighth passage in random-bred Swiss mice was inoculated intracerebrally (ic) into 2-month-old mice. Twenty-four to twenty-eight weeks after inoculation, when clinical signs were evident, the spleens were removed aseptically from 70 animals. A 20% (w/v) homogenate was prepared in a Ten Broeck homogenizer with Dulbecco's phosphate-buffered saline without calcium, magnesium, or antibiotics. The homogenate was sonically treated four times for 5-min intervals in a refrigerated Raytheon sonic oscillator at the maximum amplitude. After sonic treatment, the suspension was centrifuged at 27,000 × *g* for 30 min at 0 C. The pellet was discarded, and the supernatant fluid, which had a virus titer in mice of $10^{5.8}$ median lethal doses (LD_{50})/ml, was stored at −60 C. Another homogenate was prepared in the same way from the spleens of uninfected female BALB/cAn mice. Both preparations were tested for bacterial and mycoplasmal contamination and for the following viruses: pneumonia virus of mice, reovirus, mouse encephalomyelitis, Sendai, K, polyoma, minute virus of mice, mouse adenovirus, mouse hepatitis, lymphocytic choriomeningitis, lactic dehydrogenase, and murine leukemia virus. No evidence of contamination by any of these agents was found.

Preparation of spleen cell suspensions. Forty-two mice, weighing 16 to 18 g, were inoculated ic with 0.03 ml of the 20% homogenate prepared from spleens of infected mice. Control BALB/cAn mice were similarly inoculated with control homogenate prepared from spleens of uninfected mice. At 42, 90, and 117 days after inoculation, six infected and six control mice were killed. Spleens were removed aseptically, placed in 100-mm tissue culture dishes with about 5 ml of Hanks balanced salt solution (BSS), and finely minced with scalpels. Cells and fragments were dispersed by repeated pipetting; single cells and small aggregates were transfered to a chilled centrifuge tube and kept at 4 C.

The procedure was repeated three to four times on the larger pieces of tissue in the dish, with fresh BSS, until most of the fragments were dispersed. Larger fragments were allowed to settle in the tube; the suspension of single cells and smaller aggregates was pipetted through several layers of sterile gauze (10.16 by 10.16 cm) into a second chilled centrifuge tube and sedimented at 200 $\times$ *g* for 10 min at 4 C. The supernatant fluid was decanted, and the pellet was resuspended in 0.5 to 1.0 ml of BSS. A cell count was performed with white blood cell diluting fluid. The average number of cells per spleen from six pooled spleens was in the range of 10^8 to 2×10^8 for both infected and control groups.

Separation of cells in albumin density gradients. A method developed by R. Asofsky (*personal communication*) for the separation of spleen cells in discontinuous gradients of isotonic albumin was employed as follows. Gradients were prepared from a single lot of Path-o-cyte 5 bovine albumin of specific gravity 1.100 (an isotonic solution containing about 35% bovine albumin, Pentex, Inc., Kankakee, Ill.). Dilutions were made in BSS, *p*H 7.2, to give the following concentrations, as per cent of the commercial preparation: 100 (undiluted), 90, 80, 76, 72, 68, 64, and 60%. Average densities, as determined by weighing, ranged between 1.104 g/ml for the 100% solution to 1.050 g/ml for the 60% solution. Discontinuous gradients were made at 4 C in cellulose nitrate tubes (1.27 by 5.08 cm) by layering successively from the bottom 0.5 or 0.6 ml of the albumin solutions, starting with the 100% solution. A suspension containing 3.0×10^8 to 4.0×10^8 cells in 0.5 ml was layered on each gradient. Centrifugation was carried out at 8,000 $\times$ *g* (average) in an SW39 rotor for 30 min at 4 C. Seven fractions were collected at the interfaces with a Beckman fraction recovery system, beginning with the lowest interface. Cells at the upper and lower interfaces of the 60% step were pooled. The cells of each fraction were suspended in 5 ml of BSS and sedimented at 200 $\times$ *g* for 10 min; pellets were resuspended in 0.5 ml BSS, and cell counts were performed. A reconstruction control experiment was performed by adding 6.7×10^4 LD_{50} of scrapie virus (BALB/cAn spleen) to 2.4×10^8 spleen cells from normal, uninoculated BALB/cAn mice. This mixture (0.4 ml) was immediately layered on an albumin gradient, and the cells were treated exactly as described above.

Separation of cells by differential adherence to plastic. "Macrophage rich" and "lymphocytic cell rich" populations of cells (10) were prepared as follows. Infected and control cells (from the total cell suspensions) were added to 100-mm plastic tissue culture dishes with Medium 199 containing 50% fetal bovine serum at a final concentration of 5×10^7 to 20×10^7 cells/dish. These were incubated for 2 hr at 37 C in an atmosphere of 5% CO_2 and 95% air. Medium with unattached cells was then removed and transfered to a second dish. The cells remaining in the first dish were washed three times with Medium 199, and the loosely adherent cells were detached by shaking. Cells that remained attached were designated a "macrophage rich" population. The second dish containing the nonadherent cells was reincubated for 2 hr. Cells that did not adhere during this second incubation were designated a "lymphocytic cell rich" population. The adherent "macrophage rich" cells were scraped from dishes with the aid of a rubber policeman, washed once in BSS, resuspended in 0.5 ml of BSS, and counted. The nonadherent "lymphocytic cell rich" fractions were similarly washed and counted. In a reconstruction experiment, 6.7×10^4 LD_{50} of virus was mixed in dishes with 8.0×10^7 cells from the spleens of uninfected mice at the beginning of the adherence separation.

Infected cell assay. Each of the cell fractions obtained from albumin gradients and adherence separations was adjusted to a concentration of 10^6 cells/ml and diluted 10-fold serially in BSS containing penicillin (100 units/ml), streptomycin (100 μg/ml), and amphotericin B (0.25 μg/ml). Female (BALB/cAn) mice were inoculated ic with 0.03 ml (3×10^4 to 3×10^{-1} cells/mouse), six mice per dilution. The original total spleen cell suspensions were similarly diluted and inoculated into mice. The viral assay was read according to criteria described previously (6), and LD_{50} end points were determined by the method of Spearman and Karber (cited in reference 4).

RESULTS

Recovery and distribution of spleen cells in albumin gradients. Total cell recovery after sedimentation in albumin gradients is summarized in Table 1. With control groups, the recovery of spleen cells was 63 to 100%. Under identical conditions, only 33 to 51% of the spleen cells from scrapie virus-infected mice were recovered.

TABLE 1. *Recoveries from albumin gradients of spleen cells from scrapie virus-infected and control BALB/cAn mice*

Days after inoculation	Mice	No. of cells added/ gradient ($\times 10^8$)	No. of cells recovered/ gradient ($\times 10^8$)	% Recovered from gradient
42	Scrapie	3.3	1.7	51
	Control[a]	3.2	2.0	63
90	Scrapie	3.6	1.2	33
	Control	3.0	2.4	81
117	Scrapie	3.8	1.5	40
	Control	3.2	3.3	100
Reconstruction[b]		2.4	1.5	63

[a] Control mice were inoculated with spleen homogenate prepared from uninfected BALB/cAn mice.

[b] In reconstruction experiment, 6.7×10^4 LD_{50} of scrapie virus was added to normal BALB/cAn spleen cells before gradient centrifugation.

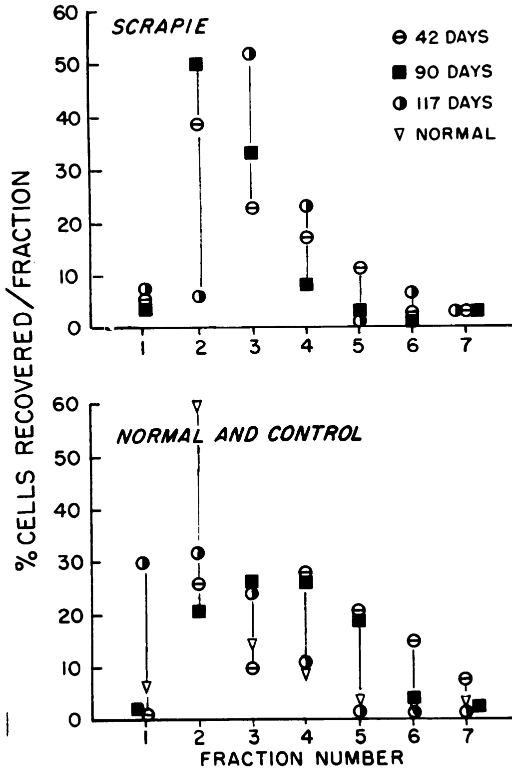

FIG. 1. *Distribution of cells from spleens of BALB/cAn mice separated by density sedimentation in albumin gradients at 42, 90, and 117 days after inoculation. Top: cells from mice inoculated with scrapie virus; bottom: cells from mice that received control inoculum and a group of normal, uninoculated mice. Ordinate: per cent of the total cells recovered from gradient. Fractions are numbered from the bottom of the tube. For total recovered cells (100%), see Table 1.*

The distribution of cells in gradients is shown in Fig. 1. While variations occurred between experiments, the pattern was consistent. Fractions 2 and 3 usually contained the largest numbers of cells. The distribution profiles of cells from infected mice (Fig. 1, top) and a reconstruction experiment (not shown) were similar to the profiles obtained for cells from normal and control mice (Fig. 1, bottom).

Distribution of virus-infected cells in albumin gradients. The recoveries of virus and cells from each fraction, expressed as per cent of total recovered, are shown in Fig. 2. Large proportions of virus were frequently found in more dense fractions that also had large numbers of cells (fractions 2, 3, 4). However, fractions having the highest specific infectivities, in terms of fewest cells/LD_{50}, were found in less dense regions of gradients. From fraction 6 at 42 days and 90 days, only 40 and 50 cells, respectively, were required per LD_{50}. These values represented 60- and 40-fold increases in specific infectivity, respectively, compared with the total spleen cell suspension before centrifugation. A 60-fold increase in specific infectivity was found in fraction 5 at 117 days. A reconstruction experiment revealed little preferential uptake of virus by any single subpopulation of normal cells.

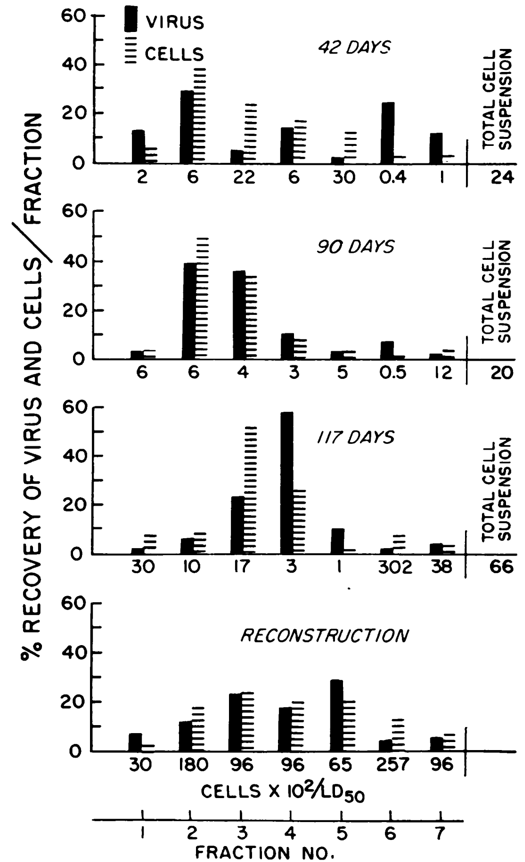

FIG. 2. *Distribution of scrapie virus among populations of cells from spleens of BALB/cAn mice, separated by density sedimentation in albumin gradients, at 42, 90, and 117 days after inoculation of virus. Solid bar, virus; hatched bar, cells. Ordinate, per cent virus and cells of the total recovered from the gradient. The ratio of number of cells $\times 10^2$ per LD_{50} is given below each fraction and on the right for the total cell suspension before centrifugation. Reconstruction: 6.7×10^4 LD_{50} scrapie virus was added in vitro to 2.4×10^8 normal BALB/cAn spleen cells before gradient centrifugation; the calculated cells per LD_{50} for total cell suspension in this instance is 36×10^2. For total recovered cells (100%) see Table 1. Total recovered LD_{50} virus (100%): 42 days, 3.5×10^5; 90 days, 2.6×10^5; 117 days, 1.9×10^5; reconstruction, 1.6×10^4.*

TABLE 2. *Scrapie virus infectivity of "macrophage rich" and "lymphocytic cell rich" populations of cells from spleens of infected BALB/cAn mice*

Days after inoculation	Mice	Cells recovered (%)		No. of cells ($\times 10^2$)/LD_{50} in total cell suspension	No. of cells ($\times 10^2$)/LD_{50}	
		Macrophages	Lymphocytes		Macrophages	Lymphocytes
1	Scrapie	14	44	Negative[a]	Negative	Negative
42	Scrapie	8	55	24	30	66
	Control	12	40			
90	Scrapie	3	32	20	3	10
	Control	5	33			
117	Scrapie	3	35	66	26	65
	Control	12	57			
Reconstruction[b]		12	47	12[c]	Negative	14

[a] Negative = no virus recovered in 3×10^4 cells.
[b] In reconstruction experiment, 6.7×10^4 LD_{50} of scrapie virus was mixed with 8×10^7 normal spleen cells at the beginning of adherence separations.
[c] Calculated value (not determined by infective cell assay).

Recovery of virus-infected cells from "macrophage rich" and "lymphocytic cell rich" fractions. After separation by differential adherence properties, 35 to 69% of the uninfected and scrapie virus-infected cells were recovered. As shown in Table 2, 3 to 14% of these cells were "macrophages." No striking enrichment of virus-infected cells (cells/LD_{50}) resulted from the procedure. (Most of the virus was associated with the "lymphocyte" fraction, which clearly also contained most of the cells.) Virus was not found in 3×10^4 "macrophages" or "lymphocytes" 1 day after infection. In a reconstruction experiment, macrophages failed to take up detectable virus in vitro. It appears, therefore, that macrophages obtained by the method used here had less association with scrapie virus than cells of other types.

DISCUSSION

Separation of cells in density gradients of isotonic albumin was introduced by Leif and Vinograd (7) and further developed by Shortman (14). Modifications of the technique have been successfully applied to the separation of functional classes of mouse spleen cells (2, 13, 15). Separation on the basis of morphologic features is less precise; in general, however, macrophages, large lymphocytes, and blast cells are found in less dense fractions, and small lymphocytes and granulocytes are found in more dense fractions (2, 9; R. Asofsky, *personal communication*).

The present experiments do not distinguish cells that are supporting virus replication from others to which virus has attached. However, high specific infectivity suggests a specific virus-cell relationship, most likely a cell host for virus replication. Fractions of cells having the highest specific infectivities (fewest cells/LD_{50}) were found in less dense regions of albumin gradients. As noted, macrophages and blast cells are among less dense cells. The specific infectivities of macrophages obtained by adherence separations, however, were not great. Blast cells, which are among those synthesizing deoxyribonucleic acid (DNA), have been shown to support multiplication of diverse types of viruses (5, 8, 12, 16, 17). It is possible that scrapie virus multiplies in association with lymphoblasts also. This view is supported by preliminary experiments which have shown that, between 6 and 9 weeks after infection, cells in fraction 6 from scrapie-infected spleens incorporated 10- to 20-fold more tritiated thymidine than this fraction of cells from nomal spleens (Lavelle, *unpublished data*). In this connection, brain cells from scrapie virus-infected mice in vitro are reported to synthesize DNA more actively than cells from uninfected mice (1), which suggests an association of virus with growing cells, or possibly, a consequence of scrapie virus infection.

In these experiments, we measured the specific infectivity of populations of cells rather than the total amount of infectious virus. Redistribution of virus that may have occurred as a result of cell lysis (suggested by the recoveries shown in Table 1) and release of virus from infected cells would introduce artifacts in the pattern of virus-cell association and the relative specific infectivity of each fraction. However, comparison with results obtained from a reconstruction experiment indi-

cates that nonspecific adsorption would tend to diminish the observed differences in specific infectivity between individual fractions. It is likely that some of the differences would be even greater if cell integrity were maintained. The possibility of a cytolytic activity of spleen cells from scrapie virus-infected mice is being examined further.

Comparison of infectivity of intact cells and a cell lysate prepared from each fraction could provide a measure of average virus content in infected cells. Furthermore, relative differences in virus titers between intact and experimentally lysed cells might provide an indication of the maintenance of cell integrity during the fractionation procedure.

The results of density gradient sedimentation studies performed thus far indicate that there is at least one class of cells associated with a high relative specific infectivity of scrapie virus. These cells may be useful in electron microscopic, immunological, and biochemical studies on scrapie virus.

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