Interaction Between 6/94 Virus, a Parainfluenza Type 1 Strain, and Unstimulated Mouse Lymphocytes

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6/94 virus was found to grow in unstimulated splenic mouse lymphocytes depleted of monocyte macrophages. Both egg-grown virus and virus produced in mouse macrophage cultures induced the development of hemadsorption and the appearance of new infectious virus in the lymphocyte cultures. An antigenic relative, Sendai virus, on the contrary, was quickly inactivated in these lymphocyte cultures, in agreement with previous reports in the literature. The T lymphocyte population seemed to be the fraction principally involved in the replication of 6/94 virus. The immunization of mice with 6/94 virus and the appearance of high levels of humoral neutralizing antibodies did not inhibit completely the susceptibility of their lymphocytes to the infectious agent.

The involvement of lymphocytes in both naturally occurring and experimentally induced viral diseases is expressed by morphological changes, alteration in their distribution in body compartments, specific humoral and cellular responses, and the production of some soluble materials that play an important role in immunity (14). It is generally accepted that, although monocyte macrophages contribute to the spread of virus throughout the body, lymphocytes play the principal role in defense against infection. Previous studies have demonstrated that cultures of mixed leukocytes can sometimes permit viral growth, whereas cultures of purified lymphocytes do not support viral replication unless stimulated by mitogens (1, 2, 4, 5, 12, 13). Most observations have been made on cultures of human lymphocytes, and little is known about animal lymphocytes. In the animal lymphocyte studies it has been shown that, although some viruses can replicate in stimulated cultures of mouse lymphocytes (3), other viruses undergo an inactivation faster than that produced by heat in acellular suspensions of virus incubated under the same conditions (16).

This paper reports the interaction of unstimulated mouse lymphocytes with 6/94 virus, a parainfluenza type 1 agent antigenically related to Sendai virus, which was recovered after lysocin-limited-identified fusion of brain cells from two multiple sclerosis patients with CV-1 cells (10).

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MATERIALS AND METHODS

Cell cultures: (i) lymphocytes. Splenic lymphocytes were obtained from inbred C3H and C57BL mice (Jackson Laboratories, Bar Harbor, Maine), weighing about 20 to 25 g. The spleens were removed aseptically and processed by the method of Koprowski et al. (9). RPMI 1640 medium (Flow Laboratories, Rockville, Md.) supplemented with 10% heat-inactivated fetal bovine serum, 50 μg of streptomycin per ml, and 100 U of penicillin per ml was used to maintain recovered cells except as noted. The viability of cell suspensions was determined by either the trypan blue or the erythrocyte B exclusion test. Suspensions of 5 × 10⁶ to 10 × 10⁶ viable spleen cells per ml in RPMI medium were introduced into plastic flasks (Falcon 3024) in the absence of serum. Lack of serum increased the adherence and spreading of the nonlymphocytic population. After 3 to 4 h, the floating cells were removed and reincubated in other plastic flasks overnight with the addition of 10% fetal bovine serum. The floating cells remaining after the second depletion of adherent cells were used for the experiments.

(ii) CV-1 cells. The CV-1 line of African green monkey kidney cells used as the assay system for infectivity titrations and neutralization tests as previously described (11) were grown and maintained in minimal essential medium with the addition of fetal bovine serum and antibiotics as above.

(iii) Separation of T and B lymphocytes. The immunocellular adsorption technique as described by Kedar et al. (8) was used to separate populations enriched in T and B lymphocytes from the spleen cultures obtained as above.

Identification of different kinds of cells: (i) macrophages. Since phagocytosis has purportedly been detected by others in a small percentage of cells supposed to be lymphocytes (6, 7), the α-naphthol acetate test as described by Yam et al. (15) was applied...
to the purified populations of splenic lymphocytes. This cytochemical reaction permits the staining of nonspecific esterases that are present in the cytoplasm of monocyte macrophages but not in lymphoid or myeloid cells.

The presence of blastic forms was morphologically determined by examining the cell smears which had been stained with Giemsa blood stain.

(ii) T lymphocytes. The complement-mediated cytotoxicity test with mouse anti-theta antigen serum was used to identify T lymphocytes. The results were determined by the erythrocyte B exclusion test.

(iii) B lymphocytes. The assay for immune rosette (EA) formation was conducted by the method of Kedar et al. (8).

(iv) Determination of specific cytophilic antibodies. Smears of splenic lymphocytes were fixed with cold acetone and then overlaid with egg-grown 6/94 virus diluted 1:2 in phosphate-buffered saline solution. After 30 min of incubation at 37°C in a moist chamber, the slides were thoroughly washed in phosphate-buffered saline and treated with guinea pig anti-Sendai virus or anti-6/94 virus serum and then with fluorescein-conjugated anti-guinea pig immunoglobulin (Sylvanian), by the standard indirect staining techniques for demonstration of immunofluorescence.

(v) Viruses and infection of cells. The 6/94 virus used was infected allantoic fluids after 5 to 6 passages of the original isolate in embryonated hen eggs or supernatant fluids derived after three to four passages of egg virus in mouse peritoneal macrophage cultures.

A laboratory-maintained strain of Sendai virus designated MN and grown in the allantoic cavity of embryonated hen eggs was used in comparative studies.

Infection was carried out by incubating the cell-virus mixtures at 37°C for 1 to 2 h and discarding the unadsorbed inoculum by a series of centrifugations and washings in phosphate-buffered saline, so that less than 10^3 TCID50 (50% tissue culture infective dose) was calculated to remain in the last supernant fluid. In some experiments, residual virus was neutralized by incubating the washed inoculated cells with specific antisera as described for macrophage infections (11). The dosage of virus used for inoculation varied with the experiments. Both the 6/94 and Sendai virus-inoculated cells were incubated at 32°C.

(vi) Viral assay. Hemagglutination titers and infectious virus (TCID50) production were measured as described previously for macrophage cultures (11).

The total production of infectious virus by lymphocytes was always determined by conducting titrations of sonicated suspensions of the infected cells. Acellular suspensions of virus were incubated and sampled under the same conditions concurrently to test for thermal inactivation of virus. Tests for hemadsorption (HAD) were performed by incubating equal volumes of lymphocyte suspensions with 0.5% guinea pig erythrocytes in phosphate-buffered saline for 45 min at 4°C.

After centrifugation, the supernatant was discarded and the pellet was resuspended in a few drops of 0.1% crystal violet in minimal essential medium. The percentage of cells showing 3 or more attached erythrocytes was determined by scoring 400 cells.

(vii) Immunization of mice and serological tests. The intranasal (i.n.) and intraperitoneal (i.p.) immunization of C3H mice and assays for development of humoral antibodies were as reported in the previous paper (11).

RESULTS

Characteristics of splenic lymphocytes used for the infection. The viability of the cells varied but was generally between 75 and 90%. Less than 1% of the population of splenic lymphocytes twice depleted of adherent cells contained the red-stained esterase cytoplasmic granules, which indicates that contamination with macrophages was minimal.

Expression of new virus in unstimulated lymphocytes. Three hours after infection, i.e., after the antiserum treatment, the cultures infected with egg-grown 6/94 virus were negative for HAD and hemagglutination. However, 24 h after infection a number of HAD-positive cells were found; the numbers depended on the multiplicity of infection (MOI) used and increased with the incubation time. Typical HAD rosettes, clumps of agglutinated lymphocytes, and agglutinated erythrocytes appeared simultaneously, the latter indicating the presence of extracellular virus. Control cultures showed no such reactions. The maximal percentage of HAD-positive cells encountered was about 30 to 40%. The hemagglutination titers of the supernatant fluids were always very low, and never higher than 1:4.

The viability of infected cells was not significantly different from that of uninfected controls sampled up to 96 h postinfection, but it decreased quickly between 4 and 7 days after infection. In Fig. 1, the titers of infectious virus obtained from C3H lymphocytes are plotted against time. In both infected cultures, using different MOI, an increase in titers occurred. Unfortunately, residual virus was not as well neutralized with antiserum at the higher MOI; therefore, the total increase of virus was less evident at the higher than at the lower MOI.

Comparison between 6/94-infected and Sendai virus-infected lymphocytes. Eustatia and Van Der Veen (3) reported that macrophages and lymphocytes from C57BL mice were resistant to Sendai infection, although phytohemagglutin-stimulated C57BL lymphocytes could be thus infected. We found that macrophages from the same mouse strain were only partially resistant to infection with 6/94 virus. Consequently, it was of interest to compare the susceptibility of unstimulated lymphocytes from C57BL mice to infection with 6/94 and Sendai viruses. An MOI of 1 was used for the lymphocyte infections with each virus. Sendai virus was completely inactivated by these cells in 4 days. On the other hand, 6/94 virus showed a log 1
increase in 2 days; then the titers decreased but the virus level at 4 days was still slightly higher than in the beginning (Fig. 2). There was no difference in the viability of Sendai and 6/94 virus-infected cell cultures for the duration of the experiment. Examination of the thermal inactivation rate of an acellular suspension of 6/94 virus revealed that the rate was directly related to the concentration of virus initially present: i.e., starting with 7 logs of virus, a 3 log loss occurred in 4.5 days; starting with 5 logs of virus, it took 3.0 days to produce the same 3 logs of inactivation. Thus, starting with 1.5 logs of virus at 3 h after infection (Fig. 2), one could not expect to find any active virus present after 2 to 4 days of incubation.

6/94 virus obtained after three and four passages in C3H macrophage cultures was also found to replicate in homologous lymphocytes.

Infection of purified populations of lymphocytes. To determine which lymphocyte population (T and/or B) supported viral replication, populations enriched for either T or B lymphocytes from C3H mice were infected with 6/94 egg-grown virus. Table 1 presents the composition of unseparated as well as of enriched populations. In Fig. 3, titers of infectious virus produced by the three populations of lymphocytes as measured in CV-1 cells are plotted against time. The titers obtained in the T-enriched population were somewhat higher than those produced by the unseparated lymphocytes. The level of virus produced by the B-enriched lymphocytes was the lowest and was significantly less than that produced by T-enriched lymphocytes.

Susceptibility of lymphocytes from immunized mice to infection. The immunization of mice with 6/94 virus and their humoral antibody responses as well as the susceptibility of their macrophages to infection with the same virus have been reported (11). Lymphocytes from the same immunized and normal mice were

FIG. 1. Production of infectious virus in unstimulated mouse lymphocytes infected with 6/94 virus. Symbols: □, C3H lymphocytes, initial viability 85%, MOI = 5; ■, C3H lymphocytes, initial viability 90%, MOI = 1.

FIG. 2. Production of infectious virus in unstimulated C57BL lymphocytes (initial viability, 80%). Symbols: ●, 6/94 virus, MOI = 1; ○, Sendai virus, MOI = 1.

TABLE 1. Composition of splenic lymphocytes before and after separation

<table>
<thead>
<tr>
<th>Lymphocytes</th>
<th>% B cells</th>
<th>% T cells</th>
</tr>
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<tbody>
<tr>
<td>Unseparated</td>
<td>24</td>
<td>52 ± 6</td>
</tr>
<tr>
<td>T enriched</td>
<td>2</td>
<td>92 ± 5</td>
</tr>
<tr>
<td>B enriched</td>
<td>68</td>
<td>ND</td>
</tr>
</tbody>
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* Values were determined by the method of Kedar et al. (8). ND, Not determined.
infected with 6/94 egg-grown virus. Figure 4 gives the titers of infectious virus produced in lymphocytes derived from mice that received an initial inoculation of 10^4 or 10^6 TCID_{50} i.n. or 10^6 TCID_{50} i.p. of 6/94 virus followed by a booster inoculation of the same amount of virus 30 days later. Lymphocytes harvested 10 days after the booster were infected with virus at an MOI of 5. In this experiment, residual virus was not neutralized with antiserum. It is believed that the decreases in titers observed at 24 h reflect, in part, thermal inactivation of such residual virus. Contrary to the findings in control cells, no viral increase occurred in any of the lymphocytes from the immunized animals until after the third day of incubation. By day 5, almost the same amount of virus was detectable in cells from the mice initially immunized i.n. as in the controls. However, at this time the cells from animals immunized i.p. showed no significant increase in detectable virus.

Uninfected cells from the group of i.p. immunized mice were tested for the presence of specific cytophilic antibodies after 5 days of cultivation in vitro. Immunofluorescence testing revealed bright spots on the membrane surface in 3 to 8% of the cells. Lymphocytes from nonimmunized mice were always negative.

Examination of stained smears of lymphocytes from any of the immunized groups of mice after infection with 6/94 virus revealed that blast transformation was present. Thus, at 6 days after infection, 23% of the cells were blast forms as opposed to 5% found in similarly infected lymphocyte cultures from normal mice. Many of these blast forms were positive for HAD. Whether the increase in blast forms was induced in vivo by the booster inoculation or in vitro is not known, since a search for such forms was not conducted before virus inoculation of the cells in vitro.

**DISCUSSION**

Growth of 6/94 virus was observed in cultures of unstimulated lymphocytes from both C3H and C57BL normal mice. This finding points to another significant biological difference between 6/94 virus and Sendai virus, a close antigenic relative. Zisman and Denman (16) found not only that mouse lymphocytes could not support growth of Sendai virus, but that they inactivated the virus. Our results with Sendai virus are in agreement with these authors. In this experiment, lymphocytes from C57BL mice were used. Although they were susceptible to 6/94 infection, they were less susceptible than lymphocytes derived from C3H mice. It is of interest that macrophages from these two inbred strains also showed the same differences in susceptibility to 6/94 infection (11).

Exposure of lymphocytes to 6/94 virus did not

![FIG. 3. Production of infectious virus in unstimulated C3H T and B lymphocytes (initial viability, 90%; MOI = 5). Symbols: ●, unseparated population; □, T-enriched population; ○, B-enriched population.](image1)

![FIG. 4. Production of infectious virus in unstimulated C3H immune lymphocytes. Cell viability equaled 75 to 80%; MOI = 5. Symbols: ●, controls; △, high dose i.n. (10^4 TCID_{50}); ○, low dose i.n. (10^4 TCID_{50}); ▲, high dose i.p. (10^4 TCID_{50}).](image2)
initially affect cell viability, for cells only began to die 96 h after infection. The number of cells producing new infectious virus was always lower in the lymphocyte than in the macrophage cultures (11) derived from the same mouse strains. For example, if the percentage of cells showing positive HAD is taken as an index of the numbers of cells producing virus in macrophage cultures, nearly 100% of cells became positive as previously described, whereas in lymphocyte cultures 30 to 40% became HAD positive under optimal conditions. These results imply that not all of the lymphocytes were able to support virus replication. Whether the cells which did not develop HAD reflect a complete resistance to infection or a lack of ability to express virus, we cannot say.

The significantly higher titers of infectious virus produced in T-enriched than in B-enriched populations suggest that the former are mainly responsible for virus growth. However, since the number of T cells contaminating our B-enriched population was sufficient to produce the amount of virus detected, it is impossible to establish the degree of susceptibility of B cells to the 6/94 virus.

Although no replication of virus could be detected for the first 3 days in cells from animals immunized initially either i.n. or i.p., by day 5 almost as much virus was being produced in cells from the i.n.-immunized groups as in control cells. As with macrophage cultures, the most resistant lymphocytes were those from the i.p. group. The delayed or decreased production, or both, of infectious virus in all of the immune cell cultures might be explained partially by the neutralization of extracellular virus by cytophilic antibodies which were detected on the surface of some cells. At an MOI of 5, such antibodies might not have been sufficient to neutralize all of the virions so that a few might still have been available for infection, especially of the T cell population. On the other hand, since the presence of cytophilic antibodies was detected in only about 8% of the cells in the most resistant cultures, part of the resistance might be cell mediated. The discovery of increased blast formation in immune cells exposed to 6/94 virus would support this explanation. However, these blast forms also showed many HAD-positive cells, which suggests that lymphocytes with immunological memory can at the same time express the presence of virus on their membrane surface.

The demonstration by studies in vitro that 6/94 virus produced by macrophages can infect homologous lymphocytes provides additional information on the interaction between this virus and cells of or related to the immune system.

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LITERATURE CITED