IMMUNOSUPPRESSIVE ACTIVITY OF A SUBLINE OF THE MOUSE EL-4 LYMPHOMA
Evidence for Minute Virus of Mice Causing the Inhibition*

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A variety of murine transplantable tumors have been found to be immunosuppressive. In the case of viral-induced neoplasms, immunosuppression could often be related to the effects of the corresponding oncogeneic type C virus (1-3). In the case of chemically induced or spontaneous neoplasms, the concept has been raised that tumor cells, or their metabolic soluble products, were immunosuppressive (4-6). In a previous study, a subline of EL-4 lymphoma, EL-4(G-), was shown to have strong inhibitory effects on allogeneic mixed lymphocyte cultures (MLC)2 in vitro. EL-4 is a chemically induced, thymus-derived transplantable tumor of C57BL mice (7, 8), which contains endogenous type C viruses (reference 9 and footnote 3), and is also contaminated by common murine viruses.

In the present study, we report on our findings of common virus contamination of the EL-4(G-) subline maintained in our laboratory. We have accumulated evidence consistent with the hypothesis that the inhibitory activity of EL-4(G-) ascites cells and the culture fluid was mediated by an infectious virus. At the present time, the best candidate for this activity is a variant of minute virus of mice (MVM).

Materials and Methods
Mice. Male BALB/c and C57BL/6 mice, 5- to 7-wk old, and pregnant NIH Swiss mice were obtained from the Animal Production Unit, National Institutes of Health (NIH), Bethesda, Md.

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1 Bonnard, G. D., and R. B. Herberman. 1975. Immunosuppressive effects of a subline of mouse EL-4 lymphoma on in vitro allogeneic reactions. Manuscript submitted for publication.
2 Abbreviations used in this study: CF: complement fixation; HI, hemagglutination inhibition; LDV, lactic dehydrogenase virus; LMC, lymphocyte-mediated cytotoxicity; MAP test, mouse antibody production test; MLC, mixed lymphocyte culture; MVM, minute virus of mice.
3 Aoki, T., R. B. Herberman, J. W. Hartley, M. Liu, M. J. Walling, and M. Nunn. Surface antigens on transplantable tumor cell lines producing mouse endogenous type C viruses. Manuscript submitted for publication.
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Male NIH Swiss mice, 3-wk old, for the mouse antibody production tests, were from the colony raised at Microbiological Associates, Bethesda, Md.

Tumor System. EL-4 is a thymus-derived, benzopyrene-induced lymphoma of C57BL mice, which has been carried as a transplantable ascitic tumor for many years (7, 8). According to the presence or absence of serologically detectable Gross cell surface antigen, we distinguish in this laboratory an EL-4(G+) and an EL-4(G-) subline (9). Two tissue culture lines of EL-4(G-), A and B, and one EL-4(G+) tissue culture line, were initiated at the onset of this study, and then maintained as described elsewhere. Each of the EL-4 ascites tumor sublines and tissue culture lines was repeatedly tested and found free of contamination by mycoplasma.

Ascitic Fluid. Ascitic fluids were harvested, 5–7 days after tumor inoculation, in 10 ml Hanks' balanced salt solution. This, depending on the volume of the ascites, resulted in an approximate 1:5 dilution. Tumor cells were removed by centrifugation at 150 g for 10 min, and the cell-free fluids were filtered through a 0.22 μm membrane (Nalge Co., Nalgene Labware Div., Rochester, N. Y.) and kept at 4°C for up to 8 wk.

Harvest and Treatments of the Tissue Culture Fluids. Early in vitro passages of both EL-4(G-) tissue culture lines A and B were fed daily. Culture fluids were harvested from many flasks and pooled in 500-ml aliquots, between the 13th day and the 25th day in vitro. Later passages of the EL-4(G-) tissue culture lines A and B were fed every 3 days, thus resulting in a considerably lower rate of growth. After centrifugation to remove tissue culture cells, all culture fluids were further filtered through 0.22 μm membranes. The culture fluids were numbered chronologically at the time of harvest. After the initial screening of the effects of these culture fluids, only the culture fluid no. 4 was studied in depth. 10-ml samples of culture fluid no. 4 were dialyzed three times against 500 ml of phosphate-buffered saline, pH 7.4, at 4°C. The material within the dialysis bag was used at the same dilutions and in parallel with the nondialyzed culture fluid. 10-ml samples of culture fluid were ultracentrifuged at 100,000 g for 1 h at 4°C (Type 50 rotor, Model L ultracentrifuge, Beckman Instruments, Inc., Fullerton, Calif.). Pellets were resuspended in 3 ml of Hanks' balanced salt solution and they and the supernate were stored at 4°C until use. 2-ml samples of culture fluid were treated with UV irradiation. This was performed by rocking the samples in a watchglass at a distance of 27.6 cm from two 15 W germicidal tubes, thus providing 67 electroretinograms/mm²/s. 1.8 ml culture fluid, or culture medium as control, were mixed with 0.2 ml chloroform (Mallinckrodt Chemical Works, St. Louis, Mo.) for 10 min at 22°C, then centrifuged 10 min at 150 g, at 4°C, and the aqueous phase overlying the chloroform was harvested.

Inhibition of MLC with Culture Fluid. For demonstrating inhibition, 1 ml of culture fluid or treated culture fluid was added to MLC (see below) at their onset; this resulted in a 1:5 final dilution. Higher final dilutions were also used, as indicated.

Detection of Lactic Dehydrogenase Virus (LDV Test). For detection of LDV (10, 11) in various materials from EL-4(G-) origin, three weanling male NIH Swiss mice were inoculated, 0.05 ml intraperitoneally and 0.03 ml intracranially, with a 1:10 dilution of the test specimen. Controls included three mice inoculated with LDV and three mice injected with diluent only. Mice were bled from the retroorbital sinus after 3 days and the plasma was tested for an increase in the lactic dehydrogenase enzyme level, as compared to the negative controls.

Mouse Antibody Production Tests (MAP Test). The testing of various materials for contamination with 11 further common murine viruses, indicated in the legend of Table III, was performed by the MAP test (12), as described in detail elsewhere (10). The EL-4(G-) ascites tumor cells were washed twice in Hanks' balanced salt solution and resuspended at 2 × 10⁸ cells/ml. The EL-4(G-) tissue culture cells were from the line A after 246 days in culture. The cells were washed twice and resuspended in Hanks' balanced salt solution at a concentration of 2 × 10⁶ cells/ml. They were representative of the later passages of EL-4(G-) tissue culture cells, which had lost the inhibitory capacity on MLC (Table I). Each material to be tested was inoculated to four weanling male NIH Swiss mice both intranasally and intraperitoneally. As a control for inoculation with each material, four littermates were injected with sterile, fresh tissue culture medium. 4 wk later, mice were bled under sterile conditions, from the retroorbital sinus. The sera were collected individually, diluted 1:5 in phosphate-buffered saline, heated at 56°C for 30 min, and tested, as reported earlier (13). Either the hemagglutination inhibition (HI) or complement fixation (CF) techniques were used for detection of antibodies against the panel of 11 common murine viruses as explained in the legend of Table III. Then the sera were pooled for each group of four mice and stored at −75°C for further use.
Kilham Rat Virus. A cesium chloride density gradient purified preparation of Kilham rat virus (14), which has been shown in our laboratory to have profound inhibitory effects on the proliferative responses of rat lymphocytes in vitro, was used at similar concentrations and tested for its ability to inhibit the cytotoxic response in mouse MLC.

Prototype MVM and Polyoma Virus. The original strain of MVM was a gift from Dr. L. V. Crawford, Institute of Virology, Glasgow, Scotland (15). The production of MVM pools was performed as described earlier on either mouse or rat embryo tissue culture cells (16, 17). These MVM pools have been used extensively as prototype MVM; so far, contamination by other viruses has not been detected. In the present study, five different pools of MVM were used, two as virus pools (no. 1 and no. 2), and three to raise the reference anti-MVM immune sera. The original strain of polyoma virus was a gift from Dr. W. L. Rowe, Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, Md. (12). The production of pools of polyoma virus was achieved as described earlier (13). In this study, three different pools were used to raise the reference antipolyoma virus immune sera. Each fresh pool of virus was tested at Microbiological Associates, Bethesda, Md., for purity by the LDV and MAP tests. Furthermore, hemagglutination titers were measured and infectivity, both in vivo and in vitro, was established for each pool. The pools were cryopreserved at -75°C until use.

Mouse Reference Anti-MVM or Antipolyoma Virus Immune Sera. The reference immune sera were raised with the viruses described above. These immune sera were tested serologically (10, 13) against the panel of viruses given in the legend of Table III. All reference immune sera have been extensively used as diagnostic reagents for MVM and polyoma virus, respectively, at Microbiological Associates, for screening biological materials for many investigators in the Virus Cancer Program. They did not appear to have any detectable activity against other viruses in the diagnostic panel. The HI titers of these sera were as follows: anti-MVM immune serum no. 7, 1:640; no. 8, 1:80; and no. 9, 1:2,560; antipolyoma virus immune serum no. 10, 1:1,280; no. 11, 1:512; and no. 12, 1:320.

Neutralization Assays. Ascitic fluids, immune sera, or control sera were tested for their ability to neutralize various inhibitory materials, usually culture fluid or EL-4(G-) ascites cells. Typically, 0.1 ml of inhibitory material was incubated for 1 h at 22°C with various volumes of ascitic fluid preparations or with 0.1 ml of various dilutions (1:10–1:1,000) of immune or control sera. The stimulatory cells were then added and the mixtures immediately transferred to the responding cells in the culture flasks (see below). Final dilutions of ascitic fluid in the MLC have been calculated, whereas dilutions of antisera used in the neutralization assay are given, and should be divided by a factor of 50 to obtain the final dilutions in MLC.

In Vitro MLC Technique. The technique of MLC was as described elsewhere.1 For each experiment, triplicate MLC with 5 × 10^6 BALB/c responding spleen cells and 2 × 10^8 C57BL/6 X-irradiated stimulating spleen cells in 4.5 ml medium served as positive control. One culture with 5 × 10^6 BALB/c spleen cells alone in 4.5 ml medium served as negative control. The experimental cultures were performed by adding various materials, usually culture fluid and/or sera, in various final dilutions to MLC. The cultures were incubated in standing 30 ml flasks and then harvested for the assessment of the generation of cytotoxic lymphocytes.

Lymphocyte-Mediated Cytotoxicity (LMC). Cytotoxicity was measured in 4-h 51Cr-release assays as described elsewhere.1, 3, 4, 5. One ml of effector cell suspension from the MLC were tested in duplicate against 2 × 10^4 51Cr-labeled RBL-5 ascites target cells, a C57BL/6 Rauscher virus-induced leukemia. Thus, the ratios of effector cells to target cells, based on the number of responding lymphocytes initially present in the MLC, (lymphocyte to target cell ratios) were 50:1, 20:1, and 5:1, respectively. The reaction mixtures were all made up to 2 ml with medium. The values of LMC are expressed as percentages of 51Cr release compared to the total 51Cr label of the target cells. The spontaneous release occurring in medium alone was always less than 10%, and this was subtracted from the experimental percent release. The standard errors of the means between duplicates rarely exceeded 3% and are not given in the results.

Results

Inhibitory Activity in Tissue Culture Fluids from EL-4(G-) Cells, Stored at 4°C or -75°C. In the previous study,1 we reported on the strong inhibitory activities of EL-4(G-) ascites cells, and culture fluids from the early in vitro...
passages of the EL-4(G-) tissue culture cells, on the proliferative and cytotoxic responses in MLC. It was important to study the stability of the inhibitory activity of the culture fluids, since consistently strong effects were needed for detailed biophysical and/or virological characterization. The culture fluids were stored in aliquots at 4°C and -75°C, and tested several times. In the typical experiment shown in Fig. 1 A, 1 ml of culture fluids stored for 11 mo at 4°C were added to BALB/c vs. C57BL/6 MLC at the onset of culture. As initially observed,1 strong inhibitory activity was present in early culture fluids. Culture fluid no. 3, from a later passage of the EL-4(G-) tissue culture line A, was virtually noninhibitory. Similar results were repeatedly obtained with the culture fluids stored at either 4°C or -75°C. In subsequent experiments, we used culture fluid no. 4, which had been harvested from EL-4(G-) tissue culture line B after 13 days in culture and was stored at -75°C. This will be subsequently referred to as "the culture fluid." The effects of the culture fluid on the cytotoxic response in MLC were strictly dose dependent, with increasing inhibition of cytotoxicity by increasing concentrations of added culture fluid (Fig. 1B). According to a simple calculation, these data revealed further that, within the range of doses tested (1:5-1:500 final dilution of culture fluid), a 10-fold increase in the concentration of the culture fluid resulted in approximately a 10-fold reduction in the number of effector cells.

Inhibitory activity could no longer be found in culture fluids of later passages of the EL-4(G-) tissue culture lines A and B (Table I). Further investigations were then performed with the culture fluid no. 4 to determine the nature of the inhibitory activity and its relationship to the inhibition by the tumor cells themselves.1

Biophysical Properties of the Inhibitory Factor in the Culture Fluid. The inhibitory activity of the culture fluid was resistant to at least four cycles of freezing and thawing in alternate alcohol-Dry Ice and 37°C water baths. It was resistant to 56°C for 1 h at least, but its activity was lost after 20 min at 100°C. All of the culture fluids tested had been passed through a 0.22 μm filter before use. Further filtration through a 0.10 μm filter had only a slight effect on the activity. When the culture fluid was dialyzed against phosphate-buffered saline, the material retained in the dialysis bag was practically as active as the original material at inhibiting a BALB/c vs. C57BL/6 MLC.

The culture fluid was then ultracentrifuged at 100,000 g for 1 h. The supernate was harvested and filtered through a 0.22 μm filter. The pellet was resuspended in one-third of the original volume in Hanks' balanced salt solution, and filtered. The effects of these materials on the MLC are shown in Fig. 2. The 100,000 g supernate had lost most of the inhibitory activity, whereas the pellet had strong activity.

The culture fluid was also exposed to UV irradiation and tested for residual inhibitory activity (Fig. 3). The UV-irradiated material had lost the inhibitory activity. Taken together, the biophysical characteristics above were compatible with the inhibitory factor being a virus.

The resistance of the inhibitory factor to chloroform was tested to determine whether the agent had a lipid envelope as in the case of mycoplasma or
enveloped viruses (18). The results of these experiments are shown in Table II. The inhibitory factor was clearly resistant to chloroform treatment.

**Evidence for Infectivity of the Inhibitory Factor.** Since culture fluids of later passages of EL-4(G−) tissue culture cells had been consistently noninhibitory in MLC, we performed experiments to determine whether these cells were susceptible to infection with the inhibitory factor. In a typical experiment, 2 ml of the culture fluid no. 4 (or 2 ml of medium as control) were added to 8 ml of medium containing 4 × 10^6 cells of a late passage of EL-4(G−) tissue culture line A. The addition of the culture fluid had no effect on the rate of growth of the cells, as assessed by regular cell counts and [3H]thymidine pulses. The cells were spun down and resuspended at 5 × 10^6/ml in fresh medium on days 3, 8, and 12, and these secondary culture fluids were tested in MLC (Fig. 4). Secondary culture fluids harvested at 8 and 12 days were more strongly inhibitory than the culture fluid no. 4. A 1:500 dilution of each secondary fluid produced almost as much inhibition as 10-fold more of the original culture fluid. It clearly appeared that the EL-4(G−) cells, upon inoculation with the inhibitory culture fluid, started anew to produce an inhibitory factor, presumably the same as that in the original culture fluid. This was consistent with the possibility of a reinfection of the later passages of EL-4(G−) cells by an immunosuppressive virus. In further experiments, we found that these infected EL-4(G−) cells themselves were strongly inhibitory, whereas the uninfected culture cells tested in parallel were not.

*Detection of Viruses in the Culture Fluid by LDV and MAP Tests.* In yet
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Table I
Absence of Inhibitory Activity in Culture Fluids of Later Passages of the EL-4(G−) Tissue Culture Lines A and B

| Culture fluid number | EL-4(G−) line | Time in culture | LMC at the lymphocyte/target cell ratios |
|----------------------|----------------|----------------|------------------------------------------|
|                      |                | days           | 20/1 | 60/1 |
| None                 | —              | —              | 33.7 | 53.6 |
| 1                    | A              | 15             | 2.1  | 7.4  |
| 9                    | A              | 41             | 40.4 | 64.2 |
| 12                   | A              | 69             | 30.6 | 53.8 |
| 4                    | B              | 13             | 1.1  | 4.5  |
| 6                    | B              | 19             | 22.4 | 46.8 |
| 7                    | B              | 19             | 40.3 | 64.2 |
| 8                    | B              | 28             | 34.7 | 56.8 |
| 10                   | B              | 56             | 41.1 | 59.4 |
| 11                   | B              | 57             | 40.2 | 59.9 |
| 13                   | B              | 145            | 31.3 | 56.7 |

The following cultures were set up and harvested on day 6 for LMC at two different lymphocyte to target cell ratios: (a) BALB/c vs. C57BL/6 MLC (triplicate cultures). (b) MLC with addition of various culture fluids at the 1:5 final dilution.

![Graph](image)

**Fig. 2.** Inhibitory activities of 100,000g supernate and pellet of culture fluid no. 4. Percent LMC were determined at the 60/1 lymphocyte to target cell ratio for BALB/c cells cultured alone (□), the MLC (●), the MLC in presence of various dilutions of culture fluid (○—○), 100,000g supernate (●—●), or 100,000g pellet (●—●). The pellet had been resuspended in a third of the original volume before testing.

Another approach to detect viral contamination, various materials used in the present or earlier experiments were tested for LDV, and screened in the MAP test for the presence of 11 common murine viruses (Table III). Polyoma virus and MVM were found in the culture fluid when tested 7 mo after harvesting. The EL-4(G−) ascites tumor was also contaminated with polyoma virus and MVM;
Reciprocal of the Final Dilution of Culture Fluid

FIG. 3. Effect of UV irradiation on inhibitory activity of the culture fluid. Percent LMC were determined at the 60/1 lymphocyte to target cell ratio, for BALB/c cells cultured alone (□), the BALB/c vs. C57BL/6 MLC (●), and MLC in presence of various dilutions of the culture fluid (○—○) or UV-irradiated culture fluid (●—●).

in addition, LDV was also detected. In contrast, later passages of the EL-4(G-) tissue culture line A did not appear to have any of these viruses. Since the culture fluid and the EL-4(G-) ascites cells were consistently inhibitory, contamination with MVM and polyoma virus correlated with their inhibitory activity. However, the role of other viruses could not be excluded on this basis.

Neutralization of the Inhibitory Factor by Ascitic Fluid from Some Mice Bearing EL-4(G-) Ascites Tumors. In preliminary experiments, we found that cell-free ascitic fluids from EL-4(G-) tumor-bearing mice, as opposed to the culture fluid, had no inhibitory activity on MLC. It was conceivable then that ascitic fluids, which were harvested 5–8 days after tumor inoculation, might already contain detectable levels of antibodies to the inhibitory factor from EL-4(G-) cells. If so, serological analysis of ascitic fluids might provide information on the inhibitory factor. We therefore performed HI or CF serological tests on 20 consecutive ascitic fluids from EL-4(G-) or EL-4(G+) ascites, from which the tumor cells were used in experiments reported earlier.1 These ascites fluids contained no detectable HI antibodies against polyoma virus or MVM; antibodies against mouse hepatitis virus and/or Sendai virus were present in only a few ascitic fluids, but this did not correlate at all with prior injection of the inhibitory EL-4(G-) ascites cells. Experiments were then performed in which various amounts of ascitic fluid were mixed with 0.1 ml of the culture fluid, incubated for 1 h at 22°C, and then added to the BALB/c vs. C57BL/6 MLC (Fig. 5). The results indicated that many ascitic fluids of EL-4(G-) mice could counteract the inhibitory factor found in the culture fluid. These data were consistent with, but did not prove, the hypothesis that the ascites fluid contained neutralizing antibodies against the inhibitory factor.

Neutralization of the Inhibitory Factor by the Immune Sera from the MAP Test. We performed further neutralization tests with the pooled sera of the three groups of mice which had been immunized for the MAP test (see Table III). The pooled normal sera of the nonimmunized littermates, which were controls
**Table II**

*Effect of Chloroform on the Inhibitory Activity of the Culture Fluid*

| Addition to MLC | Final dilution | LMC in MLC with culture fluid or control medium |
|-----------------|----------------|-----------------------------------------------|
|                 |                | Untreated | Treated with chloroform |
| None            | —              | 81.9      | —                             |
| Culture fluid   | 1:5            | ND*       | 0.1                           |
|                 | 1:15           | 3.2       | 0.4                           |
|                 | 1:50           | 7.3       | 7.8                           |
|                 | 1:150          | 70.1      | 46.8                          |
|                 | 1:500          | 81.4      | 70.9                          |
| Medium          | 1:5            | 82.0      | 0.0                           |
|                 | 1:15           | 81.7      | 79.1                          |
|                 | 1:50           | ND        | 81.7                          |
|                 | 1:150          | ND        | 80.1                          |
|                 | 1:500          | ND        | 78.8                          |

The following cultures were set up and harvested on day 6 for LMC at the 60/1 lymphocyte/target cell ratio. (a) BALB/c vs. C57BL/6 MLC (triplicate cultures). (b) MLC with the addition of culture fluid or control medium, untreated or previously treated with chloroform.

* ND, not done.

**Fig. 4.** Inhibitory activities of the culture fluid (○—○), and of two secondary culture fluids harvested 8 days (○—○), and 12 days (○—○) after inoculation of EL-4(G−) tissue culture cells with the culture fluid no. 4. Percent LMC at the 60/1 lymphocyte to target cell ratio for the BALB/c cells cultured alone (□), the BALB/c vs. C57BL/6 MLC (■), and MLC in presence of the indicated dilutions of the three culture fluids.

in the MAP test and had no activity against any virus in the panel, were also tested. None of the sera, when added alone at dilutions of 1:500 or more, were found to have any effects on the BALB/c vs. C57BL/6 MLC. The sera were then
TABLE III
Viral Contamination of the Culture Fluid, EL-4(G-) Ascites and Tissue Culture Cells, as Assessed in the LDV and MAP Tests

| Date of sampling* | EL-4(G-) derived material tested | Inhibition of MLC | LDV test† | MAP test§ | Sera pool no.¶ |
|-----------------|---------------------------------|------------------|-----------|------------|----------------|
| 07/09/74        | Culture fluid                   | +                | -         | 160        | 160 1          |
| 02/15/75        | Ascites cells                   | +                | +         | 160        | 320 2          |
| 02/15/75        | Tissue culture cells, Line A    | -                | -         | <10        | <10 3          |

* All materials were tested, by inoculation of mice, on 02/15/75.
† Tests for LDV were carried out as described and the plasma tested for a 5- to 10-fold increase in the enzyme lactic dehydrogenase (+) or normal level of this enzyme (-).
§ MAP tests were performed as described, and the sera tested against the following antigens. By HI: pneumonia virus of mice, K virus, reovirus type 3, Theiler's encephalomyelitis virus, polyoma virus, MVM, and ectromelia virus. By CF: Sendai virus, mouse adenovirus, mouse hepatitis virus, and lymphocytic choriomeningitis virus. All tests were negative, except those with polyoma virus and MVM, which are shown. All tests with control sera were negative.
¶ Once pooled, sera were given an arbitrary number, in the chronological order of their use. From the four individual mouse sera tested, the reciprocal of the second highest HI titer is reported. Titers in the three other mice were the same, or did not differ by more than one dilution. The individual sera were subsequently pooled.

Neutralization of the Inhibitory Factor by Anti-MVM Immune Sera. Experiments were performed in order to assess the neutralizing effects of mouse reference antipolyoma virus or anti-MVM immune sera (Table V). Each of the three different reference anti-MVM sera, but none of the reference antipolyoma virus sera, was able to neutralize the inhibitory effects of the culture fluid. The results indicated that inhibitory culture fluid could be neutralized by specific antibodies. This neutralizing activity was found in reference anti-MVM immune sera and could be directed against either MVM, or against an unidentified contaminant of the prototype MVM pools used to raise the reference sera.

Identity between the Inhibitory Factors in EL-4(G-) Cells and in the Culture Fluid. The above data indicated that a virus, neutralized by reference anti-
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Reciprocal of the Final Dilution of Ascitic Fluid

**Fig. 5.** Neutralization of the inhibitory activity of the culture fluid by ascitic fluid from mice bearing EL-4(G-) tumor. Percent LMC at the 60/1 lymphocyte to target cell ratio, for the BALB/c vs. C57BL/6 MLC (○), the MLC with a 1:50 dilution of the culture fluid (□), MLC with culture fluid preincubated with various doses of ascitic fluid (▲—▲), and MLC with various doses of ascitic fluid (△—△).

**TABLE IV**

| Serum no. | EL-4(G-) derived material used to inoculate mice | Known antiviral activity | Dilutions of the sera used in the neutralization phase |
|-----------|-----------------------------------------------|--------------------------|---------------------------------------------------|
|           |                                               |                          | 1:10     | 1:100    | 1:1,000   |
| 1         | Culture fluid                                 | Polyoma, MVM             | 77.8*    | 71.7     | 42.3      |
| 2         | Ascites cells                                 | Polyoma, MVM             | 75.3     | 78.2     | 77.0      |
| 3         | Tissue culture cells, line A                  | None                     | 7.7      | 0.7      | 6.3       |
| 4         | Control medium                                | None                     | 5.6      | 3.5      | ND$       |
| 5         | Control medium                                | None                     | 9.5      | 11.2     | ND        |
| 6         | Control medium                                | None                     | 12.6     | 17.1     | ND        |

The following cultures were set up and harvested on day 6 for LMC at the 60/1 lymphocyte to target cell ratio: (a) BALB/c vs. C57BL/6 MLC (triplicate cultures). Median LMC, 77.3%. (b) MLC inhibited with 0.1 ml of the culture fluid (five cultures). Median LMC, 6.0%; range, 3.8–9.6%. (c) MLC with the addition of 0.1 ml of various dilutions of normal or immune sera. The range of LMC in those cultures was 65.6–83.0%. (d) MLC with the addition of 0.1 ml of the culture fluid and 0.1 ml of the indicated dilutions of the immune or control sera. Culture fluid and dilutions of sera were preincubated 1 h at 22°C with occasional shaking, before their addition to the MLC. Mixing with the MLC resulted in a further 50-fold dilution of the immune sera.

* % LMC.
† ND, not done.

MVM immune sera, was the inhibitory factor in the culture fluid from the early passage of EL-4(G-) cells in culture. It was important to demonstrate that the inhibitory activity of the fresh ascites cells and of the sonicate of these cells was also due to the same virus. Experiments were therefore performed to determine...
TABLE V

Effects of Reference Anti-MVM and Antipolyoma Immune Sera on Inhibitory Activity of Culture Fluid on MLC

| Serum no. | Specificity of immune serum | Dilutions of the sera used in the neutralization phase |
|-----------|-----------------------------|------------------------------------------------------|
|           |                             | 1:10 | 1:100 | 1:1,000 |
| 7         | MVM                         | 57.2* | 12.4  | 5.4     |
| 8         | MVM                         | 46.6  | 44.4  | 14.5    |
| 9         | MVM                         | 42.9  | 48.7  | 40.8    |
| 10        | Polyoma                     | 5.4   | 2.6   | 1.0     |
| 11        | Polyoma                     | 3.3   | 7.4   | 4.0     |
| 12        | Polyoma                     | 9.3   | 3.1   | 3.2     |

The following cultures were set up and harvested on day 6 for LMC at the 20/1 lymphocyte to target cell ratio: (a) BALB/c vs. C57BL/6 MLC (triplicate cultures). Median LMC, 41.8%. (b) MLC inhibited with 0.1 ml of the culture fluid (five cultures). Median LMC, 3.5%; range: 2.1–4.9%. (c) MLC with the addition of 0.1 ml of the culture fluid and 0.1 ml of the indicated dilutions of the mouse antipolyoma virus or anti-MVM immune sera. Culture fluid and dilutions of sera were preincubated 1 h at 22°C with occasional shaking, before their addition to MLC. Mixing with MLC resulted in a further 50-fold dilution of the immune sera. The effects of 0.1 ml of various dilutions of all the sera themselves on the MLC were negligible.

* % LMC.

The effects of the reference anti-MVM immune sera on the inhibition produced by the EL-4(G−) ascites cells or sonicates of the ascites cells. As with the culture fluid, the inhibitory activity of the whole cells and of the sonicate was completely neutralized by anti-MVM serum and not by antipolyoma serum (Table VI). Further, anti-MVM sera also neutralized the inhibition mediated by the secondary culture fluids, obtained after infection of the later passages of the EL-4(G−) tissue culture cells.

Effects of Prototype MVM and of an Isolate of Kilham Rat Virus on the MLC. In order to find out whether MVM was immunosuppressive in allogeneic MLC, two pools of prototype MVM were repeatedly used at various dilutions in BALB/c vs. C57BL/6 MLC. Typical results are shown in Table VII. The prototype MVM had a slight enhancing effect on the cytotoxic response in MLC. In the same experiments, doses of Kilham rat virus, which have been reported to strongly inhibit rat proliferative responses in vitro (D. A. Campbell, Jr. and E. K. Manders, unpublished results), were tested for inhibition of cytotoxic response in mouse MLC, and they were found to have no detectable effect.

Discussion

There have been a considerable number of reports on the immunosuppressive properties of chemically induced or spontaneous murine tumors in vivo (4, 5, 21, 22). Very rarely did these reports elucidate the cause of such inhibition. Recently, a few investigators have studied the immunosuppressive effects of the tumor cells or their products in in vitro assays of lymphocyte responsiveness (6, 23, 24). Although potentially very useful, these studies have not yet identified the causes for inhibition in these systems. The available evidence would suggest
TABLE VI
Effects of Reference Anti-MVM and Antipolyoma Virus Sera on Inhibitory Activity of EL-4(G-) Ascites Cells, Sonicate, and Culture Fluid No. 4

| EL-4(G-) derived material used to inhibit the MLC | Final dilution or no. of cells or cell equivalent (× 10^-6) used | 1 h preincubation of inhibitor | Without serum | With serum* immune to: |
|-------------------------------------------------|-------------------------------------------------------------|------------------------------|---------------|-----------------------|
| None                                            | -                                                          | 67.2‡                        |               | Polyoma MVM          |
| Culture fluid                                   | 1:15                                                        | 0.7                          | ND            | ND                    |
|                                                 | 1:50                                                        | 11.9                         | 7.4           | 67.8                  |
|                                                 | 1:150                                                       | 42.9                         | ND            | ND                    |
|                                                 | 1:500                                                       | 65.0                         | ND            | ND                    |
| Ascites cells                                   | 4.0                                                         | -1.5                         | -0.7          | 64.8                  |
|                                                 | 1.4                                                         | 1.3                          | -1.3          | 56.0                  |
|                                                 | 0.4                                                         | 0.4                          | -0.5          | 64.3                  |
| Ascites cell sonicate                           | 4.0                                                         | -1.1                         | -1.4          | 65.1                  |
|                                                 | 1.4                                                         | -1.5                         | -0.5          | 66.4                  |
|                                                 | 0.4                                                         | -0.1                         | 1.1           | 59.2                  |

The following cultures were set up and harvested on day 6 for LMC at the 60/1 lymphocyte to target cell ratio: (a) BALB/c vs. C57BL/6 MLC (triplicate cultures). Median LMC, 67.2%. (b) MLC inhibited by various amounts of culture fluid, after 1 h preincubation without serum or with antipolyoma virus immune serum, or with anti-MVM immune serum, as indicated. (c) MLC inhibited by various numbers of EL-4(G-) ascites cells from a cryopreserved pool, after 1 h preincubation, as indicated. (d) MLC inhibited by amounts of sonicate from and equivalent to various numbers of EL-4(G-) ascites cells, after 1 h preincubation, as indicated.

* 1:100 dilutions of antipolyoma virus serum no. 12 and anti-MVM serum no. 9 were used.
‡ % LMC.

that each system may contain a different factor, and that there is no unique solution to the problem of immunosuppression by these tumor cells. We have earlier expressed the concern (reference 25 and footnote 1) that contamination of the transplantable tumor lines by infectious and immunosuppressive agents, like mycoplasma organisms (26), common murine viruses (10, 27), or the presence of immunosuppressive endogenous type C particles (3, 28, 29) may not always have been adequately ruled out. The sublines of the C57BL/6 mouse EL-4 lymphoma used in this and the preceding studies were free of mycoplasma contamination.¹ They have been characterized quite extensively for their content of endogenous type C particles (reference 9 and footnote 3) and the data in this paper document that some of the sublines used were contaminated with at least three other viruses: polyoma virus, LDV, and MVM.

Chronologically, the first observation which we had made¹ was that EL-4(G-) ascites cells inhibited allogeneic reactions, and in particular the BALB/c vs. C57BL/6 MLC, as assessed by lymphoblast counts, [³H]thymidine incorporation, and LMC. Several pieces of evidence suggested that a contaminating virus was responsible for the inhibition in that earlier study. Sonicates of EL-4(G-) cells, filtered culture fluids of early in vitro passages of EL-4(G-) cells, and spleen
### Table VII

**Effect on the MLC of Prototype Strains of MVM and of Kilham Rat Virus**

| Addition to MLC | Hemagglutination titer* | Volume added | LMC at the lymphocyte to target cell ratios |
|----------------|-------------------------|--------------|--------------------------------------------|
|                | ml                      |              | 20/1 | 60/1 |
| None           |                         |              | 54.7 | 74.0 |
| Culture Fluid  |                         | None‡        |      |      |
|                |                         | $3 \times 10^{-1}$ | -0.5 | -0.5 |
|                |                         | $10^{-1}$     | 1.3  | 6.9  |
|                |                         | $3 \times 10^{-2}$ | 18.7 | 41.7 |
|                |                         | $10^{-2}$     | 35.3 | 66.2 |
| Prototype MVM no. 1 | 256                     | $10^{-2}$   | 67.3 | 81.5 |
|                |                         | $10^{-3}$     | 68.6 | 81.2 |
|                |                         | $10^{-4}$     | 61.9 | 82.0 |
|                |                         | $10^{-5}$     | 70.6 | 81.6 |
| Prototype MVM no. 2 | 32                      | $10^{-2}$   | 56.8 | 75.1 |
|                |                         | $10^{-3}$     | 60.0 | 75.8 |
|                |                         | $10^{-4}$     | 55.3 | 79.3 |
|                |                         | $10^{-5}$     | 67.5 | 83.7 |
| Kilham rat virus | 2,512                   | $5 \times 10^{-1}$ | 51.7 | 78.2 |
|                |                         | $1.5 \times 10^{-1}$ | 44.1 | 71.3 |

The following cultures were set up and harvested on day 6 for LMC at two different lymphocyte to target cell ratios: (a) BALB/c vs. C57BL/6 MLC (triplicate cultures). (b) MLC with various doses of the culture fluid. (c) MLC with various doses of the prototype MVM strain no. 1. (d) MLC with various doses of the prototype MVM strain no. 2. (e) MLC with two doses of the Kilham rat virus preparation.

* Reciprocal of the last dilution causing partial hemagglutination of guinea pig erythrocyte (15).
‡ No hemagglutination, even by the undiluted culture fluid.

cells (or sonicates of spleen cells) of tumor-bearing mice were strongly inhibitory. The inhibitory activity was not due to the EL-4 cells themselves, since later passages of EL-4(G-) cells maintained in vitro, or another subline maintained in vivo, EL-4(G+), were not inhibitory. The acquisition by the originally noninhibitory EL-4(G+) line of inhibitory properties, 8 mo after initiation of these studies was also consistent with an infectious inhibitory agent. Kinetic studies of the responses in MLC or to the mitogen concanavalin A, and of the inhibition mediated by EL-4(G-) cells, revealed that a latency period of 3-4 days was necessary before inhibition could be observed; this suggested, among other possibilities, that viral replication was needed in these cultures before immunosuppression could be seen.

In the present study we concentrated our efforts on the characterization of the inhibitory activity of the culture fluid from an early in vitro passage of the EL-4(G-) cells. Thus, we would subsequently need to demonstrate that this inhibitory factor was identical with that in the EL-4(G-) ascites cells themselves.

The factor in the culture fluid was characterized as a virus by simple biophysical methods. The inhibitory factor was particulate (nondialyzable, sedimentable at 100,000 g for 1 h), very small (recovered after 0.10 μm filtration), sensitive to UV irradiation but heat stable, and resistant to chloroform; it was infectious for EL-4(G-) tissue culture cells. The results of the MAP and LDV tests suggested that MVM or polyoma virus might be responsible for the inhibition. But these results did not exclude other viruses. LDV was ruled out, because this virus
would not resist 56°C for 1 h or chloroform treatment (27), as did the inhibitory factor. Initial attempts to infect mouse embryo cells with the culture fluid were unsuccessful, so that an indirect characterization of the inhibitory factor by serological techniques seemed preferable.

In preliminary experiments, cell-free ascitic fluids of mice bearing EL-4(G-) ascites were tested for inhibition of MLC. This might have occurred, particularly if LDV were the agent, since LDV has been reported to infect mice chronically, with high circulating titers of infectious virus (19, 20). However, such inhibition was seen only infrequently, as opposed to the constant inhibition seen with EL-4(G-) ascites cells, another argument to exclude LDV as a cause of inhibition. We postulated then that the reason why culture fluids would inhibit, and ascitic fluids would not, might be the presence in the ascites of sufficient antibodies to neutralize the inhibitory factor in the culture fluid. Further studies are needed to demonstrate that this effect indeed was due to neutralizing antibodies and to study their specificities.

The data involving neutralization by the sera of mice immunized for the MAP test confirmed that the inhibitory factor in the culture fluid could be neutralized, and that it might be either polyoma virus or MVM. Nonspecific serum factors or natural antibodies against the inhibitory factor in these NIH Swiss mice were formally excluded, because the sera of the controls had no effects. For neutralization of the culture fluid, sera of mice injected with the EL-4(G-) ascites cells were at least as effective as sera of mice immunized against the culture fluid itself. This provided the first clear evidence that the inhibitory factor in the culture fluid and in the EL-4(G-) ascites cells were the same.

When reference immune sera against polyoma virus or MVM, and control nonimmune sera, were used, only the anti-MVM sera neutralized the inhibition by the culture fluid or by the ascites cells. This indicated that the neutralization was mediated by specific neutralizing antibodies. If one accepts the evidence that the reference anti-MVM sera were specific for the prototype MVM, this data would then indicate that MVM was responsible for the inhibition seen. However, it must be remembered that the possibility always exists with any prototype virus strain, that it is contaminated with other unidentified viruses, against which reference immune sera might then also be active (10). Thus, we cannot dismiss the possibility that the inhibitory virus in EL-4(G-) cells and in the culture fluid is a separate virus in the prototype MVM strain. Because we used three different anti-MVM immune sera produced against viruses grown in either mouse or rat embryo cells, a fortuitous contamination of one pool of MVM virus is unlikely, and we must envisage a systematic contamination of all pools. On the basis of this serological data, it is possible to rule out a role for the 10 other common rodent viruses in the MAP test panel, because the reference anti-MVM immune sera had no detectable activities against these viruses.

The reference anti-MVM sera neutralized the inhibitory activity of EL-4(G-) ascites cells, sonicates, and culture fluid equally well. This indicated that the inhibitory factor in all materials was the same virus. Similarly, it has been possible to neutralize the secondary culture fluids obtained after infection of the EL-4(G-) tissue culture cells.
Infectivity of the inhibitory factor was demonstrated by the inoculation of noninhibitory later passages of EL-4(G-) tissue culture cells, since they became strongly inhibitory upon such inoculation. Inhibition by the diluted inoculum itself was excluded on three grounds. (a) The cells were washed, and the medium was changed once or twice before harvest of the secondary inhibitory culture fluids. (b) The secondary culture fluids were about 10 times more inhibitory in MLC than the original inoculum. (c) The cells, which initially could not inhibit MLC, became inhibitory.

The serologic data provided strong evidence in favor of MVM being the inhibitory virus in EL-4(G-) derived materials, but this could not be confirmed by direct testing with two pools of prototype MVM. Although both pools had significant hemagglutination titers (Table VII) and were readily infectious for both rat and mouse embryo cells, and for a mouse fibroblast cell line, attempts to produce inhibition of MLC with these pools were unsuccessful. Attempts to infect the later passages of EL-4(G-) cells with the prototype MVM (pool no. 2) have been unsuccessful so far, as measured by the induction of MVM antigens detectable by the fluorescent antibody technique (16), or by changes in growth rate of the cells (S. P. Staal, unpublished results). Therefore the prototype MVM seems to infect lymphoid cells with much less efficiency than fibroblasts. The virus associated with EL-4(G-), however, possesses a tropism just the opposite to that of the prototype MVM. Attempts to detect infectivity in nonconfluent mouse fibroblasts by the appearance of cytopathogenicity or the induction of viral antigens detectable by the fluorescent antibody technique (using anti-MVM reference sera) have been negative; this virus, as assessed with the latter technique, readily infects virus-negative EL-4(G-) tissue culture cells and also lymphocytes in MLC (S. P. Staal, unpublished results). Therefore, the EL-4(G-) associated virus may represent a variant murine parvovirus that is lymphotropic, or a different, unidentified virus. This will need to be grown in large quantities on EL-4(G-) tissue culture cells, and characterized further. Conversely, the prototype MVM may have lost its ability to grow in lymphoid cells through forced passage in fibroblasts. Analysis of the ability of fresh isolates of MVM to grow in lymphoid cells should solve this question.

Our laboratory has recently examined in detail the immunosuppressive effects of a dimethylbenzanthracen-induced mammary adenocarcinoma in Fischer rats, and found that inhibition of lymphocyte responses in vitro was due to Kilham rat virus (14), another parvovirus (D. A. Campbell and E. K. Manders, unpublished results). Kilham rat virus does not appear to grow in mouse cells (14, 15). Neutralizing antibodies for MVM and Kilham rat virus have been reported not to cross-react (30). Therefore, Kilham rat virus could hardly be the inhibitory virus in our mouse system and might not be expected to be active here. Indeed, the data in Table VII showed that Kilham rat virus, at doses which strongly inhibited in the rat system, would not inhibit the cytotoxic mouse MLC response.

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4 Campbell, D. A., Jr., G. D. Bonnard, R. K. Oldham, and R. B. Herberman. Inhibition of proliferative responses of rat lymphocytes by tumor cells and by spleen cells from tumor-bearing animals. Manuscript submitted for publication.
Many possible mechanisms for inhibition of MLC by the virus in the immunosuppressive culture fluid need to be considered. A valuable approach with other immunosuppressive viruses, both in vivo and in vitro, has been to consider that the loss of immune response was due to the infection with replication of the virus in the particular subset of cells which are committed to respond (3, 31–34). Since it is known that thymus-derived lymphocytes are mainly responsible for the proliferative and cytotoxic responses in mouse MLC (35), a simple hypothesis is that the immunosuppressive virus infected the thymus-derived spleen cells directly. The rather selective effects of this immunosuppressive virus on lymphoblast proliferation, illustrated in detail in the previous study, and the tropism of this virus for the EL-4 lymphoma, a thymus-derived lymphoma with characteristics of T lymphocytes (7, 8), including the ability to replicate vesicular stomatitis virus (36), would be consistent with this hypothesis. The demonstration that reference anti-MVM immune sera could neutralize the inhibitory factor, and the availability of immunofluorescent techniques (16) to recognize the virus in infected cells, should be of considerable help in longitudinal follow-up of MLC infected with our immunosuppressive virus and in the characterization of the precise cells which become infected within the responding cell population. At present, the possibility that cells other than thymus-derived lymphocytes, possibly infected macrophages, play a central role in the inhibition of MLC cannot be ruled out.

If the infection of thymus-derived proliferating lymphocytes can be confirmed, it may be of some importance beyond the scope of this study. For instance, one might be able to detect antigen-reactive virus-sensitive T lymphocytes by infection with our immunosuppressive virus, and induction of antigens detectable with reference anti-MVM sera by the indirect immunofluorescence technique (16). This may represent a worthwhile addition to the use of the vesicular stomatitis virus plaque assay for the characterization of antigen-reactive thymus-derived cells pioneered by Bloom and co-workers (32, 33).

We have previously stressed the need for extensive testing of murine transplantable tumors on the one hand (reference 25 and footnote 1), and murine virus stocks on the other hand (10) for contamination by microbiological agents, and in particular by common murine viruses. If experiments are performed with contaminated materials, they may lead to erroneous conclusions. This problem is particularly important in experimental tumor immunology, because we need a clear understanding of the relationships between tumors and viruses, and between each of these and the host immune response. The present experiments demonstrated that a virus found in a subline of the EL-4 lymphoma, completely inhibited the cytotoxic response of mouse lymphocytes to alloantigens. They also raised some doubt as to the purity of the prototype MVM strain and as to the monospecificity of the widely used reference anti-MVM immune sera. Contamination of the tumor cells with this immunosuppressive virus critically affected the response of allogeneic lymphocytes to these tumor cells, which in the absence of viral contamination readily elicited the normal expected allogeneic response. The recognition of this and other potential immunosuppressive contaminants of transplantable tumor systems by the techniques developed in the
preceding studies (reference 25 and footnote 1), or used here, represents an essential step towards a better understanding of antigenicity and immunogenicity of tumor cells in various murine systems. The in vivo host response to tumors like the widely used EL-4 may be adversely affected by contamination of the tumor inoculum and the possibility exists that such viruses interfere with the experimentation on syngeneic lymphocyte responses to tumor cells in vitro.

Summary

Filtered culture fluids from the early in vitro passages of a subline of the C57BL/6 mouse EL-4 lymphoma, EL-4(G−), were strongly inhibitory for BALB/c vs. C57BL/6 mixed lymphocyte cultures (MLC). The inhibitory activity could be preserved by storage at −75°C or 4°C, thus allowing its further characterization. The inhibitory factor was particulate (nondialyzable, sedimentable at 100,000 g for 1 h), very small (recovered after 0.10 μm filtration), sensitive to UV irradiation, but heat stable (56°C, 1 h) and resistant to chloroform. It was infectious, since later, noninhibitory passages of EL-4(G−) tissue culture cells became strongly inhibitory upon inoculation with the culture fluid. This data was consistent with the inhibitory factor being an infectious virus. Virus analysis by mouse antibody production tests revealed that viruses were indeed present in EL-4(G−) ascites cells and in the culture fluid, and not in a late passage of EL-4(G−) tissue culture cells which were not inhibitory. Neutralization of the inhibitory factor was achieved by pretreatment with ascitic fluid or with the sera raised against those EL-4(G−)-derived materials which contained viruses. Mouse reference immune sera against minute virus of mice (MVM) completely neutralized the inhibitory factor in the culture fluid or in EL-4(G−) ascites cells. Two prototype MVM strains, and one Kilham rat virus preparation, did not inhibit the mouse MLC. Thus, the possibility exists that a variant of MVM, or an unidentified virus, has been grown and selected for in EL-4(G−) cells and recognized, due to its immunosuppressive characteristics. In any event, immunosuppression by EL-4(G−) cells was not mediated by the tumor cells, their metabolic products, or associated endogenous type C viruses, but by an exogenous virus, most likely a variant MVM with immunosuppressive characteristics. This adds weight to a parallel observation from our laboratory on the immunosuppressive effects of Kilham rat virus in rat lymphocyte cultures.

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