Virus Inoculation in Mice Bearing Ehrlich Ascitic Tumors: Antigen Production and Tumor Regression

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Ehrlich ascitic carcinoma, as developed in albino mice, has been used as a source of hemagglutinating and complement-fixing antigens, and it proved to be suitable for one type of antigen, or both, for at least 12 viruses of 16 tested. Hemagglutinins were obtained with members of arbovirus groups A, B, and C; complement-fixing antigens were obtained for at least one member of each antigenic group tested. Ehrlich ascitic tumor was compared with sarcoma 180 as a source of antigens; although sarcoma 180 showed many advantages over Ehrlich tumor, the latter gave, in general, better results for complement-fixing antigens. Oncolytic effect with complete recovery of the mice was observed in some instances. The highest recovery rate resulted with Congo and UNA viruses (40%), and the second highest rate resulted with dengue 2, St. Louis, Hazara, and Uukuniemi viruses (20%). The best survival was observed, in decreasing order, with Congo, St. Louis, dengue 2, Tacaribe, Sindbis, Junin, and Amapari viruses.

Previous studies (7) have shown that sarcoma 180, when propagated intraperitoneally (i.p.) in intact mice, provides an excellent system for the multiplication of some arboviruses, with the associated appearance of hemagglutinating (HA) and complement-fixing (CF) antigens in the ascitic fluid. This report describes similar studies with Ehrlich ascitic tumor, compares the two sets of results, and provides data on tumor regression associated with some of the viral infections.

MATERIALS AND METHODS

Mice. Adult mice of the Charles River CD(R)-1 strain were used throughout.

Ehrlich ascitic tumor. A mouse bearing an Ehrlich ascitic tumor was obtained from A. C. Sartorelli, Department of Pharmacology, Yale University School of Medicine, in January 1971. The ascitic tumor had undergone an undetermined number of passages in albino mice.

In the preliminary tests to determine the dose level of ascitic fluid that would most rapidly produce ascitic tumors and yet permit the mice to survive long enough for antigen development, groups of 30, 44, and 50 mice were inoculated i.p. with 0.1 ml of undiluted fluid 0.2 ml of fluid diluted 1:10, and 0.2 ml of undiluted fluid, respectively. The inoculum of 0.2 ml of undiluted fluid was chosen for the experiments, since it gave a good yield of ascitic fluid by day 8 post-inoculation in combination with an average survival time of better than 14 days (14.4 ± 4.7) (Table 1).

Viruses. The following 16 viruses (1, 6) were used: Eastern equine encephalitis (EEE) (TenBroeck), Sindbis (EgAr339), and Una (BeAr13136) viruses of antigenic group A; dengue 2 (TR1751) and St. Louis encephalitis (SLE) (Parton) viruses of group B; Apeu (BeAn8) and Marituba (BeAn15) viruses of group C; Oropouche virus (TR9760) of the Simbu group; Uukuniemi virus (S-23) of the Uukuniemi group; Crimean hemorrhagic fever virus, Congo (IbAn10200) and Hazara (JD280), of the Congo group; Amapari (BeAn70563), Junin (XJ and Noguera), Tacaribe (TR11573), and Tamiami (CDC W 10777) viruses of the Tacaribe group; and Colorado tick fever virus (Condon).

Virus stocks consisting of 10 or 20% suspension of infected newborn mouse brain tissue were used for inoculation; all were lyophilized preparations held at 4°C, except for the EEE virus stock, which was kept wet frozen at −70°C. Dilutions were made in 0.75% bovine albumin in phosphate-buffered saline (pH 7.2). The lyophilized virus stocks were known to contain at least 10⁴ suckling mouse intracerebral 50% lethal doses in the 0.1-ml volume inoculated; titers were determined for the EEE virus stock when used.

Experimental design. Mice in groups of 5 were inoculated i.p. with Ehrlich ascitic tumor. After 8 to 10 days, when marked abdominal swelling signalled the development of fluid, they were inoculated i.p. with virus. Thereafter, for as long as any mice survived, or

<table>
<thead>
<tr>
<th>No. of mice</th>
<th>Dose (ml)</th>
<th>AST*</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>0.1 undiluted</td>
<td>15.5</td>
<td>±4.8</td>
</tr>
<tr>
<td>44</td>
<td>0.2 diluted 1:10</td>
<td>16.1</td>
<td>±4.3</td>
</tr>
<tr>
<td>50</td>
<td>0.2 undiluted</td>
<td>14.4</td>
<td>±4.7</td>
</tr>
</tbody>
</table>

*AST, Average survival time in days = X.

TABLE 1. Survival of mice after i.p. inoculation of different dilutions of Ehrlich ascitic tumor

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TABLE 2. HA titers of ascitic fluid antigens obtained by i.p. inoculation of viruses into Ehrlich tumor-bearing mice*  

<table>
<thead>
<tr>
<th>Virus</th>
<th>Inoculum (ml)</th>
<th>Reciprocal of HA antigen dilution on day after inoculation:</th>
<th>Further observations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Sindbis</td>
<td>IMB</td>
<td>0.1</td>
<td>4</td>
</tr>
<tr>
<td>UPA</td>
<td>IMB</td>
<td>0.1</td>
<td>4</td>
</tr>
<tr>
<td>SLE</td>
<td>IMB</td>
<td>0.1</td>
<td>4</td>
</tr>
<tr>
<td>EEE</td>
<td>IMB</td>
<td>0.5</td>
<td>2</td>
</tr>
<tr>
<td>EEE</td>
<td>IMB</td>
<td>0.1</td>
<td>4</td>
</tr>
<tr>
<td>EEE</td>
<td>IMB</td>
<td>0.1</td>
<td>4</td>
</tr>
<tr>
<td>Apeu</td>
<td>IMB</td>
<td>0.1</td>
<td>4</td>
</tr>
<tr>
<td>Marituba</td>
<td>AF</td>
<td>0.2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

* Viruses were prepared by inoculating different hosts with various viruses. The fluids were extracted twice with 20 volumes of acetone, dried on vacuum pump for 1 h, and then rehydrated in 5 volumes of borate-saline (pH 9.0) as described previously for sarcoma 180 fluids (7). Hydration took place quickly, and the antigens could be used on the same day without being centrifuged. For statistical analysis, the significance levels for survival were determined by the ratio of the differences between the means to their standard errors (5).

HA antigens. For preparation of HA antigens ascitic fluids were thawed and separated from cellular debris. The fluids were extracted twice with 20 volumes of acetone, dried on vacuum pump for 1 h, and then rehydrated in 5 volumes of borate-saline (pH 9.0) as described previously for sarcoma 180 fluids (7). Hydration took place quickly, and the antigens could be used on the same day without being centrifuged. HA and hemagglutination inhibition assays were those of Clarke and Casals (3) adapted to a microtechnique (9). Serial twofold dilutions of antigens, starting at 1:4, were tested at pHs ranging from 5.8 to 7.2 with intervals of 0.2 pH units. Results are given as the optimum titer at any pH; if more than 1 group of mice were inoculated with the same virus suspension, the results shown in the tables are also the optimum obtained in each day. Each HA antigen was tested for specificity in the hemagglutination inhibition test with its homologous and least two unrelated immune sera or ascitic fluids. For this purpose immune fluids were extracted twice with acetone, and goose cells were adsorbed.

CF antigens. The CF antigens used were the fluids freed of cellular debris and heated for 20 min at 60°C in a water bath. Serial twofold dilutions starting at 1:2 were made in Veronal buffer and tested against homologous and unrelated immune fluids (immune fluids were sera or ascitic fluids). The CF tests were carried out in grid triturations as described by Casals (2). For each immune fluid, a control experiment was performed with a corresponding reference sucrose-acetone-extracted mouse brain antigen (mouse liver antigen in the case of Marituba virus) (3).

RESULTS

Specific HA antigens were obtained with three arboviruses of group A (EEE, Sindbis, and Una viruses), SLE virus of group B, and Apeu and Marituba viruses of group C; Apeu and
Marituba viruses were inoculated as virus-infected Ehrlich ascitic tumor fluid rather than as mouse brain (Table 2). No hemagglutinins were detected with the other nine viruses. Of the viruses used, dengue 2, Hazara, Uukuniemi, and Oropouche viruses are known to yield hemagglutinins by other techniques. The rest of the viruses used in the test never showed hemagglutinins with any system, and therefore we do not know whether they really have any.

To determine the relationship between the amount of inoculated virus and the time and amount of antigen production, five groups of mice were inoculated with different doses of EEE virus (Table 2). The highest titer was obtained with the highest inoculum in the shortest time, reaching a titer of 1:4,096 on day 2 post-inoculation.

The time of appearance of hemagglutinins was from 1 to 5 days depending on the virus and the doses inoculated. They were not detected later than day 10 after virus inoculation.

Specific CF antigens, however, were obtained with at least one virus of each antigenic group tested (Table 3). Positive results were obtained with EEE, Sindbis, Una, dengue 2, SLE, Apeu, Marituba, Hazara, Oropouche, Uukuniemi, Colorado tick fever, and Amapari viruses (Table 3). Most of the viruses gave the best CF titers between days 5 and 7 except Uukuniemi and Amapari viruses, which had optima on days 11 and 19, respectively. Completely negative results were shown by the rest of the members of the Tacaribe group and by Congo virus, and it was not because of lack of samples since tapping was performed up to 24 days post-inoculation for most of them.

By comparison, although sarcoma 180 showed many advantages over Ehrlich ascitic tumor as a source of antigens, the latter gave, in general, better results for CF antigens.

Tumor regression occurred with Congo and Una viruses (40% recovery rate) and to a lesser degree (20%) with dengue 2, SLE, Hazara, and Uukuniemi viruses (Table 4). Mice looked healthy and were kept for 3 months, after which they were discarded.

Survival time of carcinomatous mice were prolonged significantly \( P < 0.001 \) with the following viruses, given in decreasing order of probability values: Congo, SLE, dengue 2, Tacaribe, Sindis, Junin, and Amapari viruses. Other viruses had less effect \( P < 0.05 \) like Hazara, Colorado tick fever, and Oropouche viruses, and the other viruses diminished or had no effect on time of survival (Table 5).

**DISCUSSION**

Our first approach for antigens in an ascitic tumor, sarcoma 180, produced good results with...
many arboviruses (7). However, different types of tumoral cells may lead to different results.

The waiting times for obtainable sarcoma 180 or Ehrlich tumors in mice are practically the same when the same inoculum of 0.2 ml of fresh, undiluted ascitic fluid is used; the average survival times for mice after inoculation of tumor cells without further inoculation of viruses were 13.3 days for sarcoma 180 and 14.4 for Ehrlich tumor; however, the standard deviation for sarcoma 180 was ±2.6, whereas for Ehrlich tumor was ±4.7.

Ehrlich tumors seem to have disadvantages as compared with sarcoma 180 in the sense that the yield of ascitic fluid from sarcomatous mice is larger (more prominent abdomens) plus the fluid recovered from the ascites is about 90% of the total for sarcoma 180 and only 80% for Ehrlich tumor. HA antigen titer is higher for sarcoma 180, except for group C where Ehrlich tumor had higher titers; however, in the case of group C viruses infected ascitic fluid instead of mouse brain was inoculated, and this step was not attempted with sarcoma 180.

Owing to the fact that individual mice were used for antigen titers and some ascites taps were better than others, individual variation cannot be ignored. However, a similar phenomenon is observed with HA antigens in pooled infected mouse brains or sera from many newborn mice; HA antigens reach a peak that is not long lasting, and 24 h later there may be no HA activity at all, as seems to be the case with Sindbis virus (Table 2), where the HA antigen titer appeared on day 5 (1:256) and was negative from day 6 on. Routine work indicates that there is a moment in the growing curve of a virus in a host, tissue, or cell system where the production of HA antigen is best, with a range of positivity around it in time, and the same is true for CF antigens. Possible explanations for this phenomenon are many: one is the formation of hemagglutination inhibition antibodies observed in the same ascitic fluids, at least in sarcomatous mice (7); another is the presence of lipid or lipoprotein substances, since acetone removes lack of HA activity in treated sera.

The treatment we used here is like that described for mouse serum (3), and no further investigations were done to determine whether all of the lipids were removed in all instances; as a routine technique applied to the ascitic fluids it seems to be good enough.

Perhaps due to its high cellular content, Ehrlich tumor yielded better CF antigens for most of the viruses tested. Many of them produced a better yield of antigens in 5 to 6 days; these viruses generally kill newborn mice in 5 or 6 days or less. Viruses with longer times for yielding antigens, like Uukuniemi and Amapari viruses, are known for having longer average survival time in newborn mice; from 6 to 12 days and 13.7 to 18 days, respectively (1).

Multiplication of viruses in tumor cells has been known for many years from studies of virus-induced oncolysis of solid tumors (8), of ascitic tumors (4), and of tumor cells in vitro (10). However, at the time these experiments on tumors in mice were performed many of the viruses yielding the best results were not available since they were isolated later (1) like Congo, Hazara, and Uukuniemi viruses from the family Bunyaviridae and Una virus, Alphavirus (arbovirus A) from the Togaviridae family isolated in 1959. The same is true for Tacaribe, Junin, and Amapari viruses, members of the family Arenaviridae isolated in 1956, 1958, and 1964 respectively, which produced a longer survival time in carcinoma-mous mice.

Prolongation of carcinomatous mouse survival and known ability of the viruses to grow in mice seem to correlate with viruses that normally are not pathogenic for adult mice inoculated i.p. (1).
This observation seems to indicate that the viruses may have the ability to multiply and destroy the carcinomatous cells without killing the animal.

ACKNOWLEDGMENTS

We are very grateful to Robert Shope and Colin White for their advice and help in the preparation of this manuscript. N.E.M. is investigator of the Commission of Scientific Investigations from Buenos Aires Province, Argentina.

LITERATURE CITED