An Antigenic Fraction of the Lymphosarcoma 6C3HED of the C3H Mouse

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Summary

Antigenic components were solubilized from a particulate fraction of mouse lymphosarcoma 6C3HED at pH 9.0-9.5. Protein, lipid, and carbohydrate were present. Three measures of antigenicity were employed. Mouse cytotoxicity tests revealed that the tumor fraction adsorbed a greater amount of rabbit antitumor antibody than did similar fractions of normal C3H mouse lymphoid tissues. In Schultz-Dale tests, prior desensitization of immune C3H mouse uterine muscle with spleen and lymph node-thymus antigens did not eliminate a response to the tumor antigens. The immunization of C3H mice by a single injection of the tumor antigenic preparation in Freund's complete adjuvant resulted in a greater number of regressions than was observed in mice treated with adjuvant alone.

Introduction

Lipoprotein antigens isolated from normal mouse tissues (4, 12, 14, 17), cultured mouse lymphoblasts (19), and mouse tumors (6, 13) have been examined with respect to their relationship to tumor and transplantation immunity by various methods, i.e., ability to evoke the 2nd-set response to grafts, the enhancement phenomenon, the production of isoaotibodies, and the adsorption of hemagglutinins. Initial recovery was from the "small particle" fraction of the tissues; solubility with retention of antigenicity, to some extent at least, was reported by LeJeune et al. (17) and by Kandutsch and his colleagues (13, 15).

Evidence that mouse lymphosarcoma 6C3HED differed from the normal tissues of C3H mice was presented by Nungester and Fisher (20), who demonstrated greater inhibition of the tumor in C3H mice treated with rabbit anti-6C3HED pellet (small particle) antiserum than in mice treated with anti-liver pellet antiserum. Amos and Day (2) reported the presence of X antigen in 6C3HED, and Amos (1) also described alpha, an antigen also present in the normal tissues of the mice, but in much smaller quantities than in 6C3HED. Davies and Hutchison (7) did not attempt to isolate the antigen from mouse lymphosarcoma 6C3HED ascites fluid, since it appeared to be weaker than did the antigens of those tumors which were fractionated. Presumably, however, the 6C3HED antigenic component was of the same type as the others.

This report deals with the isolation and partial characterization of components of lymphosarcoma 6C3HED that appear to differ from constituents of the normal tissues of the C3H mouse. Antigenic properties of the fraction were studied by (a) adsorption of cytotoxic antibody, (b) the Schultz-Dale response, and (c) active immunization followed by challenge.

Materials and Methods

Lymphosarcoma 6C3HED (11) was grown in C3H mice, intraperitoneally or subcutaneously. Normal tissues were collected from C3H mice that had not been exposed previously to the tumor or to antitumor serum. Mice were obtained from the Roscoe B. Jackson Memorial Laboratory or from the colony maintained in this laboratory. The Michigan strain of mice was used for preliminary tests when necessary; results were confirmed with C3H/Jax mice.

Antilymphosarcoma seraums were prepared by the i.v. inoculation of ascites tumor cells into New Zealand rabbits according to several different protocols.

To obtain tissue for fractionation, subcutaneous tumors were harvested from mice 5-7 days after inoculation, the lymph nodes were removed from the tumors, and the tumor tissue was blotted between sheets of filter paper. During the performance of the fractionation procedures, the tissue preparations were kept in an ice water bath unless otherwise stated. The tumor tissue was homogenized in 2.5 volumes of M/15 phosphate buffer at pH 7.4 in a Potter-Elvehjem type of ground-glass homogenizer, and the homogenate was centrifuged in a Spinoe model L preparative ultracentrifuge at 3000 relative centrifugal force (ref) for 15 min at -4°C (Chart 1). The supernatant fraction (Sup-I) was then centrifuged at 105,000 ref for 20 hr at -4°C. After a creamy, white floating layer (Sup-I1a) was removed from the rest of the supernatant fraction by means of a 10-ml syringe and a 19-gauge hypodermic needle inserted through the wall of the tube, the pellicle was homogenized in phosphate buffer so that the final volume was equal to the weight of the tissue from which the fraction was prepared or to some convenient multiple of this weight. The sediment was homogenized in the liquid portion of the supernatant fraction and designated Res-III. Spleens, lymph nodes, and thymuses were removed from normal mice; the lymph nodes and thymuses were combined; and the same fractionating procedures were followed.

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2 Part of a thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy to the faculty of the Department of Microbiology of the University of Michigan, 1960.

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Tissue homogenate (in M/15 phosphate buffer pH 7.4)

3000 rcf, 15 min, -4°C

Residue

Supernate (Sup-I)
d=1.0 gm/ml
105,000 rcf, 20 hr, -4°C

Residue

Supernate (Sup-Il)
d=1.15 gm/ml
105,000 rcf, 20 hr, -4°C

Residue

Supernate (active)

Pellicle (white, creamy, top layer)

Res-III

Chart 1. Isolation of the pellicle antigen of lymphosarcoma 6C3HED.

One procedure for studying the antigenicity of normal or tumor tissue or their fractions was to determine their ability to adsorb the cytotoxic effect of 6C3HED antiserum. For these tests, antiserum was combined with a tissue preparation in a ratio of 1:19, respectively. Incubation of the mixture for 30 min at 37°C was followed by storage overnight at 2°-5°C. The precipitate was then removed by centrifugation at 10,400 rcf at 5°-8°C for 1 hr. The adsorbed antiserum was serially diluted in 0.85% NaCl (saline) and combined with an equal volume of freshly harvested and washed 7-day ascites tumor cells suspended in pooled normal guinea pig serum. After incubation in a 37°C water bath for 30 min, 0.2 ml (containing 2.5 × 10^6 cells) of each preparation was injected s.c. into each inguinal region of 3 normal C3H mice. The controls for each test were tumor cells in (a) antiserum treated with suspending fluids, (b) normal rabbit serum, (c) saline. The mice were examined for tumor growth for 25 days following inoculation. The reciprocal of the highest serum dilution that permitted no tumor growth was considered to be the cytotoxic titer of the serum. Because of the relatively small number of mice available, we preferred to demonstrate the reproducibility of results by repetition of the whole procedure, including fractionation, rather than to use large numbers of mice in a single test.

A 2nd approach for comparing the antigenicity of normal and tumor tissues and their fractions was to measure the ability to elicit smooth muscle contraction of uteri taken from mice actively immunized with living tumor cells.

Active immunization of C3H mice was accomplished by the s.c. inoculation of viable lymphosarcoma cells. Tumors resulting from an initial inoculum of 100-1000 ascites tumor cells regressed in about 80% of the mice in 4–5 weeks. A 2nd inoculation followed, in which the number of cells was 10- or 20-fold greater. Increasing numbers of cells were administered until the mice had been exposed to at least 1 inoculation of 1 × 10^6 tumor cells. The site of inoculation was alternately the right or left inguinal region. In a few mice (0–18%), tumors did not grow after the 1st or 2nd inoculations, and these mice were discarded. Those animals in which 1 × 10^6 cells produced tumors usually died as the result of tumor growth; survivors were discarded since the tumor was still present at the time of the next immunizing injection. Treatment with larger doses was continued each month in order to enhance resistance, if possible, and to maintain it until the mice were required for experiments. The immunity resulting was not powerful and varied with the season of the year, as did the ability of the tumor to grow in the mice.

Female C3H mice that had been actively immunized received i.m. injections of 9 μg of estradiol benzoate in oil (9). The hypertrophied uterus was removed 3–5 days later. One of the uterine horns was placed in the apparatus, shown in Charts 2 and 3, at 37°C. The horn was incubated for 15–20 min, with aeration, in 3 ml of the modification of Ringer’s solution (5) recommended by Mary A. Fink. During this time, the thread to the muscle was untied. The thread was then attached, and another 15-min period was allowed for equilibration. There were few or no normal contractions in this medium because of its low Ca²⁺ concentration. Contractility of the muscle was determined at the start of, and at various times during, the experiment by the addition of 0.02 mg of acetylcholine chloride to the tissue bath. All materials were added to the bath just below the surface of the medium with a 3-inch, 22-gauge hypodermic needle on a 1-ml syringe. The muscle was washed by repeatedly filling the tissue bath with Ringer’s solution and draining to a level just above the top of the uterine horn. Enough Ringer’s solution to give a total of 3 ml was then admitted for continuation of the test. The responses of both normal and immune uterine horns to preparations of both normal mouse tissues and the tumor were examined. The tissue fractions were dialyzed against repeated changes of distilled water at 2°-5°C before use, in order to remove ions that might elicit a contractile response.

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Active immunization of mice with the tumor's pellicle fraction demonstrated its ability to elicit resistance in C3H mice to 6C3HED tumor. Mice were immunized with a 1:1 emulsion of Freund's complete adjuvant (Difco) and 6C3HED pellicle fraction by a single s.c. injection. A 2nd group of mice received Freund's adjuvant emulsified with distilled water, and a 3rd group received no pretreatment. Fifteen days later, the mice were challenged s.c. with $1 \times 10^5$, $5 \times 10^5$, $1 \times 10^6$, or $5 \times 10^6$ ascites tumor cells. The number of tumors which grew and the number of these which regressed were noted.

Results

ADSORPTION OF CYTOTOXIC EFFECTS OF ANTISERUM. Results compiled from tests with rabbit antitumor serum adsorbed with fractions prepared from tumor and from normal C3H mouse tissues are presented in Table 1. The cytotoxicity titers of the antiserums (treated with buffer only) were 160-320. Although some ability to block antitumor activity was present in the tumor Res-III fraction, the amount of this activity in the pellicle fraction was greater; tumors grew in about 0.5 of the mice injected with undiluted antiserum adsorbed with pellicle fraction (the final serum dilution was 1:40 in the cytotoxicity tests). The pellicle fraction from the tumor also showed greater capacity to neutralize antitumor antibody than did similar fractions prepared from the normal C3H mouse tissues. If the 3000 ref supernatant fractions were centrifuged at 105,000 ref for 2 hr (no sucrose added), all antigenic activity was in the residue. For flotation, a density of 1.15 gm/ml was required to extract most of the antigenic components from the residue. At 1.18 gm/ml, there was no residue fraction; all particles floated.

In efforts to obtain a soluble antigen, lymphosarcoma pellicle fraction was treated in several ways. One method was apparently successful. Alteration of the pH to 9.0-9.5 by 1 N sodium hydoxide added in drops followed by centrifugation at 105,000 ref for 2 hr at $-4^\circ$C permitted the retention of the antigenic portion of the pellicle fraction in the supernatant fraction. Readjustment of the pH of the supernatant fraction to 7.4 with 0.5 N hydrochloric acid resulted in gradual reprecipitation of the antigenic material. Table 2 contains mouse cytotoxicity test

### Table 1

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Fraction</th>
<th>Dilution of adsorbed antiserum</th>
<th>Takes (%)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1:40</td>
<td>1:80</td>
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<tr>
<td>6C3HED</td>
<td>Homogenate</td>
<td>4/4</td>
<td>6/6</td>
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<tr>
<td></td>
<td>Sup-IIa</td>
<td>0/6</td>
<td>0/3</td>
</tr>
<tr>
<td></td>
<td>Res-III</td>
<td>1/9</td>
<td>2/6</td>
</tr>
<tr>
<td></td>
<td>Pellicle</td>
<td>20/39</td>
<td>28/30</td>
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<tr>
<td>Lymph node and thymus</td>
<td>Homogenate</td>
<td>3/3</td>
<td>3/3</td>
</tr>
<tr>
<td></td>
<td>Sup-IIa</td>
<td>0/3</td>
<td>0/3</td>
</tr>
<tr>
<td></td>
<td>Res-III</td>
<td>0/3</td>
<td>0/3</td>
</tr>
<tr>
<td></td>
<td>Pellicle</td>
<td>1/12</td>
<td>2/12</td>
</tr>
<tr>
<td>Spleen</td>
<td>Homogenate</td>
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<td>2/2</td>
</tr>
<tr>
<td></td>
<td>Sup-IIa</td>
<td>0/3</td>
<td>0/3</td>
</tr>
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<td>Res-III</td>
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<td>2/15</td>
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<td>Buffer</td>
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<td>0/5</td>
</tr>
<tr>
<td></td>
<td>Buffer-sucrose</td>
<td>0/12</td>
<td>0/6</td>
</tr>
</tbody>
</table>

* Sup-IIa obtained from the 1st 105,000 relative centrifugal force centrifugation, Res-III and pellicle from the 2nd (Chart 1).

* No. of mice in which tumor(s) grew/No. of mice inoculated.

* In the normal rabbit serum controls and cell controls, all mice developed tumors.
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<table>
<thead>
<tr>
<th>TABLE 2</th>
<th>ADSORPTION OF CYTOTOXIC ACTIVITY OF RABBIT ANTI-6C3HED SERUM WITH THE SOLUBLE PORTION (pH 9.2) OF THE PELLICLE FRACTION OF THE TUMOR</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{Treatment of 6C3HED pellicle fraction} )</td>
<td>( \text{Dilution of adsorbed antiserum} )</td>
</tr>
<tr>
<td>105,000 relative centrifugal force, 2 hr; supernatant fraction</td>
<td>( 0/3^{a} )</td>
</tr>
<tr>
<td>To pH 9.2 with 1 N NaOH, 105,000 relative centrifugal force, 2 hr; supernatant fraction to pH 7.4</td>
<td>( \text{Reagent control}^{b} )</td>
</tr>
<tr>
<td></td>
<td>( 0/2 )</td>
</tr>
</tbody>
</table>

\( ^{a} \) No. of mice in which tumor(s) grew/No. of mice inoculated. 
\( ^{b} \) In the normal rabbit serum controls and cell controls, all mice developed tumors.

Results. Dialysis against 0.075 m barbital-sodium barbital buffer, pH 8.4, dialysis against 0.05 m sodium tetraborate, pH 9.0, and the addition of 0.1 N sodium hydroxide to pH 9.2 produced inactive or weakly antigenic supernatant fractions. A rapid increase in pH seemed to be necessary. This speed may have caused less alteration in the molecular configuration of the antigen than would have resulted from a slow change. A possibly related observation was made during acid treatment. The active components of the pellicle fraction were precipitated by the rapid addition of an equal volume of 0.5 M trichloracetic acid with constant stirring. If the trichloracetic acid was added at the rate of 1 ml/min, antigenic properties remained in neither the precipitate nor the supernatant fraction. Readjustment of the active trichloracetic acid precipitate to pH 9.2 with sodium hydroxide did not solubilize the antigen.

Exposure of the tumor pellicle fraction to various enzymes was of no aid in freeing a soluble antigen, but did give some information concerning the possible chemical nature of the antigen. The pellicle fraction was treated at 35°C for various time intervals with enzymes dissolved in phosphate buffer at pH 7.4. Although these conditions were not optimal for the action of all the enzymes, they had been shown to permit some activity. The final concentration of each enzyme after addition to the tissue fraction was as follows: trypsin (twice crystallized), 5 mg/ml; DNase, 0.5 mg/ml; RNase, 0.5 mg/ml; wheat germ lipase, 0.5 mg/ml; and \( \alpha \)-amylase (proteinase-free), 1 mg/ml. Magnesium sulfate was added to the buffer in which the DNase was dissolved to a final concentration of 0.01 M. After incubation the trypsin was inactivated with soybean trypsin inhibitor (5 mg/ml). Control tests demonstrated that antitumor antibody was not affected by the enzymes under the conditions employed. Antiserum adsorbed with lymphosarcoma pellicle fraction exposed to trypsin for 6 hr had a mouse cytotoxicity test titer at least 2 tubes (4-fold) higher than did antiserum adsorbed with the pellicle fraction control preparation. Amylase and lipase destroyed adsorptive activity of the tumor fraction by 11 hr, although not by 4 hr. DNase and RNase had no effect upon antigenicity during 18 hr of incubation. These results suggested the presence of protein, lipid, and carbohydrate in the tumor antigen. Further fractionation revealed that DNA was not detectable and that the removal of RNA-containing components did not alter activity. The presence of protein, carbohydrate, and lipid was confirmed by chemical "spot" tests. Sodium sulfate fractionation at 33%, 50%, 66%, and 100% saturations resulted in partial retention of activity in the 33-50% precipitate and in the final supernatant fraction. The total activity remaining seemed to be less than 0.5 of that exhibited by the whole pellicle fraction. Efforts to solubilize the antigen with urea or with oleic acid were in vain. Although addition of urea to a final concentration of 0.002 M with immediate dilution to 0.001 M gave partial solubility, the major portion of the activity was still in the residue.

Schultz-Dale Results. The uterine muscles of actively immunized mice responded to pellicle fractions of both normal tissue and tumor tissue. The contraction after the addition of the tumor fraction was greater than the reaction to the spleen or lymph node-thymus fractions; response to the spleen pellicle fraction was the weakest. It was possible to exhaust the ability of the muscle to react to the splenic fraction. The response to the lymph node-thymus fraction was then minimal. Once the reaction to these normal tissues could no longer be elicited,
application of tumor fraction still resulted in contraction, although it was less than the reaction observed when previous exposure to normal tissue antigens was omitted. Chart 4 presents such a series of reactions. Since contraction occurred after the addition of the lymphosarcoma fraction, although the reaction to the altered normal tissue fractions had been exhausted, it was concluded that a specifically different antigen or antigenic complex might be present in the tumor preparation. Uterine muscles from normal (nonimmunized) C3H mice did not respond to properly dialyzed preparations.

**Discussion**

Both immunization of C3H mice with a pellicle fraction of lymphosarcoma 6C3HED, isolated by differential centrifugation and flotation, and the Schultz-Dale response to the fraction by uterine muscles of actively immunized C3H mice demonstrated that there was an antigenic difference between 6C3HED and the normal tissues of the C3H mice. The portion of the pellicle fraction that was soluble at pH 9.0-9.5 retained antigenicity as measured by the ability to block antitumor antibody in mouse cytotoxicity tests. The antigenic substance appeared to be a complex containing protein, lipid, and carbohydrate; cytotoxicity tests indicated that all 3 were necessary for maximum adsorption of rabbit antilymphosarcoma antigen by the fraction.

The lymphosarcoma 6C3HED pellicle preparation appeared to be similar to the antigens described by others (3, 4, 6, 12-14, 17, 19) in a number of respects. It was particulate and contained protein, lipid, and reducing sugars. Sensitivity to acid (trichloracetic) was noted when it was added slowly, but not when it was dumped in rapidly. The effect of increased pH seems to have been similar. We achieved solubilization of the active components (adsorption of cytotoxic antibody) at pH 9.0-9.5, with inactivation occurring with further increase in alkalinity. Davies (6) recorded separation of a soluble fraction from his particulate antigen at pH 9.5, with complete solution (and loss of antigenicity) at pH 10.5. LeJeune et al. (17) reported solubility of hemagglutinin-inhibiting constituents after hydrolysis of the particulate antigen from L5178Y lymphoblasts in hydrochloric acid at pH 2 and 50°C. Sensitizing activity of the soluble material was considerably less than that of the untreated antigen. Kandutsch (13) was able to solubilize the enhancement-related antigen in 5% Triton X100 with retention of activity. Treatment of the Triton-soluble material with snake venom permitted recovery of a water-soluble component, which was capable of adsorbing hemagglutinin (15). None of the biologic methods were the same as used in the present study, so direct comparison of the various antigenic preparations is not possible. To avoid known problems associated with various technics, we selected methods that relied entirely upon reactions of the inbred C3H mouse, immunization and the Schultz-Dale response, to detect antigenic specificity. However, isolation of the antigenic fraction from the tumor homogenate was followed with cytotoxicity tests in which heterologous (rabbit) antitumor antiserum was adsorbed with fractions obtained from the tissues. Blocking or removal of the antibody was detected by the inoculation of mice with tumor cells exposed to the adsorbed antiserum. This screening test would fail to detect antigens that (a) were too weak to elicit sufficient antibody production in the rabbit, (b) were identical to components of rabbit tissues, or (c) induced antibodies lacking in cytotoxic ability in the presence of normal guinea pig serum. The method would not differentiate between specific 6C3HED antigens and normal tissue antigens present in the tumor cells used to immunize the rabbits.

Fink (8) used the Schultz-Dale test to study mouse tumor antigens and anti-mouse tumor antibodies. She demonstrated the reaction in BALB/c mice immunized with the isologous Sarcoma S-621, lyophilized and emulsified in Freund's adjuvant; the “microsome” fraction of the tumor was the challenging antigen. In our work, C3H mice were immunized by repeated regressions of lymphosarcoma 6C3HED to eliminate antibody production against nontumor antigens and to reduce the alteration of cellular components by excessive manipulation of the cells, as well as to avoid lyophilization, which greatly attenuated the activity of the tumor homogenate. Foley (10) showed that induced regression of 6C3HED lymphosarcoma resulted in resistance of the mice to later inoculation of viable tumor cells. MacDowell et al. (18) immunized C58 mice to leukemia Line 1 by initial inoculation of minimum lethal doses and demonstrated the development of resistance in survivors to increasingly larger doses of tumor cells, given until high doses were completely inhibited. The method was used since it appeared that it should be satisfactory in this system, in view of Foley's results. Since any antibodies induced in the mice should be directed only against antigens specific to the tumor, the Schultz-Dale response should be observed only after the addition of the same tumor-specific antigens. Nevertheless, reactions did occur in the presence of normal lymphoid tissue preparations. Unfortunately, even the simple process of harvesting an ascites tumor will undoubtedly cause structural alterations in the cell constituents. Thus, altered normal tissue antigens and altered and/or unaltered tumor antigens were present in the immunizing preparations. For this reason, fractions of tissues from normal C3H mice, made at the same time.
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as the tumor fraction and presumably exposed to the same deleterious influences, were used to desensitize the immune uterine muscle before exposing it to 6C3HED fractions. Whether the apparent antigenic difference, observed by the persistence of the reaction to the tumor after desensitization, was quantitative or qualitative was not ascertained to our satisfaction. The tumor pellicle fraction was always capable of eliciting a greater response than were the normal tissue fractions, when similar volumes or even smaller ones were used. There was insufficient lymph node-thymus material to expose the muscle to large amounts of this fraction and then test for a response to small quantities of the tumor preparation. As indicated in Footnote 3, the tumor altered biologically in such a way that completion of these studies was impossible. The tumor became hemorrhagic, was highly toxic to the mice, and seemed to differ antigenically from earlier generations. Efforts to demonstrate microbial contamination of the tumor were not successful. As a result of this alteration, studies could not be continued. Further examination of the immunizing ability of the pellicle and the soluble fractions, continuation of Schultz-Dale testing both with the soluble fractions and after desensitization of the immune muscle with other normal tissue materials in addition to lymphoid tissue fractions, and the utilization of other biologic tests that would have permitted comparison of this component of the 6C3HED tumor with tissue antigens described by other investigators would have revealed valuable information for the determination of the true relationship of the antigenic material to the tumor cell and to the normal tissues of the mouse. Characterization of the soluble fraction, if it proved to be fully active in all tests, by analytical ultracentrifugation, immunoelectrophoresis, additional chemical analyses, and other available physicochemical methods would have provided more specific information concerning singularity or multiplicity of the antigen detected, whether all chemical components present were required definitely for antigenicity, and if specificity could be increased by the removal of some of these components.

We cannot be certain, but it seems possible that the antigen responsible for the cytotoxic action of rabbit antiserum upon lymphosarcoma 6C3HED, as reported by Kidd (16) and Nungester and Fisher (20), is present in this preparation, and it may well be the same as that which Davies and Hutchison (7) detected in 6C3HED ascitic fluid. The origin of the antigenic component is not known. It may have been present in the tumor since its induction or have arisen later during transplantation in C3H mice of various sublines (the tumor strain used was obtained from J. G. Kidd in 1955). It is possible that the component is or contains one of the normal tissue antigens present in the tumor cell, e.g., a transplantation antigen. Finally, the active substance may be a tumor-specific antigen, an antigenic complex, or a group of antigens, which has been incompletely differentiated from, and thus cross-reacts with, normal tissue antigens, at least those in lymphoid cells.

Acknowledgments

Grateful acknowledgment is extended to Dr. Mary A. Fink, National Cancer Institute, NIH, for helpful suggestions regarding the Schultz-Dale tests and to Dr. J. H. Gaddum, Pharmacology Department, University of Edinburgh, whose recommendations were incorporated into the design of the tissue bath used for these tests.

References

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