A Study of the Preparation, Localization, and Effects of Antitumor Antibodies Labeled with I\textsuperscript{131}*

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The possibility of healing a malignant neoplasm with a specific immune serum has stimulated much research. Neoplastic cells often possess sufficient antigenicity and specificity so that cellular or humoral antibodies to tumor cells may be fabricated by the animal bearing the tumor. The potency of this immune response may be great enough to prevent the "take" of the reinoculated tumor (2, 24, 29, 30). Furthermore, a specific type of local inflammatory response, which may indicate the development of spontaneous resistance to the neoplastic cells (13, 26), has been observed at the edge of some tumors.

Antibodies to neoplastic tissue in one species can be produced in another. These antibodies can be demonstrated serologically (7, 11, 12) and, under some conditions, have been shown to alter the growth or appearance of neoplastic cells in vitro (5, 9, 10). There is little evidence, however, that the growth or structure of well established neoplastic cells can be altered in vivo by immunologic methods.

More recently, Pressman and co-workers (16-21) have pioneered in demonstrating that a specific heterologous antijorgan or antitumor serum labeled with I\textsuperscript{131} shows in vivo localization (8, 20). This suggests that an antitumor serum might be effective in retarding neoplastic growth if its specificity were increased and if it were coupled with adequate quantities of a radioactive substance.

This study was undertaken to test this hypothesis by ascertaining, both in vitro and in vivo, the localization and effects of antitumor sera to a number of transplantable tumors.

The investigation is particularly timely, since progress in immunologic methods makes it appear likely that: (a) a particularly potent and specific antitumor serum can be produced by using certain adjuvant and elution procedures; (b) the potency and specificity of antitumor antisera can be measured more accurately than previously; (c) the fate of antitumor sera can be traced in vivo by means of radioactive labels; and (d) these radioactive substances might be used to localize therapeutic amounts of radioactivity in a neoplasm.

MATERIALS AND METHODS

Neoplasms and their maintenance.—Three transplantable tumors were employed: the Flexner-Jobling rat tumor, the Jensen rat sarcoma, and the Ehrlich mouse ascites tumor. The two rat tumors were maintained by weekly intramuscular transplants of 0.2 ml. of 1:2 saline homogenate of the neoplastic tissue injected into the anterior thigh muscles of 200-gm. Sprague-Dawley rats. The Ehrlich ascites tumor was preserved by weekly passage of 0.1 ml. of a 7-day growth injected intraperitoneally into CF No. 1 mice, weighing about 25 gm.

Preparation of tumor for immunization and antibody-combining capacity determinations.—The rat tumors were removed aseptically 1 week after inoculation, and the grossly bloodless areas were dissected free, frozen quickly, and placed in sterile petri dishes over P\textsubscript{2}O\textsubscript{5} in a vacuum until used. The mouse tumor used for immunization was a fresh 5 per cent suspension washed 3 times in 0.86 per cent NaCl. Washed lyophilized tumor was used for the in vitro experiments.

Production of antitumor sera.—Antisera to each of the tumors were produced by injecting normal adult rabbits with a 5 per cent suspension\textsuperscript{1} of tumor in 0.86 per cent NaCl. The cell suspensions were prepared once a week on the day of the first injection for the week. The rabbits were injected on 3 consecutive days of each week via five portals simultaneously, i.e., intraperitoneally, subcutaneously, and intradermally. The intraocular injections were given in five different areas, 0.2 ml. to an area, each day. The injection pattern was repeated for 3 consecutive weeks. In some instances, the first injection of the 1st week was given intramuscularly in Freund's adjuvant. The antiserum was collected by withdrawing blood, with sterile precautions, from the hearts of immunized rabbits on the 4th, 5th, and 6th weeks. Control serum from noninjected animals was obtained simultaneously. The antiserum was lyophilized and stored at —20° C. in the dry form over P\textsubscript{2}O\textsubscript{5} in a vacuum.

Production of gamma globulin.—The gamma globulin fraction of the sera was separated by the method of Nichol and Deutsch (15) whenever it was needed.

Iodination of the antitumor serum.—Iodine-131 was attached to the antitumor gamma globulin by the method of Talmage et al. (29). After iodination, the globulin was transferred dropwise to a 130 X 15-mm. column of Amberlite (Fisher IR-4B(OH)). This was followed by the dropwise addition of 1-ml. quantities of distilled water. The fluid emerging from the column was collected in 1-ml. amounts in glass tubes. Each tube was surveyed separately, and the washes containing the greatest quantities of radioactivity were discarded. The sera was separated by the method of Nichol and Deutsch (15) whenever it was needed.

1 Expressed as wet weight of fresh tumor.
amounts of radioactivity were combined (usually 4-ml yield). Suitable small samples of the iodinated solution were removed before and after passage through the Amberlite column to assay the degree of protein-binding of $^{131}I$. After washing the precipitates with 1:10 dilution of 20 per cent trichloroacetic acid, the radioactivity in the supernatants and precipitates was counted, and the per cent of original radioactivity in the protein precipitate was determined.

Elution of the labeled $\gamma$-globulin.—To further increase the specificity of the iodinated antitumor $\gamma$-globulin, specific elution methods similar to those described by Talmage et al. (25) for purified antigen-antibody systems were adapted to our conditions.

In vitro evaluation of the antitumor sera.—Complement fixation (27) and/or the in vitro combination of the iodinated antitumor $\gamma$-globulin with the powdered whole lyophilized tumor (23) were used to evaluate the potency and specificity of each antiserum before it was injected into the animals. Suitable controls for antibody (normal or other antitissue rabbit $\gamma$-globulin) and antigen (various lyophilized normal tissues) were employed. The latter test usually consisted of placing 10 mg. of lyophilized, pulverised tumor or control tissue in a test tube to which was added 0.5 ml. of normal rabbit serum. After the tumor was suspended in the normal serum, 0.5 ml. of a suitable dilution (for counting) of the iodinated antibody $\gamma$-globulin to be tested was added to each tube, and the tubes were shaken in the cold (4° C.) for 1 hour. They were then centrifuged in the cold. The supernatants and the final precipitates were counted separately, and the results were expressed in terms of percentage uptake by the various antigens.

In vivo experiments.—Eluted iodinated $\gamma$-globulins, both normal and antitumor, were given to animals bearing tumors of comparable size. In most instances the thyroids were "blocked" by the prior administration of Lugol's solution. In general, the anti-Jensen and anti-Flexner-Jobling preparations were given intravenously and the anti-Ehrlich ascites tumor preparations, intraperitoneally. The animals were sacrificed by etherisation, and the thorax and abdomen were opened. A little heparin was introduced into the right ventricle, and a blood sample removed to determine radioactivity. (The ascites tumor was collected first in heparin-containing tubes.) The animals were then perfused with 0.86 per cent NaCl equal to about 10 times their normal blood volume, and representative organs and tissues were removed for weighing and counting.

Iodine-131 and counting procedures.—The iodine was obtained from the Oak Ridge National Laboratory as carrier-free $^{131}I$ and was used for iodination without further processing. It usually had a specific activity of from 8 to 50 mc/ml at the time of iodination, the samples of higher specific activity giving the more efficient iodination. Two-mil. samples were placed in 8-ml plastic tubes and counted in a well-type scintillation counter and compared with suitable iodine-131 standards counted simultaneously.

**RESULTS**

**Preliminary experiments.**—Various methods of immunizing rabbits against rat tissues were studied in two preliminary experiments. The data indicated that sera with a maximum complement-fixing capacity were obtained 1 week after 3 consecutive weeks of immunization and that no fur-
tion alone, multiple-portal injection alone, and in Freund's adjuvant followed by multiple-portal injection revealed that the number of 50 per cent hemolytic units fixed by sera obtained by each of the methods were, respectively, 2.2, 8.5, and 11.0. Stimulation of the antibody-forming mechanism was also evidenced by the fact that, in a large number of γ-globulin preparations from antitumor antisera, the immune sera averaged 70–100 mg. of γ-globulin/10 ml, while the normal rabbit sera averaged about 40 mg. (Table 2).

In vitro studies demonstrating combination of iodinated antitumor γ-globulin with lyophilized tumor.—Although complement fixation has yielded many data concerning the potency and specificity of antitissue antibodies, the procedure is cumbersome when applied to a very complex antigen-antibody system such as this. It thus seemed reasonable to try to use the combining power in vitro as a guide to the potency and specificity of the antitumor sera that were being studied.

Preliminary studies with γ-globulin prepared from antirat kidney serum, anti-Jensen sarcoma serum, and normal rabbit serum revealed that it was possible to obtain a measure of specificity and potency with this procedure (Chart 1). A study of some of the characteristics of this reaction with noneluted antirat kidney serum (developed in rabbit) showed that the reaction was relatively constant, within the limits employed, when either the amount of antibody γ-globulin or the time of the reaction was varied. But when the amount of antigen was varied, with the quantity of antibody and reaction time held constant, the per cent of radioactivity bound to the antigen steadily increased at a rapid rate until 5–10 mg. of antigen were present and then increased at a slower rate (Chart 2). Subsequent study of this reaction with anti-Flexner-Jobling γ-globulin has indicated that a part of

<table>
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<th>TABLE 2</th>
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<tr>
<td><strong>YIELDS OF COLD ALCOHOL (15) γ-GLOBULIN PRECIPITATIONS (IN MG/10 ML OF SERUM) OBTAINED WITH ANTI-JENSEN, ANTI-FLEXNER-JOBLING, ANTI-EHRlich ASCITES, AND NORMAL RABBIT SERA</strong></td>
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<tr>
<td>76.3</td>
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<td>98.5</td>
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<td>Av. 70.3</td>
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**CHART 1.**—In vitro uptake of I131-labeled noneluted γ-globulin by homologous and heterologous tissue.
this increase was probably due to nonspecific absorption, since iodinated normal rabbit γ-globulin as well as specific antibody globulin was removed from the tumor by an overnight wash with non-iodinated normal rabbit serum (Chart 3). A part of this increase in combining activity appeared to be specific, however, and suggested that the test was being performed in a region of antibody excess. With the 80-mg. antigen level, there was almost complete cross-reaction between the eluted anti-tumor γ-globulin and another transplantable rat neoplasm, as contrasted with the partial cross-reaction with rat kidney or rat liver. At the 10-mg. antigen level, a greater combining capacity of the

![Graph](chart2.png)

**Chart 2.**—Effect of variation of antigen and antibody concentration and reaction time on the *in vitro* binding of 131I-labeled noneluted antirat kidney γ-globulin by lyophilized rat kidney.

![Graph](chart3.png)

**Chart 3.**—*In vitro* uptake of 131I-labeled eluted anti-Flexner-Jobling and normal γ-globulin by the corresponding tumor and other rat tissues under varying conditions.
antiserum for the homologous tumor was demonstrable. Chart 4 represents a comparison of two methods of elution that have been described by Talmage et al. (25) as effectively increasing the specificity of labeled antibody globulin in simpler antigen-antibody systems. The particular anti-Flexner-Jobling sarcoma γ-globulin employed in this experiment showed no greater avidity for its tumor than for rat kidney before elution. It did, however, show greater combining capacity than normal rabbit γ-globulin for kidney and tumor. After elution by heat plus specific antiserum, with or without acidification, the increased specificity of the eluate was evident. Although acidification during elution apparently increased the efficiency of the elution process, it resulted in little if any more specificity.

The consistency with which elution by the specific unlabeled antiserum resulted in greater in vitro localization of the labeled γ-globulin may be seen in Table 3, which gives the per cent of radioactivity that combined with the appropriate tu-

<table>
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<tr>
<th>Antigen (10 mg)</th>
<th>FJ Tumor</th>
<th>Rat Kidney</th>
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<tbody>
<tr>
<td>Liver before elution</td>
<td>4.4 ± 1.5</td>
<td>9.3 ± 1.5</td>
</tr>
<tr>
<td>Liver after elution</td>
<td>44 ± 2.1</td>
<td>55 ± 2.0</td>
</tr>
<tr>
<td>Normal γ-globulin</td>
<td>8.5 ± 2.1</td>
<td>18 ± 2.0</td>
</tr>
<tr>
<td>Anti-Flexner-Jobling</td>
<td>9.3 ± 1.5</td>
<td>9.5 ± 1.7</td>
</tr>
<tr>
<td>Anti-Ehrlich ascites</td>
<td>9.5 ± 1.7</td>
<td>9.1 ± 1.6</td>
</tr>
<tr>
<td>Anti-Flexner-Jobling</td>
<td>9.3 ± 1.5</td>
<td>9.5 ± 1.7</td>
</tr>
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* The results are expressed as the average of several in vitro experiments performed with different γ-globulin samples eluted at different times.
mor, rat kidney, and rat liver, before and after elution in a series of *in vitro* tests. The elution methods are fitted admirably for preparing material to be injected into animals, because they involve only the addition of specific antiserum to the original labeled material. However, the low efficiency of the elution process for this type of antigen-antibody system makes it difficult to retain a therapeutically effective amount of radioactivity. When the efficiency of elution with heat and unlabeled immune serum was determined, it was found that only 3–6 per cent of the original labeled γ-globulin combined with the antigen during absorption, and from 20 to 50 per cent of this combined labeled γ-globulin was released from the antigen during elution.

In vivo experiments with rat tumors.—A series of experiments was undertaken in order to ascertain whether the specific combining power of the eluted antitumor γ-globulin for its antigen could be demonstrated *in vivo*. The eluted antirat tumor γ-globulin was administered to the tumor-bearing animals 10–18 days after transplantation, and the animals were then sacrificed at intervals to determine the fate of the labeled antibodies.

It is apparent from Chart 6 that the eluted anti-Flexner-Jobling γ-globulin (AFJ) injected intravenously in doses of 3.2 million counts/min into the rats bearing 17-day tumors localized in the tumor to a greater extent than did the normal rabbit γ-globulin, which had a similar radioactivity (3.7 million counts/min) and protein content/ml. There was much greater localization of radioactivity in the spleen, liver, and lungs of both groups than in the tumors. In contrast to the *in vitro* experiments, relatively small quantities of the labeled eluted antitumor γ-globulin combined with kidney. The localization in other organs was greater with specific antiserum than with control serum. Correspondingly, the rate of excretion of radioactivity in the urine was considerably faster in the animals receiving the control serum, while the labeled specific antitumor γ-globulin disappeared somewhat more quickly from the blood stream. It is of interest that the tumors were the only tissues studied in which radioactivity increased during the interval from 1 to 24 hours. The tumors apparent-

![Chart 5](chart5.png)

**Chart 5.**—Comparison of the *in vitro* uptake of eluted and noneluted *I*²¹⁺*-labeled anti-Ehrlich ascites γ-globulin by the EA tumor and various normal mouse tissues.

Chart 5 shows the differential affinity of the eluted labeled anti-Ehrlich ascites γ-globulin for the lyophilized Ehrlich tumor. Greater apparent specificity of the antitumor γ-globulin was demonstrated with 10 mg. of antigen, as compared with 50 mg., probably because there was less nonspecific adsorption with less antigen. Again the kidney, in the mouse as in the rat, shows the greatest crossreaction with the eluted antitumor γ-globulin. Since the ascites tumor has no stroma and is presumably derived from an undifferentiated carcinoma, it seems very unlikely that this cross-reaction was the result of a common connective tissue antigen, as might be assumed in the case of the crossreaction between antirat tumor sera and rat kidney. The diminished difference between the affinity demonstrated with 10 mg. of antigen and 50 mg. of antigen, and the greater contrast between the reaction of normal rabbit γ-globulin and the reaction of the anti-Ehrlich ascites tumor γ-globulin, indicate that there is less nonspecific absorption of labeled γ-globulin by the ascites tumor than there is by the rat tumors.

Since the ascites tumor has no stroma and is presumably derived from an undifferentiated carcinoma, it seems very unlikely that this cross-reaction was the result of a common connective tissue antigen, as might be assumed in the case of the cross-reaction between antirat tumor sera and rat kidney. The diminished difference between the affinity demonstrated with 10 mg. of antigen and 50 mg. of antigen, and the greater contrast between the reaction of normal rabbit γ-globulin and the
ly also lost the radioactivity more slowly than other tissues.

Similar results were obtained in other experiments in which heat or acid plus heat-eluted AFJ antibodies were employed. When the noneluted AFJ globulin was utilized, the trends were the same, but the differences between the animals receiving the control serum and those receiving specific antiserum were less great and less consistent. No corresponding localization in the tumors was obtained in the single experiment in which eluted anti-Jensen sarcoma γ-globulin was used on animals bearing Jensen sarcoma.

A chronic treatment experiment was performed in which intravenous doses of iodinated AFJ γ-globulin eluted with excess antiserum were given tumor-bearing animals every other day for 16 days. Comparable tumor-bearing animals were given a similarly iodinated normal rabbit serum (NRS) preparation. All animals were sacrificed at 1 hour after the final injection. The greatest differences in the localization of I\textsuperscript{131} between the two groups were again observed in the spleen and liver, the animals that had received the specific antiserum showing much more localization in these organs than those receiving NRS I\textsuperscript{131}. There were no consistent differences between the two groups in the per cent of injected radioactivity that localized in the tumors, and there was no evidence of a progressively increasing concentration in the
tumors at the expense of the other organs. However, the tumors of the group receiving the specific antiserum averaged two-thirds the weight of those from the noninjected control group and showed slight but consistent histologic changes that suggested a deleterious effect on the tumor. In these animals, there appeared to be a slightly more prominent inflammatory reaction at the perimeter of the tumors.

In general, the in vivo experiments with the rat tumors gave little promise of yielding a method for retarding neoplastic growth. Although the in vitro studies indicated that a fairly specific antitumor serum was available, it would not localize effectively in the tumor when given intravenously. Apparently, the capillary membranes, tenuous as they are in these transplantable tumors, block the passage of specific globulin into these tumors.

In vivo experiments with the Ehrlich ascites tumor of mice.—With the Ehrlich ascites tumor, the problem of capillary permeability was circumvented by injecting the radioactive labeled immune material intraperitoneally. If it could be established that such labeled immune γ-globulin was an effective carcinocecidal substance, the problem of getting the material across the capillary membrane might warrant further study. In the experiments to be described treatment was initiated 5 days after the tumor was transplanted. Comparable groups of mice were studied bearing tumors of comparable size based on increment of body weight following tumor inoculation. All animals were sacrificed 20 hours after injection. The ascitic fluid was separated into cellular and supernatant portions by centrifugation, and these were counted separately.

Three variables that might affect the response of the ascites tumor in vivo were investigated and are listed below:

1. Variation in amount of initial tumor inoculum: Mice were injected intraperitoneally with 0.02, 0.5, or 0.1 ml. of ascitic fluid.
2. Variation in type of globulin: Mice that had been injected intraperitoneally with 0.1 ml. of ascitic fluid 5 days earlier were given 1 cc. of iodinated eluted anti-Ehrlich γ-globulin (AEA I\(^{131}\)) or 1 cc. of iodinated normal rabbit γ-globulin (NR I\(^{131}\)) with equivalent protein content.
3. Effect of fasting on localization of AEA I\(^{131}\) by tumor: Some of the animals mentioned above were deprived of food after the injection of γ-globulin while others were fed ad libitum.

In addition, the two following sets of conditions were studied which contributed further information about the combination of AEA I\(^{131}\) and tumor:

1. Distribution of injected AEA I\(^{131}\) combined in vitro with tumor: Ascitic fluid from mice that had been previously injected with 0.1 ml. of Ehrlich ascitic fluid was pooled. Half was mixed with eluted AEA I\(^{131}\), and the other with NR I\(^{131}\). Both were shaken for 1 hour and then centrifuged in the cold. The sediments containing the tumor cells were washed, diluted with 0.86 per cent NaCl and then injected intraperitoneally into normal animals.

2. AEA I\(^{131}\) distribution in organs of the normal mouse after intraperitoneal injection: Iodinated anti-Ehrlich ascites tumor γ-globulin and iodinated normal rabbit γ-globulin were injected intraperitoneally into normal mice, and the distribution was studied.

Results of the study of these five variables are summarized in Table 4. The tumor cells localized 2-5 times the per cent of injected AEA I\(^{131}\)/cc found in the ascitic fluid and blood, and from 5 to 10 times that found in every tissue examined except the thyroid.

The AEA I\(^{131}\)-localizing capacity of the tumor in fasted animals was 2-3 times more per gm. than in the mice that were fed ad libitum.

In those animals that were given NR I\(^{131}\), there was about 3 times as much radioactivity in the supernatant fluid as in the tumor cells. The organs still localized less I\(^{131}\) than the tumor in these cases, but the blood had about twice the radioactivity of the tumor. There was little difference between the starved and fed groups that were injected with NR I\(^{131}\).

A comparison of the localization of AEA I\(^{131}\) in animals injected with different amounts of tumor suggests that there was an excess of antibody with respect to tumor antigen, since with increasing quantities of tumor there was a progressive increase in the total radioactivity bound by the tumor.

The distribution of AEA I\(^{131}\) that was attached to tumor cells before injection suggested that an appreciable part of the radioactivity which was found in the organs in the other studies might have been associated at some time with tumor.

The distribution of AEA I\(^{131}\) and NR I\(^{131}\) in normal mice indicated a greater I\(^{131}\) uptake in organs following injection of AEA than with the normal rabbit γ-globulin and, reciprocally, a lower amount remaining in the blood. This localization cannot be accounted for by cross-reactions between stroma of tumor and organs, as it might have been in the experiments with solid rat tumors. It must be due to common or related cellular antigens, as was suggested by the results of the in vitro experiments with the ascites tumor.

Another experiment was performed to test the
### TABLE 4

<table>
<thead>
<tr>
<th>Variables</th>
<th>Tumor</th>
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<tbody>
<tr>
<td>Initial tumor inoculum (ml)</td>
<td>Injection γ-globulin-labeled with 131I*</td>
</tr>
<tr>
<td>I A</td>
<td>0.02</td>
</tr>
<tr>
<td>I B</td>
<td>0.02</td>
</tr>
<tr>
<td>II A</td>
<td>0.1</td>
</tr>
<tr>
<td>II B</td>
<td>0.1</td>
</tr>
<tr>
<td>II C</td>
<td>0.1</td>
</tr>
<tr>
<td>II D</td>
<td>0.1</td>
</tr>
<tr>
<td>III A</td>
<td>0.5</td>
</tr>
<tr>
<td>III B</td>
<td>0.5</td>
</tr>
<tr>
<td>IV A †</td>
<td>0.1</td>
</tr>
<tr>
<td>IV B ‡</td>
<td>0.1</td>
</tr>
</tbody>
</table>

* The AEA consisted of eluted anti-Ehrlich γ-globulin labeled with 3.5 million counts 131I per cc. The normal γ-globulin (N) had a similar 131I content and an identical protein content.
† No Lugol’s solution given in this experiment.
‡ Globulin + tumor combined in vitro before injection.

### TABLE 5

<table>
<thead>
<tr>
<th>Variables</th>
<th>Tumor</th>
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<tbody>
<tr>
<td>Initial tumor inoculum (ml)</td>
<td>Injected gm. γ-globulin</td>
</tr>
<tr>
<td>I A</td>
<td>0.02</td>
</tr>
<tr>
<td>I B</td>
<td>0.02</td>
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<tr>
<td>II A †</td>
<td>0.1</td>
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<tr>
<td>II B</td>
<td>0.1</td>
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<tr>
<td>II C</td>
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<tr>
<td>II D</td>
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<tr>
<td>III A †</td>
<td>0.5</td>
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<tr>
<td>III B</td>
<td>0.5</td>
</tr>
<tr>
<td>IV A †</td>
<td>0.1</td>
</tr>
<tr>
<td>IV B</td>
<td>0.1</td>
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* Two of the three animals included in this average died before time of sacrifice.
† One of the three animals included in this average died before time of sacrifice.
‡ All three of the animals in this group died before time of sacrifice.
effect of multiple injections of iodinated γ-globulin on the net localization of I\textsubscript{131} in tumor-bearing and normal mice, varying the initial inoculum size, type of γ-globulin (AEA or NR γ-globulin), and state of nutrition, as in the first experiment. About 1.2 million counts of either I\textsubscript{131}-labeled AEA or NR γ-globulin were injected on each of 3 consecutive days either into mice bearing 4-day tumors (Groups I, II, III) or into normal mice (Group IV). The mice were all sacrificed on the 8th day (24 hours following the third injection). The results, summarized in Table 5, were, in general, comparable to those of Table 4 but showed the additive effect of multiple injections, which can be seen by comparing the per cent injected dose per gram. In the latter series, the tumor localized from 40 to 100 per cent of that in the former. If there was no additive effect with three injections, one would expect to find only about one-third of the total injected dose bound to tumor.

The difference in localization of AEA I\textsubscript{131} in tumors of fasted and fed animals was marked but somewhat less than that following a single injection. This difference was seen in other tissues as well, with those of fasted animals showing greater localization. In addition, there was a similar differential localization in the fasted animals given NR I\textsubscript{131}, suggesting that the increase involved factors other than increased specific antigen-antibody combination.

Again, the per cent injected dose per gram was greatest in animals bearing the smallest amount of tumor and decreased with increasing amounts of tumor, while the per cent injected dose per total tumor varied inversely, suggesting the presence of excess antibody. The localization of AEA I\textsubscript{131} and NR I\textsubscript{131} in normal animals was also similar to that found in the previous experiment.

The other main difference between the two series of experiments was the considerable mortality in the multiple-injection mice; a number of animals died between the 2d and 4th day after the injections were begun. Mortality was greatest in the AEA I\textsubscript{131} groups that had either the smallest amounts of tumor or none at all.

Two regimens were used to evaluate the effect of the eluted AEA I\textsubscript{131} γ-globulin on the survival of tumor-bearing mice. In one, four injections of AEA I\textsubscript{131} γ-globulin, or suitable control solutions, were spaced at 12-hour intervals over a 48-hour period. In the second, the various materials were injected in three equal doses of I\textsubscript{131} at 8-day intervals. The average survival time for the groups in each of these experiments indicated that the iodinated, eluted antitumor γ-globulin was toxic to the mice. In general, the AEA I\textsubscript{131} γ-globulin-injected mice died before the noninjected controls. Furthermore, in all cases, the group receiving the I\textsubscript{131}-containing substance died before the group receiving the corresponding unlabeled material. The effect, upon survival time, of unlabeled AEA γ-globulin with or without guinea pig complement was investigated further and is reported in the following paper (Flax).

There is little doubt that closely spaced multiple injections of AEA γ-globulin, with or without I\textsubscript{131} attached, had an inhibitory action on the growth of the ascites tumor. In Chart 7, the weight of tumor cells, determined at autopsy, is summarized for four of the groups. I\textsubscript{131} alone apparently slightly diminished tumor growth during the 7 days following four closely-spaced injections, even though little iodine remained attached to the tumor cells (Chart 8). A part of this effect may have been indirect, since the I\textsubscript{131}-injected mice probably ate poorly.

Much greater effects on the quantity of tumor were produced by multiple injections of AEA γ-globulin, with or without I\textsubscript{131} labeling (Chart 7). During the first 3 days following completion of the injections, the quantity of measurable tumor cells in the peritoneal cavity decreased abruptly while those in the noninjected control groups showed steady growth. However, about 6 days afterward, the tumor again showed a net growth rate similar to that of the noninjected controls.

Histologic study of the tumor cells from the AEA and AEA I\textsubscript{131} γ-globulin-treated groups revealed large numbers of necrotic cells and few mitotic
figures during the first 3–5 days after the course of injections was completed. A detailed analysis of these changes was not possible, since many of the mice underwent post-mortem change. However, the changes were similar to those described in the following paper (Flax). No significant additive effects resulted when I131 was attached to the antibody. This might be expected because the Ehrlich ascites tumor is a relatively radioresistant neoplasm, and even with the four closely-spaced injections, no more than 180 rep could have been delivered to the neoplastic cells in a 7-day period by clear track plates. The treated cells were well separated and outlined individually by the exposed silver. However, neither this technic nor that of sectioning gave a definitive answer to the question of whether the I131 was limited to the surface of the cell.

DISCUSSION

The results of these experiments may help to explain the failure of most previous attempts to influence actively-growing neoplasms in man and experimental animals by intravenous injection of

![Chart 8](image-url)

**Chart 8.**—The localization of AEA-I131 γ-globulin following multiple intraperitoneal injections in tumor-bearing mice

the radioactive antibody. Although the differential localization of the I131 on tumor cells appears creditable, both in the tracer experiments (Tables 4 and 5) and in the survival experiment (Chart 8), it is apparent that in the case of Ehrlich ascites tumor much more I131 would have to be attached to much larger quantities of specific antibody to evaluate a possible synergism between the effects of an attached radioactive substance and the toxic action of antibody in retarding tumor growth.

Attempts were made to make radioautographs of the in vivo treated cells to gain further information about the site of localization of AEA I131 γ-globulin. It was found that good radioautographs could be obtained by gently smearing the treated ascites tumor cells onto Eastman 10-μ nu-
established neoplasm with specific heterologous antiserum utilized a tumor derived from lymphocytic tissue (14). Recent studies suggest that lymphosarcomas and bone tumors should be studied further (8, 20).

Whether the specific in vivo localization of antibodies to tumor can be facilitated by the simultaneous administration of substances that increase capillary permeability remains to be investigated. Increase in differential localization would probably depend on a selective action on the capillaries of the neoplasm, which may be impossible to achieve. The effect of injection of the labeled antitumor γ-globulin directly into the tumor is of theoretical interest but would seem to offer little more than palliative therapy for the treatment of inoperable malignancies.

The results reported in this paper are not in disagreement with the frequent observation that immunologic mechanisms may prevent the growth of a transplantable tumor if the antibodies are present at the time of inoculation and before the tumor has established a blood supply. Further evidence along these lines is presented in the following paper (Flax). The rapid decrease in neoplastic cells following injection of the specific antibody indicates that antitumor γ-globulin is potentially effective in retarding tumor growth, if it reaches the cell. Recent work (4) suggests that with relatively more antitumor antibodies, marked diminution in the quantity of a rat ascites tumor can be achieved in vivo, and cures have been reported.

The results of the in vivo experiments with the Ehrlich ascites tumor pose at least two problems. The increased avidity of both normal tissues and the tumor cells for the antitumor antibodies in fasted mice, even though the period of fasting is quite brief, may indicate that the tissues of the fasted animals metabolize the intraperitoneally-injected foreign serum protein. Such an interpretation cannot be generally applied, however, since the fasted animals treated with normal serum fractions showed either no such difference as compared with comparable nonfasted animals or only a moderately increased combination of the labeled normal γ-globulin with the tumor cell.

The increased toxicity of the I131-labeled AEA γ-globulin for animals bearing no tumors or for those receiving smaller inoculums is also apparent. Does this indicate that binding of this eluted antiserum by tumor cells decreases its general toxicity for the animal as a whole? If so, the greater toxicity of the antiserum as compared with normal serum may result from a cross-reaction of the antiserum with vital organs and tissues.

Another aspect of these experimental results has been the in vitro demonstrations that there is a more marked cross-reaction between a given antitumor serum and a second unrelated tumor tissue than there is between the antiserum and certain normal tissues. This broad specificity of an antitumor serum for neoplastic tissue, in general, suggests an immunologic similarity between neoplasms and may be evidence for a unique biochemical structure of some part of the neoplastic cell (9, 11). Hence, immunologic methods may be applicable to further investigation of the essential difference between neoplastic cells and normal cells. The recent demonstration by Kidd (6) of a serologically distinct constituent in the Brown-Pearce carcinoma supports this suggestion.

SUMMARY

Multiple injections of lyophilized rat tumor tissue given to rabbits by multiple portals and preceded by a single intramuscular injection of the same material in Freund's adjuvant resulted in unusually potent and fairly specific antitumor antisera. The characteristics of these antisera have been investigated in vitro by means of complement fixation or by labeling the specific γ-globulin with I131 and determining the amount that combines with insoluble components of the tumor. The specific antitumor antibodies became more apparent following purification of the labeled γ-globulin by means of elution with additional unlabeled antitumor sera. There was an almost complete in vitro cross-reaction between antiserum produced to Flexner-Jobling tumor and Jensen sarcoma.

Intravenous injection of the eluted I131 antitumor γ-globulin into rats bearing the homologous tumor resulted in slight selective localization of the I131 γ-globulin in the tumor as compared with the localization of similarly labeled normal γ-globulin. However, there was always much greater localization of the antitumor γ-globulin in the liver and other organs. This failure of selective localization is probably due to the low permeability of the blood vessels of the tumor to the large antibody globulin molecules. When labeled antibodies to Ehrlich ascites tumor were injected intraperitoneally into mice bearing proliferating ascites tumors, the labeled antibody globulin was clearly differentially localized on the tumor cells.

Although the growth of the Ehrlich ascites tumor can apparently be retarded by the specific eluted I131-labeled γ-globulin, no increase in survival time has been demonstrated, probably because of the toxicity of the labeled antibody.

The toxicity of the intraperitoneally injected
labeled antiserum preparation for the tumor-bearing mouse varies inversely with amount of tumor-growing in the mouse.

Fasting of the mice during the period following injection of the labeled antiserum increases the localization of the specific antibodies on the tumor cells.

A synergism between the effects of the attached radioactivity and the toxic action of the antibody has not yet been demonstrated. With a more radiosensitive tumor and with better in vitro methods of increasing the specificity of the antiserum, a study of this combined carcinocidal approach should be possible.

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