Differences between Antibodies against the Dense Sediment and Microsome Fractions of the N-2-Fluorenylacetamide-induced Rat Hepatoma

SHINZO ISOJIMA, JOSEPH BERNECKY, JACOB PLANINSEK, YASUO YAGI, AND DAVID PRESSMAN

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SUMMARY

Rabbit antibodies against the microsome and dense sediment fractions of N-2-fluorenylacetamide-induced rat hepatoma were compared in vivo and in vitro. Although both antibodies localized in the hepatomas and normal livers when injected into tumor-bearing or normal rats, clear differences in their localizing properties could be shown by various techniques. The combined use of 125I-labeled anti-microsome and 125I-labeled anti-sediment antibodies in the same mixture (paired label technic) made it possible to compare the 2 antibodies quantitatively under strictly identical conditions. The localization in vivo of the anti-microsome antibodies was greatly reduced by treatment with the hepatoma microsomes, but no effect was observed on the localization of anti-sediment antibodies. When the hepatomas from the injected rats were fractionated into subcellular fractions, it was found that the microsome fraction contained a larger proportion of the anti-microsome antibodies than of the anti-sediment antibodies, whereas the dense sediment fraction contained a larger proportion of the anti-sediment antibodies than of the anti-microsome antibodies. The 2 antibodies also behaved differently when their localization was examined as a function of time. The anti-microsome antibodies seemed to localize and be eliminated more rapidly than the anti-sediment antibodies.

These differences were clearly reflected in their sites of localization as determined by radioautography of tissue sections from rats which had been injected with 125I-labeled antiserum globulins. The anti-microsome antibodies localized preferentially on the sinusoidal walls and on the hepatoma cells and normal hepatic cells. In contrast, the anti-sediment antibodies localized heavily in the connective tissues and lightly on the sinusoidal walls, but not on the hepatoma or normal hepatic cells.

Staining tissue sections in vitro by the fluorescent antibody technic showed that the anti-microsome antibodies were directed against the components of the hepatic cells and the anti-sediment antibodies against the components of the connective tissues.

Previously, we have shown that rabbit antibodies against rat hepatoma induced by N-2-fluorenylacetamide localize preferentially in hepatoma when injected into tumor-bearing rats (2, 5). Sediments from tumor homogenate obtained by low speed centrifugation were used as immunizing antigens in these earlier studies. The sites where such anti-hepatoma sediment antibodies


2 This work was done during a leave of absence from the Department of Obstetrics and Gynecology, Osaka University Medical School, Osaka, Japan.

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The present work deals with the comparison between antibodies prepared against the microsome fraction and against the sediment fraction of the rat hepatoma with particular reference to their localizing properties in vivo. Clear differences were found in the localization properties of the two antibodies in vivo by quantitative radiolabel techniques as well as by radioautography. Strict comparison between the 2 antibodies was made possible by the use of the paired label technic, according to which each antibody preparation was labeled separately with a different radioisotope of iodine (\(^{125}\text{I}\) and \(^{131}\text{I}\)) and mixed. Then the mixture was used for further purification and assay to ensure that strictly the same experimental conditions were used for both antibodies. Both antibodies were also examined for their binding properties in vitro to hepatic tissue components by radiolabeled antibody technic as well as by fluorescent antibody technics.

The results with radiolabeled antibodies indicated that the localizing antibodies in the anti-microsome preparation are clearly different from those in the anti-sediment preparation. Antibodies in the anti-microsome preparation localized in vivo primarily on the hepatoma and normal hepatic cells and sinusoidal walls, whereas antibodies in the anti-sediment preparation were fixed primarily in the connective tissue. The staining of tissue sections in vitro by fluorescent antibody technic revealed that the anti-microsome antibodies were directed against the components of the hepatic cells, whereas the anti-sediment antibodies were directed against the components of the connective tissues.

MATERIALS AND METHODS

Hepatomas were induced in Sprague-Dawley male rats by feeding N-2-fluorenylacetamide (4). Cellular fractions of hepatoma were obtained essentially by the method described previously (4). The sediment obtained by centrifugation at 1500 \(\times\) g for 15 min was refractionated by centrifugation at 100 \(\times\) g for 20 min and at 1500 \(\times\) g for 15 min to give dense sediment and light sediment respectively (1). Mitochondrion and microsome fractions were prepared from the supernatant (at 1500 \(\times\) g) by fractional centrifugation in sucrose-containing medium (7). Under a phase contrast microscope, the dense sediment appeared to contain mainly connective tissue fibers, although some cell debris was also seen.

Rabbit antisera against the microsome fraction were those described in our previous report (4). Antiserum against the dense sediment were produced similarly in rabbits with 100 mg (wet weight) for each injection.

Procedures for obtaining purified radioiodinated antibodies of 7 S \(\gamma\)-globulin nature were described previously (4) along with procedures for determining localization of antibodies in rat organs in vivo and their binding to rat tissue components in vitro. Rats with cholangiomas as judged macroscopically by prior laparotomy were not used for assay in vivo.

Technics for fluorescent antibody staining and for radioautography of tissue sections were also described previously (5). The computation of the results from paired and triad label experiments is described elsewhere (6).

RESULTS AND DISCUSSIONS

Quantitative comparison of 2 antibodies by the paired label technic.— Antibodies prepared against the microsome fraction and the dense sediment of hepatoma homogenate were compared quantitatively in vivo and in vitro by the paired label technic.

To remove the anti-rat plasma antibody which was always present in these antisera, the globulin fractions were passed through a column packed with rat plasma proteins conjugated to diazotized polyaminopolystyrene (4). The filtrates were further passed through DEAE-cellulose\(^4\) to obtain the 7 S \(\gamma\)-globulin. The 7 S \(\gamma\)-globulin from anti-microsome serum was iodinated with \(^{131}\text{I}\), and the antibody components were concentrated by adsorption on and elution from hepatoma microsomes. The elution was done in the presence of the original unlabeled antisem globulin. In most experiments, the eluate was further passed through a column of Sephadex G-200 to obtain 7 S \(\gamma\)-globulin antibody that was free of antigen-antibody complexes formed during the purification. The 7 S \(\gamma\)-globulin from anti-sediment serum was iodinated with \(^{125}\text{I}\) and treated in the same manner, except that hepatoma sediment was used for purification instead of the microsome fractions.

These purified radioantibody preparations from the 2 antisera are referred to as \(^{125}\text{I}\)-anti-Hep-micro-Ab and \(^{131}\text{I}\)-anti-Hep-sed-Ab. These were mixed and the mixture was used for various treatments to ensure strict comparison of the 2 antibodies (paired label). In certain experiments, 7 S \(\gamma\)-globulin from normal rabbit serum was labeled with \(^{125}\text{I}\) and added to the mixture. The 3rd label served to evaluate the contribution of nonspecific \(\gamma\)-globulin in the system (triad label).

Binding of labeled antibodies on tissue components in vitro.—The mixture of the labeled antibodies was treated with microsome or sediment of hepatoma to determine immunologic specificities. Mixtures containing tracer amounts of radiolabeled antibodies, as determined from radioactivity measurements, along with 2, 10, and 100 \(\mu\)g respectively of each original unlabeled antiserum globulin (used in elution process), were shaken with 1 mg of the microsome or sediment preparation for 60 min at 37°C in the presence of 1 ml of normal rabbit serum at pH 8.0 (final volume, 4.0 ml). The amount of radioactivity was less than 1% of the total antiserum globulin present. After adsorption, borate buffer was added to fill the 12-ml tube, and the mixture was centrifuged at 105,000 \(\times\) g for 90 min. The precipitate was dissolved by heating with NaOH and counted. The amount of remaining mother liquid in the tube was negligible (<0.3%).

The results are shown in Chart 1. They clearly indicate that a much larger fraction of radioactivity was adsorbed by microsomes from the \(^{131}\text{I}\)-anti-microsome preparation (40%) than from the \(^{125}\text{I}\)-anti-sediment preparation (15%). On the other hand, sediment fixed about equal portions of

\(^4\)The following abbreviations are used: DEAE-cellulose, diethylaminoethyl cellulose; \(^{131}\text{I}\)-anti-Hep-micro-Ab, the \(^{131}\text{I}\)-labeled, specifically purified 7 S \(\gamma\)-globulin antibody to hepatoma microsomes; \(^{125}\text{I}\)-anti-Hep-sed-Ab, the \(^{125}\text{I}\)-labeled, specifically purified 7 S \(\gamma\)-globulin antibody to the hepatoma sediment; GNS, the globulin fraction from normal rabbit serum.
radioactivity from both preparations (26–30%). The values at all 3 antibody levels were essentially the same, indicating that the amount of microsome or sediment used for adsorption (1 mg) was well in excess of the amount of antibody used. In a preliminary set of experiments, it was found that at higher levels (1 mg and 5 mg) of antiserum globulin there was a decrease in binding that showed definite saturation effects at these levels.

Higher uptake of the anti-microsome preparation by the microsomes (40%) than by the sediment (26%) indicates that there are some antibodies in the anti-microsome preparation adsorbable only by the microsomes and not by the sediment. Similarly, higher uptake of the anti-sediment preparation by the sediment (30%) than by the microsome fraction (15%) indicates that there are some antibodies in the anti-sediment serum adsorbable only by sediment and not by the microsome fraction. These points were further confirmed by fluorescent antibody technic (see below). The extent of nonspecific adsorption of 7S \( \gamma \)-globulin from normal serum was reported previously to be 6–8% (4). However, the values for most batches of 7S GNS used here were about 2%. Both these values are appreciably lower than the values for the antibody preparations.

**Localization of labeled antibodies in vivo after treatment with tissue components.**—Although the above results showed a clear difference between the anti-microsome and anti-sediment preparations in vitro, further experiments were carried out to determine the effect of such treatment on the localizing properties in vivo of the radiolabeled antibodies remaining in the supernatants.

Mixtures of tracer amounts of \( ^{125} \text{I} \)-anti-Hep-micro-Ab and \( ^{131} \text{I} \)-anti-Hep-sed-Ab containing 140 \( \mu \)g each of unlabeled original antiserum globulin were treated with 2.8 mg of the microsome or the sediment fraction of hepatoma in the same manner as described above. The labeling properties of the supernatants were tested by assaying them in both tumor-bearing and normal rats. The use of the paired label technic obviated any need to assay each preparation in a separate group of assay animals and ensured that the observed differences in localization were not due to differences in the procedures or differences in assay animals.

The results are shown in Table 1. The localization of the original preparation is given and expressed as the % of the injected dose localized 20 hr after injection. The amounts of the localizing antibodies remaining in the supernatant after treatment are given as the % of the amounts present in the original mixture. It is seen that the in vivo localizing antibodies in the anti-microsome preparation were much more reduced by treatment of the mixture with the microsome fraction than were those in

**TABLE 1**

**REMOVAL OF IN VIVO LOCALIZING ANTIBODIES BY TREATMENT WITH HEPATOMA COMPONENTS**

\( ^{125} \text{I} \)-anti-Hep-micro-Ab and \( ^{131} \text{I} \)-anti-Hep-sed-Ab were mixed and the mixture was treated with hepatoma components. The original or the supernatants after treatment were assayed in 2 normal and 2 tumor-bearing rats. The % of the injected dose localized in the liver of normal rats or in the tumor and the surrounding liver of tumor-bearing rats is shown for the original mixture. Relative amounts of tumor- or liver-localizing antibody remaining in the supernatants were calculated as follows:

\[
\text{%} \text{of supernatant localized} \times \text{% recovery of supernatant} = \text{% of original localized}
\]

Average values of localization of the original mixture in 2 tumor-bearing or normal rats were taken as the bases (100%). The recoveries of \( ^{125} \text{I} \)-anti-Hep-micro-Ab and \( ^{131} \text{I} \)-anti-Hep-sed-Ab were 51 and 82% in the supernatant from microsomes and 63 and 67% in that from sediment.

**LOCALIZATION OF ORIGINAL PREPARATION**

<table>
<thead>
<tr>
<th>% of injected dose localized</th>
<th>Liver with tumor</th>
<th>Normal liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>( ^{125} \text{I} )-anti-Hep-micro-Ab</td>
<td>7.5</td>
<td>19.9</td>
</tr>
<tr>
<td>( ^{131} \text{I} )-anti-Hep-micro-Ab</td>
<td>5.5</td>
<td>18.1</td>
</tr>
<tr>
<td>( ^{125} \text{I} )-anti-Hep-sed-Ab</td>
<td>4.2</td>
<td>15.7</td>
</tr>
<tr>
<td>( ^{131} \text{I} )-anti-Hep-sed-Ab</td>
<td>3.9</td>
<td>15.9</td>
</tr>
</tbody>
</table>

**EFFECT OF TREATMENT**

<table>
<thead>
<tr>
<th>Relative amounts of localizing Ab remaining in supernatants (% of original)</th>
<th>Tumor liver localizing Ab</th>
<th>Normal liver localizing Ab</th>
</tr>
</thead>
<tbody>
<tr>
<td>After treatment with microsomes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( ^{125} \text{I} )-anti-Hep-micro-Ab</td>
<td>72</td>
<td>55</td>
</tr>
<tr>
<td>( ^{131} \text{I} )-anti-Hep-micro-Ab</td>
<td>111</td>
<td>87</td>
</tr>
<tr>
<td>After treatment with sediment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( ^{125} \text{I} )-anti-Hep-micro-Ab</td>
<td>74</td>
<td>52</td>
</tr>
<tr>
<td>( ^{131} \text{I} )-anti-Hep-micro-Ab</td>
<td>64</td>
<td>53</td>
</tr>
</tbody>
</table>

* The paired label results for a single animal are given.

\( ^{125} \text{I} \)-anti-Hep-micro-Ab, the \( ^{131} \text{I} \)-labeled, specifically purified 7S \( \gamma \)-globulin antibody to hepatoma microsomes; \( ^{125} \text{I} \)-anti-Hep-sed-Ab, the \( ^{131} \text{I} \)-labeled, specifically purified 7S \( \gamma \)-globulin antibody to the hepatoma sediment.
the anti-sediment preparation. This indicates that antimicrosome preparation contained localizing antibodies qualitatively different from the major part of antibodies in the anti-sediment preparation. On the other hand, treatment with the sediment reduced equally the localizing antibodies in both antibody preparations. This unilateral cross reaction could be caused by the presence in the sediment prepared by low speed centrifugation of a small amount of cell debris which contains components belonging to the microsome fraction. The microsome fraction obtained by high speed centrifugation, however, would contain little, if any, of the major component of the sediment—connective tissue fibers. Although the extent of contamination by microsomal antigens in the sediment fraction must be enough to cause a large reduction of localizing antibodies in the anti-microsome preparation in the large antigen excess region, the portion of localizing antibodies against contaminant microsomal antigens in the anti-sediment preparation must be quite small relative to that directed to connective tissue antigens, since there is little reduction in localizing antibody activity by the microsome fraction.

Distribution of localized antibodies in subcellular fractions. —Differences between the anti-microsome and anti-sediment preparations were further shown when the distribution of antibodies localized in vivo was determined for various subcellular fractions of the hepatic tissue.

A mixture of \(^{125}\text{I}-\text{anti-Hep-micro-Ab}\) and \(^{131}\text{I}-\text{anti-Hep-sed-Ab}\) was injected into 2 hepatoma-bearing rats. The discrete hepatomas and the remainder of the livers were homogenized separately and fractionated by differential centrifugation to give fractions of dense sediment, light sediment, mitochondria, microsomes, and soluble proteins. The amounts of \(^{125}\text{I}-\text{anti-Hep-micro-Ab}\) and \(^{131}\text{I}-\text{anti-Hep-sed-Ab}\) bound to each fraction were determined by the radioactivity present. The results are shown in Table 2. The amounts of each antibody localized in the hepatomas, the remainder of the livers, and their subcellular fractions are given. The ratios of the localized antibodies from \(^{131}\text{I}-\text{anti-Hep-sed-Ab}\) to those from \(^{125}\text{I}-\text{anti-Hep-micro-Ab}\) show that these antibodies were distributed quite differently in these subcellular fractions. High ratios indicate preferential localization of the anti-Hep-sed-Ab, and low ratios indicate preferential localization of the anti-Hep-micro-Ab. In all cases, the ratios were highest in the dense sediment fraction, which consisted mainly of connective tissue components, and lowest in the microsome fraction, which apparently contained most of the cell surface materials. The differences between ratios for the 2 fractions—dense sediment and microsomes—were great, showing a factor of 20—30. Such differences would not be possible even if some antibodies remained in the intercellular spaces of perfused organs and became fixed on tissue components upon homogenization.

The use of the ratios, which are determined accurately by the paired label technic is particularly suitable for such comparison since any loss in a subcellular fraction during the fractionation procedure does not interfere with the result. In the above experiments, about 43—56 % of the localized radioactivity was recovered in the fractions obtained. In spite of problems of recovery, the major part of the radioactivity from the anti-sediment preparation (\(^{131}\text{I}\)) was always found in the dense sediment and light sediment fractions, whereas the radioactivity localized from the anti-microsome preparation (\(^{125}\text{I}\)) was found mainly in the other fractions. Without use of paired

### Table 2

**Localization in Vivo of Anti-Hep-micro-Ab (\(^{125}\text{I}\)) and Anti-Hep-sed-Ab (\(^{131}\text{I}\)) in Subcellular Fractions of the Hepatomas and the Surrounding Livers**

<table>
<thead>
<tr>
<th></th>
<th>(^{125}\text{I})</th>
<th>(^{131}\text{I})</th>
<th>(%\text{^{125}I/^{131}I})</th>
<th>(^{125}\text{I})</th>
<th>(^{131}\text{I})</th>
<th>(%\text{^{125}I/^{131}I})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatoma</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>0.82</td>
<td>2.37</td>
<td></td>
<td>2.17</td>
<td>5.46</td>
<td></td>
</tr>
<tr>
<td>Dense Sediment</td>
<td>0.074</td>
<td>0.85</td>
<td>11.5</td>
<td>0.202</td>
<td>1.95</td>
<td>9.65</td>
</tr>
<tr>
<td>Light Sediment</td>
<td>0.032</td>
<td>0.181</td>
<td>5.7</td>
<td>0.041</td>
<td>0.311</td>
<td>7.59</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>0.110</td>
<td>0.081</td>
<td>0.68</td>
<td>0.168</td>
<td>0.137</td>
<td>0.82</td>
</tr>
<tr>
<td>Microsome</td>
<td>0.064</td>
<td>0.028</td>
<td>0.44</td>
<td>0.276</td>
<td>0.127</td>
<td>0.46</td>
</tr>
<tr>
<td>Supernatant</td>
<td>0.071</td>
<td>0.119</td>
<td>1.68</td>
<td>0.351</td>
<td>0.55</td>
<td>1.57</td>
</tr>
<tr>
<td>Remainder</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>5.97</td>
<td>17.2</td>
<td></td>
<td>5.15</td>
<td>16.5</td>
<td>15.0</td>
</tr>
<tr>
<td>Dense Sediment</td>
<td>0.217</td>
<td>3.05</td>
<td>14.0</td>
<td>0.311</td>
<td>4.68</td>
<td>7.68</td>
</tr>
<tr>
<td>Light Sediment</td>
<td>0.454</td>
<td>3.49</td>
<td>7.70</td>
<td>0.296</td>
<td>2.04</td>
<td>7.68</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>1.07</td>
<td>0.87</td>
<td>0.81</td>
<td>0.378</td>
<td>0.314</td>
<td>0.83</td>
</tr>
<tr>
<td>Microsome</td>
<td>0.82</td>
<td>0.424</td>
<td>0.52</td>
<td>1.01</td>
<td>0.70</td>
<td>0.69</td>
</tr>
<tr>
<td>Supernatant</td>
<td>0.172</td>
<td>0.279</td>
<td>1.62</td>
<td>0.244</td>
<td>0.57</td>
<td>2.23</td>
</tr>
</tbody>
</table>

* These abbreviations are explained in Footnote b to Table 1.

a Hepatoma, 6.7 gm; remainder, 9.8 gm.
b Hepatoma, 13.9 gm; remainder, 10.1 gm.
c Highest and lowest ratios for each rat are set in boldface type.
The results are shown in Table 3. The extents of localization of $^{125}$I-anti-Hep-micro-Ab, $^{125}$I-anti-Hep-sed-Ab, and $^{125}$I-7 S GNS at 1, 6, and 18 hr after injection are shown for the hepatoma and the remaining portion of tumorous livers and for normal livers. The ratios of the % of $^{125}$I-anti-Hep-sed-Ab to the % of $^{125}$I-anti-Hep-micro-Ab are listed as a comparison of the localization of the 2 antibody preparations.

Differences in the localization behavior of the 2 antibodies were clearly shown by the change of the ratios with time after injection. The increase of the ratios indicates either that the $^{125}$I-anti-Hep-micro-Ab requires less time to reach the maximum localization or that it is removed more rapidly from the sites than $^{125}$I-anti-Hep-sed-Ab, or both. During the period of 6–18 hr, the ratios of anti-sediment to anti-microsome increased significantly, but the over-all localization values decreased. Thus, the increase of the ratios in this period indicates that the localized antibody from the anti-microsome preparation decreased more rapidly in the tissues than that from the anti-sediment preparation.

During the earlier period of 1–6 hr, there was some difference between the hepatomas and normal livers. In normal livers, the ratios remained unchanged and the localization of both antibodies decreased. On the other hand, some apparent increase of the ratios could be seen in the hepatomas. Although the large variations among the individual tumors made it uncertain, the localization seemed to increase during this early period, especially for the anti-sediment preparation. These results seem to indicate that, unlike localization in normal tissues, the localization of antibodies in the hepatoma may be more rapid and the anti-sediment antibody may require more time to localize than the anti-microsome antibody. This seemed to be in line with the difference between the sites of their localization observed by radioautography (see below).

The low values obtained for $^{125}$I-7 S GNS show that the background due to unfixed or nonspecifically fixed protein is low, and the higher values observed with the antibodies are due largely to the specific fixation.

Radioautographic demonstration of localized antibodies in tissues.—The above results strongly indicated the multiplicity of the hepatoma-localizing antibodies. Although both anti-microsome and anti-sediment preparations contained antibodies which localize in hepatoma and normal liver when injected, these antibodies behave quite differently in several aspects, as shown by the use of the paired label technic. This would indicate that these antibodies were directed against different antigenic components of the tissue and strongly suggests that they localize in different parts. Therefore, microscopic examination of their sites of localization was undertaken by the use of radioautography with $^{125}$I-labeled antibody preparations.

In some of the experiments, the crude globulin labeled with $^{125}$I was used, and in other experiments the 7 S $\gamma$-globulin obtained by passage through a DEAE-cellulose column was used. In the latter case, anti-plasma antibodies were removed by treatment with rat plasma con-

### Table 3

**Localisation in Vivo of Anti-Hep-micro-Ab ($^{125}$I) and Anti-Hep-sed-Ab ($^{125}$I) As a Function of Time**

Localisation values are given as % of injected dose localized. Six tumor-bearing rats (I–VI) and 6 normal rats (VII–XII) were used.

<table>
<thead>
<tr>
<th>Livers with hepatoma</th>
<th>1 hr</th>
<th>6 hr</th>
<th>18 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hepatoma</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$^{125}$I</td>
<td>1.52</td>
<td>3.57</td>
<td>4.22</td>
</tr>
<tr>
<td>$^{125}$I</td>
<td>2.41</td>
<td>4.96</td>
<td>7.97</td>
</tr>
<tr>
<td>$^{125}$I/$^{125}$I</td>
<td>1.58</td>
<td>1.39</td>
<td>1.89</td>
</tr>
<tr>
<td>$^{125}$I-GNS</td>
<td>0.53</td>
<td>0.48</td>
<td>2.22</td>
</tr>
<tr>
<td><strong>Remainder</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$^{125}$I</td>
<td>12.83</td>
<td>14.76</td>
<td>11.92</td>
</tr>
<tr>
<td>$^{125}$I</td>
<td>22.98</td>
<td>21.09</td>
<td>18.69</td>
</tr>
<tr>
<td>$^{125}$I/$^{125}$I</td>
<td>1.79</td>
<td>1.43</td>
<td>1.57</td>
</tr>
<tr>
<td>$^{125}$I-GNS</td>
<td>1.13</td>
<td>1.08</td>
<td>1.55</td>
</tr>
<tr>
<td><strong>Normal livers</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$^{125}$I</td>
<td>10.30</td>
<td>9.24</td>
<td>8.12</td>
</tr>
<tr>
<td>$^{125}$I</td>
<td>24.29</td>
<td>22.83</td>
<td>19.32</td>
</tr>
<tr>
<td>$^{125}$I/$^{125}$I</td>
<td>2.34</td>
<td>2.47</td>
<td>2.38</td>
</tr>
<tr>
<td>$^{125}$I-GNS</td>
<td>0.47</td>
<td>0.52</td>
<td>0.68</td>
</tr>
</tbody>
</table>

$^a$ These abbreviations are explained in Footnote b to Table 1. $^b$ $^{125}$I-GNS, 7 S $\gamma$-globulin from normal rabbit serum labeled with $^{125}$I.

The extent of localization in vivo as a function of time after injection.—The localization of injected antibodies in vivo requires their contact with the tissue antigens responsible for it. Since the anti-microsome and anti-sediment antibodies seem to localize in different parts of hepatoma and liver, as shown in preceding results, the 2 antibodies may differ in the time required to reach the corresponding antigen and in the rate of removal from the site of localization. Therefore, the following experiment was designed to compare the extent of localization as a function of time. The triad label technic was used.

In this experiment, 7 S $\gamma$-globulin from normal rabbit serum labeled with $^{125}$I ($^{125}$I-7 S GNS, 1.2 mg) was added to the mixture of $^{125}$I-anti-Hep-micro-Ab and $^{125}$I-anti-Hep-sed-Ab, (containing 200 µg each of unlabeled antisemum globulin) to provide information about nonspecific localization. The mixture was injected into 6 hepatoma-bearing rats and 6 normal rats. At 1, 6, and 18 hr after injection, 2 rats from each group were killed by perfusion and the livers with tumors and normal livers were assayed for the presence of each isotope. The measurement was done by counting twice in a double-channel $\gamma$-ray spectrometer and the amounts of each isotope were calculated as described elsewhere (6).
jugated to diazotized polyaminopolystyrene. Both preparations gave similar results for each antiserum.

In all, 5 hepatoma-bearing rats and 3 normal rats were used for anti-microsome preparations and 3 hepatoma-bearing and 3 normal rats for the anti-sediment preparations. Globulins from normal serum and anti-rat plasma serum, with or without previous treatment with plasma-polystyrene conjugate, were used as controls. In all, 5 hepatoma-bearing rats and 5 normal rats were used for these controls. The results of radioautography are shown in Figs. 1–3. The silver grains indicate the presence of radiolabel. Animals were sacrificed 5 days after the injection.

The localization from each antiserum was different from control localization, and there were differences between the 2 antibody preparations. The anti-microsome preparation localized preferentially on the sinusoidal walls and on the hepatic cells of hepatoma and normal liver, although the radioactivity was not uniformly distributed (Fig. 1). The strongly anaplastic regions of hepatomas seemed to have smaller numbers of grains than the less malignant portions. In contrast, 3H-anti-sediment globulin localized heavily in the connective tissue parts of both hepatoma and normal liver and rather lightly on the sinusoidal walls (Fig. 2). No such preferential localization was seen with the control globulins—globulins from normal serum and anti-plasma serum previously treated with plasma-polystyrene conjugate (Fig. 3). There was essentially no difference between the globulin fractions of normal serum and anti-rat plasma serum preparations. In the hepatomas, some parts were not well perfused and showed many grains in the blood vessels and sinusoids for all preparations. Similar preferential localization in connective tissues was observed by Kyogoku et al. (5) with the anti-hepatoma sediment antibodies.

These results further confirm that the major portions of hepatoma-localizing antibodies in the anti-microsome and in the anti-sediment preparations are directed against different components of the hepatoma and normal liver and localize in different structures when injected. This difference in the sites of localization of the 2 antibodies seems to explain the difference observed earlier in the rates of localization in the hepatoma and in normal liver. In normal livers, the connective tissues and the hepatic cells are in good contact with the circulating blood, whereas the dense connective tissues of the hepatoma may not have direct contact with the circulating blood. Thus, the anti-sediment antibodies which are largely against connective tissue components localize rather slowly in the hepatoma and, once they are fixed, may not be eliminated so readily as the anti-microsome antibodies, which are fixed on the sinusoidal walls and on the hepatic cells.

It should also be noted that the localization of injected antibodies probably does not take place inside the cells, since penetration of antibody molecules through cell membranes would not occur readily in vivo. Therefore, the localizing antibodies in anti-microsome serum must be directed to the components of cell surface, which are exposed to the circulating blood or intercellular fluid. The observation of Wallach and Hager (8) that cell membrane components are contained in the microsome fraction of some ascites tumor cells is in accord with this concept. However, whether these antibodies are produced by the endoplasmic reticulum, which carries ribosomes in the cytoplasm and is closely associated to cell membrane, must await further investigations on the antigenic nature of these subcellular components.

Reaction in vivo of antibodies with tissue sections by fluorescent antibody technic.—Differences between anti-microsome and anti-sediment antibodies were also shown in vitro by the fluorescent antibody technic. Frozen sections (5-μ thick) of perfused hepatoma and normal liver were reacted with globulin fractions of rabbit antisera previously treated with beef liver and rat kidney sediments, twice respectively. Globulins had been prepared at 1/4 saturation of ammonium sulfate at pH 7.0 and were used as solutions at 10 mg/ml. The sections were washed and stained with the fluorescein-labeled globulin fraction of horse anti-rabbit globulin serum which had been treated twice with rat liver sediment (12,000 × g, 20 min). They were washed and examined with an ultraviolet microscope.

The results are shown in Figs. 4, 5. With the anti-microsome globulin (Fig. 4), strong yellow-green fluorescence was seen in the cytoplasm of the hepatic cells in the hepatoma as well as in normal liver. Nuclei were not stained in the hepatoma or in the normal liver. Under high magnification, the staining did not seem uniform in the cytoplasm. Staining was generally negative in connective tissues, but some fine fiber-like structures showed staining. On the other hand, the anti-sediment globulin (Fig. 5) was fixed on connective tissues and reticular fibers, but not on hepatic cells. These specific stainings were removed by treating antiserum globulin with the corresponding antigens.

Thus, the results indicate that the anti-microsome antibodies were directed against components of hepatic cells, whereas the anti-sediment antibodies were directed against those of connective tissue. These results seem to agree in many respects with the previous reports on anti-hepatoma microsome antibodies by Hiramoto et al. (3) and on anti-hepatoma sediment antibodies by Kyogoku et al. (5).

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Fig. 1.—Localization of radioactivity in hepatoma of rat injected with 131I-labeled G-anti-Hep-micro. All rats in Figs. 1-3 were sacrificed 5 days after injection. Note the silver grains on the hepatic cells and along the sinusoids. × 300.

Fig. 2.—Localization of radioactivity in hepatoma of rat injected with 131I-labeled G-anti-Hep-Sed. Note the larger number of silver grains on the connective tissue compared to those on hepatic cells. × 300.

Fig. 3.—Localization of radioactivity in hepatoma of rat injected with 131I-labeled GNS. Very little localization was seen, although the same amount of radioactivity was present per gm of tissue as in Fig. 2. × 300.

Fig. 4.—Fluorescent staining of hepatoma sections by G-anti-Hep-micro. Note intense staining of cytoplasm of hepatic cells. The connective tissue is not stained. × 300.

Fig. 5.—Fluorescent staining of hepatoma sections by G-anti-Hep-Sed. Note primary staining of connective tissue and reticular fibers. × 300.
Differences between Antibodies against the Dense Sediment and Microsome Fractions of the N-2-Fluorenylacacetamide-induced Rat Hepatoma

Shinzo Isojima, Joseph Bernecky, Jacob Planinsek, et al.

Cancer Res 1965;25:968-975.

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