

The Localization of Radioantibodies in Human Brain Tumors. III Radioiodination of Pre-purified Localizing Antibody^{1,2}

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The delivery of radioactivity to human brain tumors through the use of a specific antibody carrier would offer yet another approach to the diagnosis and therapy of a group of cancers that often still defy control. Glioblastoma multiforme, in particular, continually calls for new modes of attack, for it is a highly malignant brain tumor that more often than not causes death within eight months following diagnosis, regardless of attempts at radical surgical resection, external irradiation, or administration of anticancer drugs.

The feasibility of localizing radioantibodies in human brain tumors *in vivo* was reported in the first paper of this series (1), and a controlled base-line for assays of localized radioantibody, from which to determine the purity, concentration, and localizing properties of subsequent preparations of radioantibody, was established. In the second paper in the series (2), the histological disposition of radioantibody in brain tumors was described and compared with that of radioactive control preparations through the use of ¹²⁵I-radioautography. Antibodies were prepared in rabbits against individual human gliomas that were obtained after primary craniotomies. At a later date the antibodies were radioiodinated, partially purified by an absorption-elution process, and infused in the patients just prior to secondary craniotomies for recurring gliomas. Control proteins, labeled with a different isotope of iodine, were infused at the same time. A number of individual assay techniques were then used to determine the extent of radioantibody localization. In eleven of the first twelve cases reported, radioantibody localization was shown to have occurred over and above a base-line control.

A more difficult task now remains; to obtain, purify, and label a more specific form of antiglioma antibody. The partially purified antibodies in the past have been primarily cross-reacting types that constitute about 0.2 per cent of whole

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antiserum globulin, *i.e.*, about 40 μg and 100 μc of globulin out of an initial 20 mg and 50 mc of globulin. Based upon previous experience in animal systems (3), it can be expected that any specific antibody will probably constitute about only 0.02 per cent, *i.e.*, 1 μg and 10 μc . The actual yield will inevitably be far less, perhaps no more than 0.0002 per cent, *i.e.*, 10 ng and 100 nc.

Three problems need to be solved: how to immunize to obtain specific antibody in higher titer and with less cross-reaction; how to purify antibody in higher yield and greater specificity; and how to label submicrogram quantities of pre-purified antibody with millicurie amounts of radioiodine. The present investigation was carried out as a beginning to the solution of this last problem. It is obvious that if an eventually isolated, specific glioma-localizing antibody is to serve as a carrier of therapeutic amounts of radioactivity, it must be labeled after purification, not before.

In the case of antifibrin antibody, Spar *et al* (4) have already worked out a method for post purification radioiodination. An antiserum is incubated with homogenized fibrin and centrifuged; the residue, containing the fibrin-antibody complex, is eluted with pH 11.6 buffer and centrifuged; and the eluted antibody is dialyzed against pH 8 borate buffer and labeled by reaction with ^{131}I iodine monochloride. Antibody with a high specific ^{131}I activity can thus be obtained. Such a preparation was highly successful in causing regression of transplantable rodent tumors (5), and others have been used routinely in the exploration of therapy by means of radioantifibrin treatment (4).

One might well ask why the method of Spar *et al* does not pertain to the present problem. The answer is that fibrin is a relatively simple antigen when compared to a tissue complex, can induce antibody in high yield and with relatively little cross-reaction, can serve as an insoluble antigen system for purification of antibody by absorption and elution methods, and does not give rise to very much contaminating protein in the eluted product that might compete with antibody for radioiodine. Distinctive glioma antigens, on the other hand, have not yet been isolated and remain obscured by a highly complex tissue milieu; thus, the yield of specific antibody against gliomas is necessarily very low and highly contaminated with cross-localizing antibodies.

A second difference between tissue sediments and fibrin further complicates the problem. Though insoluble tissue sediments serve very well as a medium for purification of localizing antibody by absorption-elution methods, the eluates of solubilized antibody also contain a high concentration of tissue protein—insoluble under conditions of absorption but soluble under the changed conditions necessary for elution. As shown below, eluates of antibody may often contain a higher concentration of extraneous protein than the initial whole globulin. Such tissue proteins present no problem when antibody is labeled prior to purification, since they, of course, remain unlabeled; however, in the case of post purification radioiodination, the contaminating tissue proteins compete for more radioiodine than the initial nonantibody globulin would have. This results in antibody with lower specific radioactivity than in the case of antibody labeled prior to purification. The problem, then, is to obtain a protein product which is primarily antibody protein.

The experiments described below demonstrate first the problem of the presence of contaminating protein in antibody eluates. They then show how a partial separation of pre-purified antibody from such proteins can be obtained so that subsequent radioiodination results in as much as a fifty-fold increase in specific labeling of antibody over that of whole antiserum globulin. A thousand-fold further increase in specific labeling is the goal, but the results obtained represent an encouraging step in the right direction.

METHODS AND MATERIALS

Since the purification methods for obtaining specific antiglioma antibody against human gliomas were not expected to be substantially different from those for obtaining antibody against normal organs, nor to vary too greatly from procedures for obtaining antibody that localizes in rat tissues, the initial investigations utilized the system of rat kidney and localizing rabbit-antirat-kidney antibody as a model. They were then extended to the rat spleen-antispleen system, and finally to the human glioma-antiglioma system.

Well-washed, insoluble sediments of tissues, whether rat kidney or spleen or human glioma, were prepared as follows: Tissues were finely minced with scissors, suspended in four volumes of cold borate-buffered saline (pH 8, 0.85% NaCl), and homogenized by a Teflon homogenizer in a closely fitting Pyrex tube. The homogenates were centrifuged at the relatively low force of 100xg for 20 min at 4°C, the supernatant fluids were removed by pipette, and the sedimented materials were washed by resuspension in four more volumes of cold buffered saline and subsequent recentrifugation. The washing procedure was repeated until water-clear supernatant fluids were obtained. The sediments were then frozen-dried and stored in screw-cap vials at -20°C until needed. The dried tissue sediments served both as immunizing antigens in rabbits and as media for the purification of antibody by absorption-elution procedures.

Antiserum globulins were obtained from rabbits according to the protocol previously described (1).

Radioiodination was accomplished by the IC1 method of Helmkamp *et al* (6) with one modification. In the Helmkamp method the radioiodide is added to the IC1 reagent to effect exchange of radioiodide with nonradioactive iodide in the IC1 molecule, and the mixture is jetted into the protein solution to bring about radioiodination of the protein. In the method used here, the radioiodide in double-strength buffered saline (ionic strength = 0.3, pH = 8) was added to the protein solution (also in double-strength buffered saline) and thoroughly mixed. The nonradioactive IC1 reagent was then jetted into the protein-radioiodide solution. Isotope exchange, being faster than protein radioiodination, took place first, and as good yields of radioprotein were obtained as with the Helmkamp method. The reason for modification was to insure maximum dispersion of radioiodide before iodination took place and thus to obtain a minimum number of radioantibodies with more than one atom of radioiodine attached. To obtain an average of one atom of iodine per protein molecule in 20 mg globulin, dissolved in 5 ml buffered saline, with an iodination yield of 60 per cent, 0.1 ml 0.005 M IC1 was used with an equal volume of 2M NaCl. When lesser amounts

of proteins were iodinated, correspondingly smaller amounts of IC1 and larger amounts of NaCl were used. The total reagent volume was kept at 0.2 ml. Details concerned with the iodination of small amounts of pre-purified antibody will be described under the specific experiments.

Lewis-strain male rats, 300 gm in weight, were used for the *in vivo* localization experiments. Radioantibody preparations for *in vivo* assay were injected intravenously in 2 ml amounts, and their distribution among the major rat organs was determined one day later, after sacrifice of the rats and perfusion of the organs. Percentage of the injected radioactive dose in the various organs was obtained by counting the tissues in a dual-channel well-type scintillation counter (by which amounts of ^{125}I and ^{131}I could be separately determined) and by comparing the tissue uptakes of radioactivity with that of injection standards.

RESULTS AND DISCUSSION

Experiment One: In this experiment the question of whether eluted globulin might be simply recovered from an eluate in pure form by ammonium sulfate precipitation was investigated. Twenty mg of an antikidney antiserum globulin were labeled with ^{131}I , diluted with 5 ml normal rat serum, and precipitated in 2M ammonium sulfate. The precipitate was washed twice with 2M ammonium sulfate and dialyzed overnight against buffered saline (pH 8, 0.85% NaCl), giving a final volume of 7.8 ml. This volume, plus 5 ml normal rabbit serum, were absorbed 1 hr at room temperature with 100 mg of kidney sediment, and centrifuged at 1500xg for 20 min and 25° C. The sediment was washed three times with 10 ml portions of buffered saline, suspended in 7 ml buffered saline, and gently agitated for 25 min at 63° C. The eluted solution of globulin was separated by centrifugation, combined with 5 ml of additional rabbit serum, and reabsorbed with 100 mg additional kidney sediment. The washing and elution steps were repeated, and the doubly purified antibody eluate was divided in two parts. One part was labeled with ^{125}I , the amount of IC1 reagent having the equivalency of 10 mg globulin (the approximate amount of total protein in the eluate). The other part was kept as a single ^{131}I -labeled globulin. Each eluate portion was combined with 5 ml normal rabbit serum, and globulin was precipitated in 2M ammonium sulfate and washed twice with 2M ammonium sulfate. The precipitates readily dissolved in 5 ml buffered saline, and, after dialysis overnight against buffered saline, were diluted further with saline to 20 ml. Each eluate was assayed in a group of eight rats, four sacrificed one day after injection and four, seven days after injection. One preparation (^{131}I alone) represented globulin labeled prior to purification. The other (^{131}I and ^{125}I) represented globulin labeled both before and after purification. The results are shown in Table I.

It can be seen first of all that the relabeling process destroyed about one-third of the localizing activity of the ^{131}I -antibody (compare A and B), which would indicate that an average of about four atoms of iodine per antibody molecule was contained in preparation B (cf, Johnson, Day and Pressman (7)). Denaturation of antibody protein by re-iodination was discounted, since blood and thyroid values for A and B were similar. However, by far the greater por-

tion of the ^{125}I must have reacted with a protein different from antibody globulin in the eluate since little localization took place. The blood values for C were substantially lower and the thyroid values considerably higher than those for A and B, an indication that the ^{125}I -labeled protein was, for the most part, something other than a serum protein. The relative difference between B and C at day one was maintained practically unchanged at day seven. The bulk of the evidence, therefore, pointed to the probability that tissue-eluted antibodies were heavily contaminated with a protein, precipitable in 2M ammonium sulfate, but readily soluble in saline, that competed too greatly with eluted antibody for ICI to permit post purification radioiodination of the latter.

Experiment Two: As a result of an investigation to determine the efficiency of a column of DEAE-A50-Sephadex in separating ^{131}I -globulin from unbound radioiodide, it was also found that a partial separation of eluted globulin from eluted tissue protein could also be achieved under the same conditions—using double-strength borate-buffered saline (pH 8, ionic strength 0.3) as developer.

An antirat-kidney antiserum globulin (40 mg) was labeled with ^{125}I (5 mc), dialyzed against buffered saline, diluted to 10 ml with buffered saline, and absorbed for one hour at room temperature with 40 mg rat-kidney sediment. Normal rabbit serum was not used in this experiment. The mixture was centrifuged for 20 min at 1500xg and 25° C, the supernatant fluid was decanted, and the sediment was washed three times in 10 ml volumes of buffered saline by resuspension and recentrifugation. The sediment was then suspended in 5 ml double-strength buffered saline, brought to 60° C, and gently agitated for 20 min at that temperature. After centrifugation for 20 min at 25° C, the heat-eluted radioantibody solution was decanted, the recovered volume being 4.5 ml. The estimated recovery of eluted antiserum globulin, based upon the percentage recovery of radioactivity from the initial whole globulin (110 μc from 5 mc) was

TABLE I

LOCALIZATION IN RATS: (A) ^{131}I ANTIKIDNEY ANTIBODY, LABELED PRIOR TO PURIFICATION; (B) THE ^{131}I PORTION OF THE SAME PURIFIED ANTIBODY, ALSO RELAELED WITH ^{125}I AFTER PURIFICATION; (C) THE ^{125}I PORTION OF THE RELAELED ANTIBODY

Preparation	Percentage injected radioactive dose in tissues*		Percentage of tissue localized dose in kidney	
	1 day	7 days	1 day	7 days
A	25.4	16.4	34.7	38.8
B	17.5	11.0	32.2	36.0
C	2.2	0.8	13.2	19.8

*Kidney, liver, spleen, testes, adrenal, lung, heart, thymus—Averaged from values for 4 rats in each group.

2.2 per cent or 0.9 mg. The eluate was then relabeled with iodine, this time with about 100 μ c of ^{131}I as the isotope and with an ICl volume equivalent to 1 mg of protein.

After labeling, the volume of eluate was 5.0 ml. 0.5 ml was removed and set aside for subsequent assay. The remaining 4.5 ml was passed through a 250×10 mm column of DEAE-A50-Sephadex, using double-strength borate-buffered saline as developer. Volumes were collected in 1 ml amounts, and a total of 60 ml were collected. Recovery of ^{125}I was 70.5 per cent and of ^{131}I , 98.7 per cent. ^{125}I and ^{131}I in each tube were counted, giving the plot shown in Fig. 1. Individual fractions, representative of the peaks, were diluted with an equal volume of distilled water (to dilute the double-strength saline appropriately for injection), and injected in individual rats for assay of their localization properties one day later. The 0.5 ml of unfractionated eluate was diluted to 10 ml with 0.5 ml distilled water and 9.0 ml normal saline, and was injected in 2 ml volumes into each of three rats to assay for the localization properties of the whole unfractionated eluate. The results are tabulated in Table II.

The reduction in the amount of sediment from that used in experiment one for absorption-elution, and the omission of normal serum during absorption, helped to reduce the amount of nonantibody protein in the eluate. Also employing one rather than two absorption-elution steps maintained a higher proportion of antiserum globulin to tissue proteins in the eluate. Nevertheless, the isotope (^{125}I) bound to antibody prior to purification still had better localization activity than that (^{131}I) bound to antibody after elution. This can be seen in the first line of Table II, where the assay for diluted whole eluate is presented. The first radioactive fractions from the DEAE-A50-Sephadex column, however, showed an improvement in the ^{131}I -labeled eluate while preserving the localization activity of the ^{125}I . Moreover, the increased blood values and lowered thyroid values indicated that the fractions were serologically more compatible in the rat and less subject to catabolism. Fraction 15 was apparently, in part, a soluble complex of antibody tissue protein, and the two fractions just before it caused shock in and death of the rats after intravenous injection. Fraction 25 seemed to represent tissue protein with no localization properties. The next two fractions, however, did localize. The three fractions, 25-27, although low in yield of ^{125}I , showed the presence of a peak for ^{131}I . Fractions 36-38 were interesting in that as ^{125}I localization values decreased, ^{131}I localization values increased. The nature of these fractions, although low in yield, would certainly bear further investigation, particularly since the percentage of the total tissue-localizing antibody that was found in kidney was highest (52% of ^{131}I for fraction 37 vs. 29% of ^{131}I for fraction 5). With respect to yield, fractions 4-8 contained 40 per cent of the ^{125}I and 23 per cent of the ^{131}I introduced to the column. They therefore represented the richest antibody with the least amount of contaminating protein and with the best serological properties (that is, with highest blood values and lowest thyroid levels, indicative of best compatibility).

Experiment Three: The next step was to label eluted antibody after its passage through a DEAE-Sephadex column rather than before in order to obtain a richer fraction of antiserum globulin with higher specific radioactivity. The first

protein peak of the effluent was selected for this purpose. To increase further the relative amounts of antiserum globulin over that of eluted tissue protein, 80 mg of globulin and 20 mg of sediment were used for the initial absorption rather than 40 mg of each as in experiment two. After elution into 5 ml buffered saline, the globulin was passed through the DEAE-Sephadex column, and 1 ml portions were

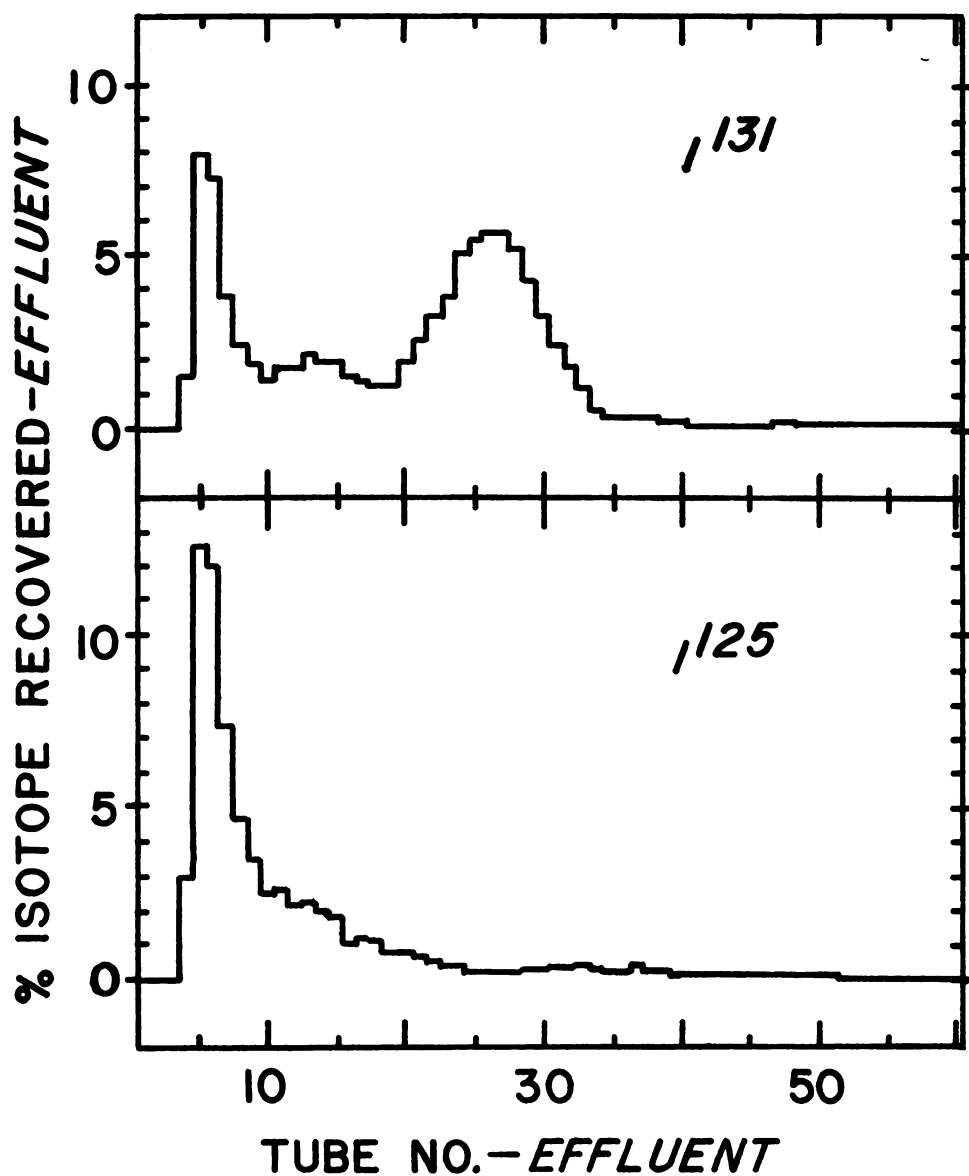


Fig. 1. Recovery of radioactivity ineffluent from column of DEAE-A50-Sephadex through which an ant kidney eluate was passed. ^{125}I labeled the antiserum globulin before globulin was absorbed on and eluted from the kidney sediment. ^{131}I labeled eluted product.

TABLE II
 YIELDS AND LOCALIZATION PROPERTIES OF ELUATE FRACTIONS OBTAINED BY PASSAGE OF AN ANTIKIDNEY ELUATE THROUGH A COLUMN OF DEAE-A50 SEPHADEX. LABELING WITH ¹²⁵I TOOK PLACE BEFORE, AND RELABELING WITH ¹³¹I TOOK PLACE AFTER ABSORPTION-ELUTION

Column Fraction	% of Total Eluate Radioactivity in Fraction		% Injected Isotopic Dose One Day After Injection						% of Tissue Localized Dose in Kidney	
	¹²⁵ I	¹³¹ I	¹²⁵ I			¹³¹ I			¹²⁵ I	¹³¹ I
			Tissues*	Blood	Thyroid	Tissues*	Blood	Thyroid		
Whole (diluted)	2.00	2.00	10.2	45	4.6	3.3	15	10.4	26.0	14.1
4	3.08	1.47	10.7	59	2.8	6.9	55	3.9	26.4	23.8
5	12.72	8.00	10.0	45	2.5	3.9	51	2.4	33.0	29.3
6	12.07	7.35	9.6	46	2.8	4.6	50	2.9	29.1	24.4
7	7.35	3.96	9.5	41	2.0	4.6	46	2.8	28.6	24.3
8	4.75	2.50	4.3	38	2.8	3.1	55	4.1	21.5	25.6
15	1.84	2.00	7.9	42	1.8	3.9	53	3.6	30.5	23.7
25	0.14	5.55	0.2	0	25.8	0.7	5	10.0	4.5	29.6
26	0.11	5.72	7.2	11	3.7	2.7	33	5.9	25.2	37.4
27	0.08	5.68	8.5	48	3.0	3.2	27	3.6	27.7	31.8
36	0.35	0.29	7.7	71	2.6	2.6	14	4.5	22.3	37.7
37	0.26	0.20	4.3	38	1.2	7.1	34	7.6	36.6	51.6
38	0.20	0.14	0.0	0	15.6	8.4	10	10.0	44.8

*Liver, kidney, and spleen.

TABLE III
 YIELDS OF RECOVERED ANTIKIDNEY RADIOANTIBODY, RADIOIODINATED AFTER ABSORPTION-ELUTION, DURING STEPS
 IN ISOLATION OF SPECIFIC KIDNEY-LOCALIZING ANTIBODY

Purification Step	% Recovery in Terms of Radioactivity Remaining at Each Step											
	1	2	3	4	5	6	7	8	9	10	11	
1 Initial ¹²⁵ I (8.98x10 ⁹ cpm or 12.0 mc)	100.0											
2 After dialysis	55.0	100.00										
3 Absorbed by kidney sediment	2.20	4.00	100.00									
4 Recovered by elution from sediment	0.880	1.60	39.98	100.00								
5 Recovered in serum, nephrec- tomized rats	0.440	0.80	19.99	50.00	100.00							
6 Localized in kidney, normal rats	0.0175	0.0319	0.798	2.00	3.99	100.0						

TABLE III—Continued

<i>Purification Step</i>	<i>% Recovery in Terms of Radioactivity Remaining at Each Step</i>											
	1	2	3	4	5	6	7	8	9	10	11	
7 Contained in kidney sediment	0.0058	0.0106	0.266	0.665	1.33	33.3	100.0					
8 Recovered by elution from sediment	0.0039	0.0071	0.177	0.443	0.887	22.2	66.7	100.0				
9 Localized in liver-kidney-spleen	0.0030	0.0054	0.132	0.333	0.666	16.7	50.1	75.2	100.0			
10 Localized in kidney	0.0024	0.0039	0.102	0.252	0.504	12.7	38.0	57.0	75.8	100.0		
11 Specific for kidney	0.0022	0.0036	0.094	0.232	0.463	11.7	34.9	52.4	69.6	92.0	100.0	

collected. These were checked for protein concentration in a Zeiss spectrophotometer, and seven successive tubes were selected for subsequent labeling. The 7.0 ml of pooled fractions, estimated to contain 1.6 ml protein, were labeled with ^{125}I and dialyzed to remove unbound iodide.

The best method to test for the amount and integrity of localizing antibody, that had thus been labeled after absorption-elution and passage through DEAE-Sephadex, appeared to be that of following the amount of radioactivity recovered at each step as purification proceeded in the direction of a specific kidney-localizing antibody.

The labeled antibody eluate was absorbed by 20 mg kidney sediment and eluted from it, injected into nephrectomized rats, and permitted to circulate and be absorbed *in vivo* for two hours. Removal of much of the cross-localizing antibody can be accomplished by this technique (8). The serum from the nephrectomized rats was then recovered and injected in normal rats. The next day kidneys from these normal rats were excised, kidney sediment prepared, and kidney-localizing radioantibody extracted by elution and by passage through a column of DEAE-Sephadex. The final preparation of pooled effluent was assayed in a series of five rats.

Certain losses were experienced during absorptions, elutions, and *in vivo* passage, but these could be held relatively equivalent to similar losses experienced in former experiments. The final yields could therefore be compared with confidence. In Table III the percentage of radioactivity retained at each step is charted, and as each step is reached the subsequent ones are computed in terms of activity at that step, the final assay, of course, showing kidney localization.

In comparison with antikidney-antiserum globulin, labeled whole and injected directly into rats, the iodination here represents a four-fold increase in specific labeling of kidney-localizing antibody. Ideally, one should like to be able to label globulin at step eight which represents 9.44 per cent of the eluate at step four. Since the eluate at step four represents 1.6 per cent of the initial pre-iodination eluate (1.6 mg) of step two, the total amount of globulin at step eight would be $1.6 \text{ mg} \times 0.016 \times 0.0044$ or $0.11 \mu\text{g}$ of globulin. Based upon the localization assay of the globulin at step eight, 52 per cent is specific kidney localizing antibody or about $0.06 \mu\text{g}$. It is evident that a much more critical procedure for separating eluted antibody globulin from eluted tissue protein must be worked out before this ideal radioiodination of purified antibody can be achieved.

Experiment Four: In the case of the rat-spleen-antirat-spleen system, a 35-fold increase in specific labeling of localizing globulin was obtained. Two double absorption-elution procedures were carried out side by side. In the one, whole antispleen antiserum globulin, 80 mg labeled with ^{131}I and dialyzed, was absorbed by 20 mg spleen sediment, eluted therefrom, reabsorbed by another 20 mg portion of spleen sediment, and re-eluted. In the other, 80 mg of unlabeled whole antiserum globulin was absorbed by and eluted from 20 mg of sediment, and the eluate was passed through a column of DEAE-Sephadex. The effluent from the column was labeled with ^{125}I , dialyzed, and absorbed by and eluted from 20 mg of spleen sediment. Both preparations were then mixed and assayed in a group of five rats for localization properties. The results are shown in

Table IV. The amount of ^{125}I (Table IVB, step 5) recovered as localizing antibody (in liver, kidney, and spleen) was greater than that of ^{131}I (Table IVA, step 7), *i.e.*, 0.38 per cent vs. 0.011 per cent of the initial isotope. The final eluate was also richer in ^{125}I than in ^{131}I localizing antibody (30.1% vs. 17.5%), no doubt due to the prior passage of the ^{125}I antibody through DEAE-Sephadex before it was labeled.

Experiment Five: It now having been established that one could label antibody after elution from tissue and subsequent passage through DEAE-A50-Sephadex, the technique was tried with the human glioma-antiglioma system, and compared with the previous technique of labeling antiglioma antiserum globulin prior to partial purification. The *in vitro* test for evaluating antiglioma antibody

TABLE IV

STEPWISE YIELDS OF RECOVERED ANTISPLEEN ANTIBODY, RADIOIODINATED AFTER ABSORPTION-ELUTION AND COLUMN FRACTIONATION, AS COMPARED WITH YIELDS OF ANTIBODY, RADIOIODINATED AS WHOLE GLOBULIN

A. Labeled Whole Globulin

Purification Step	% Recovery in Terms of Radioactivity Remaining at Each Step					
	1	2	3	4	5	6
1 Initial ^{131}I	100					
2 After Dialysis	60	100				
3 Absorbed by spleen sediment	2.4	4.0	100			
4 Eluted from sediment	0.96	1.6	40	100		
5 Reabsorbed by spleen sediment	0.14	0.23	5.75	14.4	100	
6 Re-eluted from spleen sediment	0.056	0.093	2.43	6.08	47.2	100
7 Localized <i>in vivo</i>	0.011	0.019	0.475	1.19	8.27	17.5

B. Labeled Effluent

1 Initial ^{125}I	100				
2 After dialysis	43.4	100			
3 Absorbed by spleen sediment	2.41	5.56	100		
4 Eluted from spleen sediment	1.25	2.89	52.0	100	
5 Localized <i>in vivo</i>	0.38	0.87	15.6	30.1	

activity, previously described (1), was used, and an antiserum against glioma No. 36 was selected for the test. The initial amount of globulin for each of the two tests was 80 mg, the amount of No. 36 glioma stromal sediment for each absorption step was 20 mg, and the amount of ^{125}I for each iodination was 6.4 μc . In the one test whole globulin was labeled prior to purification; in the other, an absorption-elution of unlabeled globulin and subsequent passage through a column of DEAE-Sephadex was made prior to labeling. The results shown in Table V compare the yields of recovered radioactivity obtained at each step after iodination.

The first absorption of the effluent by glioma sediment, comparable in amount of antibody globulin to the second absorption of the whole globulin after an initial absorption-elution, shows a 49-fold increase in specific radioactivity labeling of antibody (3.65/0.0744). However, in the case of brain sediment, a higher amount of eluted tissue protein was obtained than with kidney or spleen, and a second purification step was necessary to obtain greater purity of the labeled preparation. Therefore, in terms of a suitable preparation for *in vivo* studies, the comparison between yields of labeled antibody should be made between the amounts shown in the last line of Table V, *i.e.*, 0.618/0.0744 or 8.3. Even so, a substantial increase in specific labeling of antibody was still evident. A preparation of purified antibody against glioma No. 36, radioiodinated after elution according to this scheme, was used for the *in vivo* study of patient No. 36 which was described in Table VIII of the first paper in this series (1).

DISCUSSION

In the only *in vivo* system in which specific tumor localization of antitumor radioantibodies was obtained, the yield of radioantibody for assay was only 0.0002 per cent of the original whole globulin (3). Of this amount 37 per cent localized in tumor and 2 per cent in all the other tissues combined. The globulin was labeled before antibody purification, and, out of 100 mc of initial radioiodine, only 100 nc (0.0001 mc) were obtained. Since the tumors (induced rat hepatomas) were about 20 grams in weight, only about 5 nc of radioactivity per gram were localized.

It would now be possible to raise the final localization value to 25 nc/gm by present procedure of post purification iodination. This would immediately bring the tumor-localized radioactivity within the sensitivity range needed for diagnostic external scanning. However, for therapy, an additional thousand-fold increase in specific labeling would be required *minimally* in order to deliver 250 $\mu\text{c/gm}$ of tumor.

Thus, in the case of the human brain-tumor system, as soon as one learns how to eliminate cross-reacting antibodies and to obtain specific glioma-localizing antibody, comparable to the rat-hepatoma system, the present method of post purification iodination will be able to provide a diagnostic tool for specific external scanning of brain tumors. Improvement in labeling methods and purification of antibody will be needed to permit preparation of a therapeutic radio-antiglioma antibody.

TABLE V
 STEPWISE YIELDS OF RECOVERED ANTIHUMAN-GLIOMA ANTIBODY, RADIOIODINATED AFTER ABSORPTION-ELUTION AND COLUMN FRACTIONATION AS COMPARED WITH YIELDS OF ANTIBODY RADIOIODINATED AS WHOLE GLOBULIN

<i>Purification Step</i>	<i>Labeled Whole Globulin (86 mg)</i>				<i>Labeled Effluent (1.04 mg)</i>			
	<i>1</i>	<i>2</i>	<i>3</i>	<i>4</i>	<i>1</i>	<i>2</i>	<i>3</i>	<i>4</i>
Initial ¹²⁵ I (64 μc)	100				100			
After Dialysis	48.1	100			48.8	100		
Absorbed by Glioma sediment	0.961	2.00	100		3.65	7.48	100	
Eluted from Glioma sediment	0.385	0.800	40.0	100	2.13	4.37	58.4	100
Reabsorbed by Glioma sediment	0.0744	0.155	7.75	19.3	0.618	1.265	16.9	29.0

SUMMARY

If an eventually isolated, specific glioma-localizing antibody is to serve as a carrier of therapeutic amounts of radioactivity, a method for high specific labeling of antibody must be obtained. In order to obtain high specific labeling of tissue-localizing antibodies with radioiodine, the radioiodination must be done after antibody purification. However, after localizing antibodies are purified by absorption on and elution from well-washed tissue sediments, the antibody eluates also contain eluted tissue proteins. These proteins compete for radioiodine and prevent high specific labeling of antibody. Therefore, their removal is necessary before effective radioiodination of pre-purified antibody can be accomplished. The experiments reported here demonstrate this problem and show how passage of eluates through columns of DEAE-A50-Sephadex helps to separate eluted antibody from eluted tissue protein. As a result, as much as a fifty-fold increase in specific labeling is obtained, although this varies from tissue to tissue. The rat kidney-antikidney and rat spleen-antispleen systems serve as models for the experiments and finally the human glioma-antiglioma system is also tested.

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