Chromatofocusing Studies Involving a Monoclonal Fab'

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Isoelectric focusing (IEF) of the Fab' derivative of murine monoclonal antibody ZCE-025 is known to detect at least six bands having isoelectric points (pI) ranging from 5.4 to 7.8. Chromatofocusing was employed to separate these bands. Electrophoresis of the starting materials under nonreducing conditions indicated all of the materials to migrate as Fab'. The electrophoresis of urine samples obtained from Balb/c and nude mice 8 hr after the i.v. injection of the various ^{125}I bands revealed the low pI bands to migrate approximately as a ^{125}I-Fab'. The higher pI band activity was located in lower molecular weight regions. Serum samples taken at 8 hr postinjection from the above mice revealed a series of what appeared to be high molecular weight complexes and some low molecular weight species. Biodistribution studies in comparison Balb/c mice and nude mice revealed that the low pI ^{125}I-Fab' bands gave an organ and tumor uptake at 8 hr very similar to Fab', while the high pI ^{125}I-Fab' bands were rapidly excreted into the urine and feces and did not concentrate in the tumor. The data suggest that the population of molecules making up the Fab' of this antibody is heterogeneous and variably stable. Theoretically, some of the entities observed could be counter productive to successful radioimmunoimaging. It is also possible that some of the labeled molecules are associating in vivo with endogenous proteins that might, in some Mabs, affect the biodistribution of the radiopharmaceutical.

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Hamilton et al. (*1*) have examined murine monoclonal antihuman IgG subclasses by isoelectric focusing (IEF)-affinity immunoblot analysis as well as by other techniques. The results indicated isoelectric point (pI) microheterogeneity, immunoreactivity and specificity differences in these monoclonal antibodies (Mabs). As a group, the above Mabs had pIs ranging from 6.1 to 7.8 and consisted of one to five major dense bands flanked by up to four minor faint bands. The major dense bands displayed a restricted charge heterogeneity (0.1–0.6 pH units).

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The multi-pI banding pattern, observed by Hamilton (*1*), goes unnoticed by (SDS-PAGE) (10%), and thus is often unsuspected. The cause of the phenomenon could stem from post-translational changes such as amidation or deamidation of glutamate or glutamine residues (*2,3*). Glycosylation can also result in a charge shift of the protein band of selected non-IgG heavy-chain Ig myeloma proteins (*4*). Treatment of Mab IgG with neuraminidase failed to decrease gamma heavy chains to a single spot in two-dimensional gels (*4,5*). In any event, these shifts in the IgGs pI signify posttranslational modifications which appear consistent within a given hybridoma cell line. An exception, of course, is class switching, which is fairly uncommon (*6*).

Fragments of Mabs have been shown to give earlier and superior lesion-to-background ratios in tumor models when compared with intact Mabs, and to improve image quality in human radioimmunodetection (RID) (*7–10*). For these reasons, much work in radioimmunodiagnosis now focuses around Mab fragments. While Fab and Fab' fragments offer an advantage for RID, they are not without problems, one of which is a half-log decease in affinity over bivalent species and an increased probability that a randomly applied radiolabel will involve the one remaining variable region. The end result of the above could be reduced tumor targeting. Since the possibility exists that some of the pI bands described by Hamilton et al., represent none or less immunoreactive materials, we have attempted to investigate each band separately in regard to its immunoreactivity and in vivo distribution and tumor targeting. Our interest was selection of the most appropriate band for RID. The following represents the results of our efforts.

MATERIALS AND METHODS

Characterizations of Antibody ZCE-025 and Its Fragments

The original infusion that produced the hybridoma came from the laboratory of Dr. J-P. Mach in Lausanne, Switzerland and is known as Mab-35 in his publications. The ZCE-025 used in this study, however, was produced by Hybritech, Inc., San Diego, CA. ZCE-025 has an affinity of approximately 6×10^9 for carcinoembryonic antigen (CEA) when studied with either a radioiodine or ^{111}In label. It migrates at 150,000 daltons on SDS-PAGE gels and has an immunoreactivity of 70%. ZCE-025 was separated from

other proteins by sodium sulfate salt precipitation followed by diethylaminoethyl (DEAE) chromatography.

To prepare the Fab', the intact molecule was reduced to $F(ab')_2$ by pepsin digestion. This was followed by ultragel AcA-54 chromatography. The resultant $F(ab')_2$ presents as a single band corresponding to 100,000 daltons on SDS-PAGE gels and the Ka remained at 6×10^9 M/liter with the same immunoreactivity as the starting preparation.

Fab' was prepared from the $F(ab')_2$ by reduction for 30 min in excess cystine followed by blockade of the free sulhydryl groups by iodoacetamide (*10*). This preparation was dialyzed to remove excess cystine. The Fab' produced from this reaction was 95% pure by SDS-PAGE and confirmed by HPLC. The Ka was reduced, compared to the parent molecule, by half a log, i.e., 1×10^9. The immunoreactivity remained at 70%. These findings are in keeping with predicted values. Nonreducing SDS-PAGE (10%) (*11*) revealed one major band at ca. 50,000 daltons at Rf 4, which corresponded to the expected size of a Fab'. Specifically, there was no evidence of small species that would suggest Fab' had been fragmented in any way to component parts.

Chromatofocusing

Chromatofocusing was performed on ZCE-025 Fab' fragments (3.22 mg/ml) in a C10/20 column of polybuffer exchanger 74 equilibrated with 25 m*M* imidazole HCl buffer (pH 7.4). The purified ZCE-025 Fab' fragments had been extensively dialyzed against the above buffer. Following addition of the protein, the column was developed with degassed polybuffer 74, diluted 9-fold and adjusted to pH 4.0 according to the recommended protocol (*12*).

The flow rate on the column was 40 ml/hr, and 1 ml fractions were collected and examined for protein by measuring the optical density at 280 mm and multiplying by 0.71 (*10*). After determining the pH of these protein-containing fractions, they were selectively pooled and dialyzed against 80% ammonium sulfate to remove the polybuffer 74 followed by exhaustive dialysis against Tris.HCl buffer (pH 7.4). The protein samples were then concentrated and reexamined by IEF. The lowest two pI bands, 5.4 and 5.8, were in fractions 91–102, and the lowest pI band (5.4) was in fractions 103–108.

As the remaining bands had not eluted, 10 column volumes of eluent buffer containing 0.3 *M* sodium chloride (*13*) was applied and 1 cc fractions were collected. This was followed by eluent buffer containing 1.0 *M* NaCl. All fractions were again examined for protein. The protein samples were pooled, dialyzed and concentrated. Examination by IEF revealed pI bands 6.1 and 6.3 in fractions 10–14, which had been collected after 0.3 *M* sodium chloride elution. pI band 7.0 appeared in fractions 15–20. The highest pI band (7.8) eluted in fractions 11–14 after the additional 1.0 *M* sodium chloride.

Radiolabeling/Immunoreactivity Assay

ZCE-025 Fab' and its bands were labeled with ^{125}I using the lactoperoxidase technique (*14*). The immunoreactivity assay was performed by an established procedure (*15*). Briefly, an excess of a second Mab known to recognize a site sterically independent of the site recognized by the labeled Mab was immobilized on a polystyrene ball. A known number of counts per minute of the labeled antibody and either 0 or 500 ng of CEA were added to each of three tubes containing a ball. The tubes were incubated overnight at 37°C. After two washes, the percentage of labeled Mab bound to each ball was determined. From this the immunoreactivity was computed.

Animal Model Studies

The protocol used in the distribution and excretion studies in Balb/c mice and nude mice bearing the T-380 human colon tumor has been previously described (*10*). Basically the mice were injected intravenously via tail vein with less than 1 μCi of the appropriate ^{125}I fraction. At a time interval of no longer than 8 hr, the mice were killed by cervical dislocation and the organs indicated in Tables 4 and 5 were acquired and weighed on an analytical balance. Counting was performed using an auto gamma counter, the tissues being compared to a standard prepared from the injectate. The data were computed as a percentage of the injected dose per gram (%ID/g) for all organs and as a percentage of injected dose (%ID) for urine and feces. All statistical evaluations on the biodistribution studies were performed using the two-tailed Student's t-test and a p of 0.05 or less was considered significant (*16*).

Electrophoresis/Gel Strip Studies

The ^{125}I-labeled Fab' and chromatofocused bands were examined by nonreducing SDS-polyacrylamide gel electrophoresis using separating and stacking gels containing 10% and 5% acrylamide, respectively (*9,10,17*). In a similar fashion, serum and urine samples from mice injected with ^{125}I-labeled Fab' or one of the ^{125}I-labeled bands were collected after 8 hr. The molecular weights of these materials were approximated by running visual standards in a parallel lane (*18*). The radioactive strips were not stained but instead were sliced into nine segments of 1 cm each immediately after the completion of the electrophoresis. The gel segments were counted on a gamma well counter to correlate the distribution of the associated radioactivity with molecular size.

RESULTS

Isoelectric focusing confirmed earlier studies in our laboratory (*10*) that ZCE-025.Fab' fragments separate into six bands ranging in pI values from 5.4 to 7.8. These bands were located at 5.4, 5.9, 6.1, 6.35, 7.0 and 7.8 pI units, respectively. Specifically, the pre-focused material migrating electrophoretically as Fab' under nonreducing conditions indicated that the starting material was without small protein contaminants. Isoelectric focusing of the chromatofocused ^{125}I-Fab' gave five groups of protein, referred to as Bands A–E. Thus, Band A is a pI = 5.4 + 5.9 double band, Band B is a 5.4 band, Band C is a 6.1 + 6.35 double band, Band D is a 7.0 band and Band E is a 7.8 band. The tandem immunoreactivity assay revealed that ^{125}I Fab' fragments (the starting material) had an immunoreactivity of 57%, while ^{125}I-Bands A–E had immunoreactivity levels of 45%, 37%, 14%, 6% and 4%, respectively. Bands A and B accounted for 80% of the material recovered and Bands C–E 20%. These data correlate quite closely with the results of the electrophoretic (nonreducing conditions) studies of these entities, which indicated that most of the protein in bands C–E migrate at less than 50 kD.

Electrophoresis/gel slice data (Table 1) of the radiolabeled materials revealed that ^{125}I Fab' had 76% of its radioactivity located in piece 4, which is the piece proven

TABLE 1
Electrophoresis Data on ^{125}I-ZCE-025 Fab′ Bands A–E

Slice number	^{125}I-Bands pI = 5.4 and 5.9 Band A	pI = 5.4 Band B	pI = 6.1 and 6.35 Band C	pI = 7.0 Band D	pI = 7.8 Band E	^{125}I-Fab′	Visual STDS MW in K	Visual STDS distances (cm)
1	0%	0%	0%	0%	0%	0		
2	1%‡	1%†	0%	1%*	1%*	1%	180 K	1.65
3	1%†	1%‡	5%‡	17%‡	7%‡	0%	116 K	2.2
							84 K	2.7
4	69%‡	69%‡	9%‡	14%‡	19%‡	76%‡	58 K	3.4
							48.5 K	3.8
5	5%‡	4%‡	2%†	3%†	3%†	4%*	36 K	4.8
6	12%‡	8%‡	39%‡	6%‡	5%‡	8%†	26.6 K	5.4
7	8%‡	8%‡	11%‡	6%‡	8%‡	6%†		
8	3%‡	10%‡	26%‡	39%‡	40%‡	4%*		
9	1%†	1%†	8%‡	14%‡	16%‡	1%	0.67 K	8.0

Counts: * greater than 2x background; † greater than 5x background; ‡ greater than 10x background.
Data represents percentage of activity at each one cm slice of gel. The bands are those isolated following isoelectric focusing of the Fab′ of monoclonal antibody ZCE-025.

to contain the Fab′ by staining an electrophoresis strip to which nonlabeled Fab′ had been applied with Comassie blue indicator. Iodine-125 Band A and ^{125}I-Band B both had 69% of their radioactivity located in piece 4. Band C had only 9% of its activity at piece 4 and 39% located in piece 6. Bands D and E had more radioactivity located in piece 8 than any other, i.e., 39% and 40%, respectively, yet, a peak did occur at piece 4. Pieces 5 and 6 were shown to correspond to a 26.6 and 36 Kd standard respectively, while piece 8 was adjacent to the 0.67 Kd bromophenol blue front.

Serum and urine samples from comparison Balb/c mice and nude mice injected with ^{125}I-Bands A–E were subjected to electrophoresis. Levels of radioactivity sufficient to easily determine a migratory pattern was achieved in all A–C samples at 8 hr, whether serum or urine. This was true in nearly all urine samples (A–E) at 8 hr. Only band E in the nude mice failed to produce significant urine counts (Tables 2 and 3) to allow determination of migration. The peak activity of Bands A and B was found in slice 4 for both urine and serum. For all ^{125}I-Bands, urine samples indicated elevated radioactivity in piece 6 (when significant counts were recovered). Activity at this site was higher for Bands C–E than for A and B. The serum samples taken from Balb/c mice injected with ^{125}I-Bands A and B reveal significant amounts of radioactivity in pieces 1–3, which correspond to high molecular weight complexes not present in the starting materials. To a lesser extent this same phenomenon is found in the serum from nude mice injected with ^{125}I-Bands A and B.

TABLE 2
Electrophoresis Data on Serum and Urine from Balb/C Mice Injected with ^{125}I-ZCE-025 Bands A–E

Slice number	Band A → Serum	Band A → Urine	Band B → Serum	Band B → Urine	Band C → Serum	Band C → Urine	Band D → Serum	Band D → Urine	Band E → Serum	Band E → Urine
1	13%*	0%	10%*	1%	5%	0%	NO	1%	NO	0%
2	19%†	1%	19%†	1%	2%	0%		0%		0%
3	16%†	3%*	18%†	1%*	7%	0%	SIG	0%	SIG	0%
4	20%†	45%‡	20%†	56%‡	9%	8%*		1%		3%
5	18%†	15%†	15%†	8%†	18%*	4%*		1%		4%
6	15%*	24%‡	12%*	19%‡	35%*	59%‡	RADIO AC	49%*	RADIO AC	45%*
7	3%	6%*	5%	7%†	18%*	16%†		16%*		16%*
8	1%	3%*	1%	4%*	7%	8%*		13%		15%
9	0%	2%*	0%	3%*	0%	5%*		19%*		19%

Counts: * greater than 2x background; † greater than 5x background; ‡ greater than 10x background.
The bands used for injection have the same pIs as shown in Table 1. The Rfs also correspond to the molecular weights indicated in Table 1. Time of sample collections was 8 hr postinjection.

TABLE 3
Electrophoresis Data on Serum and Urine from Nude Mice Injected with ^{125}I-ZCE-025 Fab′ Bands A–E

Slice number	Band A		Band B		Band C		Band D		Band E	
	Serum	Urine	Serum	Urine	Serum	Urine	Serum	Urine	Serum	Urine
1	3%	1%	5%	1%	12%*	3%	NO	6%	NO	NO
2	5%	1%	15%*	4%*	13%*	5%	SIG	7%	SIG	SIG
3	9%*	2%	11%*	8%*	12%*	5%	RADIO AC	7%	RADIO AC	RADIO AC
4	56%†	53%‡	41%‡	39%‡	15%*	8%		8%		
5	8%*	7%*	9%*	7%*	10%*	6%		4%		
6	8%*	18%‡	11%*	20%†	22%†	45%*		27%*		
7	7%*	10%†	6%*	10%†	7%	15%		11%		
8	4%	6%*	3%	7%*	6%	6%		18%		
9	2%	2%	2%	3%	2%	7%		13%		

Counts: * greater than 2x background; † greater than 5x background; ‡ greater than 10x background.

The ^{125}I-Band C serum samples gave a smear of radioactivity, with the largest single amounts located in piece 6, 35% and 22%, respectively. All serum samples from Bands D and E failed to give significant counts.

It is important to note that there is virtually no evidence (solvent front) of free iodine excreted into the urine in Bands A–C, indicating that at 8 hr dehalogenation had not played a major role in this study.

Table 4 displays the results of the biodistribution studies in Balb/c mice 8 hr following the intravenous injection of ^{125}I-ZCE-025.Fab′ and ^{125}I-Bands A–E. These data can be used for comparison with the nude mouse data below. The highest activities observed for ^{125}I-Fab′ were in the kidneys (11.3% ID/g) and the lungs (7.46% ID/g). Bands A and B showed a trend towards diminished renal uptake and markedly diminished lung uptake ($p < 0.05$) from comparison values. Perhaps the most striking trend in the data is the increase in the amount of radioactivity found in urine when one compares ^{125}I-Fab′ with ^{125}I-Bands A–E. On a %ID basis, the amount of radioactivity increases approximately 15%–20% in ^{125}I-Bands A and B compared to ^{125}I-Fab′. This discrepancy widens to approximately 25% in ^{125}I-Band C and 30%–36% in ^{125}I-Bands D and E. In other words, for ^{125}I-Bands C–D and E, the amount of radioactivity excreted into the urine doubled in comparison to ^{125}I-Fab′.

Table 5 displays the results of the biodistribution studies of ^{125}I-Fab′ and ^{125}I-Bands A–E in nude mice hosting the T-380 human colon carcinoma. The mice were killed 8 hr after intravenous injection of the bands and 6 hr after the ^{125}I-Fab′. The highest radioactivity observed for the bands was in the kidneys followed by the blood and lung slices. This was in accordance with the distribution in comparison Balb/c mice. Of special interest is the fate of the chromatofocused material in terms of tumor uptake. Both ^{125}I-Band A and B show an enhanced trend of tumor uptake. Unfortunately, the standard deviations are wide and statistical significance is not achieved. While Band C showed evidence of tumor uptake, Bands D and E were not concentrated by the tumor.

In contrast to what was observed in comparison Balb/c mice, the percent of the dose excreted in the urine of tumor mice decreased approximately 17% for ^{125}I-Band A and 31% for ^{125}I-Band B. This is offset somewhat by an

TABLE 4
Biodistribution of ^{125}I-Labeled ZCE-025 Fab′ and Its Labeled Bands in Balb/c Mice
(Mean %ID/g ± s.d.)

Organ	^{125}I-Fab′	Band A	Band B	Band C	Band D	Band E
Blood	5.74 ± 1.66	3.82 ± 0.25	4.93 ± 0.28	2.54 ± 0.20	0.63 ± 0.02	1.40 ± 0.80
Bone	1.21 ± 0.16	1.06 ± 0.47	1.00 ± 0.13	0.59 ± 0.04	0.24 ± 0.07	0.65 ± 0.51
Heart	2.37 ± 0.76	1.39 ± 0.02	1.59 ± 0.14	0.99 ± 0.08	0.23 ± 0.03	0.56 ± 0.37
Kidney*	5.64 ± 0.85	4.42 ± 0.10	5.41 ± 0.52	2.74 ± 0.19	1.33 ± 0.08	2.41 ± 0.92
Liver	2.31 ± 0.67	1.41 ± 0.31	1.79 ± 0.21	1.88 ± 0.59	1.18 ± 0.15	2.03 ± 0.79
Lung	7.46 ± 2.92	3.30 ± 0.37	3.62 ± 0.29	2.16 ± 0.31	0.50 ± 0.03	1.24 ± 0.78
Muscle	0.91 ± 0.13	0.66 ± 0.14	0.74 ± 0.06	0.14 ± 0.02	0.09 ± 0.01	0.23 ± 0.17
Skin	2.02 ± 0.28	1.55 ± 0.26	1.70 ± 0.15	1.08 ± 0.20	0.31 ± 0.08	0.57 ± 0.36
Spleen	2.48 ± 1.28	1.66 ± 1.06	1.35 ± 0.13	0.87 ± 0.09	0.34 ± 0.16	0.75 ± 0.55
Intestine	2.03 ± 0.43	1.25 ± 0.51	1.49 ± 0.43	0.81 ± 0.18	0.35 ± 0.05	2.58 ± 2.50
Urine†	24.5	39.2	44.0	50.8	61.0	54.2
Feces†	2.4	5.1	1.5	5.8	24.0	4.2

* Represents data from a single kidney.
† Data calculated as percentage of total dose injected; all data normalized to a 20-g animal.

TABLE 5
Biodistribution of ^{125}I-Labeled ZCE-025 Fab' and Its ^{125}I-Labeled Bands in Tumor-Bearing Nude Mice

Organ	Mean %ID/g					
	^{125}I-Fab' 6-hr data	+Band A	+Band B	+Band C	+Band D	+Band E
Blood	6.33 ± 0.53	3.81 ± 0.64	3.18 ± 0.46	2.10 ± 0.74	0.84 ± 0.18	0.89 ± 0.27
Bone	0.96 ± 0.31	0.93 ± 0.30	0.53 ± 0.11	0.36 ± 0.12	0.25 ± 0.09	0.17 ± 0.04
Heart	1.75 ± 0.46	1.30 ± 0.28	0.96 ± 0.08	0.61 ± 0.24	0.31 ± 0.12	0.29 ± 0.12
Kidney*	11.44 ± 4.46	8.86 ± 3.78	12.82 ± 8.28	2.96 ± 0.64	1.54 ± 0.23	1.56 ± 0.29
Liver	1.75 ± 0.13	1.62 ± 0.81	1.36 ± 0.71	0.83 ± 0.23	0.74 ± 0.15	0.80 ± 0.20
Lung	4.07 ± 0.47	3.36 ± 0.31	2.37 ± 0.27	1.62 ± 0.36	0.79 ± 0.14	0.71 ± 0.28
Muscle	0.74 ± 0.26	0.69 ± 0.18	0.46 ± 0.07	0.26 ± 0.09	0.17 ± 0.07	0.13 ± 0.08
Skin	3.24 ± 0.71	2.29 ± 0.53	2.35 ± 0.68	1.25 ± 0.28	0.69 ± 0.04	0.47 ± 0.20
Spleen	1.96 ± 0.56	2.12 ± 1.38	1.26 ± 0.75	0.66 ± 0.21	0.39 ± 0.22	0.32 ± 0.14
Intestine	3.42 ± 1.70	1.58 ± 0.37	1.67 ± 0.67	1.26 ± 0.27	1.72 ± 0.80	1.16 ± 0.40
Tumor	4.43 ± 2.18	6.96 ± 3.99	10.19 ± 7.43	5.82 ± 2.13	0.66 ± 0.18	0.54 ± 0.21
Urine†	39.1	22.0	13.1	54.6	61.8	63.4
Feces†	4.0	10.0	14.1	7.4	5.0	16.9

* Represents data from a single kidney.
† Data calculated as percentage of total dose injected. Animals were killed 8 hr after injection; all data normalized to a 20-g animal.

increase of 5% and 13% in radioactivity in the feces of these two bands, respectively. As was the case in comparison Balb/c mice, over 50% of the injected dose of ^{125}I-Bands C–E was excreted in the urine with some fecal excretion.

Data from the electrophoresis of serum and urine collected from Balb/c mice 6 hr after injection of ^{125}I-Fab' are shown in Table 6. The peaks in slice 4 are obvious in all of the specimens. Also, a large species in the serum and a small species in the urine are observed.

DISCUSSION

Intact Mabs and their fragments have been shown to give single bands of the expected molecular weights on polyacrylamide gels developed under nonreducing conditions (*9,17*). Our results with ^{125}I-Fab' parallel these findings and are also supported by the use of visual standards. The data presented in regard to electrophoresis of the Fab'

TABLE 6
Electrophoresis Data from Serum and Urine of Balb/C Mice Injected with ^{125}I-Fab'

Slice number	^{125}I-ZCE-025 Fab'	Serum	Urine
1	0%	0%	0%
2	0%	24%‡	0%
3	7%‡	12%†	0%
4	69%‡	43%‡	59%‡
5	2%	4%	3%
6	6%‡	6%*	16%†
7	4%‡	4%	12%†
8	7%‡	4%	9%
9	4%‡	2%	0%

Counts: * greater than 2x background; † greater than 5x background; ‡ greater than 10x background.
Samples collected 6 hr after injection of radiopharmaceutical.

used in these studies were confirmed in another laboratory (Dr. Roberto Fagnani; Hybritech, Inc. La Jolla, CA). All of the work described, however, was performed in the senior author's laboratory.

Isoelectric focusing of ^{125}I-ZCE-025 Fab' indicated six bands to exist at pIs ranging from 5.4–7.8. Chromatofocusing resolved the six bands of ZCE-025 into two basic populations, low pI (Bands A and B) and high pI (Bands C–E). The former accounted for 80% of the material eluted off the column. All ^{125}I bands as well as ^{125}I-Fab' itself were examined by electrophoresis/gel slice under nonreducing conditions. Under such conditions, complexes would not be destroyed if they existed. We believe the data to hold quite well for the time period indicated, however, the possibility that alterations might vary with labeling cannot be excluded.

Zimmer et al. (*19*) published HPLC radiometric elution profiles of ^{99m}Tc-labeled anti-CEA fragments in normal saline and from the urine of athymic mice 6 hr after injection of the radiopharmaceutical. Most of the radioactivity was associated with Fab'-like material in normal saline. The major radiopeak in the urine sample corresponded to free ^{99m}Tc. A significant amount of radioactivity in the urine, however, was associated with an intermediate molecular weight species, while the ^{99m}Tc-Fab' fragments were only a minor component.

Electrophoresis of the urine 6 hr after administration of the ^{125}I-ZCE-025.Fab' into nude mice indicated 59% of the radioactivity to migrate to Rf 4. This corresponds to nonlabeled fragments. Twenty-eight percent was found in pieces 6 and 7 and represented intermediate molecular weight components, probably light and heavy chains. The small amount of radioactivity in piece 8 (9%) could correspond to free iodine. Both ^{125}I-Bands A and B show this same trend in urine samples from nude mice at 8 hr. Thus,

for these two bands, in vivo dehalogenation appears minor in all instances, i.e., 10% or less. This is in accordance with our previous findings that although eventually marked, cleavage of the iodine from the protein is minimal in vivo for about 8 hr unless immune complexes are formed (*20*).

Our data on the serum samples taken from comparison mice injected with ^{125}I-Bands A and B reveal a smear of radioactivity in pieces 1–3, which suggests the possibility that the ^{125}I-Fab′(s) or Bands A and B might be binding albumin or some other serum protein during the electrophoresis procedure itself. It is also true, however, that this complexation could be occurring in vivo (at least to some extent). Tracey et al. (*5*) have reported a tendency, in selected serum specimens from patients with monoclonal gammopathies, for light chains to bind albumin during the focusing procedure, thus forming high molecular complexes. They were able to overcome this problem by dealbuminating the serum samples prior to isoelectric focusing. Hnatowich et al. (*21*) found the formation of a single high molecular weight species when they studied serum samples of ^{111}I-labeled OC-125 antibody in cancer patients. They did not, however, find a series of high molecular weight species. Furthermore, Pimm et al. (*22*) examined ^{111}In-labeled anti-CEA gel filtration profiles on Sephacryl S300. They studied ^{111}In-labeled intact Mabs mixed with normal mouse serum or serum of mice with colon and gastric carcinoma xenografts. The analysis of all serum samples indicated that ^{111}In was still attached to the Mab in a well-defined peak coincident with an IgG. There was no evidence of antibody-CEA complexes. We have observed such complexes in our patient studies by HPLC when the level of circulating CEA was quite elevated.

The immunoreactivity assay data by itself, in conjunction with the work of Hamilton et al. (*1*) (discussed above), supports the notion that Bands A and B, i.e., the two major pI bands, correspond to monoclonal IgG Fab′. The four minor bands remain unknown, but they certainly have less capability to target tumors than bands A and B and are cleared faster by the kidney. When taking the additional electrophoresis data into account, specifically the dramatic fall in molecular weight of the major radioactive peaks in Bands C–E, the overwhelming evidence suggests fragmentation of the Fab′. Given the rate of excretion from mice and molecular sizing, it is reasonable to postulate that some of the material in Bands C–E (especially the latter two) are light and heavy chain. These entities clear the body so rapidly via the kidney that they would have little effect on imaging were it to take place in the first 24 hr, provided the same clearance phenomenon is true in humans. Interestingly, they did not appear to be acquired and held by the kidney in the manner of the Fab′ or Bands A and B. This is in keeping with current thinking since they are near electropositive or neutral. Electropositive substances would be expected to clear faster than those that are electronegative given the negative charge on the glomerular capillary. Why they were not reacquired by the proximal tubule during their transit is unknown, since protein reabsorption is an active process at this level of the kidney. The larger bands (A and B) were acquired by the kidney. Finally, removal of Bands C–E did not result in enhanced immunoreactivity over that of the starting material.

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